Hepatic potential of Reversed-age Mesenchymal Stem Cells and Endodermal Progenitors : Contribution of LGR5 and Cdc42 cell signaling pathways

Diana Chaker

▶ **To cite this version:**

Diana Chaker. Hepatic potential of Reversed-age Mesenchymal Stem Cells and Endodermal Progenitors : Contribution of LGR5 and Cdc42 cell signaling pathways. Cellular Biology. Université Paris Saclay (COmUE); Université Libanaise, 2017. English. NNT : 2017SACLS562 . tel-01865143

HAL Id: tel-01865143
https://theses.hal.science/tel-01865143

Submitted on 31 Aug 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

université PARIS-SACLAY

NNT :2017SACLS562

Thèse de doctorat

Hepatic potential of reversed-age Mesenchymal stem cells and endodermic progenitors: Contribution of Cdc42 and LGR5 signaling pathways

Thèse de doctorat de l'Université Paris Saclay et l'Université Libanaise préparée à l'Université Paris Sud (Châtenay-Malabry)

Ecole doctorale n°569: innovation thérapeutique du fondamental à l'appliqué (ITFA)
Spécialité du doctorat: Immunologie et Biothérapie
École doctorale en Sciences et Technologie-EDST
Spécialité: Biotechnologie

Thèse présentée et soutenue à Villejuif, le 18 décembre 2017, par

Diana Chaker

Christian POUS Professeur, Université Paris Sud- Paris Saclay	Président
Hélène GILGENKRANTZ Professeur, Université Paris Descartes	Rapporteur
Rihab NASR Professeur, Americain University of Beyrouth	Rapporteur
Marc KARAM Professeur associé, Université de Balamand, Liban	Examinateur
Noushin DIANAT Chargé de recherche, ESPCI, Paris	Examinateur
Franck GRISCELLI Professeur, Université Paris Descartes	Directeur de thèse
Ziad FAJLOUN Professeur, Université Libanaise, Liban	Directeur de thèse
Nehman MAKDISSY Professseur associé, Université Libanaise, FSIII	Co- Directeur de thèse

Acknowledgments

First, I would like to thank the members of my jury. I Thank PR Christian POUS for honoring me to chair this jury. PR Hélène GILGENKRANTZ and PR Rihab NASR, thank you for agreeing to be the reporters of this thesis. I would also like to thank DR Noushin DIANAT and DR Marc KARAM for agreeing to be the reviewers of this manuscript.

Also, my appreciation to Pr. Annelise Bennaceur-Griscelli for giving me the opportunity throughout the PhD research at UMR 935 of INSERM Villejuif and for all your support to achieve adequately this project. Thanks to your willingness to share your vast experience and knowledge. Without you I Havn't got to the point where I am now. I'm very thankful to provide me funding through Vaincre le Cancer and therefore for the association president MR Michel OKS and director MR Laurent BIERE.

Many Thanks to REVIVA center in Lebanon, for letting me do a part of my research in their lab. Mostly I would like to thank the CEO DR Albert Azar, to be so comprehensive and objective during my decision to continue my thesis in France. Thank you to be so reasonable and judicious concerning my professional choice and to give me a family support to complete satisfactorily my PhD. I'm recognizing for all what you offer to me in REVIVA platform.

I Foremost would like to thank my supervisors for the opportunity to study my PhD under your guidance. I express my sincere gratitude to Frank, I really enjoyed our conversations concerning my work, they were challenging and exciting. Those open questions helped me a lot when I was writing. I'm appreciative for the assistance you provided at all levels whenever it was required. Thank you also for your human and generous approaches.

Also, I show my gratitude to Ziad, I'm grateful for your continuous willingness to help me in achieving all the lab activities during my stay in Lebanon financially and humanely wises. Thank you for minimizing me always the difficulties by saying "kheir", this word gave me a new perspective at each down-feeling.

Thank you Norman for believing in me and encouraging me to continue my university cursus. Today, I realize the positive impact you added to my way. You taught me the good organization and to have always a dream for tomorrow.

I'm very grateful for Charbel Mouawad, for your caring about the progression of the thesis and the writing of the scientific paper. Thank you to be the good messenger for my Lebanese part of this project.

I would like to thank Pr. Ali Turhan for your smiley face and high respectability even I didn't work with you.

At Reviva, I would like to thank also my colleague, Charbel Khalil, especially for giving me all the support and to facilitate me the lab duties. Also many thanks for Rose Mary to offer me a lot of administrative services and for your sincere attitude. Also, I'm thankful to Mrs. Lola Azar who gave me all the push and the opportunities to progress in MEIH and REVIVA and having always confidence in my competencies.
A distinct gratitude for Mrs. Sandy Hage, you are a special project manager and you gave me the good assistance, the respectable way to write and how to develop my scientific strategy. I'm realizing that you are my guardian angel who helps me to progress in my Lebanese part. I'm so attached to the approachable and special moments we spent together during our stay in Lebanon. I wish you all the best for your moving to Chicago. Also, I would like to thank Mrs Ghada Oreibi for receiving me and for making my work easier in the genetic department of MEIH. Thank you also, for your professional collaboration way.

I thank my colleagues at U935 Villejuif, Thank you:
Eva and Lucas for being so ready to lend a hand; Afag for your Kindness. Lucie for your empathy, we experienced together the stressful moments of the thesis; Olivier for your scientific and technical advices; Jinwook for your sympathy; Hervé for your professional way of communication and to have the good answers and advices at any time. Jerome, for the big things you learned to me through a smallest number of words, for your expressive serenity and for being so caring and friendly; Adlen for giving me a distinctive idea about the research passion; Carlos for being so friendly, supportive and nice collaborator; Patricia for your sympathetic approach and sincerity; Dominique for your fast order processing, you are a very kind mami for Mila. Sara and Gladys for your funny spirit pushing me up a lot of time. Masae, you are a perseverant and friendly person and Jean for learning me the good way to do my western blots, for your gentleness and your friendly attitude.
Thank you Tony for your nice accompany and honesty. We made a good team together, I'm happy to have you a real friend. All the best for your new challenge.
Thank you Noufissa to give me the right opinions and to encourage me in 2014 for my PhD decision. You are a peaceful and enjoyable person.

I also would like to show my gratitude to Albert Tasteyre, you have a special communication skills, serenity and professionalism. You were a source of encouragement for my doubts.
I thank also Eric Rubinstein and Philippe Leclerc for their technical support.
I am very grateful for the funding provided for me by Cedres Campus de France and CRD. Those financial aids allowed me to continue my both internships without strong economic difficulties.
Thank you. Rima Haddad for your friendly approach and your caring.
Thank you Rindalah for your soft and nice assistance. .
My sincere thanks also go to Bernard and Emilie, you were my second family. You touch me by your peacefulness and humanity. As usual, I'll be by your sides.
RIP my Mamou, near or far, wherever you are, you are giving hope to my life and you will be always in my heart.
Noha, my dear friend, thank you for your prayer which was always a constant sounding board during the most difficult moments during my thesis.
My dear Pauline, you provide me a lot of sincere helps. I know you are going through difficult moments with your disease, but be sure all the shiny days are waiting for you after this hard period.
I would like to express my sincere grateful to Faty, for your support provided to me and my son.
Thank you Mira for being a faithful friend, I'm forever grateful for your love and support. You were by my sides comprehensively in all the difficult moments I spent these recent years.
Finally, I would like to thank perhaps the most beautiful individual, a special friend who recently picked me up through the lowest of times. Your support has been unconditional of which I am eternally grateful, thank you Anthony and also for your family.
Thank you my little sis Zanzoun and my "Sohri"Hamid for your continuous support especially last year. I would like to show my gratitude to Chadi, Cynthia, Fadi and Melissa for always giving me positive energy and practical answers.
Last but not the least, I would like to thank my parents for supporting me spiritually throughout my life and my studies, and,
I dedicate this work to my precious son **David.**

Sincerely,
Diana

Papers Included

Paper 1 (Submitted)

Direct reprogramming of LGR5+ Liver Progenitors cells responding to both gp130/JAK/STAT3 and Wnt/β-catenin the signaling pathways

Diana Chaker , Frank Griscelli, Tony Ernault, Nicolas Moniaux, Olivier Féraud, Noufissa Oudrhiri, Herve Le-Stunff, Lara Bellini, Guillaume Pourcher, Sylvia Sanquer, Jamila Faivre, Christophe Desterke, Ali G Turhan, Annelise Bennaceur-Griscelli

Paper 2 (Submitted)

ML141 reverses the negative impact of the RhoGTPase Cdc42-dependent donor's age on hepatogenic differentiation of hADSCs

Diana Chaker, Charbel Mouawad, Albert Azar, Didier Quilliot, Ibrahim El Achkar, Ziad Fajloun, Nehman Makdissy

ABBREVIATIONS

A

AAT anti-trypsin
ADSC Adipose derived Stem Cells
AFP Alfa Foeto Protein
Ag Antigen
Akt Protein kinase B (PKB)
Alb Albumin
AMP Adenosine monophosphate
APAP acetyl-para-aminophenol (paracetamol)
APC Adenomatous Polyposis Coli
aPKC Par6 atypical PKC
ATP Adenosine TriPhosphate

D

DA Donor Age
DDR proteins DNA damage response proteins
Dex Dexamethasone
DLK 1 Protein delta homolog 1
DP Dental Pulp
DP Doubling Period
DSH disheveled

E

EAE Experimental Autoimmune Encephalomyelitis mice
EB embryoid body
EC Endothelial Cells
E-cadherin Epithelial Cadherin
ECM Extra Cellular Matrix
EGFR epidermal growth factor receptor
EMT Epithelial to Mesenchymal Transition
EpCAM Epithelial Cell Adhesion Molecule
ERK Extracellular signal–regulated kinase
ESC Embryonic Stem Cells
EST Estrogen

F

Fah fumarylacetoacetate hydrolase
FGF Fibroblast Growth Factor
Foxa Forkhead Box A
Fzd Frizzled

B

β-FGF Basic fibroblast growth factor
BM Bone Marrow
BMSC Bone Marrow Derived Mesenchymal Stem Cell
BMP Bone Morphogenetic Protein
53-BP1 p53-binding protein 1

C

Cdc42 Cell division cycle 42
CFU-F Colony Forming Unit
CK1 casein kinase 1
CK14 Cytokeratin 14
CK14 Cytokeratin 14
CLiP chemically induced Liver Progenitor
Cre/loxP Cyclization recombinase - locus of X-over P1
CREB cAMP responsive element binding protein
CTGF connective tissue growth factor
cyp450 Cytochrome P450

H

HBG human hepatoma cell
HBL Hepatoblast
HCC HepatoCellular Carcinoma
HDAC Histone deacetylase
HepG2 hepatoblastoma-derived cell line
HepPar Hepatocyte Paraffin
HGF Hepatocyte Growth Factor
HHEX Hematopoietically-expressed homeobox protein
hiHep human induced hepatocyte
HLA-DR Human Leucocyte Antigen -Antigen D related
HLC Hepatocytes - Like Cell
hMSC human Mesenchymal Stem Cells
HNF1-β Hepatocyte nuclear factor 1β
HNF4-α Hepatocyte nuclear factor 4α
HPLSC Human Pluripotent resident Liver Stem Cell
HSC Hematopoeitic Stem Cells
HSP60 Heat shock protein 60
hTERT human Telomerase Reverse Transcriptase
Huh7 hepato cellular carcinoma cell

G

G6Pc Glucose 6 phosphatase
GAD GTPase Activating Protein
GATA4 GATA binding protein
GSK-3 glycogen synthase kinase-3
GTP Guanosine-5'-triphosphate
GDIs Guanine nucleotide dissociation inhibitors
GEFs Guanine nucleotide exchange factors

I

IGF Insulin-like growth factor
iHep Induced Hepatocytes-like
IL 6/10/2 Interleukin 6/10/2
iMPC induced multipotent progenitor cell
INFγ Interferon gamma
IRS Insulin Receptor Substrate

J

JAK Janus kinase
JNK c-Jun N-terminal kinase

K

KRT18 Cytokeratin 18
KRT19 Cytokeratin 19
KRT8 Cytokeratin-8
KC Kupffer Cells

L

LB Liver Bud
LDL Low-density lipoprotein
LEF lymphoid enhancer binding protein
LGR5 Leucine-rich repeat-containing G-protein coupled receptor 5
LIFR Leukemia inhibitory factor Receptor
Lin28 PAS D''ABBREVIATION
LP Liver Progenitor
LR Leptin Receptor
LT Liver Transplantation

M

MAPK Mitogen-Activated Protein Kinase
MEF mouse embryonic fibroblast
mEpiSC mouse epiblast stem cell

N

NANOG homeobox gene NO ABBREVIATION
N-cadherin Neural Cadherin
NFκB Nuclear factor κB
NK Natural Killer
NOD/SCID Nonobese diabetic/severe combined immunodeficiency
NPC Non-Parenchymal Cells

P

p16 cyclin-dependent kinase inhibitor
p21 cyclin-dependent kinase inhibitor
p38 mitogen-activated protein kinase
p53 Tumor suppressor protein 53
Pak4 Serine/threonine-protein kinase 4
PBSC mobilized peripheral blood cell
PDGF Platelet-derived growth factor
PDT Population Doubling Time
PEDF pigment epithelium-derived factor
PH Partial Hepatectomy
PHH Primary Human Hepatocytes
PI3K PhosphoInositol 3 Kinase
pIR phosphorylated intramembrane Insulin receptor
PKA Cyclic Camp protein kinase A
PKS Cyclic Camp Protein Kinase A
PSC Pluripotent Stem cell

T

TAT tyrosine amino transferase
TCF T-cell factor
TERT telomerase reverse transcriptase
TGF α Transforming Growth Factor alpha
TGF-β Transforming Growth Factor β
TNF Tumor Necrosis Factor
TSA TrichoStatin A
Twist1 Twist-related protein 1

U

UCB Umbilical Cord Blood
UCMSC Umbilical Cord Derived Mesenchymal Stem cell

V

VCAM Vascular cell adhesion protein
VEGF Vascular Endothelial Growth Factor

MET Mesenchymal to Epithelial Transition
MMP Matrix MetalloProteinase

O

OC Onecut
OCT4 Octamer-binding transcription factor 4
POU5F1 POU Domain, Class 5, Transcription Factor 1
OPN Osteopontin
OSM Oncostatin M
OV6 Oval Cell Marker6

R

Rb Retinoblastoma Protein
RhoA Ras-like GTP-binding protein 1
RNA ribonucleic acid
ROS Reactive Oxygen Species production
RSPO Rspondins
RUNX 2 Runt-related transcription factor 2

S

SA- β gal Senescence-associated beta-galactosidase
SASP Senescence-Associated Secretory Phenotype
Sca-1 stem cell antigen 1
SCF Stem cell factor
SDF-1 Stromal cell-Derived Factor 1
smad 2/3 Mothers against decapentaplegic homolog 2/3
SOX17 sex determining region Y-box 17
SOX2 sex determining region Y-box 2
SOX9 sex determining region Y-box 9
SSEA-4 Stage-specific embryonic antigen 4
STAT3 Signal transducer and activator of transcription 3
SVF Stromal Vascular Fraction

LIST OF FIGURES

Figure1: Prometheus's Greek 0
Figure 2: Anatomy of the Liver 12
Figure 3: The Complexity of liver cell types and their interactions 14
Figure 4: Segregation of Hepatoblasts into hepatocytes and cholangiocytes. 15
Figure 5: The cell lineage steps during hepatic development 16
Figure 6: Contribution to the human Cytochrome P450 enzymes (CYP) isoforms to drug metabolism 19
Figure 7: Hepatocyte cell cycle phases 23
Figure 8: A schematic for Signaling pathways modulating hepatocyte proliferation in injured liver. 25
Figure 9: Weakness of liver regeneration in elderly people 26
Figure 10: Schematic view of the bipotent progenitor's location in the liver 27
Figure 11: Stem cells lineage relatives in adult liver and pancreas 28
Figure 12: Regulation of ESCs pluripotency by LIFR and Wnt pathways 31
Figure 13: Generation of hepatocytes from iPSCs. Modeling of human liver disease in vitro 33
Figure 14: Schematic for hepatocytes dedifferentiation into bipotent progenitors. 36
Figure 15: Generation of liver bipotent progenitors mediated by chemical small molecules. 37
Figure 16: WNT pathway activation by LGR 5 38
Figure 17: Crosstalk between Stat3/ LIFR and LGR5/Wnt in LGR5+ stem cells. 40
Figure 18: In vitro Human Lgr5+ organoids generation without Hepatocytes reprogramming process. 42
Figure 19: Change in distribution of donor age in recent years. Source: United Network for Organ Sharing reports, 2015 ... 92
Figure 20 : Evolution of liver transplants frequency in Europe between 1968 and 2013; *From ELTR registry, 2016* 93
Figure 21: Morphological and molecular mechanisms involved in MSCs senescence 97
Figure 22: Age-Senescent Biomarkers in human MSCs 98
Figure 23: Morphological and molecular considerations within MSC differentiation toward hepatocytes 106
Figure 24: Main molecular mechanisms involved in MSCs trans-differentiation into hepatocytes 107
Figure 25: Schematic of Cdc42 activity mode.. 110
Figure 26: Multiple signaling pathways controlling the cellular polarity via Cdc42 activation. 111
Figure 27: Wnt signaling pathways 112
Figure 28: Impact of CASIN on the polarity and epigenetic behaviors of aged HSCs related to Cdc42 GTP activity. 113
Figure 29: Regulation of Cdc42 by the MAPK signaling pathways. 115
Figure 30: Mechanisms of small molecule inhibition of Cdc42 signaling. 117

Table of Contents

ABSTRACT .....
ACKNOWLEDGMENTS .....
PAPER INCLUDED .....
ABBREVIATIONS .....
ABBREVIATIONS .....
LIST OF FIGURES .....
CHAPTER 1 Thesis report structure **1**
Part I: Lgr5+ Stem Cell Generation from Primary Murine Hepatocytes 2
Part 2: Impact of Cdc42 inhibition on aged-derived ADSCs behaviors 6
CHAPTER 2 Liver organogenesis, functions and diseases **11**
2.1. Liver cellular composition 11
2.2. Ontogenesis of the liver 13
2.3. The mesenchymal to epithelial transition during liver development position 16
2.4. Liver functions 18
2.5. Liver diseases: The global health burden 20
CHAPTER 3 Mechanisms of liver regeneration: Contribution of bipotent Liver Progenitors **22**
3.1. Overview 22
3.2. Molecular mechanisms implicated in proliferation of mature hepatocytes during liver regeneration process: Wnt, STAT3 and Cdc42 pathways 22
3.3. Age-associated mechanisms in the failure of liver stem cell regeneration 25
3.4. Primary hepatocytes senescence in vitro 26
3.5. Resident Liver bipotent Stem cells: Origin and limitation in liver regenerative potential 27
3.5.1. *In vivo* strategies for bipotent stem cells lineage tracing 29
CHAPTER 4: Advances in Rerogramming of mature Hepatocytes toward Bipotent Liver Progrnitors (LPs) via the Activation of the WNT-LGR5 Pathway **30**
4.1. Latest cues for Hepatocytes development in vitro 30
4.1.1. Limitations of Primary Human Hepatocytes (PHH) 30
4.1.2. Human Mesenchymal stem cells (hMSCs) 31
4.1.3. Generation of hepatocytes from human Pluripotent Stem cells (iPSC) 32
4.1.3.1. Human Embryonic Stem Cells (hESCs) 32
4.1.3.2. Human Induced Pluripotent Stem Cells 32
4.1.3.3. Limitation of Endoderm specification from hPSCs 34
4.1.4. Hepatocytes plasticity a new era in hepatology 35
4.2. LGR5-Wnt axis a new marker of liver stem cells in association with STAT3 pathway 38
4.3. Recent progresses resulting from the Lineage tracing of LGR5 stem cells 40
4.4. LGR5 a novel marker for long term culture and expansion of stem cells in 3D specter 41
4.5. Footprints 42
CHAPTER 5: Introduction to the scientific paper 1 ..... **44**
Scientific paper 1 46
CHAPTER 6: Impact of Donor age (DA) on Mesenchymal Stem Cells functionalities **92**
6.1. Impact of DA on liver transplantation 92
6.2. Age- related biomarkers of MSCs 93
6.2.1. Cellular activity: Proliferation and Apoptosis 93
6.2.2. Surface Antigen expression 94
6.2.3. Telomere length and Telomerase activity 94
6.2.4. Reactive Oxygen Species production (ROS) 95
6.2.5. The loss of multipotency and stemness 95
6.2.6. Age-related DNA damage and tumor suppression gene profile p53/p16 ^{INK4A}/p21 95
6.3. DA impact on MSCs functionalities 96
6.3.1. Paracrine activity and niche aging: Role of MSCs derived exosomes 96
6.3.2. The deficiency of differentiation and regenerative potential 99

6.3.3. Migration and adhesion ability: Implication of Cdc42 in stem cell aging 99
CHAPTER 7: Hepatogenic potential of Adipose Tissue Mesenchymal Stem cells (ADSCs) **101**
7.1. General debate about Hepatogenic potential of MSCs and their molecular characteristics 101
7.2. Mesenchymal stem cells: safe alternative for liver resident stem cells and induced hepatocytes from pluripotent stem cells 102
7.3. ADSCs as a potent source of human hepatocytes generation: Contribution of exosomes and Wnt pathway 103
7.4. Impact of DA on regeneration potential of ADSCs.....105
7.5. Impact of DA on differentiation potential of ADSCs 105
7.6. Human MSCs to hepatocytes: the in vitro differentiation cues.....106
CHAPTER 8: Cdc42: A novel biomarker of stem cell aging **109**
8.1. Regulation of cdc42 GTPase protein 109
8.2. Cdc42 activation and stem cell aging (2001-2017) 109
8.3. Cdc42 and Wnt pathways in stem cell aging context 112
8.4. Cdc42 and Wnt pathways in the liver regeneration context 113
8.5. Main signaling pathways regulating Cdc42-GTPase activity 114
8.5.1. RAS pathway.....114
8.5.2. Mitogen-activated protein kinase (MAPK) signalling pathway.....114
8.5.3. Nuclear factor κB (NFκB) signalling pathway 115
8.5.4. The Phosphoinositol 3 kinase (PI3k) and Insuline receptor substrate pathways.....115
8.5.5. Cyclic Camp protein kinase A (PKA) 116
8.6. Pharmacological inhibition of activated Cdc42 116
8.6.1. CASIN: Unspecific Cdc42 inhibitor 116
8.6.2. ML141 A novel spatial selective non-competitive specific Cdc42 inhibitors 117
8.6.3. Trichostatin A (TSA): Non-specific Cdc42 inhibitiors 118
8.6.4. Other pharmacological indirect Cdc42 inhibitors 119
CHAPTER 9: Introduction to the scientific paper 2 .....**121**
Scientific paper 2 124
CHAPTER 10: General discussion **153**
Debate 1: The new technology of EndoPCs allowing the activation of Wnt/Lgr5 axis pathway in primary hepatocytes in absence of Wnt activators 153
Debate 2: Novel strategy to convert the age of hADSCs and their derived hepatocytes through ML141-Cdc42 inhibition 156
Debate 3: Reverse-aged hADSCs and EndoPCs technologies in the liver's engineering and regenerative context 158
CONCLUSION 162
REFERENCES 163
ABSTRACT 191
RIEFING OF THE THESIS IN FRENCH 192

.... And the soul is linked to the **LIVER** and one's rootedness in the local, circumscribed realities in which we live our daily lives.... Thus the liver was considered to contain the secret of fate and was used for fortune-telling.... The **SELF** associated to the memory function of the brain that enables us to **MAINTAIN** a sense of awareness of ourselves as being the self-same person over a lifetime, despite changes in physical appearance due to **AGING**...

Donald Capps drawing on the work of James Hillman [1]

Et depuis longtemps…….

« *Quant au rusé Prométhée il envoya contre lui un aigle aux ailes étendues qui rongeait son* ***foie immortel*** *; il en renaissait durant la nuit autant ce que l'oiseau aux larges ailes en avait dévoré pendant le jour…… »*

Figure 1: *Prometheus's Greek myth oil on canvas painting by Peter Paul Rubens, 1611-1612*

CHAPTER 1
Thesis report structure

The liver regenerative potential was hallowed millennia ago in the Greek mythology of Titan when Prometheus, who stole fire from Zeus and the gods and gave it to mortals, was punished by Zeus for his crime by having Prometheus bound to a rock, while an eagle ate his immortal liver on day after day (Fig. 1). This evokes invariably the hypothesis of the liver's capacity for self-repair and regeneration. Since this myth and nowadays, researches were in progress and gave an irrevocable hope for a partial to advanced liver regeneration.
The liver may undergo permanent organ failure after severe injuries, chronic infection, cancer and aging. Hepatic failure leads to 1-2 million deaths per year and is classified as the 5th leading cause of death around the globe. Orthotopic liver transplantation is currently the effective therapy for end-stage liver diseases. However, the shortage of donors creates serious limitation related to this treatment. Thus, the patient's waiting list to find donors has increased to more than 30% both in Europe and United States.

The availability of primary hepatocytes is a gold standard for the liver cell-base therapy. Human primary hepatocytes are not easy to be isolated from liver biopsies and have also a limited life span in culture with a progressive loss of cell function over the time. Therefore, one of the most curbs associated to primary hepatocytes' liver therapy is the availability of a sufficient number of high quality and functional hepatocytes [2]. Moreover, the isolation and culture of hepatocytes may cause major alterations in genes' expression due to cell dedifferentiation under epithelial to mesenchymal transition (EMT) or due to cell apoptosis after only few days in culture[3-6]. In addition, *in vitro* culture models of the human liver cells are crucial for drug development and pathways mechanistic inquiries [7]. To avoid human hepatocytes variability, an alternative would be to alter their *in vitro* short-life by inducing healthy hepatocytes for : (1) the establishment of high-throughput strategies allowing a better systems for susceptibility to drugs, (2) the modeling of liver development and diseases (3) a safe source for cell-based therapies and (4) the development of bio-artificial liver devices and (5) for a liver bio-printing approach [8, 9]. Cell and tissue bioengineering of the liver earned high interests during the last decades and gained momentum through considerable progress in understanding the involvement and application of stem cells in liver regeneration.

Several studies show the existence of resident stem cells in the adult liver [10]. Liver human progenitors have been observed during acute and chronic liver diseases but were found insufficient in number and were unable to perform complete liver regeneration. Stem cell- based strategies are consequently being explored as an attractive alternative approach to liver repair. Thus, various types of stem cells have been used in the recent era to produce a large number of functional hepatocytes *in vitro*. Several studies described the differentiation of the embryonic stem cells (ESC) into hepatocyte-like cells (HLCs) [11, 12]. Despite the ESCs gold standard differentiation potential, their clinical application remains restrained considering the risk of teratoma formation and ethical limitations[13]. Later on, induced pluripotent stem cells (iPS) were introduced as an innovative approach in the field of liver cell therapy [14]. However, iPSCs derived-HLCs have shown an incomplete differentiation status compared to primary hepatocytes[15] since they maintain exogenous transgene expression which can interfere with differentiation protocols[16].

Furthermore, the generation of induced Hepatocytes (iHep) from pluripotent stem cells presents high safety but ethical worries restricting their clinical uses for cell-base replacement regenerative medicine.

Thus, fundamental clinical studies attempt to open new challenges for hepatocytes generation from adult stem cells such as mesenchymal stem cells (MSCs). More recently, liver bipotent stem cells expressing the Wnt target gene called Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5) and deriving from hepatocytes dedifferentiation have emerged as a new platform for stem cell-based liver regenerative medicine; in fact, *Lgr5*$^{+}$ stem cells/progenitors actively contributed to liver regeneration via *de novo* generation of hepatocytes [17], involving a robust activation of Wnt signaling. Indeed, the contribution of the canonical and non-canonical Wnt signaling pathways governs a myriad of biological processes underlying the development and maintenance of adult tissue homeostasis, including regulation of stem cell self-renewal, cell proliferation, and instructing their differentiation fate into hepatocytes. Regulation of Wnts' pathways can trigger multiple signaling cascades, some of them result in activation of small GTPase Rho, Rac, and Cdc42 [18]. Interestingly, mice lacking the RhoGTPase Cdc42 in their hepatocytes exhibited severe liver morphological defects indicating that Cdc42 acts in hepatocytes and might be required for liver function[19]. Recently, it has been reported in normal adult stem cells that LGR5 interacts with the IQGAP1-GTPase pathway which is an effector of Cdc42 to strengthen cell-cell adhesion. This interaction leads to activate the Cdc42/Rac complex and to crosslink the actin organization[20] .Several reports discussed the role of Cdc42 in human MSC proliferation, migration and differentiation, and discussed in mice hematopeitic stem cells (HSCs) its involvement of aging[21].

Currently, there is no published data showing the significance of the role of LGR5 in iPSCs derived-HLCs, neither the impact of the Cdc42 on the rejuvenation of human adult MSCs-derived HLCs; our strategy was based on identifying first the reprogramming process that may induce Hepatocytes differentiation (Hep-*Dif*) of mice LGR5^{+} iPSCs, and second the superlative Cdc42 inhibitor able to reverse the age effect on human MSCs and stimulate their Hep-*Dif* potential of adipose tissue derived stem cells.

In the following sections, the first part describes a new tool for liver bipotent stem cell generation via primary hepatocyte reprogramming process and intended to be differentiated into hepatocytes and cholangiocytes after long-term expansion *in vitro*, and the second part constitute a new strategy to reverse senescence of aged-derived MSCs before inducing their differentiation toward functional hepatocytes.

Part I: Lgr5+ Stem Cell Generation from Primary Murine Hepatocytes *

**Completed in France*

Scientific Background # Part I

The liver presents two mechanisms for regeneration depending on the type of injury. Partial hepatechtomy (PH) involves the remained hepatocytes to restore the liver size. However, toxic injuries involve adult liver stem cells, called Oval cells in rodent, located in the biliary duct[22] and Hering canal in humans [188, 189] .Those stem cells are able to give rise to both hepatocytes and ductal cells (cholangiocytes)[23]. Several pathways were shown to regulate the liver regeneration and hepatocytes proliferations such as canonical Wnt/β-catenin pathway, E-cadherin/ β-catenin membrane complex[24], HGF/ β-catenin[25], IL6/STAT-3 phosphorylation[26, 27], and TGFβ [28]. All these pathways are far to be efficient in human hepatocytes after PH and failed to complete liver regeneration due to the accumulations of reactive oxygen species (ROS) , the DNA damage in the remained hepatocytes and also to the non-sufficient

activation of liver stem cells [29]. On the other side, these oval cells, are activated upon toxic injury when hepatocytes are prevented to proliferate and can give rise to hepatocytes and biliary duct cells (Cholangiocytes) [30, 31]. Although oval cells are activated upon injury, when liver insult is aggressive, involvement of this occasional population in liver regeneration is insufficient [32]. Thus, the liver regenerative-based research was interested in two cover up to mimic the human *in vivo* mechanisms of liver regeneration. The first, primary human hepatocytes long term proliferation and the second, bipotent human stem cell isolation, expansion and activation. However, these two closes present strong limitations and are far to be maintained ex-vivo. In fact, primary hepatocytes isolation constituted a very complicated procedure as they do not proliferate easily on plastic dishes and enter quickly in senescence [33].

On the other side, the Isolation of bipotent liver stem cells requires a molecular characterization of the surface markers. Unfortunately , these progenitors are not a distinct population of the liver since they express common markers with hepatoblasts markers (AFP and CK19) [34, 35] , hepatocytes markers (HNF4-β and Alb^{low}) , adult multipotent stem cell markers (c-kit, Sca-1, Thy1 (CD90), and CD34) [36-38] and liver epithelial marker (EpCAM) [39]. Furthermore, the better proliferative fraction of human Liver Progenitors (LP) *in vitro* was reported to constitute only 0.1% of total Epcam+ stem cell population[40]. Thereby, for long decades, regarding the multiple types of liver cells isolated from liver biopsies, the lack of oval cell- specific markers was limiting their lineage tracing.

The *in vivo* tracking of the bipotent liver stem cells was performed by using inducible-Cre promoters systems in transgenic mice such as promoters of Foxl1 , Sox9 and OPN which are reported to be expressed in only activated ductal stem cells [41,42,43] . These progenitors were able to repopulate injured liver and to give rise to hepatocytes and cholangiocytes *in vitro*. These efforts have led to show that activated bipotent stem cells contribute to 2.5% of hepatocytes regeneration during recovery [43].

Thereby, the development of strategies to isolate and expand these progenitors *in vitro* appeared to be essential for the liver stem cell-based medicine.

The lookout moved backward to several years ago where hepatocyte showed a high plasticity upon injury and to give rise to both hepatocytes and biliary duct cells [44]. Therefore, human hepatocyte lineage tracing in FAH-/- mice model showed a high capacity of hepatocytes to dedifferentiate into bipotent ductal stem cell expressing CK19+/EPCAM+/OPN+/SOX9+ upon injury and to give rise to both hepatocytes and biliary duct cells in suitable cell culture conditions[45]. Moreover, these hepatocytes derived from bipotent stem cells were shown to express leucine-rich-repeat-containing G protein-coupled receptor 5 (*LGR5*), a Wnt target gene which was reported to be highly expressed in dividing stem cells in several epithelial tissues such as small intestine and colon[46], stomach[47], hair follicles[48]. Simultaneously, LGR5 when fixed to its ligand Rspondin1 (Rspo1) promotes the activity of Wnt-Frizzled [49] mediated signaling via activation of IL6/STAT3 pathway[50,51].

More interestingly, in a 3D system culture favorable for Wnt driving pathway, isolated single Lgr5+ stem cells formed organoids retaining the gene expression of the liver over several passages[52].

In addition, these self-renewed Lgr5+ organoids were able to differentiate into functional hepatocytes when transplanted into FAH−/− mice.

Based on all these evidences , Huch et al, 2015 [53] isolated EpCAM + stem cells directly from human liver biopsies . In 3D structure and in presence of Rspo1 and some small molecules inhibiting Notch, FGF4 and BMP pathways, EpCAM+ cells were able to form organoids expressing LGR5+. Hepatocytes derived from EpCAM+LGR5+ cells give rise to functional hepatocytes and regenerate damaged CCL4-Mouse Liver. And more recently, Katsuda et al, 2017 [54], found a combination of small molecules able to convert primary hepatocytes into LGR5+ bipotent LPs so-called chemically induced LPs (CLiPs) with high hepatocytic and cholangiocytic potentialities. This strategy skipped the complexity of EpCAM+ cells isolation and organoids formation in 3D structures. However, Rspo1 and other

canonical Wnt/β -catenin activators such as Wnt3a were essential to stably expand LGR5+ Stem cells before their differentiation. In contrast, organoids LGR5+ required inhibition of BMP, TGF-β and non-canonical Wnt in *vitro*.

Specific Aim(s) # Part I

Based on these outcomes, we centered in this first part on a new research tool for reprogramming mice primary hepatocytes into endodermic progenitors, so-called EndoPCs. Our general objective is to evaluate this novel technology of liver bipotent stem cells generation *in vitro* and to suggest it as a promising tool for stem cell-based liver therapy.

Our first aim is to assess the specific molecular identity of EndoPCs compared to pluripotent stem cells, Lgr5 oval stem cells and primary liver hepatocytes

Giving the authentical Lgr5 expression in EndoPCs, we intend to find the best Wnt activation conditions to derive specific EndoPCs-organoids and maintain their self-renewal upon withdrawal of Leukemia inhibitor factor (LIF) . In a next step we aim to evaluate the hepatic potential of EndoPCs within mice model of liver injury and their graft security. Furthermore we intend to study the crosstalk between Lgr5/Wnt and IL6/LIFR/STAT3 pathways within the EndoPCs under Wnt/Lgr5 activation cell culture conditions. Besides, we will study the hepatogenic functionnality of EndoPCS in two and three matrix dimentional structures.

Strategy # Part I

Our strategy was based on generating liver bipotent progenitors through primary hepatocytes reprograming towards endodermic lineage before reaching the pluripotency state of iPSCs.

- For this end, C57BL/6 mice primary hepatocytes underwent partial reprogramming using non-replicative adenoviral vectors encoding for the four genes involved in pluripotency maintenance Oct4, Sox2, cMyc, and Klf4 as described with Crouzet J et al [55]. Since these hepatocytes-derived progenitors expressed endodermic lineage genes such as Sox17, AFP and CK19, they are so-called endodermic progenitor's cells (EndoPCs). EndoPCs are clonogenically stable in culture and show a specific molecular signature sharing with LGR+ progenitors liver stem cells. However, these endodermic progenitors were distinct from the above-mentioned progenitors by being non-dependent on Wnt/Rspo signaling and by the inhibition of Notch/BMP signaling. These EndoPCs expressing LGR5 were LIF dependent and showed stable long term self-renewal and expansion *in vitro*. They were able to differentiate into hepatocytes, cholangiocytes on collagen coated plates. Furthermore, they repopulate mice liver after partial hepatectomy. However, EndoPCs derived hepatocytes were partially functional in monolayer cell culture system. Thus, the optimization of the cell culture system was crucial to increase the hepatogenic potential of EndoPCs. Consecutively, the EndoPCs were differentiated into hepatocytes and cholangiocytes in 2D and 3D structures.
- At a second step, we were interested to activate the Wnt/Rspo in the LGR5+ EndoPCs structures in presence of the same small molecules used by Sato et al, 2009 [56] and involving Rspo-1, Noggin, EGF, HGF and Wnt3a. Canonical Wnt driven genes Lgr5, TCF4, beta-catenin and Axin2 are evaluated before and after Wnt activation. The target of this set of experiments was to assess if the WNT/LGR5 activation loop mechanism is maintained during the maintenance of multipotency of EndoPCs in culture.

- Moreover, knowing that Wnt/LGR5 is upregulated by the STAT3 phosphorylation, activation of the JAK-STAT3 cascade via IL6 was performed in order to screen the effect of JAK2 inhibitor on EndoPCs proliferation and Wnt/ LGR5 downstream proteins and genes.

Results # Part I

Our results show that EndoPCs represent a potent source for hepatocytes and bile duct generation. 3D-system culture is crucial to boost their hepatogenic potential. Wnt activation is crucial to upregulate the LGR5 transcription and then to generate liver organoids from EndoPCs. Furthermore, it has been noted that the activation of STAT3 is able to upregulate LGR5 expression even in absence of Wnt components in the culture media which is a novel input in the research field of LGR5 bipotent stem cells. This strategy will be described and discussed while introducing our first scientific paper.

Structure # Part I

In the related chapters, further explanations will be elaborating the liver organogenesis, the mechanisms of liver regeneration and the advances in programming of adult hepatocytes in order to support the overall study performed in this project. This will be reviewed in 4 chapters:

Chapter 2. Liver organogenesis, functions and diseases. The liver anatomy is introduced in this chapter. Also, the Mesenchymal epithelial transition associated to the hepatocytes specification is overviewed. Following, the liver functions and the related diseases were highlighted briefly.

Chapter 3. Mechanisms of liver regeneration; Contribution of bipotent Liver Progenitors (LPs). Two mechanisms of regeneration are described. The cellular and molecular signaling pathways involved in the hepatocyte regeneration are largely detailed. In addition, limitations blocking the regenerative ability of the liver will be mentioned thoroughly. Since the liver regeneration is the main debate in this study, numerous topics have been highlighted such as Cdc42, Lgr5, aging, primary hepatocytes, Wnt/β-catenin and IL6/STAT3. Furthermore, liver stem cells are introduced as a potential source for cell-based liver regeneration science focusing on their isolation complexity and expansion methods for optimization. Lgr5 will be hosted as a novel bipotent liver stem cells marker crucial to activate Wnt pathway and to maintain long-term self-renewal of LGR5+ in 3D- *in vitro* atmosphere.

Chapter 4. **Advances in Reprogramming of mature Hepatocytes towards Bipotent Liver Progrnitors (LPs) via the Activation of the WNT-LGR5 Pathway.** The Lineage tractability of bipotent stem cells strategies was stated in details. The human primary hepatocytes tractability will be described showing that hepatocytes plasticity can give rise to LGR5+ bipotent liver stem cells population in the biliary duct zone. Accordingly, new strategies were developed to induce the generation of LGR5+ population from liver hepatocytes which are expanded *in vitro* maintaining a stable system. Three strategies are reported: (i) Hepatocytes dedifferentiated towards bipotent progenies via pluripotency gene expression; (ii) Direct isolation of EpCAM cells from liver biopsies followed by LGR5+ induction via Wnt pathway activation. (iii) Chemically converting hepatocytes to bipotent liver stem cells.

Chapter 5. **Introduction to the scientific paper 1.**Our data presented in the first scientific paper entitled**:**
"Direct reprogramming of LGR5+ Liver Progenitors cells responding to both gp130/JAK/STAT3 and Wnt/β-catenin the signaling pathways"

Part 2: Impact of Cdc42 inhibition on aged-derived ADSCs behaviors**

***Completed in Lebanon*

Scientific Background # Part 2

Many approaches highlighted a link between MSCs and the hepatic fate. Human MSCs (hMSCs), resident in various tissues and organs such as bone marrow , adipose tissue , muscle, liver, brain, periodontal tissue and pancreas, became recently a new hope of autologous cell therapy with low ethical problems and wide range of applications in chronic and acute liver diseases [57]. Human Adipose Tissue-derived MSCs (ADSCs) showed greater proliferative potential, required less invasive procedure to be isolated and exhibited more potent immunomodulatory effects than bone marrow-derived MSCs (BMSCs) and others MSCs biological sources[58-60]. ADSCs were described as an ideal source in medical therapy for their safety and *in vivo* efficacy for degenerative repair [61]. Recently, ADSCs have been described as a promising source of MSCs to be used as a regenerative medicine treatment for hepatic failure [62]. Human ADSCs-derived hepatocytes are considered a potential tool to study drug toxicity by expressing hepatic genes involved in regulation of exogenous drug metabolism [63, 64]. In addition, to date, several human clinical studies are conducted to explore the effects of ADSCs in patients with liver disease (NCT02705742/ NCT00913289/ NCT01062750). Furthermore, in recent studies, ADSCs derived exosomes are considered a new challenge in exosome-based therapies such as neurological disorders and interestingly for liver disease underlining the strong molecular mechanisms shared between ADSCs and liver environment [65] . "Why old cells are more vulnerable to pathology and disease than of young cells?" a question asked by Hayflik L. in 1998 just after the discovery of cell immortality after insertion of telomerase catalytic unit. At this century, observations stand at the fact that age changes occur intracellularly without any added explanations[66]. Today, aging science implicates knowledge of the molecular and cellular mechanisms of diseases, called age-related biomarkers in parallel to cancer research which preoccupied the researchers in the previous decades [67, 68] and is always expanding new hallmarks [68]. Thus, researches on aging showed extraordinary advance over the last 15 years and defined new age molecular hallmarks. Nowadays, the aging is defined as the functional decline that affects living organisms and cell functionality associated to one or several of the following mechanisms: genomic instability, telomere attrition, epigenetic alterations, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. Also, each hallmark is considered when its inhibition shows a delay in the aging process and an increase of the cell lifespan and decrease of other upregulated age related biomarkers[69].

Few months ago, *Florian et al.* , 2017 [70], estimated that by 2050 the number of people over the age of 80 will triple globally, proposing that special approaches will be established to accompany the aging diseases. In liver diseases and aging contexts , it was also discussed that the decline in hepatic progenitor cell population might be one of the reasons for impaired liver regeneration in aged donors for liver transplantation [71]. Moreover, recent reports considered the donor age (DA) as a risk factor for transplant failure [72]. Besides, BMSCs constitute the hallmark example of MSCs dysfunction in aged microenvironment [73, 74] where osteogenic potential is directed to adipocytes differentiation causing the osteoporosis phenomena [73, 75]. Over the past few years, many studies reported the negative impact of adipose tissue DA on ADSCs regenerative ability in mice [76] and human disease [77, 78] demonstrated by a decrease in their differentiation potential into osteogenic, adipogenic and chondrogenic lineages [78-80]. Other age-related biomarkers, such as the tumor suppressor genes p53, p16ink4a and p21, the telomerase activity and the proliferation rate were shown to be downregulated in aged donor's derived-ADSCs[81-

[83]. Thus, the identification of age-related valid biomarkers will be a must to support stem cell - based therapies in elderly population.

Interestingly, hematopoietic stem cells aging was correlated with the high expression of a RhoGTPase protein called cell division cycle 42 (Cdc42) [84, 85] . Cdc42 was shown to strictly regulate cell migration and adhesion molecular mechanisms [86]. As all the RhoGTPase proteins family, Cdc42 acts as a binary switch complex between the active form GTP-bound and the inactive form GDP- bound[86-89]. The Knockout of GTPase Activating Protein (GAP) , a negative regulator of the Cdc42 GTP complex, results in reducing significantly the expansion time, enhances the repair of DNA damaging and suppresses the p53, $p16^{Ink4a}$, p21, and senescence-associated beta-galactosidase (SA-β-gal) expressions in several aged tissues in mice, such as heart, brain, lung, liver, spleen, kidney, and bone marrow . Consecutively, Cdc42 GTP-bound complex was suggested to regulate aging in mice stem cells [90]. Later on, with *Carrillo-Garcia et al.* (2012), the inhibition of Cdc42 activity was considered as the fountain of Youth for stem cells [91]. Based on this hypothesis, Cdc42 activity was inhibited pharmacologically with a Cdc42 activity inhibitor (CASIN). CASIN worked as a histone deacetylase (HDAC) inhibitor, in another way, repaired the epigenetic DNA damage occurring during HSC aging and ROS intracellular accumulation [92] . Thereby, upon transplantation, CASIN-treated HSCs were shown to be potentially identical to HSCs isolated from young donors [21]. To validate that Cdc42 activation can be a biomarker in human aging, the same group , *Florian et al.* (2017), reported on a cohort study of 196 aged human donors of HSCs a high correlation between the Cdc42-GTP expression and aging of hematopoietic cells [93].

Specific Aim(s) # Part II

Based on all these findings, our study will focus, in the second part of the project, on investigating the implication of the Cdc42 activity in human ADSCs aging and their hepatogenic potential. Since *in vitro* hMSCs senescence demonstrates phenotypes similar to biological aged MSCs, it was evident to support our ends by recent advances on *in vitro* aging of hMSCs. All the previous famous studies on Cdc42 and aging were done in hematopoietic stem cells (HSCs).

- We hypothetized first that a certain imbalance may occur in the Cdc42 activity between MSCs derived from young and eldery subjects. Since the presence and function of the adipose tissue are extremely dependent of age, it was important to investigate the existing mechanisms of action in undifferentiated hADSCs, correlating Cdc42 activity particularly to MAPK and Wnt(s) signaling.
- Next, we hypothetized whether any variations in the activity of Cdc42 occuring in undifferentatied hMSCs may affect the hepatocytes' differentiation, thus higher activity could be a key determinant to inhibit hepatogenesis *in vitro*.
- We aimed to correlate the reversibility of the Cdc42 activity by specific pharmacological inhibition to several stem cells related- age biomarkers and pathways and to evaluate if the down-regulation of Cdc42 in aged-hADSCs is essential to induce their hepatogenesis.

Strategy # Part II

Nowadays, there is no reported data showing the impact of the Cdc42 inhibition on reversing the age of elderly hADSCs and reducing their senescence aberrations. Thus, our strategy consisted of:

- Recruitement of healthy subjects (n=61) with different ages (21 to 64 years old) for the collection of the stromal vascular fraction enriched with MSCs, followed by *in vitro* culture and differentiation of ADSCs into hepatocyte-like cells.

- Different signaling pathways were studied such as key factors involved in hepatogenesis (endodermic, fetal liver, hepatic master and functional genes, Wnts canonical/non-canonical), aging (growth factors, transcription factors, inflammatory cytokines, kinases, others), cell's properties (proliferation, adhesion, apopotosis) and cell's functions (albumin and urea production, LDL uptake, exosomes release).
- Identifying an efficient Cdc42 specific inhibitor (CASIN, TSA or ML141) in term of the higher ability to rejuvenate aged derived-hADSCs *in vitro* and by their assessment to compete to differentiate into hepatocyte-like cells when Cdc42-GTP activity returns to baseline levels.

Results # Part II

The data of this section describe that the aging-impact's reversibility of human MSCs is possible by the use of pharmacological inhibitor of the small RhoGTPase Cdc42, ML141. Treatments of hADSCs-derived from AD with ML141 promote greater hepatogenic potential than young differentiated ADSCs counterparts. The inhibition of Cdc42-GTP activity might represent a novel target to rejuvenate ADSCs by modifying their immunomodulatory effects, decreasing apoptosis and improving the cell activity potential (adherence, proliferation, Hep-Dif and functionality). These mechanisms involved RAS/ERK/JNK MAPK pathways, CREB/NFkB/C/EBPα/PPARγ transcription factors, IGF/VEGF grow factors and Wnts signaling and exosomes release.

Structure # Part II

The supportive scientific data for the part II is elaborated in three different chapters as following:

Chapter 6. Impact of Donor age (DA) on Mesenchymal stem cells functionalities. In this section, I will discuss the age-related biomarkers of human and animal MSCs subtypes. In addition, the mechanisms involved in the impairment of cell cycle and refracted differentiation potential of aged MSCs are stated. Next, the contribution of aged niches to the negative impact of donor age on MSCs functionality and their regenerative potential are detailed.

Chapter 7. Hepatogenic potential of Adipose Tissue MSCs (ADSCs). An overview on the hepatogenic potential of hMSCs is first defined followed by a description on how aging impact negatively the hADSCs hepatic fate *in vitro*. In this context, it was evident to discuss some cell culture cues to be considered during *in vitro* MET transition of ADSCs towards Hepatocytes. At the end of this chapter, Cdc42 is introduced as a new marker for multipotent stem cells such as Hematopoeitic stem cells.

Chapter 8. Cdc42: A novel biomarker of cell aging. This chapter is crucial to support our aims; therefore I will debate the mechanism of activation of Cdc42 over age and the signaling pathways regulating the Cdc42 cycles in stem cell aging. Focusing on Wnt signaling and Cdc42 activation is crucial since Wnt is implicated in MSCs cell cycle senescence and aging. To validate the role of Cdc42 in ADSCs aging, inhibition of Cdc42 remain our tactical step. Thus, the recent strategies targeting pharmacologically the inhibition of Cdc42 aged Stem cells are projected. Several Cdc42 inhibition mechanisms using small molecules *in vitro* are described. Indirect inhibition by Trichostatin A (TSA), CASIN and PI3K, JNK and MAPK pathways inhibitors or via a novel specific inhibitors called ML141 are particularly cited.

Chapter 9. **Introduction to the scientific paper 2.** Our data presented in the second scientific paper entitled**: *"ML141 reverses the negative impact of the RhoGTPase Cdc42-dependent donor's age on hepatogenic differentiation of hADSCs"***.

Discussion and Perspectives

Chapter 10. The overall results completed as mentioned above will be described in details. Thereby, both strategies will be evaluated compared to recent advances in liver stem cell-based regenerative medicine, liver derived cells in toxicology assays and novel strategies in liver bioprinting and bioengineering

.

PART I

Completed in France

CHAPTER 2
Liver organogenesis, functions and diseases

The fascinating regenerative potential of the Liver was hallowed millennia ago in the Greek myth of Prometheus whom Zeus punished by having his liver eaten every morning by an eagle. At nightfall Prometheus liver would grow back as to be ready for the eagle's breakfast at the next sunrise [94] (Fig.1). However the liver may undergo permanent organ failure after severe injuries. To understand liver failure, a number of strategies to generate hepatocytes *in vitro* and mimicking the liver's structure and composition have been proposed but unfortunately the success has so far been limited. In the next sections, the structure of the liver, its functions and its diseases will be presented. Strategies for treatment of liver failure will be discussed, followed by an overview of strategies mirroring the liver organization, its weaknesses and limitations.

2.1. Liver cellular composition

The liver is located in the upper right quadrant of the abdomen and is considered the largest organ in the human body and being crucial for life. The liver is divided into two main lobes with a right lobe being six times the size of the left one. The liver receives blood from the gastro-intestinal tract via the hepatic portal vein while the hepatic artery supplies oxygenated blood from the heart. Both of arterial and portal vein drain into the inferior vena cava and join the right heart atrium. The bile duct canals are combined with the branches formed by the portal vein and the hepatic artery. The bile produced in the liver is channeled to the bile ducts that join the gallbladder then exits the liver via the common bile duct (Fig.2).

2.1.1. Liver Endothelial sinusoidal cells

Understanding the liver micro-anatomic structure is the key to create liver model *in vitro* and to discuss liver bioengineering and liver signaling pathways .The basic functional unit of the liver is the lobule including canals, veins and different cell types[95]. The cells form a group around the central vein and are separated by sinusoids conducting the blood flow from the artery and the portal vein to the central vein. The sinusoidal barriers are lined by fenestrae of endothelial cells (ECs) which are essential to exchange metabolites between the liver cells, particularly the hepatocytes and the blood plasma. Liver ECs play also a role in the regulation of inflammation and immune responses and coordinate hepatocyte proliferation during liver regeneration[96-98]

2.1.2. Kupffer cells

The Kupffer cells (KCs) represent the liver macrophages which were described upon liver damage to secrete pro-inflammatory cytokines, activate liver ECs and attract immune cells to exacerbate the initial damage. KCs were shown to release TNF and IL6 for the priming of hepatocyte proliferation in mice liver regeneration model[99-101]. KCs represent about 35% of the non-parenchymal liver cells in normal liver and 80–90% of the tissue macrophages present in the body[102]. The KCs reside within the lumen of the liver sinusoids, adherent to the ECs. Together and with soluble compounds such as ECM proteins, KCs and ECs represent the principal liver non-parenchymal lobule structure.

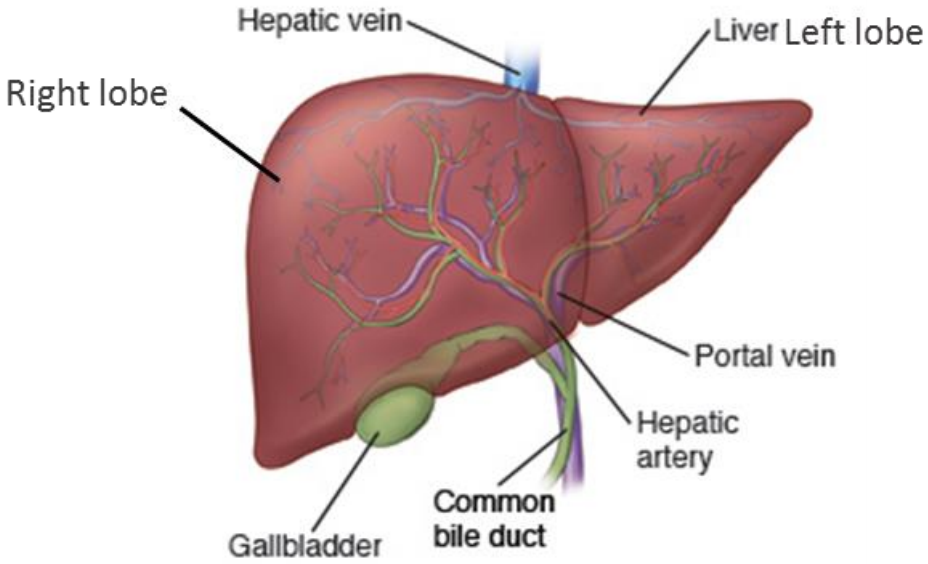


Figure 2: Anatomy of the Liver, adapted from *http://www.stanfordchildrens.org*

2.1.3. The hepatic stellate cells

The hepatic stellate cells are located outside the sinusoid in the space of Disse. Different nominations were attributed such as vitamin A storing cells, lipocytes, interstitial cells, fat-storing cells and Ito cells[103]. They are liver-specific mesenchymal cells that play critical roles in fibrogenesis. Recent studies in liver regeneration have increasingly focused on hepatic stellate cells and showed that this type of liver cells might play a critical role in liver regeneration [104].

2.1.4. Pit cells

The Pit cells are located in the sinusoids and represent liver natural killer (NK) cells. They mediate cytotoxicity and produce cytokines to defend against liver cancer [105]. Otherwise Pit cells were described to be part of liver adaptive immunity being implicated in acute and chronic liver diseases [106].

2.1.5. Hepatocytes

Hepatocytes constitute 60% of liver parenchymal cells (Fig.3), characterized by a polyhedral shape. The space between hepatocytes lines constitutes the bile canaliculi. The hepatocyte's mitochondria provides energy and contributes along with ribosomes and endoplasmatic reticulum to the metabolic mechanisms of the liver covering glycogenesis, glycolysis, lipogenesis, ketogenesis, cholesterol synthesis, blood coagulation proteins synthesis, ureagenesis and xenobiotics metabolism [107]. Hepatocytes contribute actively in liver regeneration regardless the type of injury to compensate liver mass [108]. The maturation of hepatocytes begins from the peri-portal region and is achieved in centri-lobular and peri-venular locations [109].

2.1.6. Cholangiocytes

The biliary tree is upholstered by epithelial cells called cholangiocytes. These cells are implicated in the transport of bile and production of factors preserving bile consistency. However the phenotype and the functionality are

increasingly variable within the cholangiocytes depending on their proximity from peri-biliary glands [110]. The cholangiocytes represent 3% of the cellular liver composition (Fig.3).

2.1.7. Fibroblasts

Fibroblasts are associated with large vessels and biliary epithelium. Otherwise, liver fibroblasts contribute to biliary fibrosis, cirrhosis and particularly contribute to the chronic biliary disease where bile ducts become enveloped by thick layers of fibrosis[111]. Nerveless, liver fibroblasts participate to the maintenance of cholangiocytes notably via TGF-β signaling pathway [109, 112].

2.1.8. Liver local extracellular Matrix (ECM)

Comparing to other organs, the liver is not particularly rich in ECM components. Nevertheless this ECM plays a crucial role in maintaining the liver stem cells proliferation or maintaining their stemness. The liver ECM is constituted of basement membrane proteins consisting of laminin, collagen type I, III, IV and VI, perlecan, elastin which gradient composition differs with hepatocytes distribution. Hepatocytes are connected to other cells and lateral membrane by tight junctions [113]. The laminin is required for appropriate expansion and differentiation of stem cells in response to injury. While the collagen type I have been shown to increase the proliferation of hepatic stem cells and to induce rapid differentiation of hepatoblasts (HBLs). Collagen III and IV have been shown to maintain the phenotype of hepatic stem cells and to slow their proliferation. Liver ECM is also supposed to play key roles in remodeling and chemokine/cytokine production, thus promoting differentiation into hepatocyte or cholangiocyte lineages or liver fibrosis depending on type of injury [112, 114, 115].

2.1.9. Liver Stem cells

Hepatic stem cells have been described to be present in the Canal oh Hering localized in the distal portion of the biliary tree [116]. Within the cholangiocytes carpet, the biliary tract contains a high proportion of cells expressing primitive endodermal stem cells markers (EndoSC). These progenitors have been shown to be multipotent *in vitro* giving rise to hepatocytic, biliary and pancreatic lineages[117-119]. To date, recent research highlights that all cells of the biliary tree seem to have a mark of stem/progenitor potential and plasticity explained by a multipotent fate potential [116, 120]. Liver Stem cells will be discussed in detail in Chapter 3.

2.2. Ontogenesis of the liver

2.2.1. Specification of definitive endoderm

Liver maturation is a cascade involving several complex regulatory mechanisms. In addition it concerns different cell type's harmony. Thus, the understanding of these connections has critically enriched the researcher's knowledge of stem cell maturation and improved our ability to culture, maintain and differentiate hepatocytes *in vitro*[121, 122]. The Nodal signaling is necessary for the specification of the mesendoderm from the septum transversum. Later on, low expression of WNT and FGF4 specifies the ventral foregut definitive endoderm which forms the pancreas, liver, lung and thymus. Signals of FGF1/2 and BMPs assist in the development of hepatic competence and the raise of endothelial precursors between the hepatic buds and cells of the septum transversum [123]. At this level, the key transcription factors HHEX and SOX17 become enriched in the hepatic diverticulum and activate hepatic genes such as Albumin (Alb) , AFP, HNF4, Gata4 and Gata6 [124].

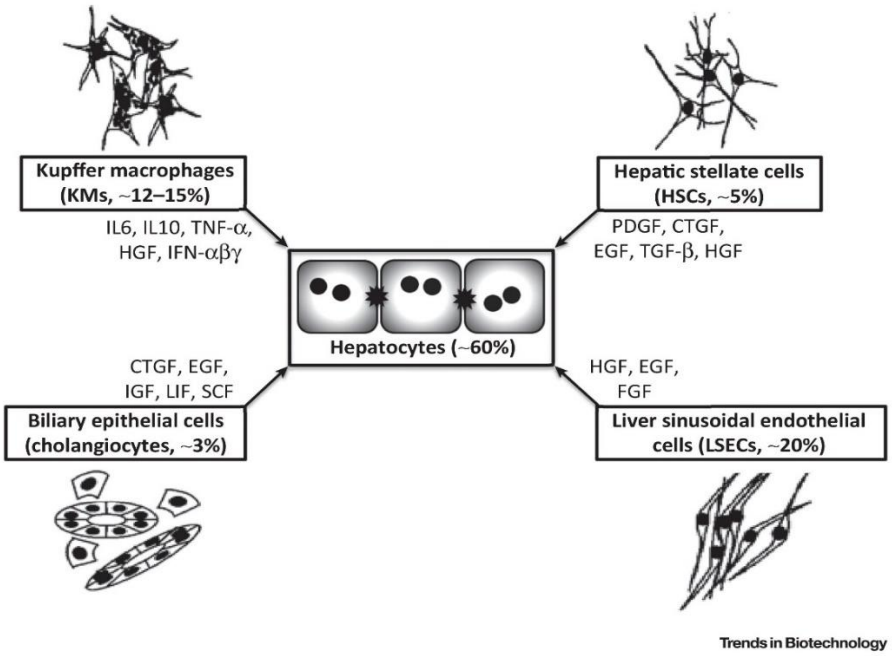


Figure 3: The Complexity of liver cell types and their interactions. From Khetani and al, 2015 [125]

2.2.2. Bipotent hepatoblasts and Biliary epithelium lineage (BEL) segregation

The endothelial progenitors play a key role in the patterning of the endodermal cells, where mesenchymal cells are engaged to form fibroblasts, stellate cells and connective tissue of the liver. Thereby these interactions and signaling between cell types are crucial to the designing of the adult lobules. In a followed step, the hepatic bud is occupied by primitive hematopoietic stem cells (HSC) originating from the umbilical cord (UC) veins and the vitelline. Sinusoidal cells arise from the earliest hepatic plates. The parenchyma is populated at this stage with a homogeneous population of bipotent HBLs giving rise to cholangiocytes in periportal liver and to hepatocytes in the centrilobular regions orchestrated by signals from ECs and mesenchyme of the portal regions. Consequently, the biliary epithelium tree patterning results from the HBLs adjacent to the portal vein where remaining HBLs commit to immature hepatocytes [123, 126, 127] .

2.2.3. Signals promoting hepatoblast migration

The Onecut-1 (OC-1, also known as Hnf6) and Onecut-2 (OC-2) regulate HBL delamination [128, 129] and the expression of extra cellular matrix (ECM) proteins and ECM remodeling enzymes such as matrix metalloproteinases (MMPs) [130, 131] . In absence of the cell-ECM interaction, the HBLs are unable to colonize the liver bud. Otherwise, the Small GTPases, well known for regulating cell migration, are shown to be closely involved in HBLs migration and maturation [132]. Endothelial signals are essential also to delaminate HBLs and promote migration via the vascular endothelial growth factor receptor gene Vegfr-2 expression [133] . Thus, recent strategies generating hepatocytes *in vitro* rely on the combination of endothelial signals and the ECM composition to improve the functionality and stability of hepatocytes[134-136] .

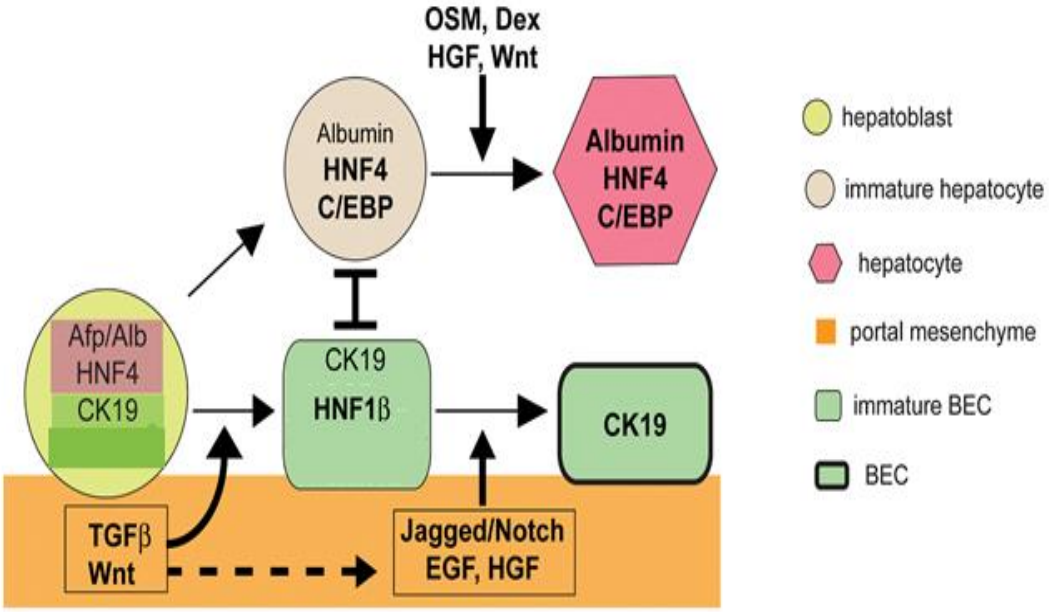


Figure 4: Segregation of Hepatoblasts into hepatocytes and cholangiocytes. *From Stem cell book.org Aaron M. Zorn -2008*

2.2.4. Hepatocytes maturation: Signaling pathways and gene expression

The bipotent HBLs express fetal liver genes (Afp) as well as markers of both hepatocytes (Albumin) and cholangiocytes (KRT19 or CK19). Opposite offence between signals of TGF-β and Wnt reinforce this lineage segregation. In the periportal mesenchyme the up regulation of these signals enhances the expression of cholangiocytes transcription factors (OC1, OC2, HNF1-β) in the adjacent HBLs, while in the parenchyma the repression of these signals up regulates the expression of hepatogenic factors (HNF4 and C/EBP) [137]. The biliary ductal plate is modulated by a continuous signaling of Notch, EGF and HGF from the periportal mesenchyme [138] while other factors such as Oncostatin M (OSM), Dexamethasone (Dex), HGF and Wnt promote hepatocyte maturation. The HSCs cells in the liver secrete the OSM, which in combination with glucocorticoid hormones (Dex), HGF and Wnt promotes Hep-Dif[139-143]. OSM is crucial for hepatocytes metabolic maturation by activating the gp130 receptor and a JAK/STAT3 signaling pathway [144] and in the same time induces morphological maturation into polarized epithelium via K-ras and E-cadherin [145, 146] . On the other hand, hepatocyte growth factor (HGF), secreted by liver MSCs or ECs, supports the differentiation of fetal hepatocytes [139]. These secreted factors upregulate the complex network of transcription factors C/EBPα, HNF1α, Foxa1–3, nuclear hormone receptors and HNF4α[147, 148] (Fig. 4). In mice models, HNF4α is first expressed in HBLs and the KO of this gene results in a failure of expression of mature hepatic enzymes [149, 150]. Furthermore HNF4α controls the mesenchymal to epithelial transition (MET) by regulating genes encoding the epithelial structure, cell adhesion and junctional proteins [151, 152] . Also, the functional hepatic maturation is achieved after birth under the continuous release of HGF by sinusoidal, stellate and ECs [143]. To highlight the importance of OSM and HGF crucial roles in hepatocytes maturation and proliferation, several transgenic mice models defective for OSM receptor gp130 or C/EBPα and HNF1α showed respectively an impairement in hepatocytes maturity and a neonatal death resulting from hypoglycemia caused by impaired glycogen storage [153, 154] . Other strategies mediating HGF induced also hepatic gene expression[140, 155, 156] .

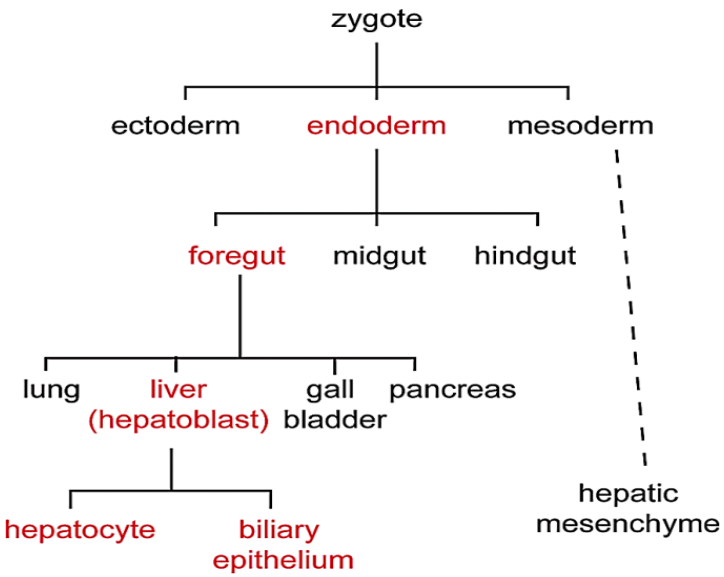


Figure 5: The cell lineage steps during hepatic development (red) from uncommitted endoderm to functional adult hepatocytes and biliary epithelium. *From Stem cell book.org Aaron M. Zorn -2008*

2.3. The mesenchymal to Epithelial transition during liver development

The epithelial to mesenchymal transition (EMT) is a physiological phenotypic shift in which epithelial cells normally interacting with basement membrane via their basal surface undergo biochemical changes that enable them to acquire a mesenchymal cell phenotype which includes enhanced migratory potential, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components [157]. Initially reported as the "epithelial to mesenchymal transformation" by Dr. Hay [158], the term "transformation" was replaced with the term "transition" as a transformation classically describes the oncogenic conversion of epithelia. Moreover, mesenchymal cells can be converted to polarized epithelial cells by a reverse process defined as the mesenchymal to epithelial transition (MET) [159]. Therefore, these two processes are central mechanisms for diversifying morphologically and functionally the cells found in complex tissues. It was admitted for a long time that it is necessary for a cell to be terminally differentiated in order to carry a specific function. However, several observations noted that terminally differentiated epithelium can change its phenotype when an EMT/MET program is activated and a transdifferentiation is carried out both at the development stages and adulthood. Such phenotypic switches are observed during tissue morphogenesis, wound healing and tissue repair as well as pathological stress including several types of inflammation and cancer progression. Hence, Lee et al, 2012 classified the MET into three different subtypes based on their biological context. First, type 1 EMT, which is associated with implantation, embryo formation and organogenesis, can generate motile mesenchymal cells (primary mesenchyme) from primordial epithelial cells. Then, these mesenchymal cells subsequently undergo a MET to form secondary epithelium noting that this type of EMT does not cause fibrosis nor induces an invasive phenotype. Second, the type 2 EMT, which is associated with wound healing, tissue regeneration and organ fibrosis, can generate resident tissue fibroblasts from secondary epithelial cells causing tissues' reconstruction after inflammatory injuries and traumas. Therefore, once the inflammation is attenuated, EMT stops; however, if the

inflammation persists, EMT can lead to organ destruction and tissue fibrosis. In addition, the third type of EMT which is associated with oncogenesis and high grade cancer, occurs in neoplastic cells that were genetically and epigenetically modified, resulting in increased invasiveness and migration. These three EMT types may be regulated by a common set of stimuli, the expression of specific cell surface markers, the activation of certain transcription factors and the reorganization of the cytoskeleton; however, specific signals delineating these subtypes are still undefined [160].

During the liver specification, EMT/MET type 1 occurred. Cells undergo several rounds of EMT and MET in order to form organs during embryogenesis. Initially, a primary EMT process is described when the three germ layers (ectoderm, endoderm, mesoderm) are formed (Fig.5) during gastrulation. The endoderm emerges from the anterior primitive streak of the gastrulating embryo when embryonic cells migrate towards the anterior of the embryo and form a monolayer of cuboidal cells surrounded by the mesoderm at day 7.5 in murine development and at 3 weeks of human gestation. During the first round of EMT, hepatic progenitors called hepatoblasts are specified within the pseudostratified ventral epithelium. Hepatoblastes (HBLs) are bipotent progenitors as they can give rise to hepatocytes in the liver parenchyma and to cholangiocytes next to the portal veins [161]. Murine HBL were identified as cells expressing E-cadherin [162, 163], Liv2 [164], CD24a, and EpCAM [165]. Also, they had been found in human fetal liver as they express factors including ALB, AFP, CK14, KRT18, KRT19, DLK1, E-cadherin, EpCAM, CD133 and the hepatocytes specific antigen HepPar1 [166, 167].

Furthermore, the second EMT type 1 process is associated with the Liver bud formation, initiated by proper cell signaling (Wnt signaling) around the fifth week of human gestation [168, 169]. HBLs, initially of an epithelial morphology and surrounded by a basement membrane, delaminate, migrate through the basement membrane and invade the surrounding septum transversum[170]. This is achieved by coordinated interkinetic nuclear migration[171], the transient loss of epithelial morphology to a more columnar shape, the degradation of the surrounding basal surface by the action of matrix metalloproteinases (MMPs) and the reduction of E – Cadherin expression[172] . Moreover, it has been demonstrated by Juan Su et al. [173] that cells deriving from the human liver bud undergo an EMT process in early human development. These cells expressed simultaneously epithelial (KRT8, KRT18 but no E-cadherin), mesenchymal (CD44 and CD29, Vimentin, Twist1, Snai2, SDF-1) and stemness (OCT4 and Nanog) markers. After the hepatic induction (week 5 of human gestation), an upregulation of EMT related genes was observed, however no information on the expression of E-Cadherin was noted.

Recently, Goldman et al. [174] validates the dual mesenchymal and epithelial features of HBLs. As a matter of fact, they demonstrated that either hepatic cells generated from human embryonic stem cells and resembling HBLs (at 5 day of differentiation) or HBLs extracted from mouse and human fetal livers (at day 9.5 or at 7-22 weeks of gestation respectively), expressed Hepatocytes (AFP and ALB) and both epithelial (EpCAM and E-cadherin) and mesenchymal (vimentin and SNAI1) markers. Moreover, it was revealed that SNAI-1 acts as a transcriptional activator for the promoters of AFP and ALB genes in hepatic specification, in contrary to its conventional role in promoting mesenchymal characteristics during EMT. Once migrating HBLs have acquired a mesenchymal phenotype, they continue to proliferate under the influence of paracrine signals from hepatic mesenchyme, such as FGF, epidermal growth factor (EGF), Hepatocyte growth factor (HGF), transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and interleukin-6 (IL-6)[175-177]. In mid-gestation, these signals promote morphological maturation of HBLs into polarized epithelium via K-ras and E-cadherin[178, 179]; HBLs aggregate around the portal vein and mature into epithelial structures: the Hepatocytes and the cholangiocytes.

Consequently, this maturation implicates a MET[171, 174]. The signaling pathways regulating this MET are the Jagged-Notch pathway controlling the differentiation of HBLs towards a biliary epithelial phenotype[180, 181], and HGF triggering pathway in conjunction with oncostatin M (OSM) promoting Hep-Dif [182]. On the other hand, Hepatocytes form a special epithelial structure which has no basement membrane, but its basal side faces the sinusoid ECs and its apical side faces the canalicular lumen[183]. Finally, hepatic stellate cells are suggested to be derived during liver development from sub-mesothelial cells that arises from EMT of adjacent primitive coelomic epithelium[184]. However, the embryonic origin of hepatic stellate cells is unresolved because they express marker genes of all three germ layers[185].

2.4. Liver functions

The liver is a multifunctional organ by performing over 5000 functions. While these functions are various and crucial, it's good to know that at microstructural lobular level, Human can sustain life even when only 10-20% of liver tissue is functioning. To simplify liver role, its functions will be summarized by the following main tasks categories.

2.4.1. Storing blood and filtration

Indeed, the blood volume in the liver circulating in hepatic veins and hepatic sinusoids is about to 450 ml, which represent 10 % of the total blood volume. KCs can clean blood from various bacteria, endotoxins and foreign antigens. In addition the liver supplies 50% of the lymphatic fluid produced in the body [186].

2.4.2. Carbohydrate and lipid metabolism

The liver is well known to participate in an active gluconeogenesis and to convert the galactose and fructose to glucose. Consequentially, the liver collaborates to maintain a normal concentration of glucose in the blood[187-189]. The crucial enzyme catalyzing the gluconeogenesis is the Glucose 6 phosphatase (G6Pc) enzyme. Furthermore , the liver converts the carbohydrates and proteins to fat, products energy by fatty acids oxidation, syntheses lipoproteins, cholesterol and phospholipids [190].

2.4.3. Protein Metabolism

The deamination of amino acids metabolism starts with Tyrosine aminotransferase (TAT) enzyme activity and leads to the increase of ammonia level in the liver. These residues are eliminated by UREA formation. 90% of plasma proteins are produced in the liver notably the AFP and Alb as fetal and adult human liver marker, Alpha-1 and gamma-2 globulins, binding and transport proteins[189, 191-193].

2.4.4. Storage functions

Besides the functions described above, the liver stores glycogen via the Glycogen synthase (GS). The liver also constitutes a reservoir for vitamins especially Vitamin A, D and B12 and stores iron in form of ferritin. [194-196].

2.4.5. Secretory and Excretory Functions

The hepatocytes secrete bile acids, cholesterol, water, bilirubin and other organic substances in 600 to 1200 ml of bile per day in the intestinal tract. Simultaneously, waste products from blood are excreted by the bile such as cholesterol and bilirubin resulting from Hemoglobin destruction in the liver[189, 193]

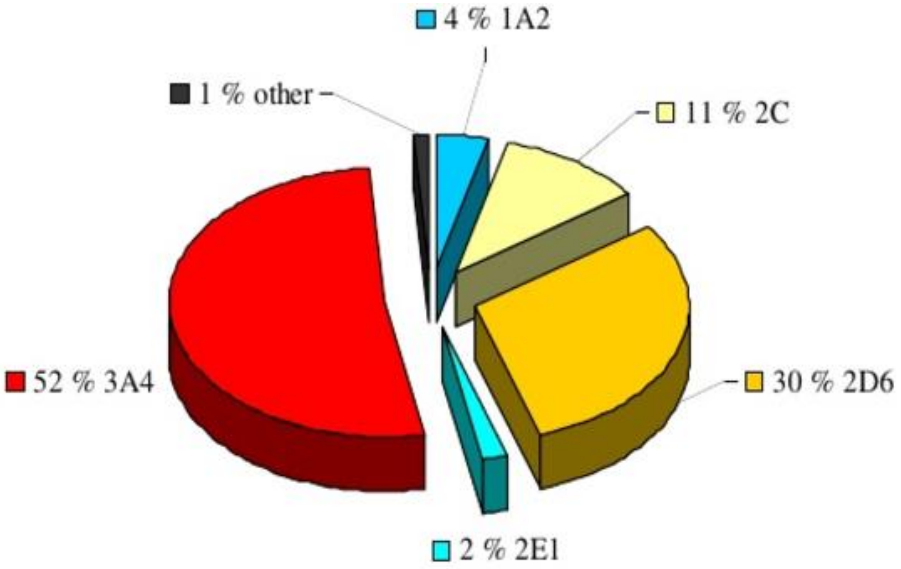


Figure 6: Contribution to the human Cytochrome P450 enzymes (CYP) isoforms to drug metabolism. The percentage of each enzyme correlates with the estimated relative size of the corresponding pie chart.

2.4.6. Detoxification functions and drug metabolism

The main function of the liver is metabolizing and processing drugs. The toxicity of the resulting metabolites can present different biological and toxicological properties [197]. Two principles categories of enzymatic metabolism reactions take place in the liver. Phase I, which is mediated by the cytochrome P450 (CYP450) families and subfamilies [198] (Fig.6) (i). Phase II, the conjugative metabolism of drugs, which is mediated through UDP-glucuronosyltransferases (UGTs) (ii). Both of Phase I and Phase II enzymes are mostly restricted to the endoplasmic reticulum (ER) of hepatocytes. Phase I CYP450 catalyzes the oxidation, reduction and hydrolysis of xenobiotics. On the other hand, Phase II enzymes work on the metabolites from Phase I through various enzymes such as glutathione S-transferases and UGTs. The results of these two enzymatic reactions are the addition of polar groups to the xenobiotics and the facilitation of their eliminations through biliary and urinary tracts [199]. Most of the drug metabolic enzymes are from CYP 1, 2 and 3 families. The activities of CYP450 are different within species [200]. CYP3A4/7 , their orthologue Cyp3A11 in mice models, are the most important human CYP450 enzymes involved in at least 422 drug metabolism [201, 202] (Fig.6) . HNF4α is considered to be the furthermost abundant transcription factor in the liver that regulates the basal expression of Cyp450 subfamilies [203]. Pharmaceutical research used the Cyp450 enzymes to evaluate functionality of hepatocyte cell lines or stem cells derived hepatocytes as cited in more than 100000 articles [202].

Consequently, screening of Cyp450 family activity *in vivo* and *in vitro* after prototype induction using CYP substrates and inducers, such as rifampicin and omeprazole, represents a crucial readout element in based pharmacogenomics, toxicological and fundamental research studies[71, 204].

2.5. Liver diseases: The global health burden

A multiplicity of diseases is linked to the liver being a multifunctional organ. Liver disease is per definition any irregular process that affects the liver. Chronic liver diseases result from severe irreversible loss of at least 75% of the liver's functions and can lead to mortality. Unfortunately, unlike other chronic illnesses, liver disease rates are progressively growing over the years [205]. Chronic liver disease arises independatly of age, sex, region or race. In the last 10 years, Liver diseases have been ranked as the fifth most common cause of death. Deeply, deaths from cirrhosis have been estimated to increase and would make it as the 12th leading cause of death [206]. In this section we summarize the liver diseases classification and some statistics provided by the World Health Organization (WHO) and the European association for the study of the liver (EASL) in their last online available reports (2017). Approximately, 20 million people in the European (EU) region suffered from chronic liver conditions and more than 30 million Americans had liver diseases. Particularly, the mortality rate from liver diseases in France is estimated to 7767 deaths per year *(www. apps.who.int/healthinfo/statistics/mortality/whodpms).*

2.5.1. Liver Cirrhosis

The Age-standardized death rates from liver cirrhosis are estimated in 2012 to 21.7 and 11.8 deaths over 100 000 patients from French and Lebanese populations respectively. Liver cirrhosis is responsible for around 170 000 deaths in Europe each year [207]. Worldwide, liver cirrhosis was estimated to be responsible of 2% of all deaths causes. Cirrhosis is also the principal cause of adult liver transplants in Europe [208]. It is an end stage consequence of many liver lesions and insults characterized by fibrosis and varied clinical indicators and complications [209]. Hepatitis B and C , chronic bile ducts destruction, liver cancer and chronic drug-induced liver diseases lead to end stage liver cirrhosis[210].

2.5.2. Liver Cancer

The common type is hepatocellular carcinoma (HCC) [211] and constitutes a serious cause of cirrhosis [207]. Liver cancer is classified as the sixth common cancer. The last WHO reports estimated 63 500 new cases of liver cancer diagnosed in Europe. Other references indicate that HCC prevalence in patients with Hepatitis C are estimated between 3 and 5% per year, where HCC represent a dominant cause of death in patients chronically infected with HCV in the Western world [212].

2.5.3. Hepatitis

Hepatitis is called also liver inflammation. Several causes can lead to hepatitis either autoimmune or exogenous insults to the liver.

2.5.3.1. Hepatitis A

Hepatitis A virus (HAV) is spread by eating food contaminated by the stools of an infected person. 1.5 million HAV infections are estimated per year worldwide. HAV infection does not lead to chronic liver disease but in some case to acute liver failure and high mortality (WHO, 2016).

2.5.3.2. Hepatitis B

Hepatitis B virus (HBV) is diffused through blood and bodily fluids and can cause either acute or chronic liver diseases. In the EU region, 13.3 million people have chronic HBV where one million American have suffered from HBV hepatitis (WHO, 2016). HBV results in 36,000 deaths every year in Europe (WHO, 2016).

2.5.3.3. Hepatitis C

Hepatitis C virus (HCV) is also transmitted through blood and bodily fluids. Egypt has the highest prevalence of HCV in the world where 40 000 people a year pass away. In the EU region, the WHO estimates 15 million people have HCV causing 86 000 deaths every year (WHO, 2016). HCV infections are associated to HCC in the Western countries [212]

2.5.3.4. Hepatitis D and E

Hepatitis D is caused by the hepatitis delta virus. 15 to 20 million people have been exposed to the hepatitis delta virus yearly; Severe Hepatitis D occurs among indigenous people. Also some Asiatic countries present Hepatitis D like Taiwan, China and India. The majority of infected patients has hepatitis evolving into cirrhosis [210]. Hepatitis E is mainly transmitted through contaminated water and it is found worldwide, but the prevalence is highest in East and South Asia. Like HAV, it's a self-limiting virus and it may develop into acute liver failure. The prevalence is of 20 million infections per year worldwide versus only three million acute hepatitis.

2.5.4. Alcohol-related liver disease (ALD)

ALD are responsible of 47 deaths per 100,000 individuals in some EU countries. The consumption of alcohol is estimated to 13 liters/person/year instead of 1.7 liters/person/year in Lebanon. The alcohol abuse is a crucial cause of liver damage. Thus in Europe, ALD is the dominant cause of end stage liver disease. Indeed, ALD is the major cause of cirrhosis in the Western world and classified as one from the first 10 causes of death worldwide (WHO, 2016)

2.5.5. Non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NASH)

The Non-alcoholic fatty liver disease (NAFLD) is an infiltration of fat in the liver and concerns up to 44% of the European population, particularly the diabetes type 2 categories. A serious evolution of the disease can lead to an inflamed liver due to accumulation of fat called NASH [213]. Basing on the guidelines of World gastroenterology Organization (WGO), the NAFLD has doubled the last 20 years and participates consecutively to be a primary cause of liver disease in the Western countries. Going to the Middle East, 10 to 30% of patients with NAFLD suffer from NASH and then cirrhosis. However in Europe, prevalence of NASH is approximately 5% [214].

2.5.6. Autoimmune and genetic cholestatic liver disease

Liver diseases that affect bile secretion are termed 'cholestatic' and they can result from autoimmune damage of the bile ducts, drugs abuse, genetic defects and congenital disorders. Although they cause less serious liver damage relatively compared to viral hepatitis and fatty liver disease, cholestatic diseases constitute a burden in terms of shortness of effective treatment. Thus, they had constituted 10% of the liver transplants causes in Europe during the past 20 years. Cholestatic and autoimmune liver diseases involve primary biliary cholangitis, primary sclerosing cholangitis, and autoimmune hepatitis. The genetic liver diseases concern cystic fibrosis, familial amyloidotic polyneuropathy, alpha 1-antitrypsin deficiency (A1AD), Wilson's disease, familial hypercholesterolemia, glycogen storage disease type 1a and Alpers Huttenlocher Syndrome.

CHAPTER 3
Mechanisms of liver regeneration
Contribution of bipotent Liver Progenitors

3.1. Overview

Several hypotheses have been established to explain liver regeneration process and its failure during serious injuries. The very old suggestion was a migration of young hepatocytes from the portal to the central zone thanks to mitochondria. This migration was shown to be regulated by lobular zonation gene expression profile [215, 216]. Other suggestion implicates a direct cell division of hepatocytes and cholangiocytes upon occurrence of injury [217]. Actually, thymidine labeling studies showed that remaining hepatocytes in the liver divided once or twice to restore the liver size [218, 219].

Interestingly , it was observed that 75 % of surgical liver mammals resection can be restored within 1 week [220, 221]. The departments of pathology and surgery in University of Washington**,** described in a very snapshot paper the mechanisms involved in liver regeneration after partial hepatectomy (PH) in mammalian cells [222]. They explain that the self-renewal of hepatocytes believed from decades to be the key element of liver regeneration after PH, represents now a controversy when acute or chronic liver injury occurred. They highlighted on recent reports hypothesizing that new hepatocytes in the regenerating liver can derive from hepatic stem cells or circulating stem cells[41, 223, 224]. On the other hand, they underlined the role of the ECM, the non-parenchymal cells (NPC) such as the ECs and the macrophages providing critical signals to hepatocytes and progenitors during regeneration [134]. Also, they showed that the type of injury and many immune modulator parameters can shorten the self-renewal capacity of liver cells. The most crucial signals are HGF, IL6, TNF α, TGF α and EGF; some hormones are shown also to mediate liver regeneration [225].

During liver regeneration, different types of liver cells divide but not together. Indeed, hepatocytes are the first cells to divide followed by the bilary ductal cells then by the NPC. In human, evidences suggested that 2 weeks after hepatectomy, regeneration had started and the liver regained 75% of its original volume 6 to 12 months later[28]. The recovery of the liver loss depends on the proliferation rate of hepatocytes, the age, the severity of the injuries, NPC signals and paracrine stimuli. In addition to these parameters, the implication of liver stem cells and the signaling pathways that maintain the progenitor fate within the liver regeneration are still confusing.

Briefly, the liver has two mechanisms for regeneration depending on the type of injury. PH involves remained hepatocytes to restore the liver size. However, toxic injuries involve liver stem cells that get up in the portal areas and migrate into the lobules giving rise to hepatocytes[22]. Both of regeneration pathways present a limited regenerative potential when aggressive insult occurs. All these clues will be discussed in the further sections.

3.2. Molecular mechanisms implicated in proliferation of mature hepatocytes during liver regeneration process : Wnt, STAT-3 and Cdc42 regulation pathways

Mature hepatocytes proliferation leads to liver regeneration in response to PH, chemical or viral injuries. This regenerative process was summarize by Mangnall et al. [28], in three defined areas: (1) Changes in the ECM (2) changes in cytokines and growth factors (3) changes in gene expression associated to the activation and passage through the cell cycle. These processes involved a series of different phases (Fig 7):

During the **starting phase**, the release of cytokines from the remained liver and other organs via the portal blood boost the hepatocytes and progeny cells to enter the cell cycle.

Later during the **priming phase** the hepatocytes enter the G1 cell cycle level following HGF release by the ECM. The matrix degradation upon injuries initiates the division of hepatocytes.

The **proliferation step** requires a cocktail of cytokines and growth factors. The proto-oncogenes such as c-myc and c-fos, c-jun and cyclin-D1 are the first genes associated with hepatocytes proliferation [10] (fig.7). Furthermore, STAT-3[26] [27], NF-kβ [226] and C/EBP [227] transcriptional program were shown to be activated during hepatic regeneration. The transcription of β-catenin was reported to form an adhesion complex with E-cadherin down flowing the Wnt signaling pathway during the regeneration process [24]. Indeed, post PH, an increase of the degradation of the Wnt downstream gene β-catenin leads to a high accumulation of β-catenin levels in the hepatocytes and thereby their translocation to the nucleus. Later on, the degradation of β-catenin turns on for several days leading to proliferation of hepatocytes. An Orchestra between HGF and β-catenin has been shown during proliferation of primary hepatocytes *in vitro* [25]. In fact, HGF redistribute the intra-nucleus translocated β-catenin independently of Wnt signaling cascade. Briefly, HGF regulates the phosphorylation of the β-catenin than promotes its translocation to the nucleus. β-catenin binding to E-cadherin isn't regulated by HGF [228] (Fig.8).

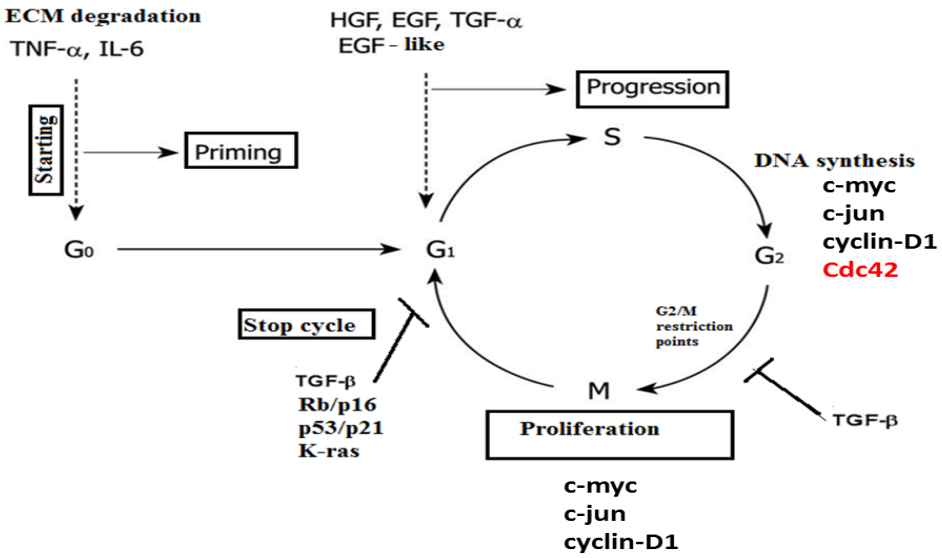


Figure 7: Hepatocyte cell cycle phases; Effect of cytokines and growth factors on transcriptional programs regulating cell mitosis in injured liver. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF-α: Transforming growth factor-α; Cdc42: cell division cycle42; TGF β -: Transforming growth factor

Always during the proliferation phase, the STAT-3 is captivatingly activated in hepatocytes post injury with a significant increase of 30 folds in animal model [26, 27]. The IL6 family cytokine binds to his receptor gp-130 in order to activate JAK tyrosine kinases and consecutively the STAT3 phosphorylation (Fig.8); once activated, p-STAT-3 translocate to nucleus to regulate proliferation transcription genes. In injured adult liver, C/EBP β, IGF-BP1 (Insulin growth factor binding protein1) and HNF-1 (Hepatocyte nuclear factor-1) are crucial transcription factors regulated during hepatocytes regeneration [229-231] via IL6/STAT3 signaling cascade. After liver injury, necrosis or hepatectomy, TNFα and IL6 levels increased in liver and plasma. These inflammatory cytokines trigger the NF-kβ and STAT3 pathways in addition to increasing the DNA synthesis[232-234]. IL6- Knockout mice are unable to respond to liver injury before IL6 injection [235]. The suggesting signaling pathway is that TNFα activate NF-kβ and increase IL6 production in liver macrophages KC. The release of IL6 activates STAT3 phosphorylation in liver hepatocytes [236] (fig.7-8). Interestingly, TNFα regulates apoptosis in normal liver but stimulates hepatocyte proliferation in liver injury concept. Other pathways were shown to be implicated in liver regeneration such as PI3K and MAP Kinase pathways [29] (Fig.8).

During the progression phase, the growth factors involved in hepatocyte cell cycling comprise HGF, epidermal growth factor (EGF), transforming growth factor (TGF α) and heparin-binding EGF-like growth factor [237]. The liver cells continue the passage from G1 to S phase then to G2/M restriction points for division then they return back to G1 and a new cell cycle until restoring the liver mass (Fig.7); during this phase the $p21^{CIP1,}$ a tumor suppressior gene inhibitor, is downregulated to allow hepatocytes proliferation[234] and JNK pathway is activated [238]. The stop point after liver recovery was reported to be regulated by TGFβ pathway [28]. Recently, the Cell division cycle 42 Cdc42, a member of Rho GTPase family small protein, was reported to play a critical role in liver regeneration after PH. Cdc42 is shown to be required in the progression phase by triggering the expression of Cyclin D1 (fig.7). Cdc42 activity regulates the cell polarity and proliferation via several downstream signaling events, such as the p21-activated kinase (PAK) and JNK cascades. Mouse model with liver-specific knockout of Cdc42 exhibited a decrease in liver weight and a delay in liver regeneration post PH. Indeed, Cdc42 deletion downregulated the activation of ERK, JNK and proliferation transcription factors in the remained hepatocytes post hepatectomy[239] . In addition, a proteomic analysis post PH proved that Cdc42 is mandatory to the termination of liver regeneration[240].

Unfortunately, this flow of cell regulation discussed previously is far to be shared by human hepatocytes after severe injuries or some toxic or viral infections after which the regeneration is shown to be incomplete. Liver cirrhosis is associated with senescence biomarkers in all liver cells and branded by a significant short hepatocyte telomeres[241]. In addition, the accumulations of ROS , the DNA damage, the senescent status of remained hepatocytes, the level of necrosis and atrophy, the non-activation of liver stem cells [29] , all together impact negatively the regenerative process of hepatocytes. Moreover, in severe liver injury, adult liver stem cells, called "oval cells," are activated when the ability of remained hepatocytes is reduced following a huge insult of the liver. These stem cells were shown to express Cdc42 in liver-injured rat model, suggesting also that Cdc42 play a role in liver stem cell proliferation and differentiation into hepatocytes [31]. Whether these stem cells can achieve a complete liver regeneration or not? This cue will be discussed in the section (3.4).

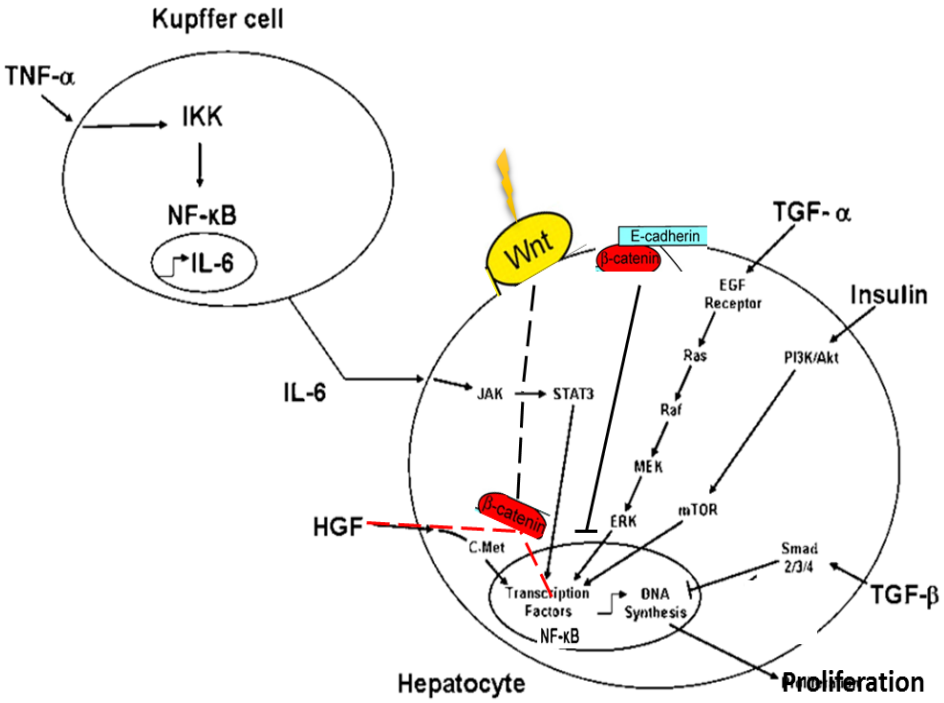


Figure 8: A schematic for Signaling pathways modulating hepatocyte proliferation in injured liver. The involving of IL6/STAT3, HGF/ β-catenin, Wnt/ β-catenin, Insulin and TGFβ, in liver proliferation process. HGF, hepatocyte growth factor; IL-6, interleukin-6; TGFα, transforming growth factor alpha; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor alpha.

3.3. Age-associated mechanisms in the failure of liver stem cell regeneration

Ono Y et al, [71] suggest that the declining hepatic progenitor cell population might be one of the reasons for impaired liver regeneration in aged donors. Indeed, they have correlated the number of these progenitors with liver age and accordingly with the liver regeneration potential in both aged and young human donors 'groups obtained after liver lobe resection. In addition, recent reports have showed that old mice's liver fails to activate the S-phase specific genes that are normally up-regulated by TNFα and IL6 resulting in a late DNA synthesis and in down-regulation of STAT-3 (Signal Transduction and Activators of Transcription) signaling pathway (Fig.9a, b). The down-regulation of PI3K-AKT signaling pathways also play a crucial role in affecting the regenerative potential of liver hepatocytes and stem cells [242]. Furthermore, the DNA polymerase-α is shown to be dramatically inhibited in association of some cell-cycle proteins such as FoxM1B, c-Myc, c-Fos and cdc2 (Cyclin D cycle-2) in old mice liver post injury or PH. Contrary to the mice liver cells, senescent human liver cells revealed telomere shortening which can alter the proliferation potential of the hepatocytes [243]. In addition to this, the alteration of the chromatin structure , the increase of protein levels of histone deacetylase 1 (HDAC1), and the epigenetic silencing of cell-cycle genes lead to a failure of regeneration in elderly liver [244, 245]. Furthermore, the accumulation of fat in old liver participates in reducing the hepatocytes regenerative capacity [246]. Also hepatocytes derived from old donors were shown to be unresponsive for repopulating PH [247, 248]. All these evidence support the decrease of regeneration ability with aging.

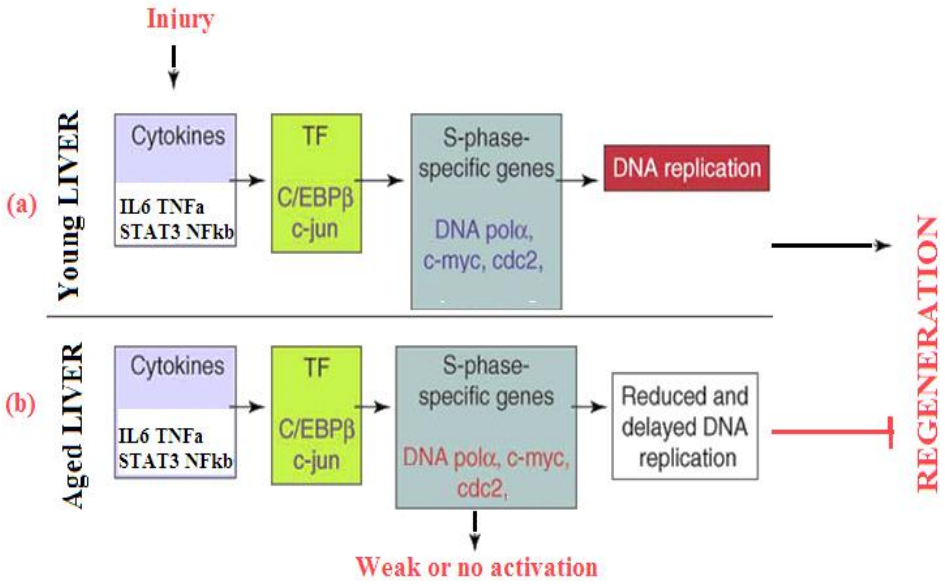


Figure 9: Weakness of liver regeneration in elderly people. Transcriptional program of liver regeneration is regulated by the IL6/STAT3 and TNFα /NF-kβ signaling pathways [249]

3.4. Primary hepatocytes senescence *in vitro*

The isolation of primary hepatocytes from liver biopsy constitutes a very complicated procedure in term of quality and number of isolated cells. Digestion enzymes, such as collagenase, are required for this end and are a critical step. Freshly isolated hepatocytes express highly *c*-jun and *c*-fos driving the hepatocyte in a priming phase G0/G1 (Fig.7) of the cell cycle [250]. In addition, these hepatocytes express NF-kβ and TNFα as well as the protein kinase of MAPK pathways[3]. Once these hepatocytes are plated, p53, K-ras and c-myc are upregulated indicating that they underwent a G1 phase of the cell cycle [251] . Going up to this step, the microenvironment can or not support hepatocytes proliferation. Progression phase *in vitro*, is completely different from its counterpart *in vivo,* and is regulated by tumor suppressor retinoblastoma protein (pRB) [252] . After culture in plastic dishes, surviving primary hepatocytes remain at G1 restriction point, do not proliferate and lead to growth arrest [253] . Indeed, primary hepatocytes enter a senescent pathway by expressing ROS, DNA damaging and expressing the tumor suppression inhibitors genes via the p53/p21 pathways [33] . Like any somatic cell, human telomeres DNA undergo an erosion after several proliferation rounds and hepatocyte growth is stopped irreversibly, when the telomeres reach the minimal length which was first described by Hayflick 50 years ago[254]. Despite avoiding the telomere-dependent senescence and the weakness in DNA synthesis of primary hepatocytes *in vitro,* some strategies tried adenovirus infection of exogenous hTERT [33] and interestingly Cdc42 activation pathway[255].

Another alternative of long time span live hepatocytes *in vitro* are hepatic cell lines derived from liver tumor biopsies. The majority of these hepatocytes lines have unlimited growth potential but do not exhibit sufficient functionality when used in pharmaco-toxicological applications neither can be used in therapeutically applications[256].

In contrast to humans, rodents display high regeneration capacity due to the durable telomerase activity[257]. Nevertheless, *in vitro*, both human and rodent primary hepatocytes showed a limited proliferation activity even under good culture conditions. In this way, the MAPK pathway is implicated in primary hepatocytes senescence by

p21 induction. Thus the pharmacological inhibition of MAPK *in vitro* succeeded to allow better hepatocytes proliferation rate and survival[33, 258] .

When orthotopic liver transplantation is not agreed in the playing field of liver therapy, human primary hepatocytes exemplify the 'gold standard' for the founding of the bioartificial liver systems [259], also serve research development strategies and *in vitro* pharmaco-toxicology assays [260]. Beside the increase in the need of the human primary hepatocytes, the throughput of production are limited by the complexity of isolation, the inadequate human supply, the high cost, the excessive variability and the low "*in vitro*" proliferation potential.

3.5. Resident Liver bipotent Stem cells : Origin and limitation in liver regenerative potential

The ability of liver cells to proliferate in response to transitory injuries is efficient. However, when defect concerns mature liver cells such in cirrhosis and massive insults, the involvement of hepatic progenitors was reported, but unfortunately was also not sufficient. Although, the liver contains facultative stem cells that can be activated in answer to different stimuli [32] and the majority of liver regeneration are shown to be progenitor independent , however these stem cells play an interesting role in some chronic liver failure [261-263]. This population was shown to interfere in liver regeneration when hepatocytes are prevented to proliferate via chemical toxic molecules [30].

Liver stem cells were observed first in rat liver, upon chemical injuries, a small group of cells with high nuclear cytoplasmic ratio with ovoid nucleus called "oval cells" migrating from portal zone into lobule, proliferating broadly and differentiating into hepatocytes[34, 264]. The most likely origin of the precursors of oval cells in adult tissue was reported to be the biliary ductals of the Canal of Hering[23, 265] and so-called "intermediate hepatocytes" (Fig.10). Thus, based on their liver arising place, they were considered as bipotential progenitor cells, capable of generating mature hepatocytes and cholangiocytes [31]. Interestingly, oval cells were also differentiated into different endoderm derived tissues such as intestinal epithelium [266] and pancreatic acinar cells [267] under optimized conditions.

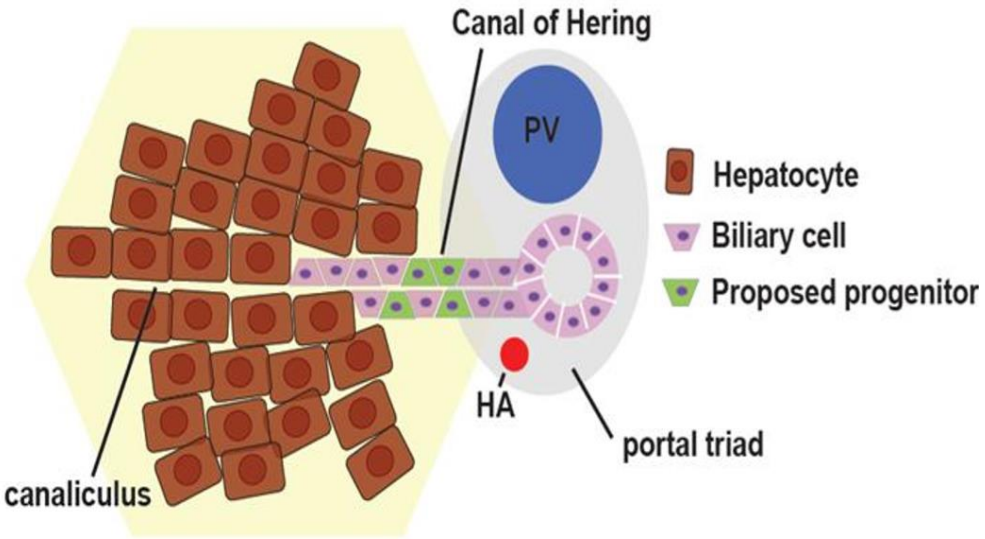


Figure 10: Schematic view of the bipotent progenitor's location in the liver

Stimulatingly, in human liver pathology, liver stem cells have been shown as bipotent intermediate epithelial hepatobiliary cells yielding to both hepatocytes and bile ducts cells [266, 268]. These progenitors present a robust similarity to oval cells in rodent. Thus the general use of "oval cell" indicates in some reports the LPs for all species. Human LPs/stem cells differentiation has been observed during acute and chronic liver diseases but was insufficient to perform complete liver regeneration. Unfortunately, little is known about the homogeneously aspect and the molecular mechanisms regulating the oval cell response to injuries. The complexity of the cell liver composition, hepatocytes, bile ducts, hematopoietic cells, ECs, macrophages, stellate cells, and others avoids to reach information regarding the activation of liver stem cells [269].

On the other hand, Petersen and coworkers were the first to describe in human that resident liver stem cells termed "ductal reactions", express markers previously believed to be hematopoietic stem cells (HSCs) and might be derived from Bone Marrow[270] . Four years later, they report same information in mice oval stem cells[271]. Basing on these evidences several reports isolated hepatic population expressing hematopoietic markers such as c-kit (the receptor for stem cell factor), Sca-1 (stem cell antigen 1), Thy1 (CD90), and CD34 and processed for further surface markers investigation [36-38]. Oval stem cells isolated from rat were the typical model, where the discovery of OV-1 surface marker was very useful in the study of LPs [272, 273] . In addition rat and human oval stem cells co-express KRT19 and Alb the markers of bile duct epithelium and hepatocytes respectively with high expression of fetal hepatoblasts marker AFP [34, 35]. In a next step, activation of oval cells after hepatectomy was investigated in mice induced- chronic liver injury models which are considered more genetically controllable than the rat[262, 274, 275]. Conversely, mice oval cells differ from their rat and human equivalents by not expressing AFP[220]. Consecutively, based on immunostaining data and surface markers, oval cells don't represent a distinct cell type that is not present in the normal liver.On the other side, human liver biopsies harbored *in vitro* colonies expressing EpCAM+ and CD133+ progenitors with high capacity of proliferation. Unfortunately these progeny cells were shown to constitute only 0.1% of total Epcam+ stem cell population involving hepatocytes and hepatoblastes [40]. Although hepatic stem cells are activated during liver injury, they are not able to complete regeneration in chronic diseases.

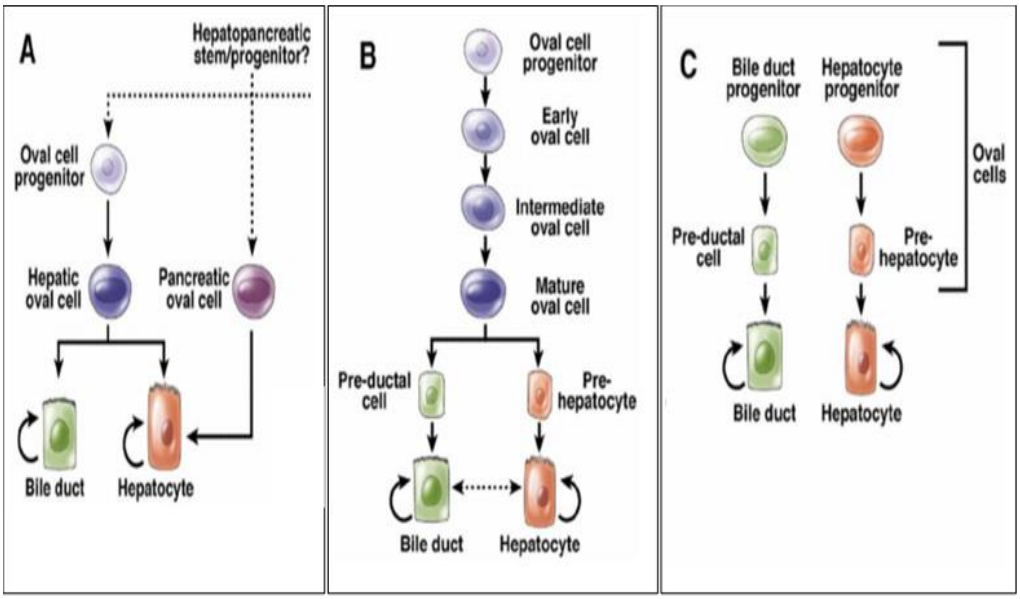


Figure 11: Stem cells lineage relatives in adult liver and pancreas. (A) Classic model depicting one single hepatic oval cell type, for both lineage (B) Oval cell heterogeneity model. Different stages of oval cell maturation. The most mature oval cell is bipotential and gives rise to hepatic and duct cell. (C) Unilineage model for each biliary and hepatic tissue

Hence, different hypothesis were established to design the lineage relationships during oval stem cells activation and liver regeneration. It's possible to have one type of oval bipotent hepatic stem cells (A), or multiple classes of oval progenitors (B) or distinct precursors for hepatocytes and bile ducts (C) (Fig.11). Based on this suggestion regarding the identification markers of bipotent LPs, the liver was classified as a non-classic stem cell system. One of the difficulties in doing lineage experiments with oval cells has been the lack of oval cell-specific markers for long decades.

3.5.1. *In vivo* strategies for bipotent stem cells lineage tracing

Wang et al [23], in 2003 showed that liver bipotent progenitors have a great hepatocyte reconstitution capacity when transplanted to fumarylacetoacetate hydrolase mutant (FAH-/-) mice without addressing whether cholangiocytes arisen from transplanted oval stem cells .

Several trials were conducted to track the oval cells *in vivo and* were based on labeling the embryonic bilary tractus genes in LPs cells by inducible-Cre promoters systems in mice transgenic model such as Foxl1, Sox9 and Osteopontin (OPN). Foxl1-Cre labeling using the β–GAL as a reporter didn't distinguish a single labeling of bipotent oval cells ; Indeed, the activation of the gene promoter upon injury, leaded to the expression of β-GAL simultaneously in the hepatocytes and biliary cells[276]. More recent report, used Sox9-CreER and always failed to track the oval bipotent at single-cell levels[41, 42] but confirmed their implication in the hepatocytes recovery. Recently, the labeling of OPN showed that oval bipotent stem cells contribute only to 2.5 % of hepatocytes during recovery of liver injury[43].

Despite all these limitations for liver regeneration success, several points seem to be an attention-grape for stem cell- liver regenerative medicine such as (i) the impact of aging on stem cell hepatic potential (ii) the modeling of the bipotent liver stem/progenitors (iii) the Cdc42 as a novel gene involved in oval cell regeneration .

Thus, the establishment of a reproducible method to generate bipotent liver stem cell lines *in vitro* will provide a new tool for (1) Discriminating the hepatocytic and cholongiocytic markers within this heterogeneous population, (2) Optimizing stem cell differentiation for liver cell-base replacement strategies and (3) Microengineering of liver models for drug screening toxicity.

Recently the Wnt target gene LGR5 (*Leucine*-rich repeat-containing *G*-protein coupled *receptor5*) was reported to mark mitotic stem cells in self-renewing tissues such as small intestine, stomach, colon and liver and hair[46-48]. Interestingly, in a 3D system culture favorable for Wnt driving pathway, isolated single Lgr5+ stem cells co-expressed sox 9 and formed organoids retaining the structure of the original epithelial tissue[47, 56, 277] .

In the next chapter, I will discuss the recent strategies of modeling bipotent liver oval stem cells *in vitro* and their differentiation into hepatocytes. Also, I will focus on the LGR5 signaling pathway implicated in the hepatogenic potential of stem cells.

CHAPTER 4
Advances in Reprogramming of mature Hepatocytes towards Bipotent Liver Progenitors (LPs) via the Activation of the WNT-LGR5 Pathway

As far as the increasing effort to develop *in vitro* hepatic stem cells and cell lines recapitulating *in vivo* metabolic features with a cost reduction, Soldatow et al [278], reported during 2013 criteria of the faithful hepatotoxicity cell models *in vitro*. Although these liver cell models mainly presented reduced function compared to *in vivo* liver, they are allowing the screening of several molecules in a shorter time and are contributing to the liver microengineering strategies and the lineage tracking of liver development. The liver is well known for its strong damage-repair response. However the mechanisms responsible for its regenerative potentials follow different schools of concept. Different types of strategies were used for lineage tracing of bipotent liver stem cells to mimic the human *in vivo* mechanisms of liver regeneration. In human, LPs are detected close to hepatocytes expressing epithelial ductal markers[279]. Thus, because of the lack in tracking human cells *in vivo*, it's very difficult to know if the ductal progenitors derive from hepatocytes or inversely. Recently, hepatocyte plasticity (dedifferentiation than differentiation) emerged with Huch et al,[280] as **"an old player in the new viewpoint of looking at liver repair**" [280]. Indeed, hepatocyte lineage tracing showed a high capacity of hepatocytes to dedifferentiate into stem cell state upon injury and to give rise to both hepatocytes and biliary duct cells[44]. The new viewpoint was that hepatocytes-derived bipotent stem cells showed expression of LGR5, known to be Wnt-driven receptor and downstream effector. In addition, these bipotent liver stem cells are reflected to be an alternative source for liver autologous stem cells based liver therapy.

That's why, in this chapter, I will discuss the latest advances in liver stem cell generation from primary hepatocytes besides the limitations of use of primary hepatocytes, liver cancer cell lines and pluripotent stem cells. Then, I will report the recent evidences of both hepatocyte's reprogramming and plasticity *in vitro and in vivo* exposing the different strategies recently conducted in this field (labeling, lineage tracing, chlonogenicity, and transplantation). In addition, the LGR5-Wnt axis, implicated largely in embryonic liver development and liver regeneration, will be described in order to clearly understand the role of this pathway in self-renewal, maintenance and differentiation of these liver bipotent stem cells LGR5+ liver bipotent stem cells derived from adult liver hepatocytes.

4.1. Latest cues for Hepatocytes development *in vitro*

4.1.1. Limitations of Primary Human Hepatocytes (PHH)

As mentioned in the previous chapter, PHH present a lot of limitations even though they are the best source of hepatocyte *in vitro*. In most cases, the greatest liver resections quality is reserved exclusively for transplantation, however PHH isolated from other biopsies are minor in quality. The single donor derivation limits their genetic homogeneity for a whole population. Additionally, their lifespan varies from several hours to several days only restricting their long usage. Furthermore their isolation procedure defines their quality *in vitro*. Indeed, the digestion of biopsies disturbs the cell-cell interactions and leads to ECM membrane transporters destruction[281]. Even by trying to create 3D organoids models for PHHs, the Cyp450 activity is dramatically lower than the *in vivo* activity. Immortalized HPP cell lines by the Simian vacuolating virus 40 (SV40) Large T or telomerase reverse transcriptase (TERT) present limited functionality , genomic instability and lack in androstane receptor (CAR)[282]. Additionally, their activity must be verified frequently before any expressive information can be measured[278].

On the other hand, liver cancer cell lines such as Hep3B, Huh7, HBG, and HepG2 lines[282], present an instability of Karyotype and a decrease in functionality even though they are CYP450 inducible [283]. Therefore, they do not present a stable alternative for *in vitro* models to study hepatocyte functionalities[282]. Some cancer line include stem cell characteristics and plasticity potentials: HepaRG cells , derived from a female patient presenting an hepatoma reserve liver glycolysis and the majority of xenobiotic- induced CYP450 enzymes similar to the *in vivo* activity of PHHs[284] . Interestingly, HepaRG cells are a bipotential cell line capable of trans-differentiation to both hepatocytes and cholangiocytes depending on culture media conditioning and media composition[197]. However, they are limited by the fact that they are derived from a single donor and having a single genetic background and thereby, they can't be used in hepatobiliary development strategies for liver disease treatments.

Due to all these unsecured limitations, the appearance of alternative sources for hepatocytes is a must. Human pluripotent stem cells appear to be an interesting tool for both pharmaceutical industry and stem cell-base science.

4.1.2. Human Mesenchymal stem cells (hMSCs)

MSCs are multipotent progenitor cells found in several body tissues [285]. Although bone marrow was the first source described to contain MSCs, it has been reported that MSCs can be isolated from Adipose tissue, human UC tissue and blood, synovium, dental pulp, amniotic fluid, skeletal muscle lung, liver[64, 286-289]. Interestingly, MSCs offer another advantages in comparison to adult multipotent hematopoietic stem cells (HSCs): they have immunomodulatory or immunosuppressive properties [289]. Moreover, MSCs can be clinically translated into the ability to induce tolerance after liver transplantation. On the other side, MSCs emerge as an attractive stem cell source for cell liver therapy presenting low ethical risks, easy availability , safe plasticity and great use for allogeneic organ and tissue repair [290]. Many approaches illuminate a link between MSCs and hepatic fate, and explain why MSCs represent a feasible alternative to human adult hepatocytes, which is giving a new hope for autologous cell therapy. The hepatogenic potential of hMSCs and recent findings for MSCs-based liver therapy will be discussed largely in the chapter 6 and 7.

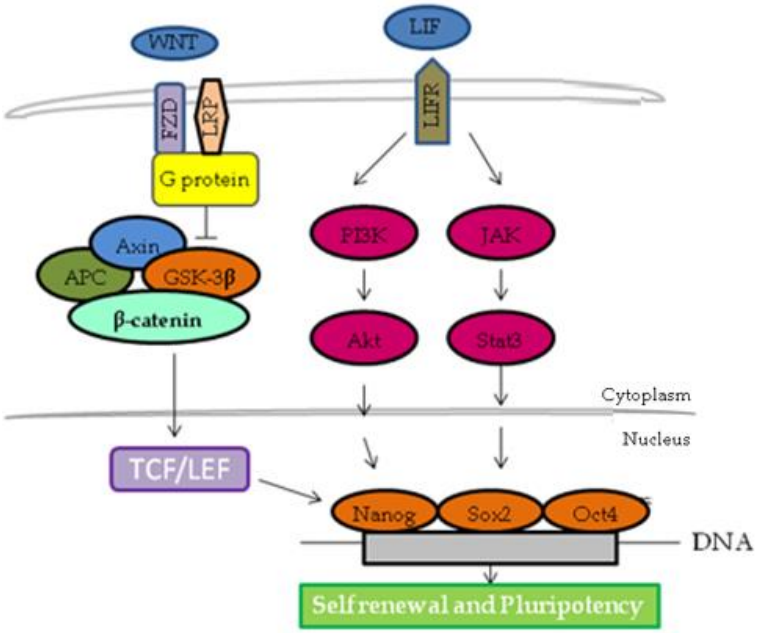


Figure 12: Regulation of ESCs pluripotency by LIFR and Wnt pathways [291]

4.1.3. Generation of hepatocytes from human Pluripotent Stem cells (PSC)

4.1.3.1. human Embryonic Stem Cells (hESCs)

hESCs are pluripotent stem cells derived from the inner cell mass of blastocysts[292]. hESCs have the ability to proliferate indeterminately *in vitro* . Contrarily to mouse ESCs, the hESCs' pluripotency is not LIF/gp130/STAT3 pathway dependent [293]. Leukemia inhibitor factor activates the JAK-STAT3 pathway through the class I cytokine receptor gp130 and others downstream signaling pathways such as PI3K/Akt and Erk/Mapk[294] . It was shown that STAT3 play a crucial role in the mouse ES self-renewal via induction of c-myc expression [295] . Interestingly, hESCs are shown to be more similar to mouse epiblast stem cells (mEpiSCs) rather than to mouse ESC presenting a primed pluripotency state. Thus, hESCs require in their medium bFGF instead of LIF to maintain self-renewal and pluripotency, growing either alone or on mouse embryonic fibroblast (MEF). Although hESCs are shown to express LIFR and the signaling subunit gp130 which are able to activate the STAT3 phosphorylation and its nuclear translocation, human LIF is unable to maintain the pluripotent state of hESCs.[293]. On the other side, canonical Wnt signaling involving β-catenin has also been suggested to control mESCs and hESCs stemness via activation of the adhesion pathway through E-cadherin/β-catenin associated PI3K/AKT signaling[294 296 297] (Fig.12) . hESCs present the capacity to differentiate into the three embryonic germ layers: mesoderm such as cardiomyocytes [298], ectoderm such as neurons and endoderm such as hepatocytes [299, 300]. Particularly, the hESC derived hepatocytes are able to repopulate injured liver and restore its functionalities [301]. The use of hESCs is mainly limited by the ethical concerns of the embryos destruction and the immune incompatibility between donors and recipients of ESC transplants [302, 303]. Several studies have described the differentiation of the embryonic stem cells (ESC) into hepatocytes- like cells (HLCs). Different contributions have been made for optimizing the combinations and sequences of inducers used for the process of ESCs Hep-Dif [11, 12, 149]. Indeed, when injected into mice, ESCs can generate teratocarcinoma and differentiate towards heterogeneous cell populations when undergoing differentiation strategies *in vitro* [13]. To overcome the hESCs limitations and solve the problem of allogeneic rejection, Shinya Yamanaka , 2007[304] and colleagues converted mice fibroblasts using just four transcription factors into so-called induced pluripotent stem cells (iPSCs).

4.1.3.2. Human Induced Pluripotent Stem Cells

The iPSCs were generated initially by transmission via retroviral mediated gene transfer of a large number of transcription factors highly upregulated in ESC. However, four of the initial factors were essential for the generation of iPSCs: namely POU Domain, Class 5, Transcription Factor 1 (POU5F1, also known as OCT4), SRY (sex determining region Y box 2 (SOX2), Kruppel-like Factor 4 (KLF4), and the c-myc avian myelocytomatosis viral oncogene homolog (c-MYC). The resulting iPSCs, like ESCs, were able to tri-lineage potential *in vivo* through teratoma formation and *in vitro* via embryoid body (EB) formation[305]. This work conferred to Yamanaka and John Gurdon the Nobel Prize in Physiology or Medicine in 2012. Later on, this reprogramming process was reproducible by many groups on human somatic cells [306]. The major advantage of working with hiPSCs is that the ethical issues related to human blastocysts using are avoided. Outstandingly, hiPSCs can be derived from somatic cells of diseased patients and thus can be used to model human disease *in vitro* [307] (Fig. 13).

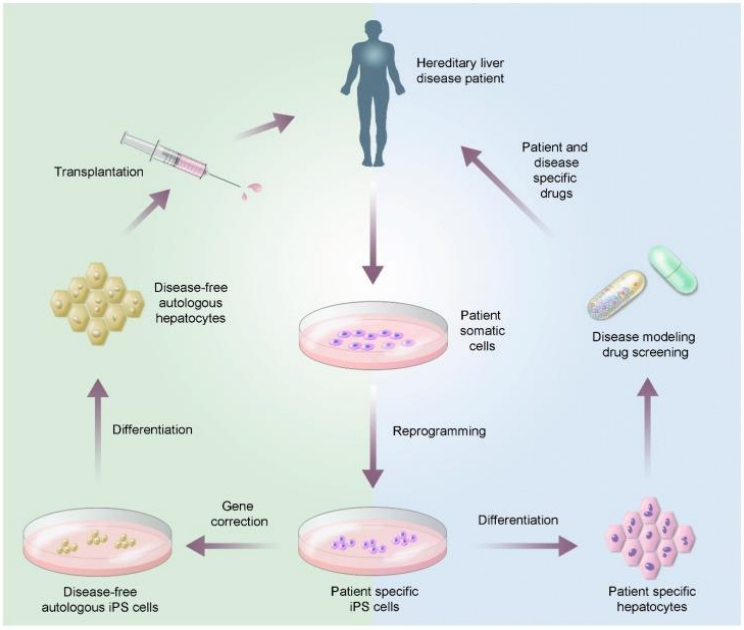


Figure 13: Generation of hepatocytes from iPSCs. Modeling of human liver disease in vitro[308]

The permanent presence of viral mediated gene transfer make them unsuitable for cellular therapy approaches[309]. Additionally, hiPSCs harbor sometimes spontaneous mutations refractory to complete reprogramming [310] or conserve mutations from parental fibroblasts [311]. That's why the reprogrammed cell source of iPSCs plays a crucial role in their differentiation potential. Although fibroblasts are the common source of human iPSCs (hiPSCs), these cells have also been reprogrammed successfully to primary hepatocytes [312].

Furthermore evidences of successful generation of hepatocytes from different sources of iPSCs have been largely reported[313]. Although, over the past three years, the strategies of iPSCs generation implied non-integrated vectors [314]or small molecules compounds[315], they don't have the ability to differentiate into fully mature hepatocytes. Indeed, the iPSCs derived hepatocytes were always termed hepatocyte-like cells (HLCs) showed to have lower levels of albumin production, CYP 450 activity, urea production and a persistent AFP expression[316].

Several protocols were conducted to differentiate iPSCs towards hepatocytes and were optimized in term of culture time, differentiation steps and specific hepatocytes functionality. After a lot of trials yielding to non-functional hepatocytes[15], recent strategies showed better efficiency with a 3 three-steps differentiation protocol [317] and an average of 12 days in culture. Thereby, the hiPSC-derived hepatocytes generated by Asgari et al[318] expressed albumin, glycogen storage capacity and CYP450 activity and were able to repopulate CCl4-injured mouse liver. Advances in this field used 3D culture suspensions for iPSCs to increase their expansion's yields and the maturation of the derived hepatocytes [319 320, 321].

Latest strategies tried to directly reprogram fibroblasts into hiHep (human induced hepatocytes) or to completely avoid pluripotency and teratoma formation through the induction of multipotent progenitor cells (iMPCs) before their differentiation towards the hepatocyte lineage[322-324].

Despite that, teratoma formation and immune response to pluripotency antigens in the non-differentiated iPSCs constitute always a cutoff of their translation into the clinical[325-327]. However, Hep-Dif from iPSCs is of high interest

in stem cell research field due to their importance as a tool in hepatotoxicity as well as modeling liver development and diseases [328, 329].

4.1.3.3. Limitation of Endoderm specification from hPSCs

To differentiate hPSCs into hepatocytes, it is crucial to recapitulate the *in vivo* developmental process *in vitro* and to take into consideration the main signaling pathways driving the Hep-Dif.
During **the definitive endoderm** (DE) specification, Activin/Nodal signaling is implicated in model organisms such as mouse as well as humans [330]. At this step of differentiation, Activin A, a member of the TGF β superfamily of proteins, coordinates with Fibroblast Growth Factor FGF and WNT/β-catenin signaling pathways [331]. Indeed , first the activation of ERK, the downstream of FGF, leads to SOX2 expression to maintain pluripotency of PSCs [332].
Next, this triggered axe promotes the induction of the DE [333]. That's why, Activin and FGF were used universally to induce DE from PSCs. The endodermal differentiation could be completed by the activation of the downstream Mothers Against Decapentapligic Homolog 2/3 (SMAD2/3) complex [334].
WNT is reported to be required only to initiate endodermal differentiation [335]. Indeed, canonical WNT /β-catenin induces and maintains the endodermal regulator SRY (Sex Determining Region Y) Box 17 (SOX17) expression in coordination with Smad2/3 and
upregulates the specific endodermic genes such as FOXA2, Sox17 and HHEX [336]. Therefore, the inhibition of GSK3β, a component of β-catenin destruction complex, enhanced the endodermal differentiation of PSCs[337].

During the **hepatic specification** hepatoblasts can differentiate into either hepatocytes or cholangiocytes. This progression involves the specific transcription factors FOXA2 and GATA Binding Protein 4 (GATA4) [338] which are capable of binding to DNA and activating the key hepatocyte markers transcription such as ALB .
Conversely, Notch activation pathway mediates the cholangiocytes development from hepatoblasts. The Notch pathway represents at present a great tool to differentiate cholangiocytes from endodermal progenitors derived from iPSCs[339]. The table 1 below shows the variability of recombinant growth factors used to direct the initial stage of differentiation from the pluripotent state towards the endoderm. In addition, to date, a lot of small molecules were used to optimize the endodermal step differentiation from hPSCs[337], however they block the further differentiation steps to endodermal tissues such as hepatocytes or cholangiocytes. Unfortunately, the exit from pluripotency to DE regulatory pathways are not yet clear and constitute a strong limitation for *in vivo* and *in vitro* success of hPSCs differentiation towards hepatocytes.

Besides all the limitations to obtain functional hepatocytes *in vitro* from PHH or transformed liver cell lines and overcoming the ED or hPSCs differentiation step, developing new models especially in hepatoxicity areas is a must. Interestingly, the HepaRG hepatoma cell line was showed to present bipotent progenitors in a manner of DE derived progenitors after plating. These bipotent progeny coexpress cKit, KRT18, KRT19, CD34, OV6, LIF receptor and Integrin β1[340, 341]. In addition, at subconfluence, they are able to lose progeny potential and to differentiate into normal adult hepatocytes and cholangiocytes with higher potential of longevity *in vitro* than PHH in culture. On the other side, researchers suggested that the iPSCs have a greater differentiation potential to their cell type of origin due to their lineage-specific epigenetic memory[342]. Basing on all evidence, reprogramming primary hepatocytes backwards to their endodermic lineage emerged as a new challenge to expand bipotent LPs *in vitro*.

Table 1: Variability in available growth factor and endoderm lineage differentiation steps time reported for hPSC-derived hepatocyte generating *in vitro*. ActvA: Activin A; Na: sodium; DEX= Dexamethasone; HGF= Hepatocyte Growth Factor; KGF=Keratinocyte Growth Factor; RA=Retinoic Acid

	Days in culture																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21+	
hESCs	DMSO									HGF							HGF+OSM					[343]
	Actv A					FGF4+BMP4				HGF					OSM+ Dex							[122]
	Actv A+ Na butyrate									DMSO					HGF+ OSM							[344]
	Actv A									HGF+ FGF4					HGF+ FGF4+ OSM+ Dex							[11]
	Actv A+Wnt3a					DMSO				HGF+ OSM												[345]
hiPSCs	Actv A					FGF4+BMP4				HGF+KGF					OSM+ Dex							[346]
	Actv A+Wnt3a					Actv A				DMSO					OSM+ HGF							[347]
	Actv A									FGF2					HGF					OSM		[37]
	Actv A					ActvA +FGF2+BMP4				FGF1/2/4+BMP4										HGF+ EGF+ OSM+ Dex		[348]
	Actv +FGF2					Actv +FGF2+ PI3k inhibitor				FGF10+RA+ TGFβ receptor					FGF4+HGF+EGF							[121]
	Actv A									FGF4+HGF					FGF4+HGF+OSM+Dex							[312]

4.1.4. Hepatocytes plasticity a new era in hepatology : Hepatocytes can give rise to bipotent Liver Stem cells

Largely discussed and described in the previous chapter, bipotent liver stem cells (oval cells) are also reported to be able to differentiate into hepatocytes [349] as well as their potential to repopulate the liver in mice liver failure models[350] [351]. Liver bipotent progenitors (oval cells) were isolated by immunoselection of the epithelial cell adhesion molecule positive (EpCAM+) cells. These cells present high self-renewal ability and a phenotypic stability after expansion for 150 population doublings. Freshly isolated or after their expansion in culture, transplanted

EpCAM+ cells repopulate livers of NOD/SCID and are able to express human specific proteins[352]. Furthermore, another report showed that the bipotent progenitors involved also Sox9+EpCAM-population and gave rise to cholangiocytes. A subpopulation of Sox9+ cells were shown to express interestingly HNF4α and potentially to supply with new hepatocytes in order to repair damaged tissues[353]. However, the reticent number of LPs representing 0.1% of the liver cell mass makes their isolation and expansion difficult and restricts their application to small-scale use [40, 354]. Thus the availability of a large number of bipotent LPs cells was suggested as a key factor to overcome all the limitations associated with ethical and efficiency of hepatocytes generation *in vitro* from hPSCs. As discussed in the previous chapter, the liver is described as special among other regenerative organs in a manner that the type of injury supposed to order the cellular recovery[355]. For many years, the question mark concerned the identity of the atypical bipotent ductal cells accumulated after toxin mediated injury, and if these cells have been derived from expansion of resident liver stem cells within the ductal zone/canals de Hering or upon spontaneous reprogramming of mature biliary duct cells and hepatocytes.

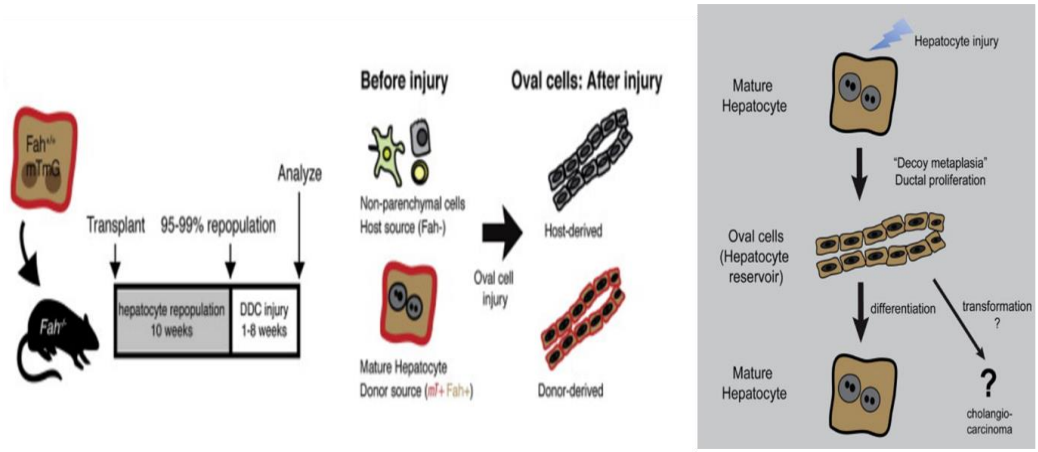


Figure 14: Schematic for hepatocytes dedifferentiation into bipotent progenitors able to give rise to new hepatocytes. Fah+/+ functional hepatocytes are transplanted in chimeric liver mice knockout for FAH [45].

Nowadays, good indications stand up that hepatocytes can "transdifferentiate" into ductal biliary epithelial cells in certain injury models[353, 356, 357] and/or by required modulation of Notch [358]and Hippo pathways[359]. Also, several reports have shown that hepatocytes can be converted into a cholangiocarcinoma originating exclusively from genetic alterations of cholangiocytes[360]. It was suggested by lineage tracing that these biliary bipotent epithelial cells are derived from reprogrammed hepatocytes through a cascade similar to the flow reprogramming described for inducing iPSCs from fibroblasts. In additions, these progenitors gave rise to new cholangiocytes and hepatocytes to recover liver cell loss in animal model[361] (Fig.14). This phenomena was explained clearly with Tarlow et al[45], in 2014, since they conducted an interesting *in vivo* lineage tracing for liver bipotent progenitors. Fascinatedly, they showed that fluorescent protein mTomato- labeled hepatocytes Fah+/+ transplanted in Fah-/- mice repopulated chimeric livers and gave rise to KRT19+EPCAM+OPN+SOX9 biliary duct progenitors.They also showed that hepatocyte-derived progenitors from donor hepatocytes were distinct from the host mice biliary-derived counterparts; Indeed, the conversion of hepatocytes into bile duct progenitors was associated to an increase in KRT19 and Epcam expression compared to the parental hepatocytes but always lower than the host

biliary ducts progenitors . Furthermore, these hepatocytes derived progenitors reserved a memory of the donor hepatocytes of origin and were able to differentiate back into hepatocytes after the complete regeneration process through Notch and Wnt regulation pathways. Hepatocyte and Bile Duct-Derived oval Cells were shown to be functionally distinct *in vitro*. After progenitor's isolation and incubation with Rspo-1 expansion medium for organoid assay, hepatocytes derived progenitors were not able to form organoids compared to the host mice biliary ducts progenitors. However, human hepatocytes derived progenitors, once exposed to Hep-Dif medium containing Dex and OSM, were able to differentiate into Alb^{high} hepatocytes distinctly to the host mice LPs[45].

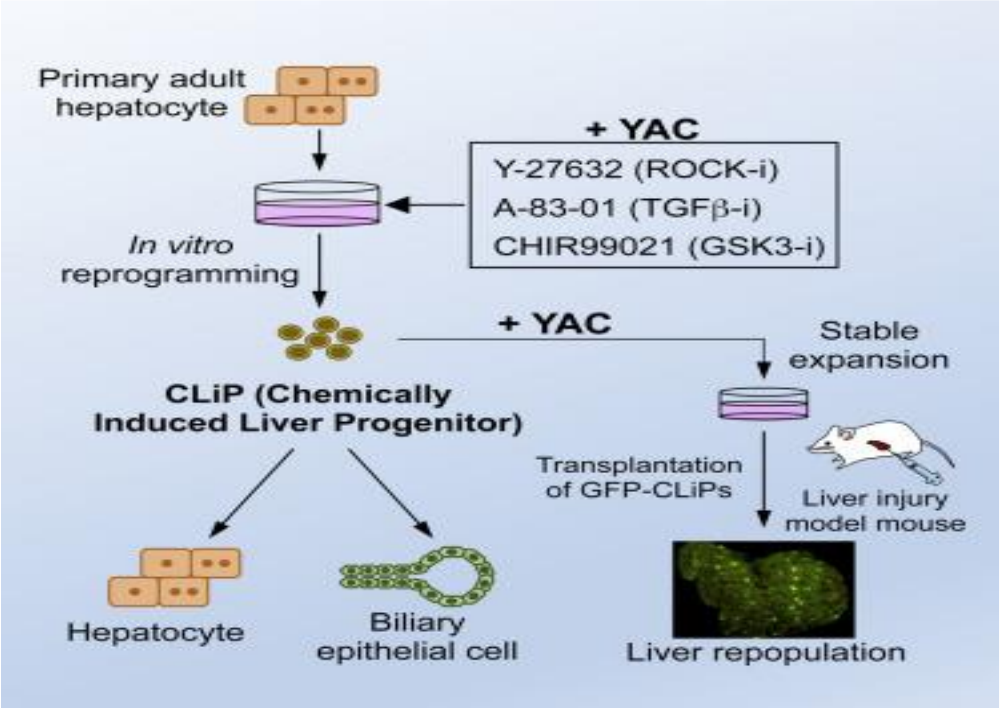


Figure 15: Generation of liver bipotent progenitors mediated by chemical small molecules. YAC cocktail mediating primary adult hepatocytes reprogramming into bipotent progenitors able to repopulate injured livers and to differentiate into hepatocytes and cholangiocytes in vitro [54]

Based on that evidence and on the failure of maintaining the bipotentiality of LPs within stable culture, the development of an *in vitro* experimental model to improve the understanding of adult hepatocytes reprogramming was a crucial additional tool. In this context, Katsuda and his crew recently found that a combination of small molecules, Y-27632 (non -canonical Wnt pathway inhibitor), A83-01 (β-FGF signaling inhibitor), and CHIR99021 (GSK-3 inhibitor/canonical Wnt activator), so-called (YAC) permitted a stable culturing of adult mammary progenitor cells and multipotent mammary tumor cells. They were based on this discovery and showed that YAC- treated rat and mouse primary hepatocytes can be reprogrammed into bipotent LPs which they refer to as chemically induced LPs (CLiPs) (Fig.15). These CLiPs were able to differentiate in presence of OSM, Dex on matrigel into functional hepatocytes expressing Alb and HNF4α. Furthermore, CLiPs on MEF feeder layer then in iPSc feeder-free medium supplemented with YAC molecules give rise to biliary duct cells expressing KRT19[54] .
Interestingly, *in vitro* induced CLiPs regenerate with high efficiency chronically injured liver tissue in urokinase-type plasminogen activator (uPA) transgenic mice crossed with severe combined immunodeficiency disease (SCID)

mice. These findings confirmed the hypothesis that human hepatocytes reprogramming could today serve as a novel source of LPs and overcome the limitations of cell source lack for use in liver regenerative medicine [362].
To spot, hepatocytes derived progenitors were shown to express higher levels of the putative progenitor marker leucine-rich-repeat-containing G protein-coupled receptor 5 (*Lgr5*) [45]. LGR5 is a Wnt target gene highly expressed in dividing stem cells in Wnt-driven, self-renewing tissues such as small intestine and colon[46], stomach[47], hair follicles[48] and recently in liver. This issue will be discussed in the following section.

4.2. LGR5-Wnt axis a new marker of liver stem cells in association with STAT3 pathway

LGR5 is a G-protein-coupled receptor with a 7-transmembrane domain termed Frizzled (FZ/Fzd)[363] crucial for maintaining proliferating progenitors in epithelial tissues such as mammary epithelium, the developing kidney and the ovarian epithelium[49] . The low-density lipoprotein (LDL) receptor-related protein 5/6 (LRP5/6) is a co-receptor and involved in the activation of the canonical Wnt pathway[364]. R- spondins were reported to be the ligands of LGR5[49] . Following bonding to the LGR5, R-SPO strongly promotes the activity of Wnt-Frizzled mediated signaling (Fig. 16).

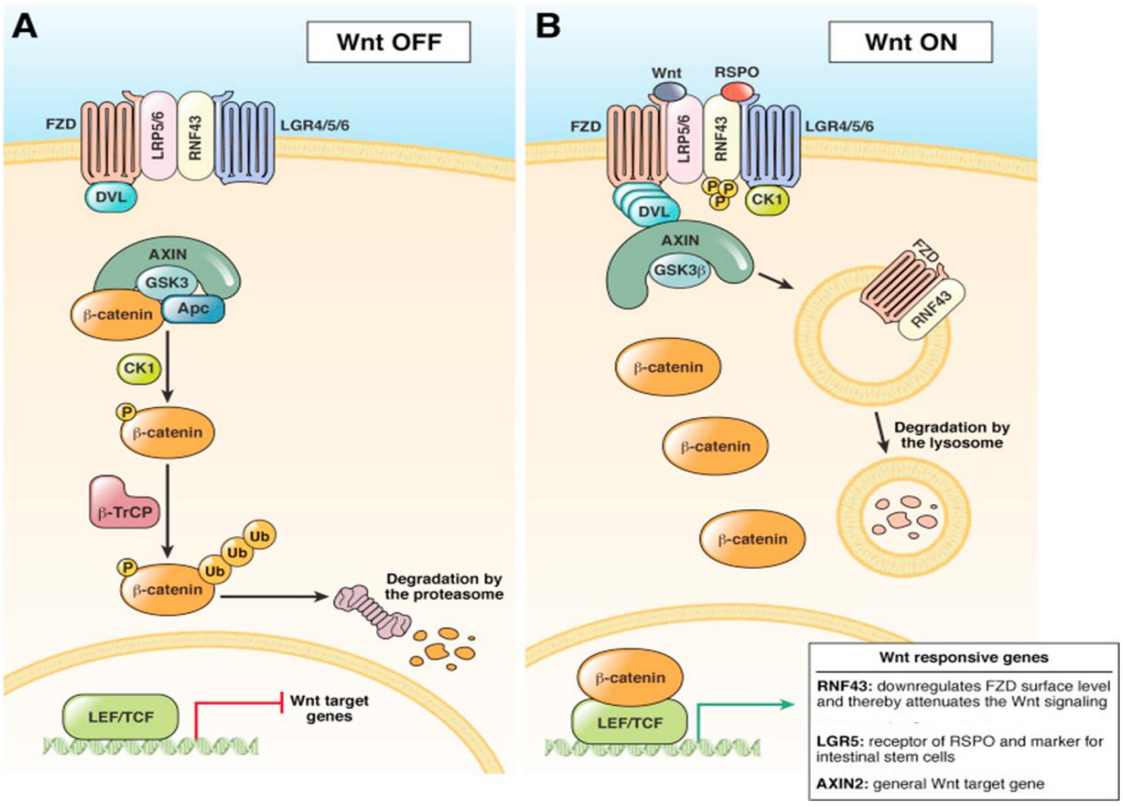


Figure 16: WNT pathway activation by LGR 5. (A) In the absence of Wnt, β-catenin is phosphorylated by the destruction complex formed of APC, AXIN, GSK3, and CK1. RNF43 removes the Frizzled/LRP receptors from the cell surface, resulting in their degradation (B) Wnt, binds to Frizzled and RSPO to LRP5/6, LRP5/6, Disheveled (DVL) is recruited to the plasma membrane to interact with Frizzled then with AXIN preventing the destruction of the triple complex . B-catenin is stabilized, and translocated into the nucleus mediating the transcription of Wnt responsive genes. LGR5-RSPO inhibits the action of RNF43 to degrade frizzled

When the Wnt ligand is present, the destruction complex that includes Adenomatous Polyposis Coli (APC) and AXIN down regulates β-catenin levels. In this case AXIN mediates the phosphorylation of β-catenin via casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK-3) [365]. Conversely, when R-SPO ligand binds to Fzd and the LRP5/6 co-receptor, AXIN cooperates with the plasma membrane and promotes the phosphorylation of LRP5/6 by GSK-3 and CK1. Thereby, AXIN is despoiled, and GSK-3 prevented phosphorylating of β-catenin. The translocation of β-catenin to the nucleus facilitates its interaction with T-cell factor (TCF) and lymphoid enhancer binding protein (LEF) transcription factors to induce the downstream transcriptional factor [366]. Indeed, LGR5-Rspo binding causes two E3 ubiquitin ligases removal from RNF43/ ZNRF3 protease-associated domain and thereby prevents the degradation of Frizzled and leads to a prolonged and a loop of Wnt signal activation[367] . Under a loop mechanism, RNF43 and LGR5 are re-encoded by the target genes of β-catenin / TCF Wnt downregulating transcriptional process. LGR5+ stem cells were reported to express Wnt receptors (Frizzled), FGF receptor 4, Insulin receptor and NOTCH receptor 1, Sox9 and a lot of genes regulating apoptosis, genome integrity, and the cell cycle and transcriptional regulation[367]. Thus Wnt downstream genes were considered associated stem cell markers such as Axin2 and β-catenin. Unfortunately, LGR5 is expressed in a small number of cells in many tissues [46 47] , therefore the isolation and expansion of these progenitors is crucial to study the molecular mechanism regulating their regenerative potential.

To maintain pluripotency in stem cells, LGR5+ cells require absolutely a healthy STAT3 pathway function. Indeed the knockout of STAT3[368] in intestinal and hair[369] LGR5+ stem cells leads to their apoptosis or to an asymmetric division. More interestingly, upon induction of injury *in vitro*, STAT3 phosphorylation is upregulated in LGR5+ stem cells and its activation was essential to generate LGR5+ organoids [50]. For several years, the crosstalk between these two pathways was unclear. Recent findings with Tian et al, 2017[51] reported that under injury, p-STAT3 is crucial to upregulate the canonical LGR5/Wnt signaling and to repress BMP/TGFβ signaling pathways in intestinal LGR5+ stem cells in a manner to standup pluripotency and self-renewal (Fig.17) . This hypothesis supports the use of BMP, TGFβ and non-canonical Wnt pathways inhibitors during the induction of organoids from LGR5+ stem cells in presence in canonical Wnt activators such as Rspo1 and Wnt3a.

Several years ago, the crosstalk between LIFR /gp130/ STAT3 and LGR5/Wnt was not discussed notably in liver stem cells. Interestingly, few months ago, Shih et al[370] studied this crosstalk within isolated bipotent liver stem cells. First, their aims were based on the evidence that IL-6 / STAT3 pathway activation was shown to upregulate the Wnt/ β-catenin pathway in pancreatic cancer cells [371] . Second, they report that pigment epithelium-derived factor (PEDF) was also able to induce the LIFR/gp130/AK/STAT3 phosphorylation cascade in $Lgr5^+$ stem cells [372]. On the other side, they showed in their experiments that PEDF is in parallel a crucial parameter for triggering the LGR5/Wnt axis in liver $Lgr5^+$ stem cells. Moreover, they showed that the inhibition of STAT3 in liver stem cells leaded to the attenuation of the self-renewal and the blocking of the PEDF-Wnt-Lgr5+ activation axis. These new discoveries allowed the liver stem cells to be added on the list of $Lgr5^+$ progenitors regulated by STAT3/Wnt axis as well as the Lgr5+stem cells derived from intestinal , pancreas and hair. Thereby, they expose the implication of some miR-31 regulators in LIFR/gp130/STAT3/Wnt/LGR5 stand axis . Thus these regulators can be considered new targets for the liver stem cell - based researches.

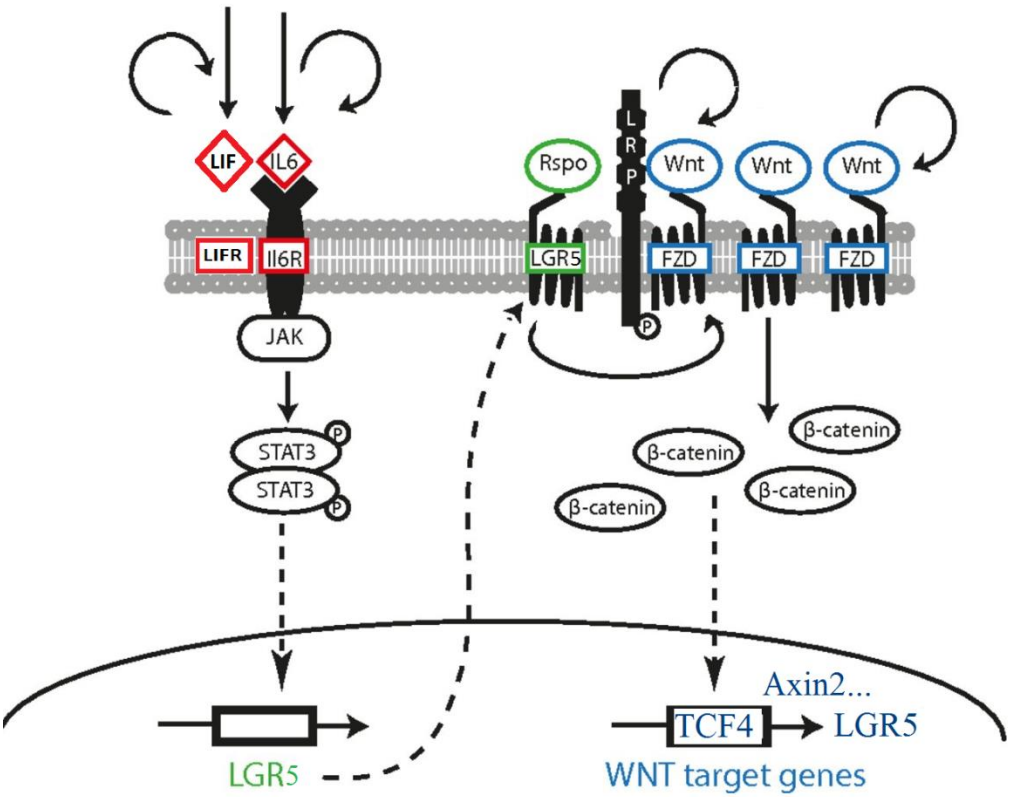


Figure 17: Crosstalk between Stat3/ LIFR and LGR5/Wnt in LGR5+ stem cells. *Modified from* ***Van Andel et al, PNAS, 2017***[373]

4.3. Recent progresses resulting from the Lineage tracing of LGR5 stem cells

Interestingly, intestinal Lgr5^{+} stem cells in presence of a cocktail of growth factors containing epidermal growth factor, the BMP inhibitor NOGGIN, and Rspo-1 (called Rspo cocktail) on 3D-matrigel scaffold grew into organoid respecting the structure of the *in vivo* epithelial architecture[374]. The addition of Wnt3a to the above- mentioned cocktail was essential to give rise to mice colon organoids with long term maintenance in culture[277]. Furthermore, The Rspo cocktail endorsed the induction of organoids from liver and pancreas[52, 375]. Distinctly, these two organs do not contain Lgr5^{+} stem/progenitor cells in normal homeostasis. Upon tissue damage, ductal cells express LGR5 and participate in regeneration. On 3D matrix, LGR5 liver stem cells formed cystic organoids retaining the gene expression of the liver over several passages. Furthermore liver Lgr5^{+} organoids can differentiate into functional hepatocytes. Transplanting these progenitors *in vitro* generated hepatocytes into Fah$^{-/-}$ liver damage models resulted in liver regeneration. However, organoids derived from human colon Lgr5 [374, 376]required modifications *in vitro* and the addition of inhibitors of some pathways involved in the self-renewal and apoptosis such as transforming growth factor β, Notch [377, 378]and p38-MAPK. These findings proposed the tissue-specific organoids as a novel tool for regenerative medicine purposes.

Based on this evidence, Clevers with Huch et al, [52] 2013 conducted a lineage labeling strategy using a novel Lgr5-ires-CreERT2 knock-in allele. Upon damage, Lgr5^{+} cells generate both hepatocytes and cholangiocytes *in vivo*. Isolated single Lgr5^{+} cells from damaged liver expand *in vitro* as clonal organoids in presence of Wnt activator in the culture over several months. Moreover, the self-renewed Lgr5^{+} organoids were able to differentiate into functional hepatocytes when transplanted into FAH−/− mice. Conversely to other dynamically self-renewing tissues

exhibiting a continuous Lgr5 stem cells division, the liver is considered a tissue with low rate of proliferation and cells rise in the ductal track only upon liver damage. Thus Lgr5 is expressed early after damage than it acts as a switch between on and off states depending on the levels of Wnt in the environment in a manner to order the cells whether to proliferate or not. Thereby, perturbation in Lgr5-Wnt driven regenerative response was shown to be responsible of hyper-proliferation in liver cancer and excessive degeneration in cirrhosis[379]. Overall, these findings support the fact that the Lgr5/Wnt pathway is a novel liver stem cell axis, crucial to induce LPs during the establishment of liver engineering and liver cell-based therapy strategies. I will discuss in the next sections the role of Lgr5 in liver stem cells regenerative potential within the latest findings in this filed.

4.4. LGR5 a novel marker for long term culture and expansion of stem cells in 3D field

In adult liver Wnt pathway is active only in perivenous hepatocyte[380]. However in bile duct Wnt is activated upon injury[381]. Distinctly, in liver, Lgr5 progenitors are not expressed in healthy adult liver, however they appear near the biliary duct upon injuries in association of Wnt signaling activation [382]. This evidence was confirmed by mouse lineage tracing knock outing for Lgr5, where LPs Lgr5+ generate hepatocytes and bile ducts *in vivo*. Fascinatedly, single Lgr5+ cells isolated from damaged mouse liver showed stable organoids structures in Rspo1-based culture medium for numerous months. Rspo-1 is the Wnt agonist discovered as a ligand of Lgr5[383]. Furthermore Lgr5 progenitors expressed Wnt target genes and some markers of other Lgr5 Wnt-driven tissues. In a next step, these Lgr5-derived organoids give rise to hepatocytes *in vitro* and repopulate livers of Fah -/- mice [52]. Accordingly, the axis Lgr5-Rspo1 was reported to be crucial to indefinitely expand LPs *in vitro* and to maintain the long-term growth of derived organoids without the need for genetic modifications or introduction of reprogramming factors[384]. Besides mice liver stem cells, long-term expansion of Lgr5+ adults stem cells were also been confirmed in intestine[374] , stomach[47], and pancreas [375]. Interestingly, in these cultures, the derived– organoids remained committed genetically to their tissue of origin. In addition, new advances in human regeneration tissues, reported that culturing patient intestinal stem cells give rise to organoids mimicking the intestinal diseases[385-387]. On the other hand, clonogenic assays showed that EpCAM-positive human liver cells showed bipotent stem cells characteristics in vivo and *in vitro*[117,352].

All these findings lead Huch et al in 2015 [53] to establish a new organoid culture system for human liver stem cells. In fact, they isolate Epcam+ (ductal) cells from human biopsies derived from failure livers. These Epcam+ progenitors were able to originate organoids whereas hepatocytes (Epcam-) couldn't (Fig.18). Furthermore, these organoids were cultured in Rspo-1 medium for 3 months and maintained normal chromosome numbers without genomic aberrations. They co-expressed Lgr5, Sox9 and KRT19. However, to induce Hep-Dif from these $Lgr5^{+}$ organoids, it was crucial to remove the Rspo1 and to add Notch inhibitor. Hepatocytes derived from Epcam+ $Lgr5^{+}$ organoids presented a high capacity to store glycogen, to uptake LDL, to secrete Alb and to metabolize drugs via both phase I and II detoxifying reactions. In addition, the transplantation of these human liver organoids into damaged CCL4-Mouse Liver induced mice liver regeneration and high expression of Alb similar to human primary hepatocytes.

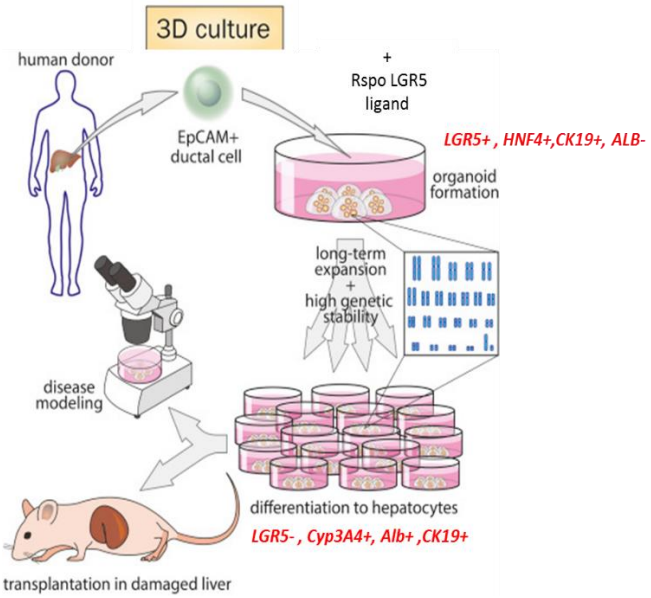


Figure 18: In vitro Human Lgr5+ organoids generation without Hepatocytes reprogramming process. *Modified from the graphical abstract of Huch et al 2015*[53]

4.5. Footprints from this chapter

These discoveries extend our understanding of the implication of liver stem cells in homeostasis and after liver injury induction. Although hepatocytes have been thought only able to generate hepatocytes and duct cells only duct cell, current evidence suggest that liver cells are capable of interconverting between cellular types of distinct phenotypic behaviors. In addition these discoveries showed that Stemness is not limited to stem cells but rather to a cellular state in which hepatocytes exhibit a high degree of plasticity and can move back to bipotent state of liver stem cell, similar to the hepatoblasts arising during the embryonic development able to differentiate into both hepatocytes and cholangiocytes.

Here also we investigate two type of dedifferentiation of hepatocytes towards bipotent Lgr5+ stem cells either by reprogramming to a primitive state or by direct plasticity in 3D microenvironment after isolation, which is considered as a stressful situation to hepatocytes similar to the destruction of biological ECM *in vivo* upon injury.

These findings elucidate also different cellular markers that are used to isolate bipotent liver stem cells such as EpCAM [352], Lgr5 [52], Foxl1[388], OPN[42], Sox9 [389]or to enrich for cells in culture and transplantation. Inopportunely, the aforesaid markers are expressed also in biliary duct progenitors which complicate somehow their isolation. Thereby, standardizing the isolation strategy and getting the same progenitors population is shown to be difficult and impossible. Interestingly, Lgr5 only appears after liver injury *in vivo* and also after the reprogramming or the study plasticity of hepatocytes *in vitro* but never during homeostasis.

Basing on all these observations, even in absence of tracing lineage in human, Lgr5 can be considered to be a novel marker for plasticity *in vivo* and *in vitro*. Lgr5+ derived organoids can constitute an alternative source instead the above mentioned PHH and PSCs limitations and thus contribute to a new human tool to study the human liver regeneration, liver disease mechanism, cell therapy transplantation and toxicology studies. Furthermore, the regulation of the Wnt/Lgr5 axis in bipotent stem cell-derived hepatocytes instructs their long-term proliferation or their differentiation fate.

SCIENTIFIC PAPER 1

Chapter 5 -: Introduction to the scientific paper 1

Nowadays, there is no system culture maintaining hepatocytes proliferation and functions *in vitro*. Recently, a novel approach has derived liver bipotent stem cells from human liver hepatocytes. These cells were shown to express Lgr5 which is the Wnt activator controlling many important biological processes. In adulthood, Wnt signaling is essential for the maintenance of somatic stem cells and committed progenitor cell compartments. Wnt/β-catenin is involved in liver tissue regeneration processes following liver damages. Thus, Clonal long-term expansion of $Lgr5^+$ liver progenitors allows new possibilities for disease modeling, toxicology studies and stem cell based regenerative medicine.

TITLE:

"Direct reprogramming of $LGR5^+$ liver progenitors cells responding to both gp130/JAK/STAT3 and Wnt/β-catenin the signaling pathways"

OBJECTIVES OF THE STUDY

- Reprograming murine liver hepatocytes into $Lgr5^+$ endodermic lineage progenitors (EndoPCs) via non integrative viral vectors in absence of Wnt small molecules activators.
- Providing a distinctive molecular and cellular characterization of the $Lgr5^+$ EndoPCs.
- Assessing the hepatogenic potential of the $Lgr5^+$ EndoPCs in 2D and 3D biocompatible scaffolds.
- Evaluating the impact of Wnt /STAT3 pathway activation on the EndoPCs proliferation rate and Lgr5-organoid's Wnt downstream effectors expression[5]

STRATEGY and RESULTS

Endodermal Progenitor Cells (EndoPCs) were produced by infecting mouse hepatocytes with recombinant adenovirus containing human Oct3/4, Sox2, Klf4, and c-Myc and STAT3 activators (LIF and IL6).

In order to analyze the influence of liver stem cell compartment in EndoPCs samples, we performed SAM supervised analysis between EndoPCs and primary hepatocytes by using $Lgr5^+$ cell gene expression profile previously identified. We have also identified a list of genes that are up-regulated in EndoPCs as compared to primary hepatocytes. Crossing the information of Lgr5 related genes up-regulated in the EndoPCs allowed to see that the majority of genes are common with $Lgr5^+$ liver stem cells. Functional enrichment on Wikipathway database was performed showing that several pathways regulating stem cells proliferation were significantly enriched in EndoPCs including the WNT pathway, the genes belonging to the PluriNetWork and the TNF-β and NF-Kβ signaling pathway.

EndoPCs are responded to the gp130/JAK2/STAT3 signaling pathway:

To determine whether LIF/JAK2/STAT3 pathway maintains self-renewal of EndoPCs, we selected clones expressing all endoderm progenitors' markers and cultured them in low serum-containing LIF with and without JAK kinase inhibitor. Our results showed that after IL6 exposure, phosphorylation of STAT3 increased within 30 min and then gradually decreased after 1 to 4h showing that gp130/JAK2/STAT3 pathway contributes actively to EndoPCs self-renewal. EndoPCs are shown to be LIF dependent *in vitro* and to not express mESCs and MSCs markers such as Oct-4, SSEA1, CD90, CD105, CD34, CD45 and CD117. The clonal EndoPCs are very stable at the chromosome level.

EndoPCs clones readily expressed definitive endodermic markers such as *Sox17 CXCR4*, *GATA4* and the *FOXA* subfamily.

EndoPCs are responded to Lgr5/Rspondin and WNT/β-catenin pathways:

EndoPCs were further characterized for the expression of Lgr5 and their capacity to respond to Lgr5/Rspondin and WNT/β-catenin pathways using small molecules activating Wnt and inhibiting Notch and BMP pathways.

To optimize the best conditions to activate Wnt/Lgr5 axis in EndoPCs, we found that the addition of 5 small molecules including Wnt3a, Rspo1, Noggin, EGF, and HGF was associated with a high expression of Lgr5, compared to other culture conditions. The addition of 5 factors was associated with the up-regulation of *Lgr5*, *Axin2* and *Tcf4* and has permitted to form organoid-like structures in low attachment plate after 7 days of culture where the absence of these molecules lead to cell growth arrest and failed to generate organoid-like structures expression LGR5 and Wnt downstream effectors .

***In vivo* intra muscular and intra splenic injection of EndoPCs give rise to hepatocytes and bile duct structure:**

To prove that EndoPCs are restricted to endoderm differentiation, EndoPCs were directly injected intramuscularly into NOD/SCID mice and tissues were analyzed. The tissue derived-EndoPCs produced the emergence of tissues showing two types of tissues, liver parenchyma-like structures and substantial areas bile duct-like expressing CK19+ (KRT19) and CK7 (KRT7)+ structures after histological analysis. On the other side, in order to document EndoPCs potential to colonize liver, EndoPCs-Luc expressing the luciferase gene were intrasplenically injected into adult C57BL/6 mice which had undergone a 30% partial hepatectomy.

EndoPCs are bipotent cells that are able to differentiate into both hepatocyte and cholangiocytes in vitro:

Finally, to evaluate the differentiation of EndoPCs into both hepatocytes and cholangiocytes *in vitro,* we have optimized two different differentiation media (DM) permitting to commit EndoPCs into hepatocytes (hDM) or cholangiocytes lineages (cDM) respectively. For hepatocytes differentiation, 2D and 3D culture systems were applied. We developed a 10-days protocol aiming to commit EndoPCs into hepatocytes like cells. Either 2D or 3D – derived hepatocytes derived-EndoPCs expressed high levels of *ALB* without any detection of *AFP* transcripts in comparison to other culture conditions. Cholangiocytes derived hepatocytes expressed CK7. Also, derived hepatocytes were able to uptake easily the LDL. Although the 3D scaffold culture system gives rise to higher levels of hepatocytes markers, their amounts were lower compared to isolated mouse hepatocytes producing. Also *Ttr, Tat*, Cytochrome P450 and *Cyp3A11* mRNA were expressed after differentiation and were able to metabolize midazolam to its main metabolite, 1-hydroxymidazolam by glucuronidation.

Direct reprogramming of LGR5^{+} liver progenitors cells responding to both gp130/JAK/STAT3 and Wnt/ β-catenin the signaling pathways

Diana Chaker [1] , Christophe Desterke [1,2] , Tony Ernault [1] , Nicolas Moniaux[3], Noufissa Oudrhiri[1], Guillaume Pourcher [4], Sylvia Sanquer [5], Jamila Faivre [2,3], Ali G Turhan [1,2,6] , Annelise Bennaceur-Griscelli [1,2,6] , Frank Griscelli [1,2,7]

[1] Institut National de la Santé et de la Recherche Médicale (INSERM) U935-Human Embryonic Stem Cell Core Facility, ESTeam Paris Sud-INGESTEM, Villejuif, France
[2] Université Paris-Sud, Faculté de Médecine Kremlin Bicêtre, France
[3] Institut National de la Santé et de la Recherche Médicale (INSERM) U785, Villejuif, France
[2] Université Paris Descartes, Sorbonne Paris Cité, Faculté des Sciences Pharmaceutiques et Biologiques, Paris, France,
[4] Service de Chirurgie Digestive Minimale Invasive, Hôpital Antoine-Béclère- Assistance Publique-Hôpitaux de Paris (AP-HP), Clamart, France
[5] Service de Biochimie Métabolomique et Protéomique, Hôpital Universitaire Necker-Enfants Malades-Assistance Publique-Hôpitaux de Paris (AP-HP), Paris, France
[6] Service d'Hématologie Biologique, Hôpital Universitaire Paris Sud, (AP-HP) Kremlin Bicêtre, France
[7] Institut Gustave Roussy, Département de Pathologie et de Morphologie Médicales, Villejuif, France

Running title: generation of new endo-iTS multipotent stem cells
Keywords: endodermal progenitor, multipotency, liver, pancreas
To whom correspondence should be addressed: Professor Frank Griscelli
INSERM 935, University Paris 11
7 rue Guy Moquet 94802 Villejuif, France
Tel : 33 1 42 11 51 93

SUMMARY
Leucine-rich repeat (LRR)-containing G-protein-coupled receptor 5 (LGR5) is a recently discovered marker of mouse liver stem cell population that appears near bile ducts exclusively upon liver damage. Damage-induced LGR5$^+$ could be clonally expanded as organoids in a three-dimensional long term culture system with LGR5 and Wnt/ β-catenin ligands, able to produce functional hepatocytes *in vitro*. Here we succeeded in converting adult mouse hepatocytes into expandable and stable LGR5$^+$ endodermal progenitor cells (EndoPCs) *in vitro* after exposure of 4 transcriptional factors (Oct-4, Sox2, Klf4 and c-Myc) and STAT3 activators. EndoPCs are dependent on three interrelated signaling pathways including gp130/JAK/STAT3, LGR5/Rspondin and WNT/ β-catenin controlling their proliferation and self-renewing capacities. EndoPC have clonogenic potential and can differentiate into liver restricted lineages *in vitro* in two- and three-dimensional long term culture systems. EndoPCs are bipotent cells with the ability to generate hepatocytes-like cells and bill duct structures *in vitro* and to repopulate injured liver tissue *in vivo*. After intra-muscular injection EndoPCs have readily generated two kinds of tissues including Albumin and Cytochrome (Cyp3A11) positive areas similar to parenchyma-like structures and substantial bile duct-like structures positive for KRT19 and KRT7. Clonal long-term expansion of LGR5$^+$ adult liver stem cells opens up experimental avenues for disease modeling, toxicology studies and regenerative medicine.

INTRODUCTION
The liver has an important latent regenerative capacity. After extreme stress or chronic injury a population of atypical ductal bipotent progenitor cells (BPCs) emerges from the bile ducts (1-2) and is able to differentiate into both hepatocytes and biliary cells as evidenced by lineage tracing after liver injury (3-4). However, the identification of liver BPCs and the signaling pathways that maintain the progenitor fate within the liver are still confusing. Surprisingly in contrast to the conventional BPCs theory, several groups have demonstrated that hepatocytes can be efficiently reprogrammed into proliferative BPCs in response to chronic liver injury (5-8). Recently the use of three small chemical molecules (Y-27632, A-83-01, CHIR99021) was shown to convert rat and mouse mature hepatocytes into proliferative liver progenitors cells that differentiate into both matures hepatocytes and biliary epithelial cells and are able to repopulate chronically injured liver tissue in urokinase- type plasminogen activator (uPA) transgenic mice (9). However these reports have not clearly identified the signaling pathways that maintain these chemically induced liver progenitors in culture *in vitro,* which were shown to give rise preferentially to hepatocytes and more rarely bile duct structures *in vivo*. In addition these biliary epithelial cells were shown to express only KRT19, which is also a hallmark of non-differentiated ductal bipotent progenitor cells and liver cancer cells (10)
The leucine-rich repeat (LRR)-containing G-protein-coupled receptor 5 (LGR5) were recently shown to mark murine liver adult stem cells (4) , which were isolated *in vitro* through the development of organoids using a three-dimensional long term culture system with high doses of Wnt/ β-catenin and LGR5 ligands. Human LGR5$^+$ liver stem cells expressing both ductal (KRT19, SOX9, OC2) and hepatocyte markers (HNF4a) were also recently isolated and expanded into 3-D organoids that were efficiently converted into functional hepatocytes *in vitro* and upon transplantation *in vivo* (11).
Here we have shown for the first time that after transient exposure *in vitro* of several embryonic transcriptional factors, mature hepatocytes can be directly converted into LGR5$^+$ proliferative liver adult stem cells, called endodermal Progenitors Cells (EndoPCs).. We have evidenced that EndoPCs are dependent on STAT3 activators regulating gp130/JAK/STAT3, LGR5/Rspondin and Wnt/ β-catenin signaling pathways enabling their proliferation and their self-renewing capacities in 2-D long-term culture conditions. We have shown that EndoPCs can be readily converted into both functional hepatocytes and biliary cells *in vitro* and upon transplantation *in vivo*.

MATERIALS AND METHODS

Establishment of EndoPCs from hepatocytes

Approximately one million hepatocytes, generated from adult mice, were infected with 20 MOI of each adenovirus encoding Oct4, Sox2, cMyc and Klf4. After 5 days of infection, cells were manually pooled and plated on mitomycin C-arrested MEFs in ES cell culture medium changed daily; DMEM medium was supplemented with 1000 U/ml of LIF. As control, 10^6 hepatocytes were transduced with an adenovirus expressing the GFP gene and 10^6 non-infected cells were plated on MEFs in the presence of LIF. Murine somatic cells were supplemented with 15% SVF, 1% Non-Essential Amino Acids, 1x Penicillin-Streptomycin, 100 µM β-mercaptoethanol. After 10 to 20 days, colonies were selected and cultured in MEF-free condition on collagen I-treated plates in ES cell culture medium. After 10 passages the cells were qualified as EndoPCs and further characterized.

EndoPCs FACS analysis and cell immunostaining

EndoPCs were observed by Transmission electron microscopy, as previously described (12). Flow cytometry was performed for the detection of SSAEA1, MSC and oval cell markers using direct conjugate antibody: anti-SSEA1 (catalogue no. 560142; BD Pharmingen), anti-CD117 (catalogue no. 553356; BD Pharmingen), anti-Sca1 (catalogue no. 553335; BD Pharmingen), anti-CD29 (catalogue no. MCA 2298PET, AbD serotec), anti-CD105 (catalogue no. 120411, Biolegend), anti-CD90 (catalogue no. 105308, Biolegend), anti-CD51 (catalogue no. 551187; BD Pharmingen), anti-CD166 (catalogue no. 560903; BD Pharmingen), anti-CD73 (catalogue no. 127205, Biolegend), anti-CD200 (catalogue no. 123807, Biolegend). Standard immunostaining was performed as reported previously (13). Primary antibodies were anti-HNF4 (catalogue no. K9218, Abcam), anti-Foxa2 (catalogue no. AB4125, Millipore), anti-Sox17 (catalogue no. AF1924, R&D Systems).

Public transcriptome dataset

Transcriptome matrix dataset GSE32210 (Whole Mouse Genome Microarray 4x44K G4122F), normalized by background subtraction and LOWESS algorithm (4) was downloaded at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32210, web link and annotated with platform file GPL4134 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL4134). Supervised analysis in order to find differential expressed genes between sorted LGR5+ cells and unsorted Hepatocytes was realized with Significance Analysis for Microarray (SAM) algorithm with a threshold of False Discovery Rate (FDR) less than 0.05 (14).

Microarray

The transcriptome of EndoPCs was analyzed with Affymetrix microarray technology (GeneChip ® Mouse Gene 2.0 ST Array) in parallel with the transcriptome of control hepatocytes (triplicate in each group). The microarray raw data were normalized by the RMA method (15). Supervised analysis with SAM algorithm was performed between each ITS and Hepatocyte control. The graphical representation of the gene expression profile and the unsupervised classification was performed with gplots R-package by using heatmap2 function. Functional enrichment was performed with standalone application Go-Elite version 1.5 on Wikipathway database (16). Interactions between enriched functions and genes obtained with GO elite were drawn as network in Cytoscape version 3.0.1 (17). Microarray data were publicly submitted on Gene Expression Omnibus (GEO) as dataset GSE51782.

Proliferation Assay (MTT Assay) and Cell viability

Cells were placed in 24-wells tissue culture plates (10^4 cells per well) and treated with mouse LIF (1000 units/ml, ESG1107, Millipore) and pan JAK inhibitor (5µM, CAS 457081-03-7, Millipore) throughout the assay. Respectively at 72, 120, 144 and 168 hrs end-points incubation, 20µl of the stock Thiazolyl Blue Tetrazolium Bromide (MTT, 5mg/ml, M2128, Sigma- Aldrich) dye solution was added to each well. After 4 hrs, 100 µL of DMSO (D2650, Sigma- Aldrich) was added to solubilize the MTT. The Modulus TM II Microplate reader (Promega) were used to read the absorbance. Each experiment was repeated at least five times to obtain the mean values. The viability of the cells was evaluated by 7AAD staining assay by flow cytometry. The cell events were captured on MACSQuant analyser (Miltenyi Biotech) and the percentage of the 7AAD negative fraction was calculated using the MACSQuantify software. EndoPCs were plated in 6-well plates. LIF was replaced by 100 ng/ml of murine IL6 (Peprotech, France). Proteins and mRNA were isolated at 0.5, 1 and 4h respectively. The gp/JAK/STAT3 pathway was inhibited with 5 µM of pan JAK inhibitor (CAS 457081-03-7, Millipore) in the presence or the absence of IL6.

LGR5 activation and cell culture medium

A total of $5x10^4$ cells were plated in 6-well plates. When cells have reached 80 % of confluence, DMEM/F12 containing 1% BSA (Sigma), and murine cytokines (all from Peprotech) including Wnt3a (30 ng/ml), RSPO1 (500 ng/ml), EGF (20 ng/ml), HGF (50 ng/ml) and Noggin (50 ng/ml) that were added separately or together to each well for 5 days. For protein extraction, attached cells were incubated in trypsin /EDTA (Sigma) at 37°C for 5 min. The released pellet was washed in PBS containing 0.5% BSA. The pellet was suspended in RIPA buffer containing anti-protease and anti-phosphatase, and vigorously shaken at 4^0C for 30 minutes and centrifuged at 12000xg for 20 minutes. For Real time PCR, the released pellet was suspended in TRIZOL (Thermo fisher scientific) and processed for RNA isolation. Organoid like-structures were performed on low attachment plate for 7 days of culture in the presence or the absence of 5 cytokines including Wnt3a, Rspo1, Noggin, EGF and HGF.

Western Blot

Protein samples were exposed to reduce SDS-PAGE and transferred to nitrocellulose membranes (LC2009 thermoFisher scientific). Membranes were blocked in 5% BSA in TBS-T (0.25% Tween-20 in Tris-buffered saline (TBS) for 1 hr and probed overnight at 4 C with primary antibodies (anti-STAT3, anti-STAT3-p; Cell signaling and anti-LGR5 Abgent) in 3% BSA/TBS-T following the manufacturer's dilution instructions. After washing, membranes were incubated for 1 hr with HRP-conjugated secondary antibody diluted 1:5000 in 3% BSA /TBS-T. Immunoreactive bands were pictured by automated image acquisition for chemiluminescence signals (G: BOX Chemi XT4; SYNGENE). Each blot was repeated in duplicate. Comparative calculation of protein expression between the blotted bands was based on the normalization to the β actin band for each sample.

LDL uptake assay

Cells were changed to serum-free media in 96 tissue culture microplate. Differentiated and control cells were incubated with 70 µg of fluorescent labeled-LDL (ab204716) for 4 hr at 37 °C in the dark. After incubation, the cells were washed following the supplier instructions. Finally, the cells were examined with fluorescence microplate reader at Ex/Em = 540/575 nm

PAS staining

Glycogen storage was detectable using Sigma-Aldrich™ Periodic Acid Schiff Kit staining (SD395B). Fixed cells were incubated with periodic acid solution for 5 min at room temperature. After rinsing, cells were immersed in Schiff's reagent for 15 min at room temperature. Cells were counterstained in hematoxyline solution.

Intramuscular injection of EndoPCs

EndoPCs were harvested by trypsinization and injected intramuscularly into the flanks of NOD/SCID mice, using ~ 5 million cells per injection. As a control, 100 million primary hepatocytes were similarly injected. Mice were sacrificed 3 months later and tissue were isolated and processed for histological analysis. The tissue were dissected, fixed in 4% paraformaldehyde, embedded in paraffin and stained with hematoxylin and eosin in association with immunohistochemistry. KRT19 and KRT7 were detected by immunohistochemistry using anti-KRT19 rabbit antibody (ab52625) and anti-KRT7 rabbit antibody (ab181598) as recommended by the manufacturer. In addition, immunoreactive albumin was detected using prediluted anti-Albumin Mouse FITC antibody and counterstaining using 1% Evans blue. Glycogen and acid muco-substances were revealed after Periodic Acid Schiff (PAS) and Alcian blue staining respectively.

Hepatectomy and transplantation in mice

C57BL6 mice were obtained commercially (Janvier-Europe, France) and used as experimental model and hepatocyte donors. Animal studies were approved by the French Ministry of Agriculture. Mice (age range: 4-8 weeks, weight: 20-30 g) were anesthetized by an intraperitoneal injection of ketamine/xylasine. The skin in the upper abdomen and flanks was sterilely prepared and a median incision of 2.5 cm was made. A partial hepatectomy of the left and median lobes was performed without any post-operative complications or morbidity. After exposure of the spleen, $3x10^6$ EndoPCs-

luc cells expressing luciferase were suspended in 100 µl of physiological serum and were injected using a 29 G needle into the spleen of C57BL6 mice. Firefly bioluminescence imaging was used to detect graft cells over a period of 7 days. In a second experiment, $3x10^6$ EndoPCs-luc were directly injected in the parenchyma liver of three mice and the Firefly bioluminescence imaging was used to detect graft cells over a period of 9 days to 35 days (Xenogen Ivis 50).

Hepatic differentiation of EndoPCs into hepatocytes and cholangiocytes-like cells

To drive endoPCs to differentiate directly into hepatic cells without embryonic body formation, $4x10^5$ endoPCs were seeded onto collagen-coated 6-well plates and maintained in mES culture media containing LIF. When the cells reached approximately 80% of confluence, the media was replaced with DMEM medium supplemented with 15% SVF (ThermoScientific), 1% Non-Essential Amino Acids, 1x Penicillin-Streptomycin, 100 µM β-mercaptoethanol with 1% B27, 25 ng/ml of HGF and 1 µM of Dexamethazone during 5 days following by 5 days of culture using 1% B27, 1µM of Dexamethazone, 20 ng/ml of VEGF and 20 ng/ml of Oncostatin M (OSM). EndoPCs were differentiated into cholangiocytes-like cells using a 5 steps protocol: step 1; 3 days with 25 ng/ml of HGF and 50 ng/ml of EGF. Step 2: 5 days with 10 ng/ml of IL6. Step 3; 2 days with 10 µM of Na+taurocholate. Step 4; 2 days with 1.8 µM Na+ butyrate. Step 5; 2 days with 20 ng/ml of VEGF. After completion of the protocol, the supernatants were immediately frozen and RNA was extracted for further characterization. Albumin was quantified in supernatant by a commercial ELISA kit (Abnova). The methodology we used to quantify Cyp3A11 activity was derived from that described previously (18).

Three-Dimensional (3D) hepatocytes differentiation system

In order to differentiate the endoPCs into hepatocyte like lineages in 3D culture system, we used 200 µm thick polystyrene scaffold Alvetex® (Reinnervate, Durham, UK) in 6 well (AVP005-3, 15 mm diameter, Reinnervate) in-well inserts. Inserts were plunged in 70 % ethanol for 10 min then washed twice with sterile water and incubated with MaxGel™ ECM (E0282 SIGMA) for 24h at RT. The excess of coating was removed with PBS washes. EndoPCs cells were plated at a density of 5×10^6 cells and seeded by applying the cell suspension to the top of the membrane. Cells underwent proliferation for 48 hr in LIF medium. At day 3 cells underwent hepatic differentiation protocol following two steps: (1) induction step using the LIF –free medium supplemented with 0.1% BSA, 100 ng/ml of IL6 , 20 ng/ml of HGF, FGF-4 and 10 -8 M of Dexamethasone (Dex) for 7 days and (2) maturation step using LIF-free medium supplemented with 100 ng/ml of IL6 50 ng/ml of OSM, 20ng/ml of HGF, and 1µM of Dex for another 7 days. Media was exchanged every 2 days. All murine growth factors and supplements were purchased from (PEPROTECH). Cells were isolated from Scaffold for LDL uptake assay and RNA extraction following the manufacturer instructions.

Immunofluorescence on 3D Alvetex 3D scaffolds

To detect Alb expression and cell distributions in Alvetex, cells were fixed using 4 % paraformaldehyde (PFA; 30 min at room temperature, Sigma) blocked in 1 % normal goat serum and permeabilized in 0.2% TRITON-X (Sigma), for 1 h at room temperature.Afterwards , cells were incubated with a mouse polyclonal Alb primary antibody (1/200; ab106582, Abcam) overnight at 4 °C. Cells were further incubated with a secondary Rabbit antibody conjugated to Alexafluor 488 (1:200; Invitrogen; Life Technologies), for 90 min at room temperature. After cells were washed, incubated with DAPI (4 µg/ml) for 5 min and mounted on microscope slides using Vectashield (Vector Laboratories, UK). Image were captured on inverted spinning disc confocal Leica TCS SP8 40× Plan- objectives were used and images were collected using a color camera and processed using LAS X software.

Statistical Analysis

Each experiment was performed at least twice. Statistical significance was evaluated using the Student's t test (unilateral and unpaired).

RESULTS

Establishment of Endodermal Progenitor Cells

Endodermal Progenitor Cells (EndoPCs) were produced by infecting 10^6 mouse hepatocytes with 20 MOI of recombinant adenovirus containing human Oct3/4, Sox2, Klf4, and c-Myc transgenes (Fig. S1*A*), a dose enabling the infection of more than 90% of hepatocytes with an adenovirus expressing GFP (Ad-GFP) (Fig. S1*B*) with a satisfactory expression of the 4 factors by Western Blot analysis (Fig. S1*C*). AdGFP-transduced hepatocytes and non-infected hepatocytes were used as control. Five days after the infections, cells were manually pooled and plated on mitomycin C-arrested MEFs in ES cell culture medium supplemented with Leukemia Inhibitory Factor (LIF). After 10 to 20 days, several clones appeared (Fig. S1*D*) and were transferred in MEF-free condition culture on collagen I-treated plates. Clones appeared only when cells were transduced with the four viral vectors whereas no colonies emerged in both control conditions. All clones possessed the typical morphological phenotypes of epithelial cells (Fig. S1*E*), a decrease in cell size after 10 passages, with a high nuclear-to-cytoplasmic ratio as shown after May-Grünwald-Giemsa (MGG) staining (Fig. S1*F*) and a normal karyotype (Fig. S2). Transmission electron microscopy (TEM) analysis of the EndoPCs showed the presence of fusiform cells with abundant euchromatin and heterochromatin was observed in the nucleus (Fig. S1*G*) as well as abundant lipid droplets in the cytoplasm (Fig. S1*H*).

Endodermal Progenitor Cells possess the characteristics of endodermic progenitors and have a LGR5 signature

Three clones were selected for further characterization. All have shown to be constantly negative for murine Embryonic Stem Cells (ESCs) markers such as stage-specific embryonic antigen (SSEA)-1 at more than 10 passages (Fig. S3) and were found constantly negative for CD90, CD105, CD236, CD133, and CD117 by flow cytometry and were found $CD29^{high}$, $CD73^{high}$, $CD51^{high}$, $CD166^{high}$ and $Sca\text{-}1^{low}$ (Fig S3, Table S1).

We then selected 15 different clones to verify whether they expressed definitive endodermic markers. All EndoPCs clones were quite similar and more than 70% of EndoPCs clones readily expressed *Sox17 CXCR4*, *GATA4* and the *Foxa* subfamily by RT-PCR analysis (Fig. S4*A*) and sox17 and Foxa2 by immuno fluorescence staining, which are all important transcriptional factors, known to regulate the transcription of endodermal and hepatic genes (Fig. S4*B*).

To further characterize the EndoPCs, we performed an overall gene expression profile using affymetrix microarray technology on EndoPCs in triplicate and on primary murine hepatocytes. In order to analyze a LGR5 liver stem cell progenitor signature, we have first analyzed $LGR5^+$ sorted expression profile as compared to primary hepatocytes in a published dataset GSE32210 (4). Supervised analysis performed between the 2 classes of samples with Significance Analysis for Microarray (SAM) algorithm allowed to identify 337 gene probes differentially expressed with an up regulation in $LGR5^+$ sorted cells as compared to hepatocytes (Table S2). These probes identified 268 genes with a fold change superior to 2 and a false discovery rate less than 0.05. Expression of these up-regulated genes in $LGR5^+$ sorted cells allowed to discriminate transcriptome sample groups by unsupervised classification (heatmap with Euclidean distances, Fig. 1*A*). Expression of these genes represented as cord plot, highlighted their important positive regulation in these LGR5 positive cells (Fig. 1*B*).

In order to analyze the influence of liver stem cell compartment in our EndoPCs samples, we performed SAM supervised analysis between EndoPCs and primary hepatocytes by using $LGR5^+$ cell gene expression profile previously identified (Fig. 1*A* and Table S2). We identified 88 genes that are up-regulated as compared to primary hepatocytes and their expression allowed to well discriminate sample groups by unsupervised classification (heatmap with Euclidean distances, Fig. 1*C*, Fig. 1*D*, and Table S3).

Functional enrichment on Wikipathway database was performed with the set of the 88 LGR5 related genes, showing that several pathways regulating stem cells proliferation were significantly enriched in EndoPCs including the WNT pathway, the genes belonging to the PluriNetWork and the TNFa /NF-KB signaling pathway (Fig. 1*E*).

EndoPCs were further characterized for the expression of LGR5. All tested EndoPCs clones were found positive for stem cell marker LGR5 by flow cytometry (Fig. 1*G*, Table S1), Immuno fluorescence staining (Fig. 1*H*) and by RT-PCR (Fig. 1*I*).

EndoPCs expansion and self-renewal depends on gp130/JAK/STAT3, LGR5/Rspondin and WNT/ β-catenin signaling pathways

All EndoPCs clones were continuously cultured with STAT3 activators (mLIF or mIL6) able to activate the gp130/JAK/STAT3 pathway. LIF at the concentration of 10^3 U/ml or IL6 at a concentration of 100 ng/ml have enabled EndoPCs to expand for over 50 passages. To verify the contribution of gp130/JAK/STAT3 pathway for their proliferation and their self-renewal capacities EndoPCs were cultured in low serum-containing medium with and without LIF. LIF withdrawal was followed by a significant ($p<0.00001$) inhibition of their proliferation capacity (Fig. 2*A*) without apparent mortality (Fig. S5). The addition of a pan JAK inhibitor into the culture medium in the presence of LIF was followed by a significant inhibition of EndoPCs proliferation ($p<0.00001$) similarly to the culture condition without LIF (Fig 2*A*) confirming that the gp130/JAK/STAT3 pathway is involved in EndoPCs proliferation and self-renewal. We further examined the gp130/JAK/STAT3 pathway in EndoPCs by evaluating the STAT3 Tyr705 phosphorylation after the addition of 100 ng/ml of IL-6 at different time intervals. After IL6 exposure, phosphorylation at Tyr705 of STAT3 rapidly increased within 30 min and then gradually decreased after 1 to 4 hr showing that STAT3 transcriptional factor play a vital role in EndoPCs proliferation and self-renewal (Fig. 2*B*).

We also showed that EndoPCs responds to both LGR5/Rspondin and WNT/ β-catenin signaling pathways. We found that unlike other culture conditions, the addition of 4 or 5 molecules in serum-free medium including Wnt3a, Noggin, EGF, HGF or Wnt3a, Rspo1, Noggin, EGF, HGF enabled to maintain EndoPCs in culture cells that highly expressed LGR5 by western blot analysis (Fig. 2*C*) allowing the generation of organoid-like structures in low attachment plate after 7 days of culture (Fig. 2*E*). The cells without the 5 molecules stopped to grow efficiently and failed to generate organoid-like structures (Fig. 2*E*). Addition of 5 factors (Wnt3a, Rspo1, Noggin, EGF, HGF) was associated with the up regulation of *LGR5*, *Axin2* and *Tcf4* mRNA (Fig. 2*D*). In addition, we found that LIF/STAT3 signaling can efficiently activate WNT/ β-catenin signaling pathway by controlling the expression of *Lgr5*, *Axin2*, *Tcf4* and β- *catenin* (Fig. 2*F*). Indeed, the addition of IL6 acting on the gp130/JAK/STAT3 pathway, into the culture medium was followed by a drastic increase of *LGR5*, *Axin2*, *Tcf4* transcripts. This effect was abolished by the addition of a pan JAK inhibitor in the culture medium (Fig. 2*F*).

Differentiation of EndoPCs into both hepatocytes and bile duct structure *in vivo*

To prove that EndoPCs are restricted to endoderm differentiation, $5x10^6$ EndoPCs and 10^8 hepatocytes used to produce the EndoPCs were directly injected intramuscularly into NOD/SCID mice. Whereas the injection of large numbers of mouse hepatocytes did not generate any tissue, EndoPCs produced after 49 to 101 days two types of tissues, represented by areas of liver parenchyma-like structures and substantial areas bile duct-like structures after histological analysis (Fig. 3*A*). An investigation of serial sections revealed that EndoPCs-derived cells expressed a series of hepatocytes and bile duct markers, including albumin (ALB) and cytokeratin makers KRT19 and KRT7 and exhibited glycogen storage and accumulated acid muco-substances (Fig. 3*A*). Bile duct like-structures derived from EndoPCs appeared very similar to bile ducts of the liver with the same levels of KRT7 and KRT19 markers (Fig. 3*A*). We confirmed by RT-PCR the commitment of EndoPCs into both ductal (*KRT7/19*) and hepatic structures (*KRT8/18*, *Alb*, *G6P*, *Cyp2E1*, *Cyp3A11*, *Cyp2D22 and Cyp2C55*) (Fig. 3*B*). The pharmacokinetics of midazolam and of its main metabolite, 1-hydroxymidazolam by glucuronidation, were investigated by mass spectrometry on tissues extracts from two mice in comparison to murine D3-derived teratoma obtained from murin Embryonic Stem Cells. Our results showed a 5-fold increase of 1-hydroxymidazolam in extract from EndoPCs-derived tissue compared to the control confirming functional Cyp3A11 presence within both organs (Fig. 3*C*).

In order to document EndoPCs' potential to colonize liver, PBS or 3 million EndoPCs-Luc expressing the luciferase gene were intrasplenically injected into 5 adult C57BL/6 mice, which had undergone a 30% partial hepatectomy. Firefly bioluminescence imaging (Xenogen Ivis 50) was used to detect graft cells over a period of 7 days after injecting EndoPCs. Luciferase activity was detected exclusively in the liver and in the spleen of all animals (Fig. 4*A*). Up to 8 and 5 different sites of the luciferase gene could be detected after assessing the dissected liver (Fig. 4*B*) and the spleen (Fig. 4*C*) respectively. These cells have the capacity to efficiently migrate from the spleen to the liver as documented in Fig. 4*B* showing two nodules present in the bloodstream.

We identified in all mice several areas in the liver parenchyma and in the spleen corresponding to the engraft cells. Morphologically, the nodules showed the two types of structures confirming the previous results with the identification

of larges areas of bile duct-like structures in both organs (Fig. 4*B* and Fig. 4*C*), expressing cytokeratin makers KRT19 and KRT7 and liver parenchyma-like structures expressing albumin that were easily observed in the spleen of all animals (Fig. 4*C*).
In addition, after intra-hepatic injection of the EndoPCs-luc the luciferase activity could be detected up to 21 days, followed by a rapid decrease of the signal with a probable rejection of these cells (Fig. S6).

Differentiation of EndoPCs into both hepatocytes and cholangiocytes *in vitro*
We have optimized two different differentiation medium (DM) permitting to commit EndoPCs into hepatocytes (hDM) or cholangiocytes lineages (cDM) respectively. We hypothesize that all EndoPCs clones tested are bipotent liver progenitors since ductal (*KRT19*, *Hnf6*, *Hnf1*□,) and hepatocytes (*KRT18, Hnf4*□□ *Hnf1*□, *AFP*, *ALB*) transcripts were readily expressed showing that all EndoPCs clones have some of the specific functional features of both hepatocytes and bile duct cells (Fig. S7*A*). Quantitative RT-PCR performed on 6 different EndoPCs clones confirmed their bipotency (Fig. S7*B*). In addition EndoPCs highly expressed KRT19 and KRT18/KRT8 by flow cytometry analysis (Table S1).
All EndoPCs clones were positive for Hnf4□□□a□definitive endoderm marker that is an important regulator of liver development and of their proper function compared to cell line hepa 1-6 mouse hepatoma cells (Fig. S7*C*). We developed a 10 days protocol aiming to commit EndoPCs into hepatocytes like cells (HLC). HLC expressed higher levels of *Alb* mRNA without any detection of *AFP* transcripts in comparison to other culture conditions (Fig. S7*D*). Quantification of albumin by RT-PCR (Fig. 5*A*), ELISA in supernatant (Fig. 5*B*) and immunofluorescence (Fig. 5*C*) showed a significant increase of *Alb* after differentiation.
Differentiated cells possessed the typical morphological phenotypes of epithelial cells and several cells were found to be bi-nucleated (Fig. 5*D*). Immunohistochemical analysis indicated that the cells accumulated glycogen and lipids that were highly expressed compared to the control (+LIF) (Fig. 5*E*) and showed greater LDL uptake activity (Fig. 5*F*). *Ttr*, *Tat*, Cytochrome P450 oxidoreductase (*Por*) and *Cyp3A11* mRNA were expressed after differentiation (Fig. 5*G*) showing that they are able to metabolize midazolam to its main metabolite, 1-hydroxymidazolam by glucuronidation which was doubled when a cytochrome P450 inducer such as 20µM of pregnenolone-16a-carbonitrile (PCN) was added (Fig. 5*H*).
EndoPCs were seeded directly onto coated Alvetex and maintained for three days in their conditioned LIF medium to proliferate. Next, they were driven to hepatic lineage following two-steps differentiation protocol comprising altogether OSM and dexamethasone so-called hDM medium. Non-differentiated EndoPCs were used as negative control in absence of the differentiated medium. At day 15, cells were fixed or lysed for appropriate experiments. First 3D-HLCs were characterized using immunofluorescence assay for the detection of Alb (Fig. 5*I*). Differentiated cells showed greater Alb expression and LDL uptake activity (Fig. 5*J*) compared to cells cultivated in both LIF and basal culture media (-LIF) on 3D-scaffolds. Interestingly, when cultured in 3D-Alvetex scaffold, the morphology changed for both endoPCs and their HLC counterparts adopting a more protoplasmic phenotype, larger in size throughout the scaffold (data not shown). Cells showed also much spreading compared to 2D culture (Fig. 5*C*) due to the increased inner capacity of the Alvetex scaffolds. Next we analyzed mRNA levels to compare the hepatogenic potential on 3D-Alvetex scaffold comparing to 2D cultures from HLC derived–EndoPCs. HLC in 3D cultures exhibited marked levels of Alb (Fig. 5*K*) compared to 2D cultures system (Fig. 5*A*). On the other side LIFR transcript showed a dropped mRNA level in the HLCs in favor of a hepatogenic commitment of the LIF-dependent EndoPCs. Finally KRT19 was dramatically downregulated after differentiation within the 3D-HLC ruling out the risk of cholangiocytes or biliary duct progenitors growing from these bipotent EndoPCs when cultivated on 3D-Alvetex scaffolds (Fig. 5*K*).
We then used a cDM to differentiate EndoPCs into duct-like phenotype using a 2-D and a 3-D culture system and collagen I as a matrix and a 14 days protocol. Histologically differentiated cells displayed a duct-like phenotype presenting a single-layered non-polarized epithelium and bile duct polarized outgrowths expressing KRT7 in the 2-D and 3-D culture systems respectively (Fig. 5*L*) confirming the potential of Endo-PCs to efficiently differentiate to bile duct like- structures that have occurred spontaneously after several weeks after intra-muscular injections (Fig. 3*A*).

DISCUSSION

In the present study, we have succeeded in converting adult mouse hepatocytes into expandable and stable endodermal progenitors (EndoPCs) after having transduced the hepatocytes with low doses of non-integrative vectors harboring Oct-4, Sox2, klf4 and c-Myc transgenes in the presence of STAT3 activator. As adenoviruses allow only a transient expression of the 4 factors, EndoPCs were not able to reach a pluripotent state. Thus they have never been found positive for SSEA1, nor for Oct-4 (data not shown) and are thus unlikely to represent a novel source of induced Pluripotent Stem cells (iPSCs). Furthermore, they are also unlikely to characterize a novel source of oval cells since all clones were always found to be $CD34^{neg}$, $CD117^{neg}$, $Sca1^{low}$. We rather believe for several reasons that EndoPCs derived-hepatocytes result from an epithelial–mesenchymal transition (EMT). Although they do not express usual Mesenchymal Stem Cells (MSCs) markers such as CD90 and CD105, they highly express other MSCs-associated markers such as CD29, CD51, CD73 and CD166. Like MSCs they have clonogenic and *in vivo* migration capacities. They have a high proliferation capacity and can be maintained in 2-dimensional long term culture system for more than 50 passages by being passed twice a week at a 1:5 ratio. Various pathways are known to activate and maintain the EMT program including the Wnt/ β-catenin (19) and TNFa pathways (20). Our gene set analysis ascertained that both the Wnt/ β-catenin and the TNFa signaling were associated with hepatocytes-to-EndoPCs conversion, which strengthens the hypothesis that the direct conversion of mature hepatocytes to EndoPCs is consistent with an EMT-like process. We thus concluded that hepatocyte-derived EndoPCs have acquired mesenchymal features and could result from an epithelial–mesenchymal transition (EMT).

In our experimental protocol LIF or IL6 sharing the same gp/JAK/STAT3 signaling pathways were introduced to the culture medium. LIF and IL-6 are closely related cytokines that bind to the common LIF-receptor–gp130 heterodimer (gp130 is also known as IL6ST), leading to the phosphorylation of STAT3 by JAK (21). LIF receptors are found in a number of different organs including the liver (22). LIF was identified as the differentiation inhibiting activity for mouse Embryonic Stem Cells (ESCs) that stimulated mESCs self-renewal (23). As for ESCs we hypothesize that EndoPCs are dependent on LIF to stimulate their self-renewal capacity. Indeed, EndoPCs are able to proliferate exclusively under LIF, leading to the phosphorylation of STAT3 by JAK.

Gene expression profile analysis by microarray-based technology have shown that EndoPCS shared a set of 88 transcripts with $LGR5^+$ liver stem cells progenitors, which were previously isolated in mice under CCL_4 treatment (4). Indeed, $LGR5^+$ liver stem cells were recently isolated *in vivo* near bile ducts, exclusively under conditions of injury and were shown to give rise to hepatic organoids *in vitro* (4), which were shown to display extensive self-renewal capacity and to express hepatocyte-like properties after *in vitro* differentiation. R-spondin (RSPO1) was shown to be the ligand of LGR5 (4, 24). The association of RSPO1 on $LGR5^+$ was shown to amplify Wnt/ β-catenin pathway by promoting the activity of Wnt-Frizzled mediated signaling receptors (24) and by promoting the Wnt receptor turnover, from the plasma membrane by clearing the cell-surface transmembrane E3 ubiquitin ligases, zinc and ring finger 3 (ZNRF3) and its homologue ring finger 43 (RNF43) (25,26). In a previous report, murine $LGR5^+$ liver progenitor cells were exclusively generated in a 3-D culture system using a matrigel containing a cocktail of factors including EGF, RSPO1, FGF10, HGF and nicotinamide (4).

In our experimental setting, $LGR5^+$ liver progenitor cells can be generated without using Wnt and LGR5 ligands as previously described (4). We showed that $LGR5^+$ EndoPCs were able to be maintained in culture and to form organoide-like structures in 3-D culture system after 7 days after LIF withdrawal and the addition of 5 molecules including Wnt3a, Rspo1, Noggin, EGF, HGF showing the important roles of both signaling pathways to promote cell-cycle progression and cell proliferation of EndoPCs. In addition we found that there is a cross talk between both gp130/JAK/STAT3 and WNT/ β-catenin signaling pathways. Indeed, we have demonstrated that the activation of the gp130/JAK/STAT3 pathway by IL6 can efficiently trigger an increase of *LGR5*, *Axin2*, *Tcf4* and β- *catenin* transcripts which were not found when a pan JAK inhibitor was added in the culture medium. These results suggest a possible cross talk between both gp130/JAK/STAT3 and WNT/ β-catenin signaling pathways. Indeed, LIFR/STAT3 phosphorylation was shown to be activated in intestinal (27), hair (28) and liver (29) LGR5+ stem cells and to be crucial for tissue-specific organoids formation (30). Furthermore, recently LIFR/p-STAT3 axis was shown to upregulate the Wnt/β-catenin in liver LGR5+ stem cells via an upregulation of specific regulator mi-RNA (29). These observations support the above-mentioned STAT3/WNT linked- axis within the EndoPCs. Nevertheless, further

mechanistic studies in well-defined conditions is needed to understand more precisely the molecular mechanisms underlying this cross talk

In overall our data suggested that as for mESCs (31,32), both gp130/JAK/STAT3 and WNT/β-catenin pathway signaling promote EndoPCs self-renewal thanks to the presence of high doses of STAT3 activator. Our results also show that hepatocytes possess a phenotypic plasticity *in vitro* and change their phenotype by inducing an EMT program. Our data confirm that mature hepatocytes possess significant phenotypic plasticity and reinforce previous studies (33-36).

EndoPCs comply with most of the phenotypic criteria of $LGR5^+$ bipotent liver progenitor cells that may differentiate into both hepatocytes and bile duct cells with a capacity to form duct structures *in vitro* and *in vivo* and to repopulate injured liver tissue. Currently, the differentiation of iPSCs and ESCs into hepatocytes and cholangiocytes requires stepwise protocols comprising the sequential application of growth factors and the introduction of transcription factors to mimic hepatocyte (37) and cholangiocytes (38) differentiation during liver development. EndoPCs technology represents an attractive alternative and an easy way to produce both liver cell lines. Only limited steps of differentiation were sufficient to produce *in vitro* hepatocytes producing albumin and adult liver-specific cytochrome P450 (Cyp3A11) and KRT7-expressing cholangiocytes. In addition, the engraftment of EndoPCs *in vivo* in a liver environment has shown an exclusive commitment of EndoPCs into KRTK19 and KRT7 positive structures resembling to biliary duct and into liver parenchyma-like structures positive for albumin.

As such, the EndoPCs may represent a promising source for the production of endodermic lineages and opens a vast field of applications in many areas. Furthermore, EndoPCs represent an alternative for the study of physiological liver stem cells and a new source to produce at high scale of hepatocytes and cholangiocytes for drug screening and toxicity assays.

ACKNOWLEDGMENTS

We sincerely thank, Olivier Feraud, Dominique Divers, Christelle Remus, Emilie Gobbo and Olivia Bawa for technical assistance. We thank Karim Benhioud for the kind gift of PCA350 plasmid, Ibrahim Casal for animal care, Paule Opolon for anatomopathological analysis, Anne-Marie Courtot for transmission electron microscopy. This work was performed with grants from ANR Programme d' Investissements d'Avenir of INGESTEM National Infrastructure (ANR-11-INBS-0009-INGESTEM) and Inserm, University Paris Sud.

REFERENCES

1 Oertel M, and Shafritz D (2008) Stem cells, cell transplantation and liver repopulation. Biochim. Biophys. Acta 1782:61–74.

2 Turner R, Lozoya O, Wang Y, Cardinale V, Gaudio E, Alpini G, Mendel G, Wauthier E, Barbier, C, Alvaro D, Reid LM (2011) Human hepatic stem cell and maturational liver lineage biology. Hepatology 53:1035–1045.

3 Espanol-Suner R, Carpentier R, Van Hul N, Legry V, Achouri Y, Cordi S, Jacquemin P, Lemaigre F, Leclercq I (2012) Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 143:1564–1575.

4 Huch M, Dorrell C, Boj SF, van Es JH, Li VS, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ, Haft A, Vries RG, Grompe M, Clevers H (2013) In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. Nature 494:247-50.

5 Tanimizu N, Nishikawa Y, Ichinohe N, Akiyama H, Mitaka T (2014) Sry HMG box protein 9-positive (Sox9+) epithelial cell adhesion molecule-negative (EpCAM-) biphenotypic cells derived from hepatocytes are involved in mouse liver regeneration. J Biol Chem 289:7589–7598.

6 Tarlow BD, Pelz C, Naugler WE, Wakefield L, Wilson EM, Finegold MJ, Grompe M (2014). Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. Cell Stem Cell 15:605–618.

7 Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, Thung SN, Wells RG, Greenbaum LE, Stanger BZ (2013) Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes Dev 27:719–724.

8 Yimlamai D, Christodoulou C, Galli GG, Yanger K, Pepe-Mooney B,Gurung B, Shrestha K, Cahan P, Stanger BZ, Camargo FD (2014) Hippo pathway activity influences liver cell fate. Cell 157:1324–1338.

9 Katsuda T, Kawamata M, Hagiwara K, Takahashi RU, Yamamoto Y, Camargo FD, Ochiya T (2017) Conversion of Terminally Committed Hepatocytes to Culturable Bipotent Progenitor Cells with Regenerative Capacity. Cell Stem Cell 1:41-55.

10 Jain R, Fischer S, Serra S, Chetty R (2010) The use of Cytokeratin 19 (CK19) immunohistochemistry in lesions of the pancreas, gastrointestinal tract, and liver. Appl Immunohistochem Mol Morphol 1:9-15.

11 Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Wenum M, Fuchs SA, de Ligt J, van de Wetering M, Sasaki N, Boers SJ, Kemperman H, de Jonge J, Ijzermans JN, Nieuwenhuis EE, Hoekstra R, Strom S, Vries RR, van der Laan LJ, Cuppen E, Clevers H (2015) Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell 160:299-312.
12 Serres C, Peoc'h K, Courtot AM, LesaffreC, Jouannet P, Laplanche JL (2006) Spatio-Developmental Distribution of the Prion-Like Protein Doppel in Mammalian Testis: A Comparative Analysis Focusing on Its Presence in the Acrosome of Spermatids1. Biology of reproduction 74 :816–823.
13 Zhu S, Li W, Zhou H, Wei W, Ambasudhan R, Lin T, Kim J, Zhang K, Ding S (2010) Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7:651-655.
14 Tusher VG, Tibshirani R, Chu G (2001) Significance Analysis of Microarrays Applied to the Ionizing Radiation Response. Proc Natl Acad Sci USA 98:10515.
15 Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31, e15.
16 Zambon AC, Gaj S, Ho I, Hanspers K, Vranizan K, Evelo CT, Conklin BR, Pico AR, Salomonis N (2012) GO-Elite: a flexible solution for pathway and ontology over-representation. Bioinforma Oxf Engl 28:2209–2210.
17 Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campilo I, Creech M, Gross B, et al. (2007) Integration of biological networks and gene expression data using Cytoscape. Nat Protoc 2:2366–2382.

18 Lahoz A, Donato M, Picazo L, Gomez-Lechon MJ, Castell JV (2007) Determination of major human cytochrome P450 activities in 96-well plates using liquid chromatography tandem mass spectrometry. Toxicology in vitro 21:1247-1252.
19 Kalluri R, and Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin. Invest 119:1420–1428.
20 Bates RC, Mercurio AM (2003) Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids. Mol Biol Cell 14:1790-800.
21 Boulanger MJ, Bankovich AJ, Kortemme T, Baker D, Garcia KC (2003) Convergent mechanisms for recognition of divergent cytokines by the shared signaling receptor gp130. Molecular cell 12:577–89.
22 Hilton DJ, Nicola NA, Metcalf D (1991) Distribution and comparison of receptors for leukemia inhibitory factor on murine hemopoietic and hepatic cells. Journal of cellular physiology 146:207–15.
23 Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, et al. (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336:684–7.
24 Carmon KS, Gong X, Lin Q, Thomas A, Liu, Q (2011) R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/-catenin signaling. Proc Natl Acad Sci USA 108:11452–11457.
25 HaoH X et al. (2012) ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. Nature 485:195–200.
26 Kinzel B et al (2014) Functional roles of Lgr4 and Lgr5 in embryonic gut, kidney and skin development in mice. Dev Biol 390:181–190.
27 Tian, Y. et al. Stress responsive miR-31 is a major modulator of mouse intestinal stem cells during regeneration and tumorigenesis. eLife 6 (2017).
28 Chen Q, et al (2017) Inactivation of STAT3 Signaling Impairs Hair Cell Differentiation in the Developing Mouse Cochlea. Stem Cell Reports 9:231-246.
29 Shih SC, Ho TC, Chen SL, TsaoYP (2017) Pigment epithelium-derived factor (PEDF) peptide promotes the expansion of hepatic stem/progenitor cells via ERK and STAT3-dependent signaling. American journal of translational research 9:1114.
30 Lindemans CA, et al. (2015) Interleukin-22 promotes intestinal stem cell-mediated epithelial regeneration. Nature 528:560-564.
31 Ye S, Zhang D, Cheng F, Wilson D, Mackay J, He K, Ban Q, Lv F, Huang S, Liu D, Ying QL (2016) Wnt/β-catenin and LIF–Stat3 signaling pathways converge on Sp5 to promote mouse embryonic stem cell self-renewal. Cell Sci 129:269–276.
32 Tang L, Wang M, Liu D, Gong M, Ying QL, Ye S (2017) .Sp5 induces the expression of Nanog to maintain mouse embryonic stem cell self-renewal. PLoS One 29:e0185714.
33 Tarlow BD, Pelz C, Naugler WE, Wakefield L, Wilson EM, Finegold MJ, Grompe M (2014) Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. Cell Stem Cell 15:605-18.
34 Michalopoulos GK, Barua L, Bowen WC (2005) Transdifferentiation of rat hepatocytes into biliary cells after bile duct ligation and toxic biliary injury. Hepatology 41:535–544.
35 Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, Thung SN, Wells RG, Greenbaum LE, Stanger BZ (2013) Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes Dev 27:719–724.
36 Yimlamai D, Christodoulou C, Galli GG, Yanger K, Pepe-Mooney B,Gurung B, Shrestha K, Cahan P, Stanger BZ, Camargo FD (2014) Hippo pathway activity influences liver cell fate. Cell 157:1324–1338.
37 Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297-305.

38 Dianat N, Dubois-Pot-Schneider H, Steichen C, Desterke C, Leclerc P, Raveux A, Combettes L, Weber A, Corlu A, Dubart-Kupperschmitt A (2014) Generation of functional cholangiocyte-like cells from human pluripotent stem cells and HepaRG cells. Hepatology 60:700-14.

FIGURE LEGENDS

Figure 1: LGR5+ dependent expression profile of the EndoPCs cells by microarray: (A) unsupervised classification on expression profile of LGR5+ sorted cells as compared to hepatocytes GSE32210, (B) cordplot of gene expression with genes up regulated in LGR5+ sorted cells as compared to hepatocytes, (C) Venn diagram of overlapping genes up regulated LGR5+ sorted cells but also in EndoPCs cells, (D) Expression heatmap of genes up regulated in EndoPCs cells and also found up regulated in LGR5+ sorted cells, (E) Barplot of Z-scores obtained during functional enrichment on wikipathway database with LGR5 dependent genes found over expressed in EndoPCs cells, (F) Functional enrichment network of LGR5 dependent genes up regulated in EndoPCs and enriched on wikipathway database, (G) Expression LGR5 by flow cytometry analysis for Hela cells and EndoPCs, (H) Immunocytochemistry showing the expression for LGR5 in EndoPCs. DNA was stained with DAPI, (I) Quantitative RT PCR for the detection of LGR5 mRNA in 5 individual EndoPCs clones, in Hepa 1-6 and in murine fibroblastes.

Figure 2: EndoPCs are depended to both gp130/JAK/STAT3 and Wnt/βcatenin the signaling pathways. (A) Proliferation Assay (MTT Assay) performed on EndoPCs up to 168 hr of culture in culture medium containing or not LIF in comparison to the condition with LIF and 5µM of pan JAK inhibitor, (B) Western blot analysis for STAT3, pSTAT3 (Y705) and β actine on EndoPCs expanded with or without 100 ng/ml of IL6 performed after 0.5, 1 and 4 hr, all bands were quantified relative to the intensity to the β-actine, (C) Western blot analysis for LGR5 and □□actine on EndoPCs expanded with or without 5 different cytokines. All bands were quantified relative to the intensity to the β-actine, (D) Results of quantitative RT-PCR used to detect LGR5, Axin2 and Tcf4 in EndoPCs expanded with or without 5 different cytokines, (F) Results of quantitative RT-PCR used to detect Stat3, Lgr5, β-catenin, Axin2 and Tcf4 in EndoPCs expanded 8 hr with or without 100 ng/ml of IL6 in comparison to EndoPCs expanded with IL6 and 5µM of pan JAK inhibitor.

Figure 3: Intra muscular injection of EndoPCs generates liver like structure: (A) Histological analysis of the tissues 101 days after an intra-muscular injection of $3x10^6$ cells showing parenchyma-like structures after Hematoxylin and Eosin (HES) staining; Albumin (Alb) is revealed by immuno-fluorescence and immuno-histochemistry. Branching-like structures are shown immunoreactive for KRT7 and KRT19 and containing acid mucosubstances and polysaccharides/glycogen after Alcian Blue (AB) and Periodic acid–Schiff (PAS) staining respectively. Normal liver was used as control showing biliary ducts positive for KRT19 and KRT7, (B) RT-PCR analyses of hepatic markers in two different liver buds (LB1 and LB2) in comparison to EndoPCs and murine hepatocytes (Hep), (C) The pharmacokinetics of midazolam and of its main metabolite, 1-hydroxymidazolam by mass spectrometry on extracts from EndoPCs-derived liver buds (LB1 and LB2) compared to ESCs (D3)-derived teratoma.

Figure 4: Incorporation of EndoPCs into mouse hepatic parenchyma
(A) Bioluminescence imaging for the detection of the EndoPCs expressing the luciferase gene 7 days after injection of $3x10^6$ cells in the spleen in 5 adult C57BL/6 that had undergone a partial hepatectomy. PBS was injected into five other mice that were used as controls. Firefly bioluminescence imaging (Xenogen Ivis 50) was used to detect graft cells. (B) Localization of the luciferase gene in the dissected liver (LUC). Two nodules present in the bloodstream were identified after Hematoxylin and Eosin (HES) showing that EndoPCs have the capacity to efficiently migrate from the spleen to the liver. The engrafted tissue shows similarity with liver sinusoid structures after HES staining, immunoreactive for KRT19 and KRT7 resembling to biliary ducts structures. (C) Localization of the luciferase gene in the dissected spleen showing liver sinusoid structures after Hematoxylin and Eosin staining (HES) immunoreactive for KRT19 and KRT7 resembling to biliary ducts structures and immuno-reactive for Alb.

Figure 5: Differentiation of EndoPCs into hepatocytes and cholangiocytes-like cells
(A) Bar graphs showing albumin mRNAs levels determined by real-time qRT-PCR for EndoPCs before (-hDM) and after differentiation using a hepatocyte differentiation medium (+hDM), (B) Albumin secretion by murine hepatocytes and by EndoPCs-derived hepatocytes after 8 days of culture in medium by ELISA before (-hDM) and after differentiation (+hDM). (C) Immunofluorescence analysis of EndoPCs for albumin before (-hDM) and after differentiation (+hDM), (D) Morphology of an EndoPCs after differentiation (+hDM) showing binuclear cells (arrow) after May-Grünwald-Giemsa (MGG) staining and store glycogen and lipids revealed by Periodic acid–Schiff (PAS) and Oil Red O staining. (E) Quantification of lipids content in EndoPCs before (-hDM) and after differentiation (+hDM), (F) Quantification of LDL uptake in EndoPCs before (-hDM) and after differentiation (+hDM), (G) Results of RT-PCR used to detect mature hepatocyte-specific transcripts (Cyp3A11, POR, TAT, TTR) in EndoPCs cultured with LIF and before (-hDM) and after differentiation (+hDM), (H) The pharmacokinetics of midazolam and of its main metabolite, 1-hydroxymidazolam by mass spectrometry on extracts from non-differentiated EndoPCs (+ LIF and without hDM) and from differentiated EndoPCs. (I) Representative images of EndoPCs that were seeded onto coated Alvetex 3-D culture system at 15 days, without (-hDM) and after differentiation (+hDM), (J) Quantification of LDL uptake of EndoPCs before (-hDM) and after differentiation (+hDM), (K) Results of RT-PCR used to detect LIFR, Albumin and KRT19 in Alvetex 3D-derived hepatocytes after 15 days of differentiation in comparison to Alvetex 3D seeded with non-differentiated EndoPCs (-hDM), (L) Immunofluorescence analysis for cholangiocytes marker KRT7 on EndoPCs at 14 days of differentiation using a cholangiocyte differentiation medium (cDM). DNA was stained with DAPI.

Supplemental Figure 1: Induction of EndoPCs from adult hepatocytes
(A) Time schedule of EndoPCs cell generation and diagram of the experimental procedures. (B) GFP expression by flow cytometry after transduction of mouse hepatocyte cells with 20 MOI of adenovirus expressing GFP (AdGFP). (C) Expression of Oct-4, Sox2, Klf4 and c-Myc by Western Blot analysis 5 days after infection of mouse hepatocytes with recombinant adenoviruses. Adenovirus expressing GFP was used as a control (AdGFP). (D) Morphology of EndoPCs at passage number 2 after amplification, (E) Morphology of EndoPCs clones on a collagen-coated 6-well plate and at passage number 5, (F) EndoPCs after after May-Grünwald-Giemsa (MGG) staining with a high nucleus/cytoplasm ratio, (G) Transmission electron microscopy analysis (TEM) analysis of EndoPCs showing cells with high area of eurochromatin, (H) Transmission electron microscopy analysis (TEM) analysis of EndoPCs showing the cytoplasm with abundant lipid droplets (LD).

Supplemental Figure 2: Karyogram of EndoPCs showing 40 normal chromosomes. Twenty metaphases were analyzed for each sub-clones and were captured using METAFER SOFTWAR (Metasystem version 3.8.6). The Karyotype was obtained after G-bandes staining and the chromosome classification was performed using IKAROS Software.

Supplemental Figure 3: Flow cytometry analysis on EndoPCs. Quantification of EndoPCs positive for SSEA1, CD90, CD105, CD236, CD133, CD51, CD29, CD73, CD117, CD166 and Sca1. 10^5 cells were stained with PE conjugated anti-mouse antibodies analyzed with a MACS Quant flow cytometer (Miltenyi Biotech) using the MACS Quantify software.

Supplemental Figure 4: (A) RT-PCR analysis for the detection of definitive endodermic markers in the 16 individual EndoPCs clones, (B) Immunocytochemistry showing the expression for Sox17 and Foxa2 in hepa 1-6 control cells and in EndoPCs. DNA was stained with DAPI.

Supplemental Figure 5: Flow cytometry analysis for the quantification of 7AAD on EndoPCs expanded up to 168 h with and without LIF.

Supplemental Figure 6: Quantification (A) and detection (B) of luciferase activity over a period of 35 days after the injection of $3x10^6$ EndoPCs in the liver of C57BL/6 mice.

Supplemental Figure 7: RT-PCR analysis for the detection of markers involved in the liver development in the 8 different EndoPCs clones, (B) Real-Time Reverse Transcription-PCR for the detection of KRT8/18, KRT7/19, Afp and Alb in hepatocytes (hep), and in 8 different EndoPCs clones. Target gene expression was normalized relative to the S18 ribosomal endogenous mRNA, (C) Immunocytochemistry showing the expression of HFN4□-positive cells in hepa1-6 control cells and in EndoPCs. DNA was stained with DAPI, (D) Results of RT-PCR used to detect Albumin and Alpha-fetoprotein (Afp) transcripts in adult hepatocyte, fetal liver cells and in EndoPCs before (+LIF) and after differentiation in the absence of LIF and the addition hepatocyte differentiate medium (hDM).

Supplemental Table 1: Flow cytometry analysis on 3 different EndoPCs clones showing the percentages of positive cells for each markers analyzed

Supplemental Table 2: Genes overexpressed in liver LGR5+ sorted cells as compared to primary hepatocytes: Supervised analysis performed by SAM algorithm in GSE32210 dataset identified over expression of 337 microarray probes in LGR5+ sorted cells (columns describe identification of these genes through different databases and their fold change determination).

Supplemental Table 3: LGR5 related genes overexpressed in EndoPCs as compared to primary hepatocyte: Supervised analysis performed by SAM algorithm on EndoPCs transcriptome identified 88 LGR5 related genes overexpressed in EndoPCs as compared to primary hepatocytes (columns describe identification of these genes through different databases and their fold change determination)

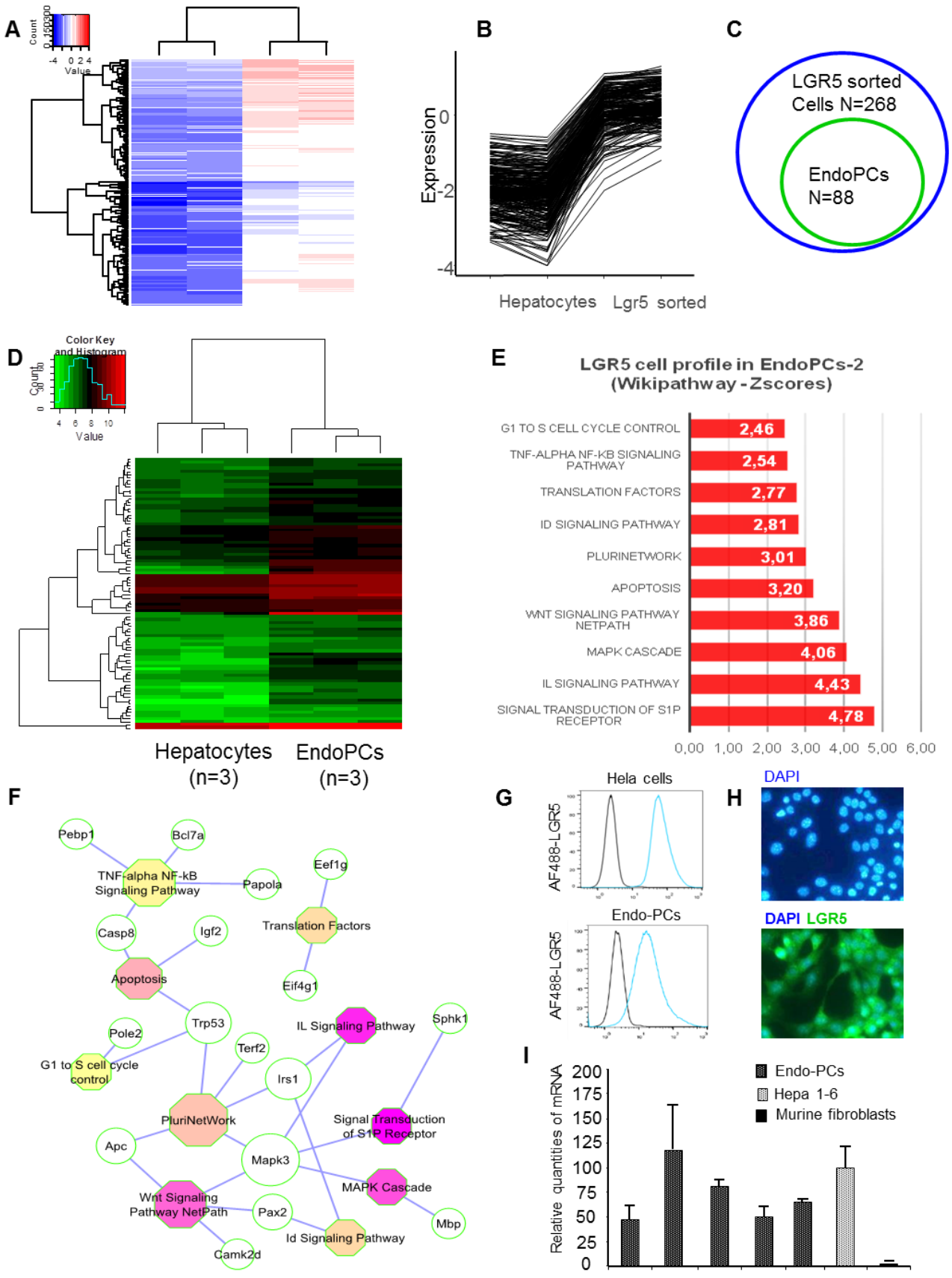

A
Count
Value
B
Expression
Hepatocytes
Lgr5 sorted
C
LGR5 sorted
Cells N=268
EndoPCs
N=88
D
Color Key
and Histogram
Count
Value
Hepatocytes
(n=3)
EndoPCs
(n=3)
E
LGR5 cell profile in EndoPCs-2
(Wikipathway - Zscores)
G1 TO S CELL CYCLE CONTROL
TNF-ALPHA NF-KB SIGNALING PATHWAY
TRANSLATION FACTORS
ID SIGNALING PATHWAY
PLURINETWORK
APOPTOSIS
WNT SIGNALING PATHWAY NETPATH
MAPK CASCADE
IL SIGNALING PATHWAY
SIGNAL TRANSDUCTION OF S1P RECEPTOR
2,46
2,54
2,77
2,81
3,01
3,20
3,86
4,06
4,43
4,78
0,00 1,00 2,00 3,00 4,00 5,00 6,00
F
Pebp1
Bcl7a
TNF-alpha NF-kB Signaling Pathway
Papola
Eef1g
Casp8
Igf2
Translation Factors
Apoptosis
Eif4g1
Trp53
IL Signaling Pathway
Sphk1
Pole2
Terf2
G1 to S cell cycle control
Irs1
PluriNetWork
Signal Transduction of S1P Receptor
Apc
Mapk3
MAPK Cascade
Wnt Signaling Pathway NetPath
Pax2
Mbp
Id Signaling Pathway
Camk2d
G
Hela cells
AF488-LGR5
Endo-PCs
AF488-LGR5
H
DAPI
DAPI LGR5
I
Relative quantities of mRNA
200
175
150
125
100
75
50
25
0
Endo-PCs
Hepa 1-6
Murine fibroblasts

Figure 1

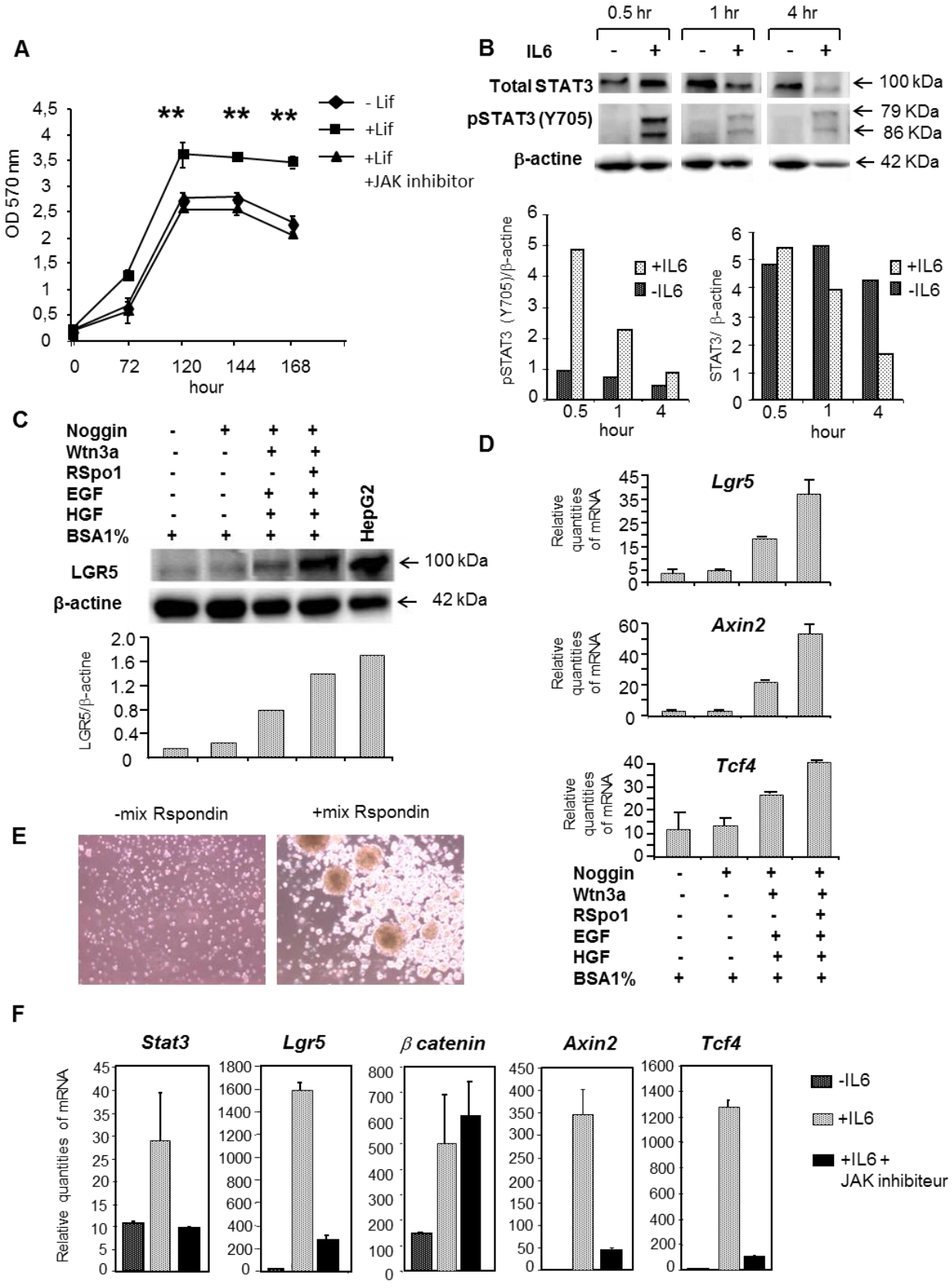

A
OD 570 nm
hour
- Lif
+Lif
+Lif
+JAK inhibitor
B
IL6
0.5 hr
1 hr
4 hr
Total STAT3
pSTAT3 (Y705)
β-actine
100 kDa
79 KDa
86 KDa
42 KDa
pSTAT3 (Y705)/β-actine
STAT3/ β-actine
+IL6
-IL6
C
Noggin
Wtn3a
RSpo1
EGF
HGF
BSA1%
HepG2
LGR5
β-actine
100 kDa
42 kDa
LGR5/β-actine
D
Lgr5
Axin2
Tcf4
Relative quantities of mRNA
E
-mix Rspondin
+mix Rspondin
F
Stat3
Lgr5
β catenin
Axin2
Tcf4
Relative quantities of mRNA
-IL6
+IL6
+IL6 + JAK inhibiteur

Figure 2

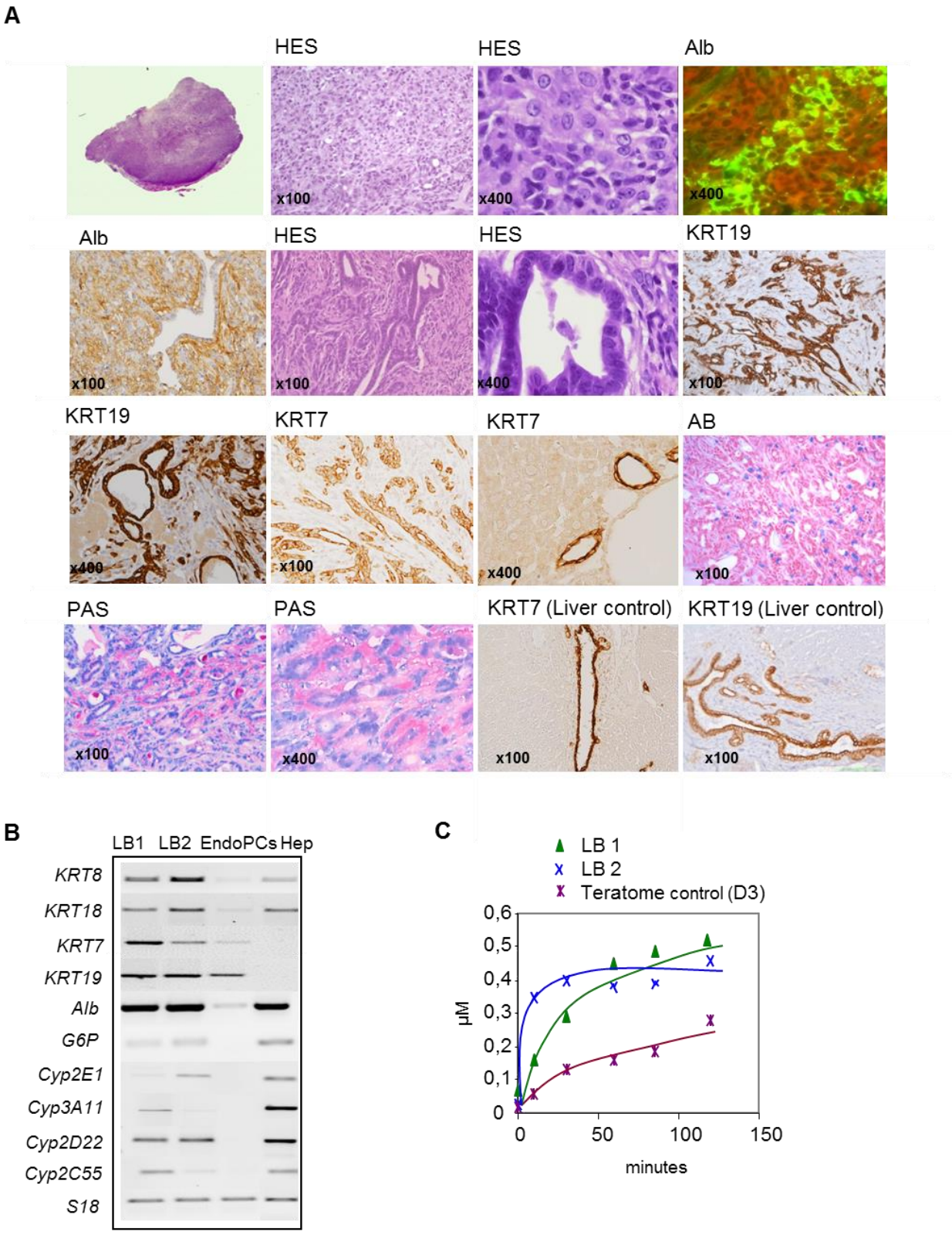

A
HES
HES
Alb
x100
x400
x400
Alb
HES
HES
KRT19
x100
x100
x400
x100
KRT19
KRT7
KRT7
AB
x400
x100
x400
x100
PAS
PAS
KRT7 (Liver control)
KRT19 (Liver control)
x100
x400
x100
x100
B
LB1 LB2 EndoPCs Hep
KRT8
KRT18
KRT7
KRT19
Alb
G6P
Cyp2E1
Cyp3A11
Cyp2D22
Cyp2C55
S18
C
LB 1
LB 2
Teratome control (D3)
µM
0,6
0,5
0,4
0,3
0,2
0,1
0
0
50
100
150
minutes

Figure 3

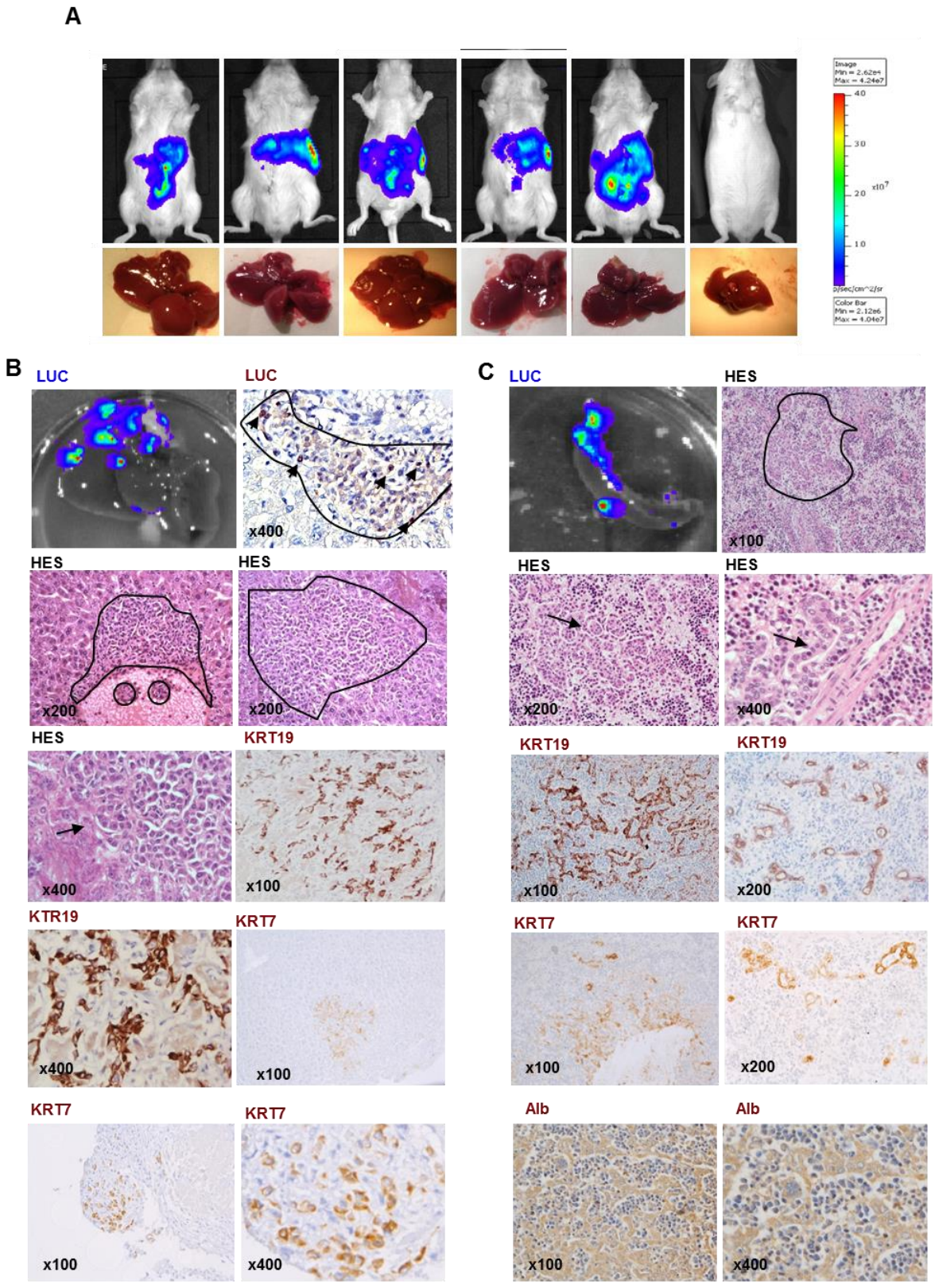

A
B
C
LUC
LUC
x400
HES
HES
x200
x200
HES
KRT19
x400
x100
KTR19
KRT7
x400
x100
KRT7
KRT7
x100
x400
LUC
HES
x100
HES
HES
x200
x400
KRT19
KRT19
x100
x200
KRT7
KRT7
x100
x200
Alb
Alb
x100
x400

Figure 4

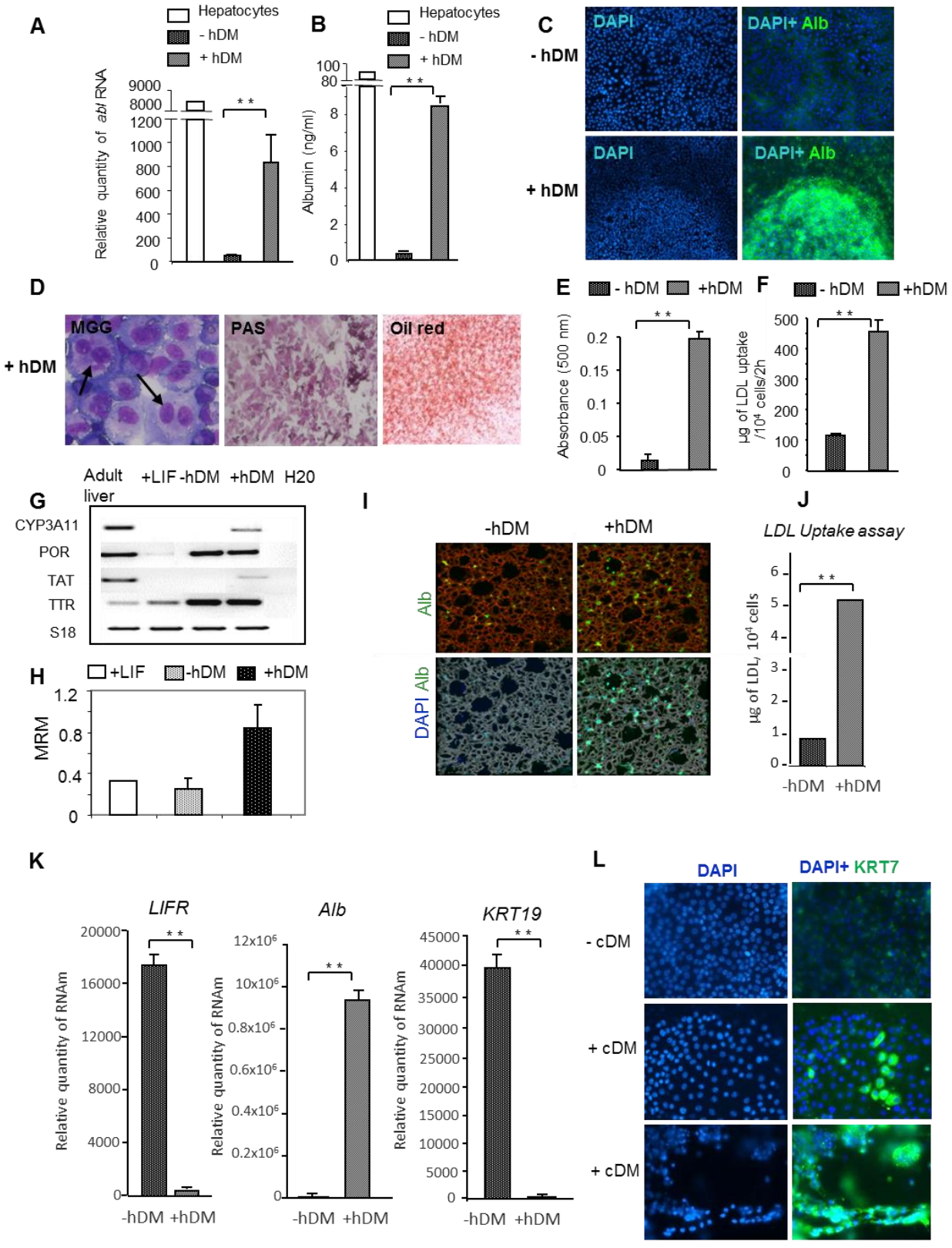
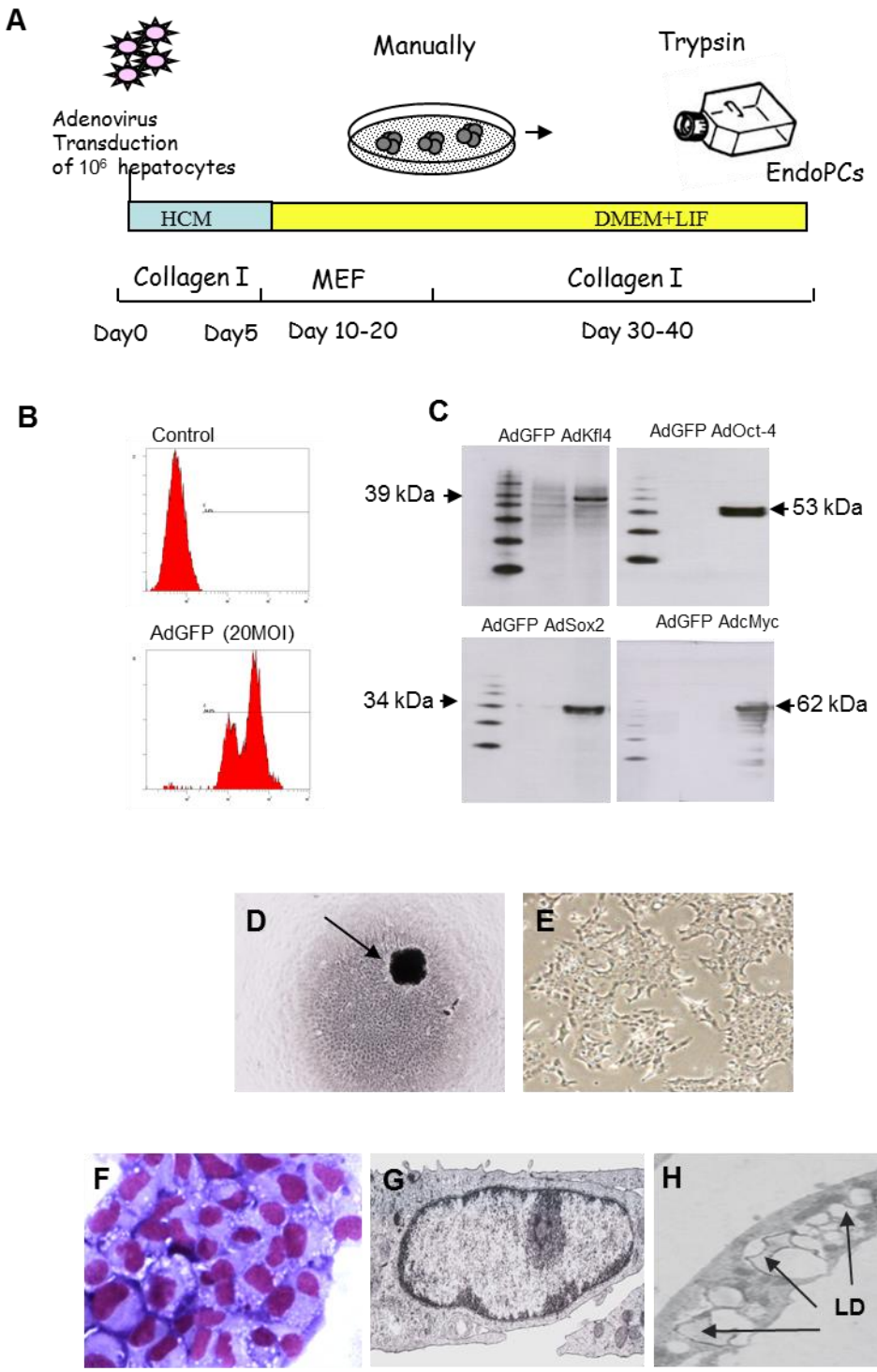
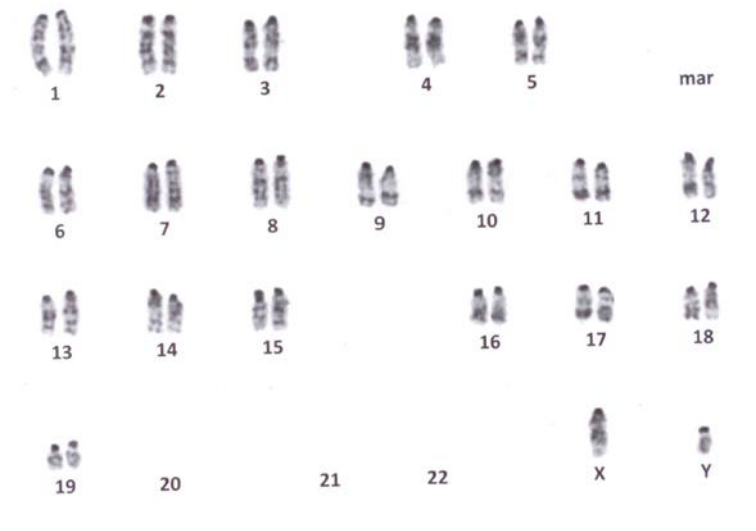

A
Hepatocytes
- hDM
+ hDM
Relative quantity of abl RNA
B
Albumin (ng/ml)
C
DAPI
DAPI+ Alb
- hDM
+ hDM
D
+ hDM
MGG
PAS
Oil red
E
- hDM +hDM
Absorbance (500 nm)
F
µg of LDL uptake /10⁴ cells/2h
G
Adult liver
+LIF -hDM +hDM H20
CYP3A11
POR
TAT
TTR
S18
H
+LIF -hDM +hDM
MRM
I
-hDM +hDM
Alb
DAPI Alb
J
LDL Uptake assay
µg of LDL 10⁴ cells
-hDM +hDM
K
LIFR
Alb
KRT19
Relative quantity of RNAm
-hDM +hDM
L
DAPI
DAPI+ KRT7
- cDM
+ cDM
+ cDM

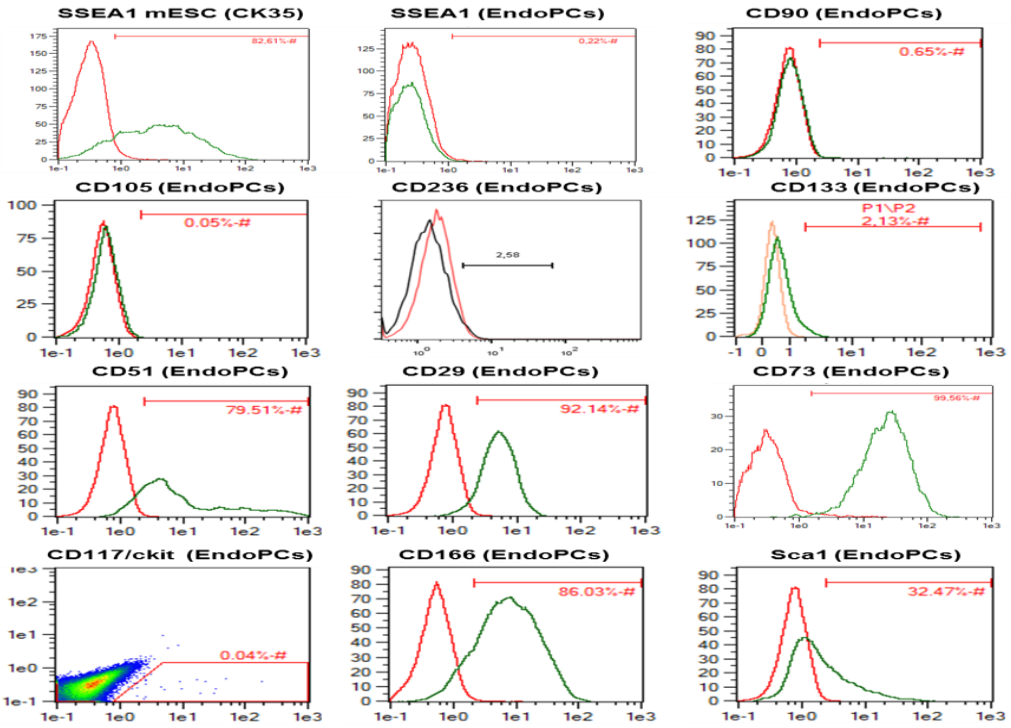
Figure 5



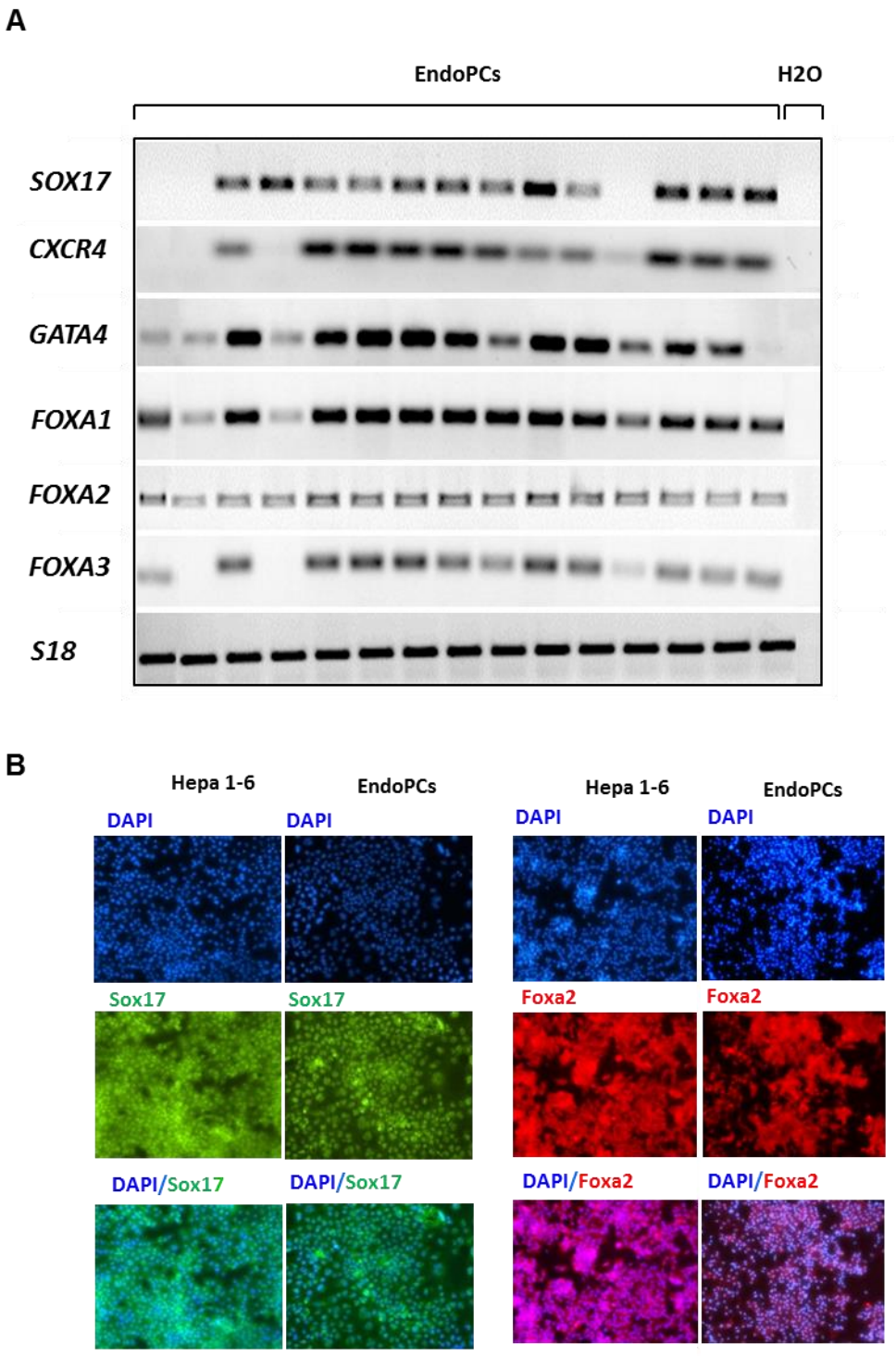
Supplemental Figure 1



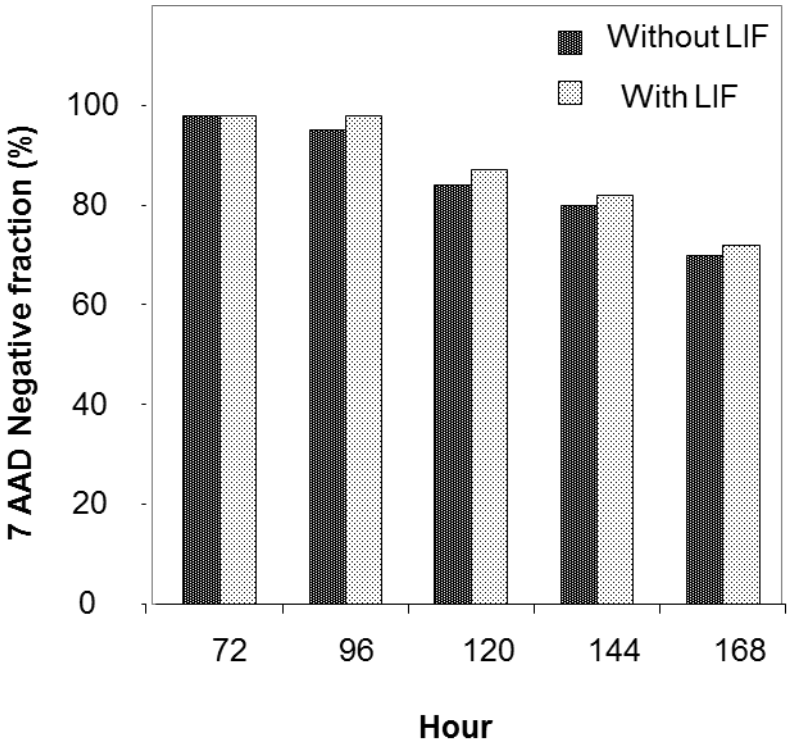
Supplementary Figure 2



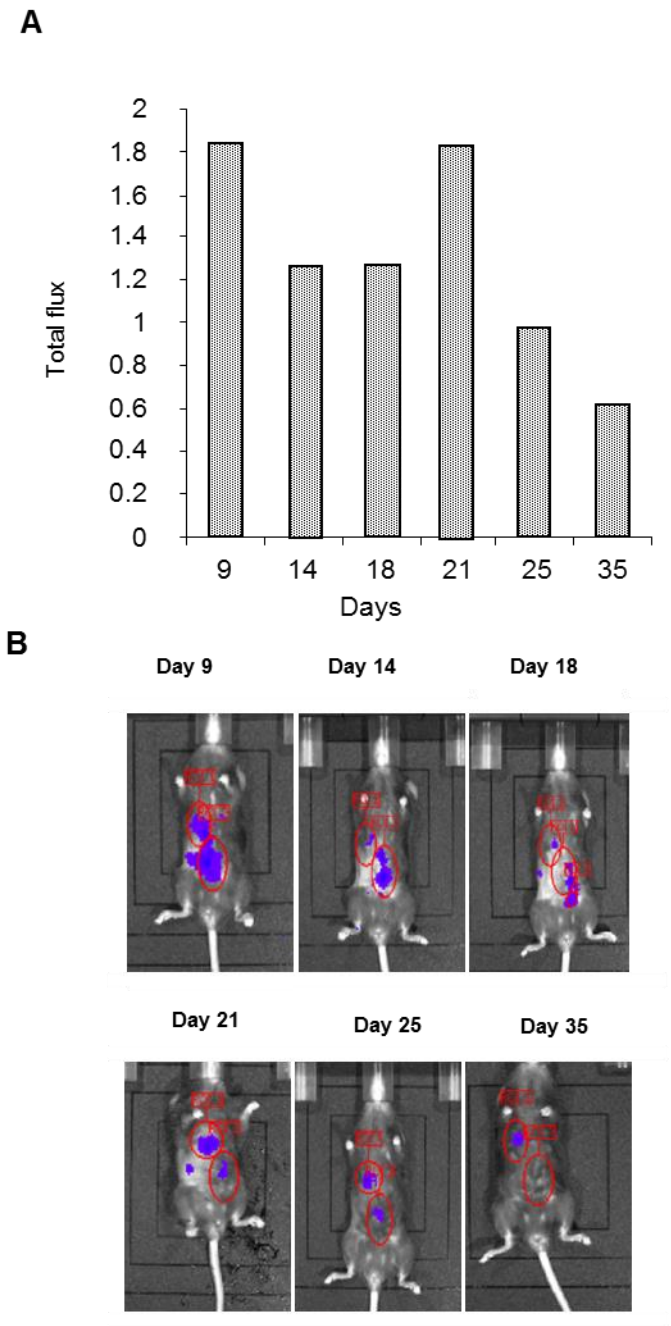
Supplemental Figure 3



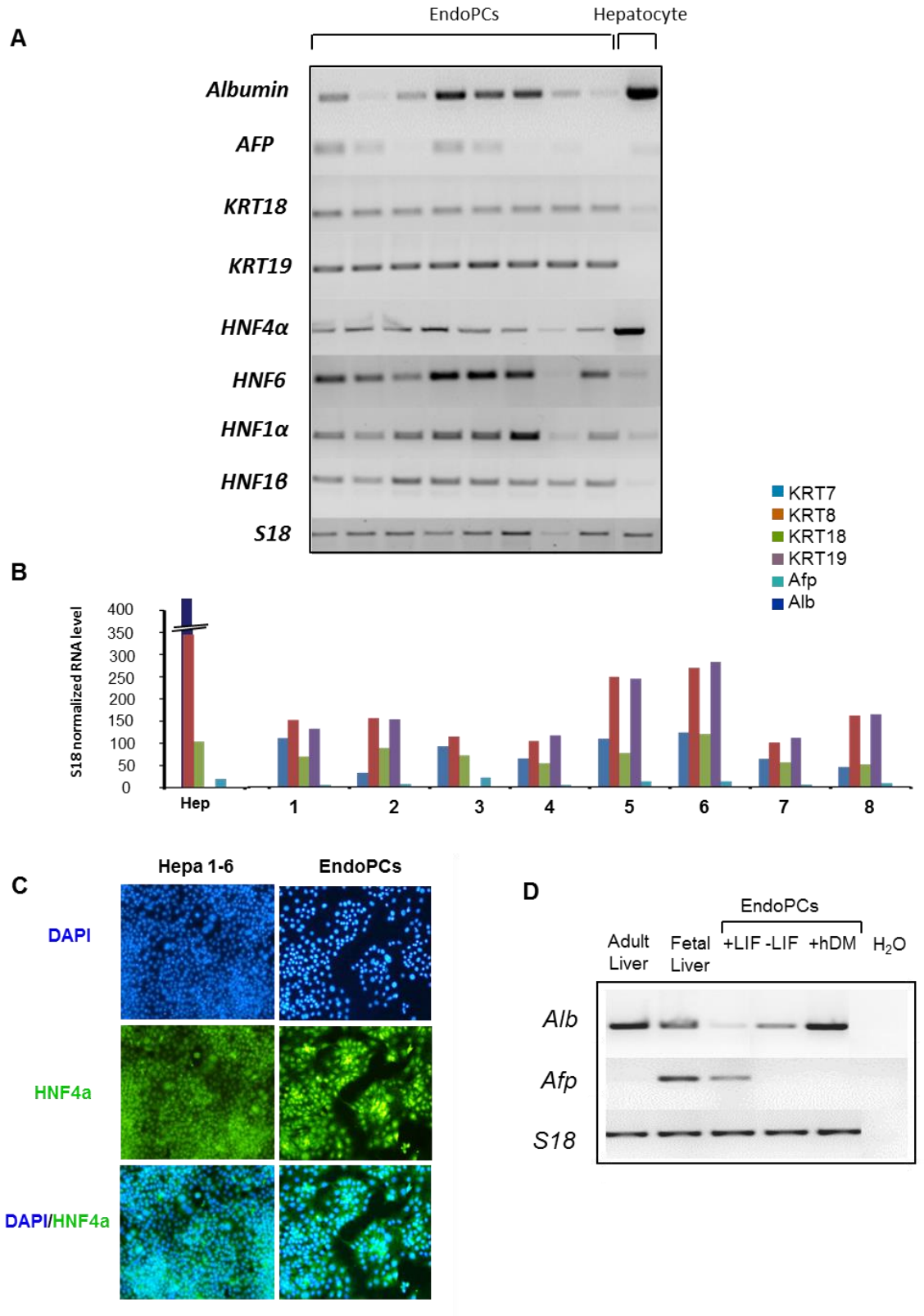
Supplemental Figure 4



Supplemental Figure 5



Supplementary Figure 6



supplemental figure 7

	EndoPCs		
	#1	#2	#3
LGR5	83.1	99.4	94.8
SSEA1	0.22	0.20	0.18
KRT18	97.2	97.1	86.4
KRT19	96.7	98.8	90.3
KRT7	0.1	1.1	0.02
Sca1	32.5	30.6	25.35
CD236	1.1	0.02	2.49
CD133	0.06	0.11	0.06
CD73	98.8	96.5	88.6
CD90	0.05	0.04	0.62
CD105	0.05	0.10	0.62
CD166	92.3	83.7	86.03
CD51	79.5	75.5	46.23
CD29	92.0	85.5	70.29
CD117	0.04	0.03	0.04
CD34	0,01	0,02	0,01

Supplemental Table1

Supplementary table 2

Gene title	Gene symbol	Gene ID	GenBank Accession	Fold change(Unlogged)	Hepatocytes1	Hepatocytes2	Lgr5-sorted cells1	Lgr5-sorted cells2
			AK082407	14.156652	-3.6660938	-4.0	0.0	0.0
myosin, light polypeptide kinase	Mylk	107589	AK044527	11.12279	-3.1490996	-3.8978798	0.0	0.0
RIKEN cDNA 4921530L21 gene	4921530L21Rik	66732	NM_025733	10.456048	-3.3047035	-4.0	-0.49072713	0.0
ring finger and WD repeat domain 2	Rfwd2	26374	NM_011931	10.39401	-3.1606684	-3.633216	0.0	0.0
				10.277037	-3.066347	-3.5457487	0.0	0.14653386
ribosomal protein S27-like	Rps27l	67941	NM_026467	10.143431	-2.930928	-3.597091	0.0	0.22443509
				9.960008	-3.1975584	-3.4453633	0.0	0.0
glutamic pyruvate transaminase (alanine aminotransferase) 2	Gpt2	108682	NM_173866	9.78981	-3.2533882	-3.1637988	0.0	0.16220845
				9.311508	-2.9050038	-3.620953	0.0	0.0
				8.878274	-3.3463788	-3.50908	-0.615464	0.0
eukaryotic translation elongation factor 1 gamma	Eef1g	67160	AK011951	8.826178	-2.8034997	-3.5845137	0.0	0.0
zinc finger and BTB domain containing 14	Zbtb14	22666	NM_009547	8.781705	-3.333042	-3.543284	-0.6787876	0.0
solute carrier organic anion transporter family, member 6d1	Slco6d1	70866	AK014872	8.75045	-2.9595554	-3.3218439	0.0	0.0
				8.730652	-3.306524	-4.0	-0.9409468	-0.14048843
autophagy related 4D, cysteine peptidase	Atg4d	235040	AK044798	8.405915	-3.0424535	-3.100949	0.0	0.0
				8.402659	-3.4642463	-3.2995255	-0.0759489	-0.58633924
zinc finger protein 36	Zfp36	22695	NM_011756	8.039652	-2.8153334	-3.228399	0.0	0.0
Rho guanine nucleotide exchange factor (GEF) 4	Arhgef4	226970	NM_183019	8.011314	-2.363419	-2.514918	0.483972	0.64144915
			AK050382	7.8880696	-3.0778193	-2.887779	0.0	0.0
HECT domain containing 3	Hectd3	76608	NM_175244	7.7225957	-2.8225427	-3.087805	0.0	0.0
			AK043926	7.6814103	-2.7763643	-2.976417	-0.015393069	0.14767812

cyclin-dependent kinase 12	Cdk12	69131	AK137262	7.600463	-2.8311863	-3.0276732	0.0	0.0
spermatogenesis associated multipass transmembrane protein 3	Samt3	73495	NM_028554	7.5035214	-2.7988894	-2.7969382	0.08240439	0.13639909
general transcription factor II A, 1	Gtf2a1	83602	NM_175335	7.2962193	-2.701409	-3.0544312	0.0	0.0
olfactory receptor 976	Olfr976	258364	NM_146367	7.2840195	-2.744539	-2.995862	0.0	0.0
				7.2768173	-2.7198815	-3.0225825	0.0	0.0
vesicle-associated membrane protein 1	Vamp1	22317	NM_009496	7.219609	-2.059887	-2.093587	0.549316	0.9706017
ST3 beta-galactoside alpha-2,3-sialyltransferase 6	St3gal6	54613	NM_018784	7.192924	-2.07744	-2.1320128	0.5210719	0.93373835
			AK078248	7.152966	-2.8099458	-2.6089966	-0.14423425	0.36471173
RIKEN cDNA 9430076C15 gene	9430076C15Rik	320189	AK035031	7.13408	-2.785677	-2.460516	0.23747104	0.20387809
transmembrane protein 242	Tmem242	70544	NM_027457	7.109999	-1.928504	-2.0776844	0.63679314	0.9980195
strawberry notch homolog 2 (Drosophila)	Sbno2	216161	NM_183426	7.1074624	-2.577634	-3.134427	0.0	0.0
				7.1028843	-3.0475416	-3.6028295	-0.7784227	-0.21642207
olfactory receptor 341	Olfr341	258952	NM_146950	7.081101	-3.2796402	-2.994552	-0.5094355	-0.12790029
C-type lectin domain family 11, member a	Clec11a	20256	NM_009131	7.063682	-3.1068509	-2.7189975	0.0	-0.16365674
				7.0275016	-2.602861	-3.0590808	0.0	0.0
				7.0222597	-3.067838	-2.7546065	-0.18770641	0.0
RIKEN cDNA 4930555G01 gene	4930555G01Rik	108978	NM_175393	7.000219	-2.5329673	-3.0446196	0.0	0.081199445
syntabulin (syntaxin-interacting)	Sybu	319613	AK051222	6.926813	-1.9837266	-2.0825	0.58548105	0.9155306
insulin-like growth factor 2 mRNA binding protein 3	Igf2bp3	140488	NM_023670	6.905001	-2.5804152	-2.9645007	0.0	0.05532534
adenosine deaminase, RNA-specific, B2	Adarb2	94191	BC059822	6.884169	-2.6370769	-2.9459934	0.0	0.0
olfactory receptor 575	Olfr575	259118	NM_147114	6.8309608	-2.6115024	-2.9528146	0.0	0.0
transformation related protein 53	Trp53	22059	NM_011640	6.796575	-2.8099394	-2.872057	0.0	-0.15592285

coiled-coil domain containing 167	Ccdc167	68597	AK003900	6.781127	-2.1858938	-2.3827543	0.4679393	0.49306378
vomeronasal 1 receptor 22	Vmn1r22	171196	NM_134178	6.7021904	-2.590232	-2.9021134	0.13158979	-0.1296576
DNA segment, Chr 3, ERATO Doi 751, expressed	D3Ertd751e	73852	NM_028667	6.6864624	-2.8263214	-2.660904	0.0	0.0
glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	Gapdhs	14447	NM_008085	6.6563315	-2.206223	-2.480244	0.29215395	0.49658948
mitochondrial ribosomal protein L3	Mrpl3	94062	NM_053159	6.622602	-1.7907743	-1.9104869	0.7542537	0.9919818
ATPase, class VI, type 11B	Atp11b	76295	NM_029570	6.558009	-1.9352307	-2.1151512	0.5025069	0.8574556
caspase recruitment domain family, member 11	Card11	108723	NM_175362	6.40839	-2.7168174	-2.6440246	0.0	0.0
			AK046413	6.3310065	-2.5814674	-2.3604655	0.0	0.36798975
			AK038761	6.266537	-2.648112	-2.444476	0.0	0.20280725
mitochondrial ribosomal protein S10	Mrps10	64657	NM_183086	6.25892	-2.1780689	-1.9155192	0.46083105	0.73621553
deafness, autosomal dominant 5 (human)	Dfna5	54722	NM_018769	6.2239785	-2.0116725	-2.4141629	0.46232924	0.41510472
ribosomal protein L35A	Rpl35a	57808	AK160963	6.209253	-2.0151248	-2.0918307	0.36058944	0.77294797
RAS p21 protein activator 2	Rasa2	114713	NM_053268	6.194932	-1.8702133	-2.0643268	0.4516536	0.8544797
crumbs homolog 3 (Drosophila)	Crb3	224912	NM_177638	6.1859603	-2.7375731	-3.315199	-0.86514443	0.0
histocompatibility 2, O region alpha locus	H2-Oa	15001	NM_008206	6.1620564	-2.3326898	-2.6781673	-0.26576072	0.43749416
				6.157489	-1.9750276	-2.0938933	0.42056653	0.73999876
zinc finger protein 37	Zfp37	22696	NM_009554	6.1498914	-2.8389733	-2.7935429	-0.05212117	-0.35475764
eukaryotic translation initiation factor 4, gamma 1	Eif4g1	208643	NM_145941	6.1322117	-1.9543796	-2.111391	0.41427314	0.7388263
				6.0903573	-2.7988768	-2.394917	0.0	0.047061928
			AK089644	6.074029	-2.4719205	-2.6159184	0.0	0.11862271
				6.0469446	-2.5371788	-2.3827503	0.0	0.2645073
				6.0425673	-2.763884	-2.4091387	0.0	0.038792122
proline/serine-rich coiled-coil 1	Psrc1	56742	NM_019976	6.0071325	-2.5878584	-2.2837644	0.0	0.30195403
insulin-like growth factor 1	Igf1	16000	NM_010512	5.9456697	-2.8212602	-3.1467328	-0.5805784	-0.24490012

predicted gene 10700	Gm10700	100038748	AK136892	5.927533	-2.5390522	-3.1555674	-0.545681	0.0
ubiquitin protein ligase E3C	Ube3c	100763	NM_133907	5.904339	-2.2034001	-2.7635915	0.0	0.20343544
mannoside acetylglucosaminyltransferase 1	Mgat1	17308	NM_010794	5.9007883	-2.5354054	-2.586869	0.0	0.0
phosphatidylinositol glycan anchor biosynthesis, class N	Pign	27392	AK082387	5.8987184	-2.6738062	-2.356626	0.0	0.10582871
adenomatosis polyposis coli	Apc	11789	AK137301	5.8764305	-1.836942	-1.9922297	0.60956347	0.6745891
transmembrane emp24 domain containing 3	Tmed3	66111	NM_025360	5.8564634	-2.4878526	-2.9288068	-0.2984431	0.0
protein kinase, cGMP-dependent, type I	Prkg1	19091	AK051624	5.826863	-2.0678098	-2.3156981	0.36840695	0.34405625
				5.8218737	-2.4035716	-2.3440971	0.0	0.31838134
			AK044879	5.8176193	-2.4521027	-2.40695	0.16515045	0.05490222
X-linked lymphocyte-regulated 4B	Xlr4b	27083	NM_021365	5.816419	-2.451852	-3.0114424	-0.3503201	0.0
forkhead box B1	Foxb1	64290	NM_022378	5.7879267	-1.8064843	-2.2102644	0.27505803	0.76161635
				5.762577	-2.4128995	-2.7702048	-0.16913535	0.052984934
nerve growth factor receptor (TNFRSF16) associated protein 1	Ngfrap1	12070	NM_009750	5.7487187	-1.9252189	-2.3344483	0.26539794	0.5375256
caspase 8	Casp8	12370	NM_009812	5.72024	-2.4860282	-2.7447743	0.0	-0.1935521
prolactin family 6, subfamily a, member 1	Prl6a1	19111	NM_011166	5.668376	-2.776721	-3.215197	-0.63598573	-0.33277473
lysocardiolipin acyltransferase 1	Lclat1	225010	NM_001081071	5.606109	-2.8320954	-3.0635135	-0.5016379	-0.41209045
calcium/calmodulin-dependent protein kinase II, delta	Camk2d	108058	NM_023813	5.533771	-2.6331475	-2.2541673	0.0	0.07310238
TATA box binding protein (Tbp)-associated factor, RNA polymerase I, A	Taf1a	21339	NM_021466	5.5145407	-1.9892751	-2.4205477	0.20559402	0.34004468
			AK037619	5.5094385	-2.357831	-2.4469655	0.0	0.117979266
vomeronasal 1 receptor 9	Vmn1r9	171203	NM_134185	5.494063	-2.1622415	-2.6031375	-0.19404991	0.3305447
polymerase (DNA directed), epsilon 2 (p59 subunit)	Pole2	18974	AK042113	5.4620275	-3.2558765	-3.3324685	-0.83778906	-0.8506952

N(alpha)-acetyltransferase 20, NatB catalytic subunit	Naa20	67877	NM_026425	5.454114	-1.9508704	-2.1734486	0.2515646	0.5153421
zinc finger and BTB domain containing 8a	Zbtb8a	73680	NM_028603	5.4539447	-1.9937238	-2.5206795	0.052701037	0.35911056
poly (A) polymerase alpha	Papola	18789	NM_011112	5.3740783	-2.3133156	-2.548275	0.0	0.0
fatty acid binding protein 4, adipocyte	Fabp4	11770	NM_024406	5.354635	-2.1472976	-2.1283276	0.15550049	0.40015337
uncharacterized LOC102632160	LOC102632160	102632160	AK033297	5.353326	-2.5058823	-2.2691731	-0.11144275	0.17296305
			AV323315	5.3389716	-2.3381884	-2.3260975	0.0	0.16419384
feminization 1 homolog b (C. elegans)	Fem1b	14155	NM_010193	5.323753	-1.9722719	-2.3935223	-0.22222616	0.5971505
tubulin, gamma complex associated protein 6	Tubgcp6	328580	AK052441	5.31417	-2.017028	-2.4885168	0.068483606	0.27651885
ankyrin repeat domain 27 (VPS9 domain)	Ankrd27	245886	NM_145633	5.31067	-1.2868905	-1.3207009	0.9008984	1.2841216
TNNI3 interacting kinase	Tnni3k	435766	AK084817	5.29715	-2.1628792	-2.6294556	0.0	0.055131532
poly(A) binding protein, cytoplasmic 4	Pabpc4	230721	NM_148917	5.255092	-2.619612	-2.356441	0.0	-0.1823971
solute carrier family 6 (neurotransmitter transporter), member 20B	Slc6a20b	22599	NM_011731	5.2550316	-2.6749136	-2.8888524	-0.41826046	-0.3509672
tolloid-like 2	Tll2	24087	NM_011904	5.227288	-2.4964888	-2.4007788	0.0	-0.12631957
				5.2015634	-2.1923287	-2.532356	0.052713253	0.0
dystrophin, muscular dystrophy	Dmd	13405	NM_007868	5.179143	-1.7670088	-1.7374225	0.5602077	0.67851466
uncharacterized LOC102632051	LOC102632051	102632051	AK076935	5.167849	-1.784164	-1.8273059	0.4096954	0.7033662
antisense Igf2r RNA	Airn	104103	AK048015	5.1641064	-3.1271038	-3.5082672	-1.2246686	-0.69661397
aldehyde dehydrogenase 16 family, member A1	Aldh16a1	69748	NM_145954	5.1512604	-2.2065682	-2.9334881	-0.33952063	0.0
sex hormone binding globulin	Shbg	20415	NM_011367	5.128382	-2.7880433	-2.6709602	-0.2254772	-0.53020537
RIKEN cDNA 2810427C15 gene	2810427C15Rik	69979	AK013171	5.1272664	-2.2692096	-2.7470803	-0.27345672	0.0
				5.1271524	-2.3540394	-2.1819682	0.27008945	-0.109558284
MAX-like protein X	Mlx	21428	NM_011550	5.0975246	-1.5266212	-1.7081442	0.5349114	0.9111612

paired box 6	Pax6	18508	BC036957	5.0825667	-1.7253469	-1.9299071	0.33662143	0.68544954
				5.0570292	-2.6412	-3.114609	-0.95295846	-0.18787421
nucleoporin 50	Nup50	18141	NM_016714	5.0281053	-2.0128703	-2.493452	0.0	0.18746036
anterior gradient 3	Agr3	403205	NM_207531	5.0113797	-2.1179392	-2.5673318	0.0	0.0
Smg-6 homolog, nonsense mediated mRNA decay factor (C. elegans)	Smg6	103677	NM_001002764	4.9909496	-2.0603952	-2.079867	0.14983773	0.34218794
PARK2 co-regulated-like	Pacrgl	66768	NM_025755	4.9886513	-2.345268	-2.1876793	0.0	0.10668189
protein phosphatase, EF hand calcium-binding domain 2	Ppef2	19023	NM_011148	4.98448	-2.0020418	-2.2803001	0.0	0.3453251
			AK052970	4.981796	-2.0039902	-2.0997398	0.103286736	0.41147578
ACN9 homolog (S. cerevisiae)	Acn9	71238	AK046436	4.961547	-1.8298796	-2.1186945	0.19230542	0.48073843
insulin receptor substrate 1	Irs1	16367	AY169784	4.9577665	-2.403705	-2.4950218	0.0	-0.29272428
sirtuin 2	Sirt2	64383	NM_022432	4.9030337	-2.190267	-2.385398	0.0	0.018219866
cytokine-dependent hematopoietic cell linker	Clnk	27278	NM_013748	4.8728204	-2.1405942	-2.4433608	-0.14536944	0.13334404
forkhead box C2	Foxc2	14234	NM_013519	4.870955	-2.1062813	-2.3575501	0.042586662	0.07276026
huntingtin interacting protein 1 related	Hip1r	29816	NM_145070	4.8693295	-2.201144	-2.7867537	-0.3873506	0.0
coiled-coil domain containing 115	Ccdc115	69668	NM_027159	4.859238	-2.1449645	-2.4291942	-0.1492556	0.13641547
yippee-like 1 (Drosophila)	Ypel1	106369	NM_023249	4.8050175	-3.117756	-3.0269003	-0.9612411	-0.66779745
MAS-related GPR, member B3	Mrgprb3	404238	NM_207537	4.7801266	-1.6783082	-1.6598347	0.7642386	0.38721257
				4.762627	-2.2143455	-2.0389507	0.09349246	0.16125849
oxidative stress induced growth inhibitor 1	Osgin1	71839	NM_027950	4.758358	-1.6993957	-1.7096418	0.36077815	0.71004313
cadherin 11 pseudogene	2610005L07Rik	381598	BC086760	4.748304	-2.2859595	-2.2098682	0.0	0.0
				4.7429295	-1.7399614	-2.3244665	0.0	0.4508504
SPT2, Suppressor of Ty, domain containing 1 (S. cerevisiae)	Spty2d1	101685	NM_175318	4.7101974	-2.157983	-2.1422236	0.0	0.16660412
			AK050325	4.7098303	-2.3143265	-2.1610906	0.0	0.0
olfactory receptor 390	Olfr390	258344	NM_146347	4.6966043	-2.251961	-2.058783	0.0	0.15480317

RIKEN cDNA 4930519H02 gene	4930519H02Rik	75058	AK076692	4.6909575	-2.3538072	-2.909203	-0.8832438	0.0
trafficking protein, kinesin binding 1	Trak1	67095	BC058971	4.682474	-2.170772	-2.2055142	0.0	0.07742637
neugrin, neurite outgrowth associated	Ngrn	83485	NM_031375	4.679105	-3.1275163	-3.8553472	-1.7094308	-0.85486794
crystallin, gamma D	Crygd	12967	NM_007776	4.667646	-2.0358818	-2.499899	-0.053739913	0.0
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6	St8sia6	241230	NM_145838	4.6553526	-2.156812	-2.2837608	0.0	0.0
			AK089374	4.649651	-2.3948224	-1.912581	0.16238718	0.0
RIKEN cDNA 4930403O15 gene	4930403O15Rik	73814	AK015069	4.6363525	-2.0814595	-1.8177896	0.20264436	0.33316645
G patch domain containing 2	Gpatch2	67769	NM_026367	4.6349173	-2.2751029	-2.1525846	0.0	0.0
BMP2 inducible kinase	Bmp2k	140780	NM_080708	4.6333885	-2.3122942	-2.1183538	0.0	0.0
F-box protein 28	Fbxo28	67948	NM_175127	4.625149	-1.6324089	-1.8701864	0.4967446	0.42864227
solute carrier family 5 (sodium-dependent vitamin transporter), member 6	Slc5a6	330064	NM_177870	4.6222134	-2.0100412	-2.1720548	0.0	0.23042677
neuropeptide Y receptor Y2	Npy2r	18167	NM_008731	4.6220875	-1.5008425	-2.0467744	0.21857479	0.6674617
				4.614935	-1.9413325	-2.319111	0.0	0.17173028
RIKEN cDNA 1700003E16 gene	1700003E16Rik	71837	AK005628	4.589794	-2.1975539	-2.038522	0.0	0.16069123
metal response element binding transcription factor 1	Mtf1	17764	NM_008636	4.5759306	-2.3874352	-2.0235765	0.0	0.0
lymphocyte transmembrane adaptor 1	Lax1	240754	NM_172842	4.572322	-2.179358	-2.2066247	0.0	0.0
RIKEN cDNA A230072C01 gene	A230072C01Rik	320742	AK029359	4.5579877	-2.6313624	-3.1131475	-1.0587927	-0.35409734
pleckstrin homology domain-containing, family A (phosphoinositide binding specific) member 2	Plekha2	83436	NM_031257	4.553572	-1.2121836	-1.5868095	0.8032808	0.79596627
coiled-coil domain containing 176	Ccdc176	72873	AK032247	4.5284176	-1.4744847	-1.5211842	0.4809282	0.857315

myeloblastosis oncogene	Myb	17863	NM_010848	4.5209937	-2.1618345	-2.3424852	-0.11801534	-0.028753558
			AK046946	4.516101	-1.8075911	-2.141631	0.0	0.39347956
vascular endothelial growth factor A	Vegfa	22339	NM_009505	4.5132346	-2.1716185	-2.764229	-0.6867235	0.06324499
Rab geranylgeranyl transferase, b subunit	Rabggtb	19352	BC057661	4.5109606	-1.4610386	-1.6335343	0.5531554	0.700532
alcohol dehydrogenase, iron containing, 1	Adhfe1	76187	NM_175236	4.493729	-1.8966362	-2.0957644	0.047493283	0.29241577
B cell CLL/lymphoma 7A	Bcl7a	77045	NM_029850	4.492284	-2.1464088	-2.5304716	-0.5809074	0.16823338
beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	B3gat1	76898	NM_029792	4.483408	-1.877117	-2.2268598	0.0	0.23666446
BMP2 inducible kinase	Bmp2k	140780	CO802710	4.468369	-2.2888253	-2.0412774	0.0	0.0
activator of basal transcription 1	Abt1	30946	NM_013924	4.4661746	-2.116566	-2.0824342	0.0	0.11691303
translocase of outer mitochondrial membrane 70 homolog A (yeast)	Tomm70a	28185	NM_138599	4.4623437	-1.840354	-2.2103288	0.011782176	0.2656404
mitogen-activated protein kinase 3	Mapk3	26417	NM_011952	4.4603176	-2.1802058	-1.9968991	0.22986247	-0.10640641
				4.4377294	-1.6793505	-1.9606124	0.18269868	0.47580668
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	Galnt3	14425	NM_015736	4.4125323	-1.994279	-2.2895854	-0.14276098	0.14306197
structural maintenance of chromosomes 2	Smc2	14211	NM_008017	4.410322	-2.1372614	-2.1445158	0.0	0.0
			AK089607	4.393663	-1.9104649	-2.0230834	0.27121085	0.060607474
oligodendrocyte myelin glycoprotein	Omg	18377	NM_019409	4.3348017	-0.956986	-1.2725358	0.99543816	1.0240505
trans-golgi network vesicle protein 23B	Tvp23b	67510	NM_026210	4.328774	-1.4299413	-1.4015824	0.5613848	0.82327867
			AF177345	4.31866	-1.4267446	-1.3782228	0.5524501	0.84895056
transformation related protein 53	Trp53	22059	NM_011640	4.3178544	-1.0033402	-1.1497626	0.9239012	1.139306
shisa homolog 5 (Xenopus laevis)	Shisa5	66940	NM_025858	4.3045015	-3.3790429	-4.0	-2.0111804	-1.2018971

UBX domain protein 7	Ubxn7	224111	BC062904	4.2939243	-1.9366149	-2.2895048	0.0	0.0
				4.268756	-2.4874299	-2.6448758	-0.64622754	-0.31331608
RIKEN cDNA 2900006A17 gene	2900006A17Rik	72913	AK013487	4.2530084	-1.4117671	-1.5849626	0.66316366	0.5186515
junctional sarcoplasmic reticulum protein 1	Jsrp1	71912	NM_028001	4.2146263	-2.178951	-2.6959136	-0.7829336	0.0
RAN binding protein 3-like	Ranbp3l	223332	NM_198024	4.209243	-1.2526375	-1.4207072	0.6421939	0.8303449
				4.205492	-1.2716005	-1.5170763	0.38286364	0.93157554
			AK047796	4.204894	-1.999895	-2.505561	-0.61348355	0.18669198
protocadherin beta 17	Pcdhb17	93888	NM_053142	4.2037396	-2.0831325	-2.3271875	-0.1401972	-0.11656382
			AK045955	4.200755	-1.9154646	-2.1588128	-0.03534541	0.109005764
solute carrier family 27 (fatty acid transporter), member 4	Slc27a4	26569	NM_011989	4.18794	-1.9515213	-1.8414	0.32355785	0.0
			AK141074	4.155496	-1.1608245	-1.2436969	0.79086834	0.91324717
somatostatin	Sst	20604	NM_009215	4.151683	-1.418011	-1.5928848	0.43178025	0.66092336
G protein-coupled receptor 98	Gpr98	110789	AK081823	4.1513643	-2.1466386	-2.8246377	-0.687957	-0.1475307
family with sequence similarity 204, member A	Fam204a	76539	AK044853	4.1500626	-1.3548404	-1.6371105	0.2818286	0.7999929
				4.148568	-1.9617354	-2.8735743	-0.66409826	0.0
RIKEN cDNA F630048H11 gene	F630048H11Rik	100038492	AK170335	4.1484456	-1.3051901	-1.3898362	0.61297566	0.79278255
phosphofurin acidic cluster sorting protein 2	Pacs2	217893	AK122326	4.1358986	-2.0959144	-1.9465487	0.0	0.057235368
WW domain containing E3 ubiquitin protein ligase 2	Wwp2	66894	NM_025830	4.1145067	-2.0690887	-2.0128973	0.0	0.0
kinetochore associated 1	Kntc1	208628	NM_001042421	4.1025925	-1.8357054	-2.0874717	-0.15093525	0.27977246
ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	Atp5b	11947	NM_016774	4.0952024	-1.2592545	-1.3432913	0.7143381	0.7519639
WAP four-disulfide core domain 2	Wfdc2	67701	NM_026323	4.0915227	-2.110705	-1.9585793	0.0	0.0
RIKEN cDNA 4930540M05 gene	4930540M05Rik	112414	AK016012	4.0831776	-2.044747	-2.014793	0.0	0.0

sex comb on midleg homolog 1	Scmh1	29871	AK083889	4.080706	-2.1826787	-2.094586	-0.12774798	-0.09077134
paired box 2	Pax2	18504	NM_011037	4.0772996	-1.888592	-2.1691568	0.0	0.011076824
				4.0756497	-1.7250577	-2.0005221	0.06514918	0.26924795
chondroitin sulfate synthase 3	Chsy3	78923	NM_001081328	4.052078	-1.4678885	-1.3482739	0.80650026	0.38670966
solute carrier family 25 (mitochondrial oxodicarboxylate carrier), member 21	Slc25a21	217593	AK044945	4.049129	-1.1628642	-1.3068889	0.657171	0.9015551
ephrin A4	Efna4	13639	NM_007910	4.019653	-1.7296734	-2.079507	0.0	0.21789718
raftlin family member 2	Rftn2	74013	BC038341	4.0142965	-2.3210483	-2.027138	-0.09078292	-0.23580869
methionine-tRNA synthetase 2 (mitochondrial)	mars-02	212679	NM_175439	4.012314	-2.0768962	-1.8807952	0.05726784	0.0
CLIP associating protein 2	Clasp2	76499	AJ276961	4.0069156	-1.0902455	-0.9979367	0.90400344	1.012245
small nuclear ribonucleoprotein 70 (U1)	Snrnp70	20637	BC049128	3.9896255	-1.7726123	-1.9480747	0.0	0.26499942
vasodilator-stimulated phosphoprotein	Vasp	22323	NM_009499	3.98485	-1.9582609	-1.9716271	0.0	0.058598723
				3.9819221	-2.5122297	-2.848982	-0.8203876	-0.54720086
RIKEN cDNA 1110001J03 gene	1110001J03Rik	66117	NM_025363	3.9818718	-1.4440786	-1.6761668	0.34372264	0.5264666
polymerase (RNA) III (DNA directed) polypeptide G	Polr3g	67486	AK037264	3.9771025	-1.9908463	-1.9925898	0.0	0.0
				3.969031	-0.83308095	-0.9946799	0.94743764	1.1961888
transmembrane protein 260	Tmem260	218989	NM_172600	3.9574082	-2.0629113	-2.1679251	-0.24484752	-0.023451975
kelch-like 2, Mayven	Klhl2	77113	NM_178633	3.9494336	-1.849945	-1.7053405	0.20068449	0.2109253
SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	Sys1	66460	NM_025575	3.9428585	-1.9051063	-2.0573945	0.0	0.0
phosphorylase kinase gamma 1	Phkg1	18682	NM_011079	3.9312572	-2.6477935	-3.0297813	-1.0876143	-0.6481114
RELT-like 2	Rell2	225392	NM_153793	3.9282918	-1.796814	-1.8880246	0.0	0.25330287
predicted gene 3877	Gm3877	100042510	AK080592	3.92112	-1.817084	-1.7775604	0.2891798	0.048992693
potassium inwardly-rectifying channel, subfamily J, member 6	Kcnj6	16522	NM_010606	3.92036	-0.8449949	-1.0689981	0.9572126	1.0769687
tetratricopeptide repeat domain	Ttc30b	72421	AK011097	3.9186523	-0.97257155	-1.2069138	0.827319	0.9411709

30B								
methionine-tRNA synthetase	Mars	216443	NM_001003913	3.9140494	-1.5065548	-2.0312295	0.0	0.41695952
				3.9125464	-1.2375522	-1.351621	0.5816865	0.76198095
mindbomb homolog 1 (Drosophila)	Mib1	225164	NM_144860	3.9037557	-1.5393102	-1.9740746	0.046481702	0.38292032
CD80 antigen	Cd80	12519	NM_009855	3.8976727	-1.912723	-2.01429	0.0	0.0
Ras-related associated with diabetes	Rrad	56437	NM_019662	3.89504	-1.1885272	-1.4199506	0.39473835	0.88746357
				3.8896706	-2.024474	-1.8844295	0.06945278	-0.05849898
phospholipase C, beta 2	Plcb2	18796	NM_177568	3.8838763	-2.0519624	-1.749351	0.3191086	-0.2442359
				3.883583	-1.1098685	-1.2897853	0.7643977	0.7563195
NIPA-like domain containing 2	Nipal2	223473	NM_145469	3.8806407	-2.0403004	-2.4127681	-0.573037	0.0
synaptotagmin XIII	Syt13	80976	NM_030725	3.8800952	-1.1652819	-1.2137136	0.85855675	0.6688034
DEAH (Asp-Glu-Ala-His) box polypeptide 15	Dhx15	13204	NM_007839	3.866127	-1.9932117	-1.9050101	0.0	0.0048999526
				3.8559208	-1.8791806	-2.018324	0.0	0.0
ubiquitin C	Ubc	22190	NM_019639	3.8383827	-1.9944079	-2.6183803	-0.6785182	-0.053496573
fibronectin type 3 and SPRY domain-containing protein	Fsd1	240121	NM_183178	3.8297477	-1.9404005	-1.9341052	0.0	0.0
nucleoporin 88	Nup88	19069	NM_172394	3.8290303	-1.1737679	-1.3092117	0.6192026	0.7709644
Holliday junction recognition protein	Hjurp	381280	NM_198652	3.8262255	-1.0229428	-1.4621638	0.54905623	0.85481244
olfactory receptor 620	Olfr620	258808	NM_146812	3.8073316	-1.362677	-1.2694911	0.5377252	0.6853946
G protein-coupled receptor 137C	Gpr137c	70713	NM_027518	3.8041728	-1.028331	-1.2307898	0.7007591	0.89579594
solute carrier family 38, member 4	Slc38a4	69354	NM_027052	3.798648	-1.1986443	-1.3704413	0.45750943	0.8082262
solute carrier family 25, member 42	Slc25a42	73095	AK049593	3.7941992	-0.79010123	-0.91705155	1.0060533	1.1343263
intersectin 2	Itsn2	20403	NM_011365	3.7904477	-1.9922566	-1.8557097	0.0	0.0
platelet/endothelial cell adhesion molecule 1	Pecam1	18613	NM_008816	3.7888916	-1.01953	-1.3604257	0.4379221	0.9927473
insulin-like growth factor 2	Igf2	16002	NM_010514	3.7795153	-1.8040226	-1.7867361	0.08875636	0.15615214

interleukin 1 receptor accessory protein	Il1rap	16180	NM_008364	3.776532	-2.099893	-1.9217522	0.0	-0.18815623
RIKEN cDNA 1700112J05 gene	1700112J05Rik	68246	AK018912	3.7653525	-1.6508776	-1.6614962	0.25383195	0.25937834
predicted gene 12185	Gm12185	620913	BC022776	3.7652488	-1.9715263	-2.7751844	-0.97115725	0.0
capicua homolog (Drosophila)	Cic	71722	NM_027882	3.744131	-2.324362	-2.4010098	-0.44086912	-0.474418
				3.7429771	-1.9419277	-1.867407	0.0	0.0
RalBP1 associated Eps domain containing protein	Reps1	19707	AK042993	3.7382588	-2.1063883	-2.5668354	-0.5457137	-0.29690722
myelin basic protein	Mbp	17196	NM_010777	3.7083793	-0.8242046	-0.8450212	0.9329015	1.1698104
chromodomain helicase DNA binding protein 3	Chd3	216848	NM_146019	3.708205	-0.85316706	-0.94890547	0.9564265	1.0237458
protein phosphatase 1, regulatory (inhibitor) subunit 2, pseudogene 7	Ppp1r2-ps7	76705	AK133428	3.6903412	-1.6109649	-1.8212762	0.08106651	0.2565271
polymerase (RNA) III (DNA directed) polypeptide F	Polr3f	70408	AK171146	3.685033	-0.7384662	-0.90391815	1.0181485	1.1062194
guanylate binding protein 7	Gbp7	229900	NM_145545	3.682043	-2.1729283	-1.826026	0.0	-0.22597772
transformation related protein 53 binding protein 1	Trp53bp1	27223	NM_013735	3.6814513	-1.9171826	-2.2469568	-0.28715706	-0.103471614
			AV229918	3.6771219	-1.9160967	-1.8420084	0.0	0.0
fatty acid binding protein 9, testis	Fabp9	21884	U96149	3.669733	-1.6938022	-1.8583666	0.0	0.19714208
			AV209602	3.6443639	-1.23996	-1.4724238	0.4004349	0.6195573
transmembrane 9 superfamily member 1	Tm9sf1	74140	AK149247	3.6439538	-1.6364256	-1.6356679	0.32193682	0.13064258
lectin, galactoside binding-like	Lgalsl	216551	NM_173752	3.6318376	-1.1821125	-1.2723526	0.5011307	0.7559749
RIKEN cDNA D030028A08 gene	D030028A08Rik	319371	AK050871	3.6294997	-1.7641265	-1.8683501	0.08761655	0.0
porcupine homolog (Drosophila)	Porcn	53627	NM_023638	3.6273923	-1.6616862	-1.7926524	0.0	0.25522503
				3.6261675	-2.3017812	-2.1664894	-0.24497159	-0.515942
zinc finger protein 664	Zfp664	269704	NM_001081750	3.6203725	-1.9259161	-2.077534	-0.14983658	-0.1373823
protein tyrosine phosphatase 4a2	Ptp4a2	19244	NM_008974	3.6202872	-1.3462822	-1.6328796	0.23581897	0.49941388
RAD51 homolog D	Rad51d	19364	NM_011235	3.6109262	-1.4658617	-1.5860581	0.3461633	0.30891705

cytohesin 4	Cyth4	72318	NM_028195	3.595358	-2.5843964	-2.8024898	-1.0601697	-0.6546185
UDP-glucose glycoprotein glucosyltransferase 2	Uggt2	66435	NM_001081252	3.5919564	-1.5728593	-1.5450218	0.23568347	0.3344203
vomeronasal 1 receptor 229	Vmn1r229	171224	NM_134190	3.5714705	-1.8980181	-1.777533	0.0	0.0
heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Hs3st1	15476	NM_010474	3.551712	-1.7792522	-2.074624	-0.18012425	-0.0068314597
additional sex combs like 3 (Drosophila)	Asxl3	211961	NM_001167777	3.5221162	-1.5493448	-2.2125163	-0.28826192	0.19461673
SEC13 homolog (S. cerevisiae)	Sec13	110379	NM_024206	3.5181472	-0.6306934	-0.83088225	0.9613105	1.2035297
			AK031438	3.516916	-1.8007321	-1.7204368	0.16393249	-0.06438654
ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	Atp8a1	11980	NM_001038999	3.5150223	-1.4470346	-1.2857405	0.46191606	0.43677282
spindle and kinetochore associated complex subunit 1	Ska1	66468	NM_025581	3.5143807	-1.850165	-1.7324221	0.1699807	-0.14027342
				3.5005255	-1.3078524	-1.8204052	-0.055588856	0.5289517
ubiquitin specific peptidase 30	Usp30	100756	NM_001033202	3.4945323	-1.2652608	-1.4116942	0.42634606	0.5094162
expressed sequence AI413582	AI413582	106672	NM_001002895	3.4828522	-2.433534	-3.1474633	-1.4518368	-0.5730034
lipolysis stimulated lipoprotein receptor	Lsr	54135	NM_017405	3.4733129	-0.93677735	-1.1340483	0.6959812	0.82946956
ATPase type 13A1	Atp13a1	170759	NM_133224	3.4660652	-1.728575	-1.800281	0.0	0.05804863
glial fibrillary acidic protein	Gfap	14580	K01347	3.464584	-2.2615197	-1.960433	-0.19699696	-0.4336008
SRY (sex determining region Y)-box 7	Sox7	20680	NM_011446	3.4568613	-1.7854643	-1.7934722	0.0	0.0
small nuclear RNA activating complex, polypeptide 3	Snapc3	77634	NM_029949	3.4538512	-2.1794217	-2.4167404	-0.5801434	-0.43357906
				3.43753	-1.7238799	-1.8412513	0.0	0.0
ring finger protein 19A	Rnf19a	30945	NM_013923	3.4227085	-1.715331	-1.81977	-0.020329587	0.03682913
WD repeat domain 90	Wdr90	106618	BC043315	3.4164555	-1.6067481	-1.3924139	0.35779166	0.19119355
DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	Ddx1	104721	NM_134040	3.4074156	-1.4896678	-1.9457297	-0.096918754	0.21765631
Notch-regulated ankyrin repeat protein	Nrarp	67122	NM_025980	3.399508	-1.6650763	-1.4783106	0.22295813	0.16985868

cDNA sequence BC1179090	BC117090	100038854	NM_001001332	3.3860202	-1.783462	-1.7361076	0.0	0.0
potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1	Kcnn1	84036	NM_032397	3.3845835	-1.6273736	-1.8264611	0.0	0.07013258
spectrin beta, non-erythrocytic 1	Sptbn1	20742	NM_009260	3.3652217	-1.8057846	-1.6807873	0.0131677175	0.0043566064
interleukin-1 receptor-associated kinase 4	Irak4	266632	NM_029926	3.361651	-0.76789445	-1.0398074	0.7531965	0.943935
transmembrane protein 69	Tmem69	230657	NM_177670	3.3511887	-1.6592692	-1.8320749	-0.091720365	0.08922388
				3.3473003	-1.8824319	-2.19569	-0.51860774	-0.088487215
zinc finger protein 868	Zfp868	234362	AK079745	3.3424897	-1.7448205	-1.7370363	0.0	0.0
telomeric repeat binding factor 2	Terf2	21750	NM_009353	3.3309846	-1.2869987	-1.1548356	0.5565053	0.47544506
multiple endocrine neoplasia 1	Men1	17283	NM_008583	3.3303554	-0.87981606	-0.91127723	0.7841716	0.8941631
TSR1 20S rRNA accumulation	Tsr1	104662	NM_177325	3.3231409	-2.0679715	-1.9654006	-0.19826747	-0.37350494
Rho GTPase activating protein 24	Arhgap24	231532	AK002660	3.3132532	-1.7230731	-1.6531113	0.0	0.08005023
calmodulin 2	Calm2	12314	NM_007589	3.2965856	-1.7906251	-1.6545284	0.0	0.0
				3.2949724	-1.159982	-1.3596145	0.32176545	0.5933125
ATPase family, AAA domain containing 1	Atad1	67979	NM_026487	3.2946699	-1.4551157	-1.4704313	0.19380859	0.31826943
RIKEN cDNA 0610042G04 gene	0610042G04Rik	68380	AK002909	3.27517	-1.6000806	-1.7778739	0.0	0.05022235
			AV080718	3.2720559	-1.7279354	-1.6926748	0.0	0.0
angiomotin-like 1	Amotl1	75723	AK016526	3.261146	-0.872723	-0.7946528	0.8780833	0.86633104
ATPase family, AAA domain containing 2	Atad2	70472	AK037651	3.2597084	-1.5750016	-1.753518	0.0	0.08522655
				3.2314727	-1.6517588	-1.7337909	0.0	0.0
angiotensin II receptor, type 1b	Agtr1b	11608	NM_175086	3.2300644	-1.5285312	-1.9537979	-0.2307304	0.13911498
aristaless related homeobox	Arx	11878	NM_007492	3.2285624	-1.069108	-1.2913437	0.4462313	0.58052653
zinc finger protein 592	Zfp592	233410	NM_178707	3.2259815	-0.49487254	-0.59545976	1.0954843	1.19374
biliverdin reductase A	Blvra	109778	NM_026678	3.2122946	-2.2912524	-2.8431752	-1.1319728	-0.6267806
RIKEN cDNA 4930589P08 gene	4930589P08Rik	67748	AK017048	3.2069905	-1.460527	-1.6449372	0.0	0.2518847

high mobility group AT-hook 2, pseudogene 1	Hmga2-ps1	15365	AK033703	3.2030365	-1.4026278	-1.5764494	0.06669046	0.30824608
unc-45 homolog B (C. elegans)	Unc45b	217012	NM_178680	3.1903477	-1.6388756	-2.434879	-0.7026057	0.0
desmin	Des	13346	NM_010043	3.178275	-1.4970404	-1.4351367	0.22808361	0.17642899
sphingosine kinase 1	Sphk1	20698	NM_025367	3.1775534	-1.5566369	-1.4314106	0.28618994	0.055065043
transportin 2 (importin 3, karyopherin beta 2b)	Tnpo2	212999	NM_145390	3.1541429	-1.6912712	-2.1466765	-0.5147488	-0.015833253
serine protease inhibitor, Kunitz type 1	Spint1	20732	NM_016907	3.1481166	-1.9068993	-2.6165965	-0.91831577	-0.27985674
RIKEN cDNA 2210016L21 gene	2210016L21Rik	72357	NM_028211	3.1382368	-1.6094453	-2.0327077	-0.41984215	0.06755956
coiled coil domain containing 178	Ccdc178	70950	NM_027616	3.133577	-1.4097131	-1.6119285	0.07090273	0.2069506
mitogen-activated protein kinase associated protein 1	Mapkap1	227743	NM_177345	3.1080291	-1.4020783	-1.425442	0.25435323	0.18949229
interleukin 12b	Il12b	16160	NM_008352	3.1012793	-1.5872145	-1.6183504	0.0	0.059712194
apoptosis-inducing, TAF9-like domain 1	Apitd1	69928	NM_027263	3.0936913	-0.7237882	-0.95002854	0.7178975	0.8717073
protein phosphatase 1, regulatory subunit 9B	Ppp1r9b	217124	NM_172261	3.0802414	-1.6230232	-1.5387673	0.077846214	0.0068057934
nebulin	Neb	17996	AK086142	3.0257704	-1.5013807	-1.7500092	-0.1738398	0.11347276
interleukin 12b	Il12b	16160	NM_008352	3.0172136	-0.6140344	-0.6668401	0.91796976	0.987241
regulator of calcineurin 1	Rcan1	54720	NM_019466	3.0007324	-1.5368806	-1.5489309	0.0	0.08363127
ubiquitin family domain containing 1	Ubfd1	28018	NM_138589	2.9940362	-1.473197	-1.5578538	0.0	0.13138397
CAS1 domain containing 1	Casd1	213819	BC010201	2.9746714	-1.986063	-2.4307573	-0.82610995	-0.43724182
protease, serine 52	Prss52	73382	NM_028525	2.9647543	-1.5717577	-1.5640776	0.0	0.0
RIKEN cDNA C130013H08 gene	C130013H08Rik	100327264	AK081392	2.9401562	-1.9024111	-1.5048468	0.039860193	-0.3319147
solute carrier family 35, member F5	Slc35f5	74150	NM_028787	2.9343903	-1.2247223	-0.8627656	0.6694871	0.354647
apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3	Apobec3	80287	NM_030255	2.9285905	-1.0103849	-0.9012393	0.6538483	0.53453827
				2.9285858	-1.51805	-1.5424625	0.0071996516	0.03268682

				2.9237368	-0.7882115	-0.942179	0.58989155	0.7736062
ubiquitin C	Ubc	22190	NM_019639	2.9202397	-0.55624646	-0.7175187	0.80913866	1.0069959
phosphatidylethanolamine binding protein 1	Pebp1	23980	NM_018858	2.9046772	-2.066221	-2.6320224	-1.0844096	-0.53411573
sodium channel, nonvoltage-gated 1 alpha	Scnn1a	20276	NM_011324	2.8742418	-0.7309387	-0.9360032	0.5777956	0.8003331
MX dynamin-like GTPase 2	Mx2	17858	NM_013606	2.857441	-1.3480943	-1.0361612	0.47498453	0.17105849

Supplementary table 2

gene ID	mean Hepa	mean EndoPCs	unlog Hepa	EndoPcs	fold change EndoPCs/hepa	Hepa1	Hepa2	Hepa3	EndoPCs 1	EndoPCs 2	Endo PCs 3
Ngfrap1	6,40	11,21	84,54	2378,33	28,13	7,60	6,34	5,26	11,33	11,18	11,14
Galnt3	4,97	9,27	31,39	620,04	19,75	5,31	5,07	4,54	9,17	9,25	9,41
Pcdhb17	3,46	6,58	11,03	96,15	8,72	3,35	3,49	3,55	6,45	6,52	6,79
Pax2	4,27	7,37	19,29	166,21	8,61	4,48	4,06	4,27	7,36	7,46	7,31
Atad2	5,39	8,44	42,02	347,53	8,27	5,55	5,20	5,43	7,85	8,72	8,75
Smc2	5,54	8,57	46,45	380,34	8,19	6,17	5,37	5,08	8,17	8,77	8,78
Kntc1	4,46	7,45	21,97	175,25	7,98	4,39	4,32	4,66	7,02	7,58	7,76
Pole2	3,63	6,62	12,41	98,56	7,94	3,54	3,58	3,78	6,29	6,71	6,86
Plekha2	4,33	6,95	20,15	123,54	6,13	4,69	4,56	3,74	7,10	6,93	6,82
Fem1b	6,31	8,73	79,46	423,18	5,33	6,31	6,49	6,14	8,87	8,61	8,70
Trp53	6,57	8,87	95,10	466,67	4,91	6,85	6,41	6,45	8,68	8,79	9,13
Ska1	4,82	7,02	28,26	129,38	4,58	5,17	4,30	5,00	6,72	7,18	7,15
Psrc1	5,20	7,30	36,84	157,37	4,27	5,60	4,46	5,55	7,45	7,02	7,42
Eef1g	8,40	10,29	338,90	1249,27	3,69	8,59	8,29	8,33	10,19	10,23	10,44
Rasa2	5,42	7,26	42,90	153,51	3,58	5,62	5,62	5,02	7,22	7,36	7,21
Igf2	5,71	7,44	52,42	173,64	3,31	5,71	5,50	5,92	7,08	7,34	7,90
Zfp37	4,00	5,73	16,04	53,05	3,31	4,03	3,68	4,30	5,22	5,92	6,05

Ppp1r9b	4,86	6,55	29,06	93,86	3,23	4,76	4,89	4,93	6,40	6,94	6,32
Hs3st1	4,20	5,88	18,39	58,76	3,20	3,80	4,65	4,16	5,88	5,98	5,77
Uggt2	4,32	5,95	19,92	61,91	3,11	4,47	3,96	4,52	5,86	5,93	6,06
Camk2d	5,43	7,02	43,22	129,35	2,99	5,74	5,39	5,17	7,18	6,95	6,92
Mapk3	8,07	9,65	269,12	803,35	2,99	7,90	7,91	8,41	9,89	9,39	9,67
Pign	6,20	7,77	73,44	217,79	2,97	6,40	6,00	6,20	7,58	7,70	8,01
Efna4	4,62	6,08	24,61	67,46	2,74	4,39	4,60	4,87	6,40	5,72	6,11
Rabggtb	5,25	6,68	37,96	102,88	2,71	5,47	5,64	4,63	6,37	6,70	6,99
Hmga2-ps1	4,54	5,94	23,29	61,37	2,64	4,49	4,24	4,89	5,23	6,40	6,19
Fbxo28	6,28	7,68	77,83	205,05	2,63	6,41	6,17	6,27	7,53	7,59	7,92
Zfp664	5,63	7,00	49,56	127,94	2,58	5,66	5,60	5,63	6,94	6,95	7,11
Pacs2	6,53	7,84	92,53	229,03	2,48	6,60	6,74	6,26	8,03	7,57	7,92
Snmp70	8,70	9,94	415,21	979,63	2,36	8,70	8,71	8,69	9,85	10,01	9,95
Naa20	6,83	8,07	113,66	267,98	2,36	6,68	7,08	6,72	8,02	7,88	8,30
Zfp868	4,86	6,04	29,10	65,99	2,27	5,11	4,43	5,04	6,19	6,07	5,87
Amotl1	5,51	6,68	45,50	102,71	2,26	5,46	5,58	5,49	6,70	6,48	6,87
Cic	6,66	7,81	101,26	225,17	2,22	6,71	6,69	6,58	7,91	7,73	7,80
Tubgcp6	5,41	6,55	42,55	93,42	2,20	5,22	5,38	5,63	6,26	6,66	6,72
Clasp2	6,03	7,14	65,32	140,70	2,15	5,88	6,09	6,11	7,03	7,07	7,31
Snapc3	6,27	7,37	77,04	165,51	2,15	6,21	6,43	6,16	7,12	7,49	7,49
Apc	6,42	7,52	85,81	183,10	2,13	6,53	6,52	6,22	7,48	7,52	7,55
Men1	7,07	8,16	134,48	285,48	2,12	7,27	6,89	7,05	8,32	7,90	8,26
Cdk12	6,97	8,04	125,51	264,01	2,10	7,25	6,92	6,75	7,94	7,89	8,30
Dhx15	8,99	10,05	508,31	1063,00	2,09	9,05	8,98	8,93	9,86	10,17	10,14
Pebp1	5,86	6,89	57,93	119,00	2,05	5,85	5,94	5,78	7,03	6,84	6,81
Chd3	10,70	11,73	1657,89	3385,38	2,04	10,80	10,83	10,46	11,82	11,78	11,57
Terf2	5,92	6,95	60,72	123,32	2,03	5,85	5,86	6,06	7,07	6,63	7,14
Calm2	11,03	12,04	2095,87	4215,92	2,01	11,12	11,15	10,83	12,05	12,10	11,98

Trp53bp1	5,85	6,84	57,85	114,84	1,99	5,87	5,62	6,07	7,00	6,78	6,74
Mbp	4,36	5,35	20,54	40,66	1,98	4,43	4,20	4,45	5,41	5,23	5,39
Tnpo2	7,48	8,44	178,01	348,35	1,96	7,47	7,65	7,31	8,18	8,45	8,70
Sbno2	6,12	7,07	69,47	134,07	1,93	6,35	6,08	5,92	7,20	6,95	7,04
Porcn	6,09	7,01	68,09	128,70	1,89	5,84	6,03	6,40	7,54	6,62	6,87
Bmp2k	6,45	7,36	87,15	163,96	1,88	6,81	6,50	6,03	7,19	7,52	7,36
Wdr90	4,92	5,80	30,23	55,84	1,85	4,81	4,95	5,00	5,68	5,76	5,97
Atp13a1	6,61	7,49	97,63	179,23	1,84	6,59	6,62	6,61	7,87	7,03	7,55
Hjurp	8,11	8,97	276,76	500,16	1,81	8,29	8,14	7,91	8,58	9,05	9,27
Omg	4,45	5,29	21,79	39,01	1,79	4,34	5,20	3,80	5,05	5,06	5,75
Nup88	7,40	8,23	168,47	299,32	1,78	7,59	7,19	7,41	8,10	8,29	8,28
Atad1	9,06	9,88	533,26	942,08	1,77	8,96	9,05	9,17	9,78	9,85	10,01
Rfwd2	8,90	9,71	476,79	835,21	1,75	9,05	8,91	8,73	9,55	9,74	9,82
Tsr1	7,39	8,20	168,13	293,52	1,75	7,48	7,54	7,16	7,91	8,27	8,42
Airn	4,38	5,17	20,84	36,12	1,73	4,68	4,08	4,39	5,29	4,88	5,35
Casp8	7,51	8,29	181,77	313,60	1,73	7,85	7,56	7,12	8,04	8,56	8,28
Bcl7a	6,24	7,02	75,69	130,11	1,72	6,38	6,35	5,99	6,76	7,11	7,21
AI413582	5,95	6,73	61,85	106,07	1,71	5,60	6,08	6,17	6,78	6,60	6,81
Ddx1	9,29	10,07	627,62	1072,16	1,71	9,28	9,37	9,23	10,02	10,07	10,10
Polr3f	5,54	6,31	46,57	79,20	1,70	5,70	5,67	5,25	6,22	6,15	6,55
Nup50	7,01	7,77	128,51	218,51	1,70	6,85	7,20	6,97	7,65	7,77	7,90
St3gal6	4,52	5,28	22,97	38,76	1,69	4,58	4,46	4,52	5,07	5,40	5,36
Shisa5	5,33	6,08	40,35	67,49	1,67	5,30	5,11	5,60	6,32	5,76	6,15
Ypel1	5,31	6,03	39,76	65,50	1,65	5,40	5,31	5,23	6,23	5,88	6,00
Arhgap24	5,22	5,93	37,23	61,15	1,64	5,06	5,59	5,01	6,01	5,95	5,84
Vasp	5,76	6,46	54,29	87,99	1,62	5,27	6,10	5,92	6,48	6,47	6,43
Rrad	4,98	5,68	31,61	51,15	1,62	5,15	4,63	5,17	6,16	5,50	5,38
Irs1	6,38	7,04	83,07	131,61	1,58	6,42	6,18	6,53	7,39	6,58	7,15

Wwp2	6,56	7,21	94,28	148,32	1,57	6,34	6,78	6,56	7,04	7,17	7,42
Apitd1	5,06	5,67	33,28	51,05	1,53	4,92	5,34	4,91	5,43	5,64	5,95
Ubxn7	7,35	7,97	163,54	250,43	1,53	7,19	7,46	7,41	8,12	7,62	8,16
Pacrgl	6,75	7,34	107,34	162,30	1,51	7,00	6,70	6,54	7,49	7,21	7,32
Mib1	8,10	8,68	274,07	410,92	1,50	8,11	8,21	7,98	8,61	8,69	8,75
Sphk1	4,76	5,34	27,18	40,45	1,49	5,07	4,49	4,74	5,63	5,13	5,26
Papola	8,24	8,76	301,94	432,31	1,43	8,30	8,33	8,09	8,69	8,82	8,76
Usp30	6,77	7,29	109,24	156,25	1,43	7,20	6,86	6,26	7,46	7,01	7,40
Gtf2a1	8,31	8,80	316,48	446,44	1,41	8,32	8,40	8,20	8,90	8,65	8,86
Taf1a	5,67	6,16	50,99	71,27	1,40	5,64	5,49	5,89	6,02	6,17	6,28
Eif4g1	9,46	9,94	705,20	980,84	1,39	9,51	9,44	9,43	9,83	9,98	10,00
Itsn2	7,25	7,72	152,28	211,09	1,39	7,67	7,19	6,89	7,71	7,71	7,75
Smg6	6,26	6,73	76,87	106,06	1,38	6,09	6,05	6,64	6,77	6,70	6,72
Des	4,34	4,77	20,21	27,35	1,35	4,43	4,34	4,25	4,73	4,61	4,98
Ube3c	7,66	8,08	202,88	270,39	1,33	7,63	7,71	7,65	8,05	7,99	8,20

PART 2

Completed in Lebanon

Chapter 6
Impact of Donor age (DA) on Mesenchymal Stem Cells functionalities

6.1. Impact of DA on liver transplantation

Twenty years ago, due to shortage in liver donor , an increase of transplanting livers from aged donors in United States and European countries was observed [390] (Fig.19) . Moreover, based on the latest European Liver Transplant Registry (ELTR) the growing of the waiting list has successfully contributed to attain a plateau of liver transplantation (LT) achieved all over Europe mainly between 2007 and 2013 (Fig.20).

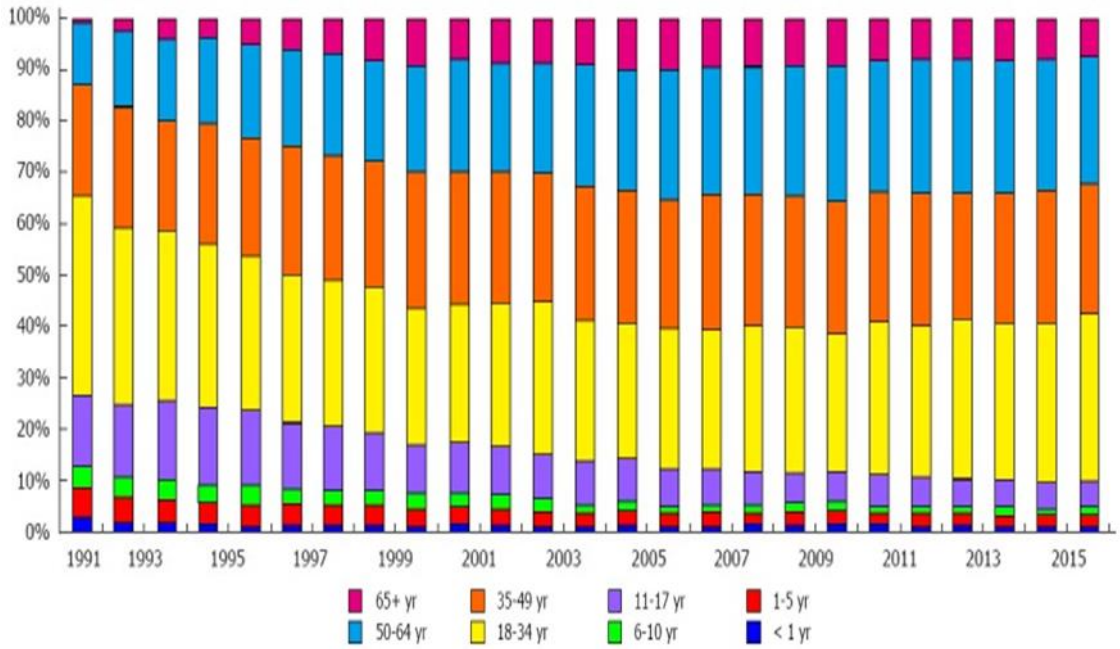


Figure 19: Change in distribution of donor age in recent years. Source: United Network for Organ Sharing reports, 2015

In addition, ELTR data associates the increasing of liver percentage coming from donors older than 60 years to a decrease of transplant survival [391]. Furthermore, following LT, several factors may affect liver regeneration related to donor and receiver age [390, 391]. Hence, liver graft survival was shown to be significantly increased when the organ was collected from a young donor. These results involved the implementation of donor age risk index concept for donors with specific LT criteria[72]. On the other hand, the impact of aging on human liver regeneration has been shown to be significantly poorer in elderly patients compared to younger patients [392]. Liver Stem cells were shown to play certain significant role in liver regeneration; Age-related senescence of liver stem cells was already discussed in the Chapters 3 and 4.

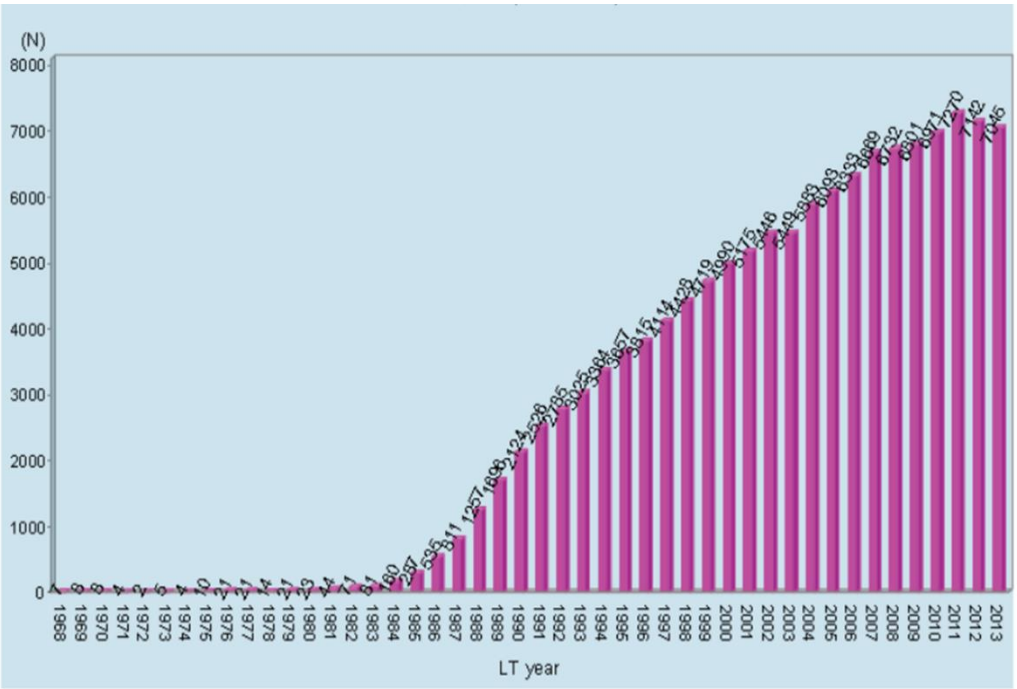


Figure 20 : Evolution of liver transplants frequency in Europe between 1968 and 2013; *From ELTR registry, 2016*

I will debate in this chapter also the impact of DA on the senescence of human MSCs. These stem cells represent a promising tool in cell-base liver diseases and they can reach senescence biomarkers *in vitro* similar *to those of individual chronologic aging in vivo*. Thus we will be highlighting on the senescence biomarkers of aged MSCs and their age-related niche status. .

6.2. Age- related biomarkers of MSC

In this context, the main research activities were based on the fact that regenerative potential of MSCs is downregulated with age leading to a limitation in their regenerative and hepatocytes' differentiation potentials. In the past few years, several studies suggested that DA influences various MSC properties[78, 393, 394]. Unfortunately, at present, there is no specific molecular marker specifying the grade of cellular aging in MSCs. Therefore, MSCs from human, mice, rat, monkey and pig were isolated from different sources such as BM, AT, UCB, DP, urine and nasal tissues to study the impact of DA on molecular and functional levels of MSCs. In this context, we will be reviewing the principal age-related biomarkers in MSCs discussed recently.

6.2.1. Cellular activity: Proliferation and Apoptosis

While the MSCs undertake senescence *in vitro* after 20 - 40 rounds of division called the "Hayflick" phenomenon [395], also cultured primary MSCs derived from aged people displayed a long time for expansion followed by an early stop of growth after 18 passages *in vitro*. This process is called MSCs senescence[396-398]. The proliferation activity was screened over different studies comparing both young and aged hMSCs' groups showing higher potential of the young hMSCs[79 399] . Other study on human nasal tissue-derived MSCs showed no significant variation in

proliferation rate over different age groups [400]. In 2014, this evidence was confirmed on human ADSCs where DA was shown to negatively impact expansion of MSCs derived from aged donors of fat tissue[78]. MSCs derived from aged donors of UCB have revealed a low growth with a less proliferation rate compared to the young group. The colony formation assay showed non-significant difference between both groups, however the young group had the ability to form better distinct colonies [401] . More recently, young human dental pulp MSCs showed an average of DP time significantly higher than aged population [402]. The decrease in proliferation rate give rise to an increase in cell size of elderly MSCs *in vitro* [398]. Choudhery et al [403] , 2012 showed that mice aged BM derived MSCs reached saturation density at a later stage than young MSCs with a lower number of cells. In fact, several factors control these processes: the cell cycle regression is related to a transcriptional up-regulation of genes regulating for example the tumor suppressor p53 signaling pathway. This pathway which promotes a decrease in proliferation rate and apoptosis in aged MSCs [403, 404]. Furthermore, Caspase-3 activity was upregulated in aged MSCs and then leaded to apoptosis[405]. Oct- 4 and telomerase activity were shown to be a key regulation parameter for cell activity , self-renewal and differentiation of MSCs [406, 407].

6.2.2. Surface Antigen expression

The modulation of the surface Ag expression of MSCs play important role which can be associated to DA. In both adult and young BM derived MSCs CD29 (unit of Fibronectin receptor), CD44 (receptor III of ECM), CD73, CD90 (membrane glycoprotein), CD105 (endoglin), CD106, CD166 (cell adhesion molecules) were positive while the hematopoietic surface markers CD45, CD34 and CD14/HLA-DR were shown to be negative [79, 402] similarly to the profile of human nasal MSCs [400] and human periodontal MSCs [408]. However, Age-related decrease of CD90 and CD105 expression was described in some references [409]. Otherwise, aged Monkey BM-derived MSCs showed a loss in CD44 expression similarly to aged human umbilical cord blood (UCB) and aminiotic fluid derived MSCs [401, 410, 411]. Furthermore, senescent human periodontal MSCs displayed a decrease in CD146 expression [402, 412] and positive CD271 (receptor of nerve growth factor) expression within the low proliferative CD90+/CD271^{low} MSCs subpopulation [408] . Thus, there is a lack of reliable surface markers for evaluating senescence status within MSCs[79, 399, 405]. Recently, aged MSCs have been shown to express leptin receptor (LR) promoting osteoporosis and adipogenesis in bone niche [413].

6.2.3. Telomere length and Telomerase activity

Telomeres are structures localized on the end of eukaryotic chromosomes. It was well observed that for the majority of cell types, cellular senescence is a result of telomere-dependent senescence, regarded as progressive telomere shortening and the loss of TTAGGG repeats, due to repetitive cell divisions [414-416]. Indeed, in senescent cells, telomeres reach a low threshold length [417]. However, stem cells and progenitors like tumor cells are known to express the human telomerase catalytic subunit (hTERT) which is a transcriptase responsible of the replication of telomere ends and maintaining the proliferative life span via the use of RNA to synthesize the G-rich repeats [418, 419]. In this way, human infant MSCs displayed long telomere length compared to older MSCs[80]. Moreover, Baxter *et al*. [420] correlated the loss of telomere during aging of hMSCs to the decrease of replicative rate , the stemness and the regenerative potential of MSCs. Furthermore, a negative correlation was widely cited between the long term span of adult hMSCs *in vitro* and the shortness of the telomere when compared to young hMSCs [398, 421]. On the other side, the ectopic expression of telomerase in MSCs enhanced their differentiation and regenerative potential compared to the same non-transduced MSCs [422]. In fact, the shortness of telomere avoids the binding of telomeric proteins on the chromosomes' ends and enhances DNA damage response and cell senescence. These aberrations originate p53 activation pathway and subsequently stem cell ageing *in vivo.* Conversely, the extinction of p53 in aged stem cells with telomere shortening is directly implicated in genome instability and oncogenic transformation by fusion of two chromosomes ends, broken chromosomes, translocations, and aneuploidy[407, 423-425].

6.2.4. Reactive Oxygen Species production (ROS)

The amount of oxidative damage increases with cell aging leading to senescence[426]. Production of mitochondrial and cytosolic ROS is well recognized to be key factors in MSC aging and the loss of stem cell properties. ROS accumulation results from DNA damage and down-regulation of a major group of genes related to mitochondria activating the p53 signaling pathway [79, 427]. Upregulation of ROS production in human MSCs impaired ATP production and telomere length and then promoted low proliferation and cell aging [428]. The estimation of the level of ROS particles was considered a crucial outcome to evaluate the use of hMSCs for cell-based therapies [429]. Furthermore, ROS has been shown to reduce the differentiation potential of MSCs and not their proliferation rate. Oxidants species result from endogenous or exogenous superoxide anion (O2 −) and hydroxyl radical (OH^- radicals) and damaged tissue MSCs by (i) increasing intracellular toxicity (ii) causing mutagenic damage and (iii) promoting cell ageing and senescence behaviors [430]. Phosphorylation of p38 a member of the MAPKs family, is described to be the regulator of ROS intracellular accumulation and thus activation of p53 [431, 432] . On the other side, phosphorylation of p53 is considered to be related also in ROS signal transduction. ROS promote the decrease of DNA binding activity of p53 by blocking the cysteine residues of p53 and lead to cell cycle arrest[433].

6.2.5. The loss of multipotency and stemness

In human ESCs and iPSCs, the intracellular stress (ROS) was associated with loss of proliferation and pluripotency [434]. Oct 4 and Nanog are essential to the self-renewal of MSCs and their differentiation and have been shown to be less expressed in senescent MSCs [406]. Loss of Nanog and Oct-4 are associated with organismal aging. Thus, their knockdown diminishes MSC proliferation rate and ROS production [406] while ectopic overexpression was shown to increase cell cycle division in addition to their osteogenic differentiation potential [435, 436] . Also in human ESCs and iPSCs, alterations in mitochondrial proliferation and development were associated with loss of pluripotency gene expression. Furthermore, the MSCs display ageing *in vitro* after a long span culture time when ESCs can be maintained in culture for a long period. In fact, in microarray analysis, the target genes regulated by Oct4 in ESCs and MSCs are different, explaining their variable culture conditions and the tentative of MSCs for senescence with aging or after long passages *in vitro* [406].

6.2.6. Age-related DNA damage and tumor suppression gene profile p53/p16 ^{INK4A}/p21

Histone deacetylases (HDACs) are a class of enzymes that catalyze the removal of acetyl groups of lysine rests in the histone tail regulating the balance between the chromatin status as euchromatin, the relaxed and transcriptionally active form, or the heterochromatin, the condensed packed form [395]. The inhibition of ESCs by high dose of trichostatin A (TSA) induces morphological and gene expression changes promoting differentiation resulting from repressive H3K27 trimethylation of H3K4 [437] . On the other hand, the p16 ^{INK4A} controls the G1-S phase and down regulates retinoblastoma protein (Rb) phosphorylation. Conversely, Rb phosphorylation leads to increased $p16^{INK4A}$ expression which is associated with aging in most mammalian tissues [438]. In aged MSCs, the HDAC inhibition is spontaneous epigenetic alteration and leads to activation of the p53 tumor suppressor and the (Rb) pathways, which trigger the cyclin-dependent kinase inhibitors $p16^{INK4A}$ and p21 respectively and accumulate damage DDR proteins such as H2AX and 53-BP1[430, 439-443]. In fact, the knock down of the expression of $p16^{INK4A}$ in aged MSCs, via DNA methylation, leads to recover their proliferation rate and spindle shape[444]. Also, the stimulation of senescence in human MSCs *in vitro* by irradiation, exhibits a senescence-associated SA- *β* gal with low p53, p21, and p16 gene profile expression [408, 445, 446] (Fig.21).

In this way, several studies were conducted to compare aged and young donors derived MSCs and showed an up regulation of cell cycle inhibition genes p53, $p16^{INK4a,}$ p21 with the increasing of the donor aging [403]. Particularly in human, periodontal MSCs $p16^{INK4a}$ expression was shown to impair the high proliferation in non-senescent MSCs and thus p53 was only expressed in senescent MSCs [408]. Recently, amniotic fluid derived MSCs showed, after induced senescence *in vitro,* an increase in SA- *β* gal activity and an enlargement of cell size [411]. These data demonstrated that the HDAC activity and the derived methylation of DNA are involved in the *in vivo* aging of MSCs. Moreover, at genomic level, 31 over 5000 genes are shown to be miRNA expressed exclusively in senescent MSCs and regulating the inhibition of cell growth and proliferation, cell cycle, cell death and cellular movement [447].

Parallel to the epigenetic alterations, a lot of reports showed that age-related senescence in hMSCs is highly influenced by their secretion of ROS triggering the activation of p53, p21 and p16 pathways promoting apoptotic response. The activation of DNA damage response (DDR) maintained the senescence in Aged MSCs when simultaneously the shortness of telomeres sustained this damage[448-450]. The understanding of these mechanisms is crucial to evaluate the functional consequences of these stem cells when used for tissue regeneration and for pharmacological assays [451]. Indeed, even aged MSCs remain alive *in vitro or in vivo*; they lose their function, self-renewal and proliferation capacity and show apoptosis state.

Functionally wise, several studies were conducted to prove the impact of age-related paracrine effects on immunomodulatory cytokines secretion, the tumor progression and the differential potential of the aged hMSCs. Thus, the impact of DA on MSCs functionalities will be discussed thoroughly within the next sections.

6.3. DA impact on MSCs functionalities

6.3.1. Paracrine activity and niche aging: Role of MSCs derived exosomes

Tissue renewing ability drops with age in muscle, blood, liver and brain. These cues are attributed to a limited response of tissue-specific stem and progenitor cells. To restore the in situ aged tissue and successfully perform the cell-replacement therapy in aged niches, it will be crucial to understand and discuss the age-related factors leading to organ environment deterioration. The organogenesis approach is to differentiate MSCs *in vitro* into the cell type of interest such as neuronal, endodermic, endothelial, hepatic, and pancreatic and others. These MSCs derived differentiated cells are intended to replace the dysfunctional counterparts *in vivo.* Recent data have shown that tissue replacement therapy failed in repairing organs and functions in elderly people. The extracellular niche seems to be less hostile for stem cells *in vivo*. From a long time, the age of the host niche interfere in the success of regenerative potential of precursor cells in old animals[452]. Since the regenerative potential of liver was well shown to be impaired with aging in human and animals, studying the biological niche was crucial to identify molecular mechanisms involved in stem cell properties, cell cycle and differentiation potential of stem cells. Indeed, aged niche displayed strong negative impact in hESCs and human adult stem cell muscle regenerative potential [452]. Aging inducs hormonal deregulation and especially Estrogen (Est) in human. The decrease in Est promotes mainly osteoporosis, neurodegenerative and age related diseases [453]. It was shown that this hormonal disturbance induces spontaneous differentiation of MSCs to adipocytes into osteoporosis Bone Marrow niche[454]. On the other hand, this systematic humoral deregulation is associated to inflammatory changes with increasing of DA. Human serum levels of inflammatory cytokines IL6 and TNFα are elevated in aged people and associated with disorders of MSCs differentiation [455]. Thus, Pawelec et al [456], called this mechanism "inflammaging".

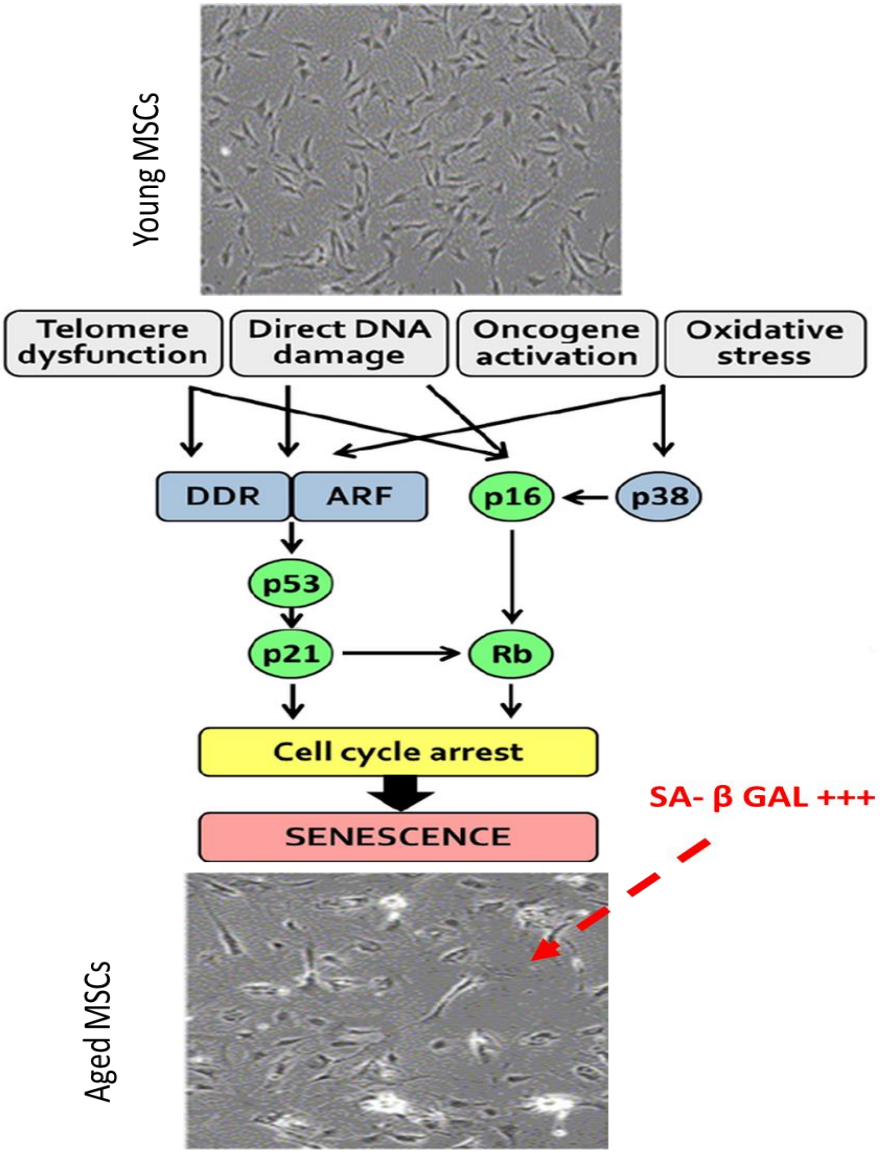


Figure 21: Morphological and molecular mechanisms involved in MSCs senescence

The observation that the decline of MSCs regenerative potential in periodontal tissues in elderly was associated with loss of teeth [457, 458] tapped the question mark about the signals affected in situ [73] . Aged MSCs in bone niche exhibited a misdirected differentiation into adipocytes leading to osteoporosis [75]. Moreover, recent observations showed that transplanting young mice BMSCs in old bone niche displayed adipogenic differentiation instead of osteogenesis [459]. However, ADSCs and dental pulp MSCs didn't show osteogenic defect in another microenvironmental niche[460]. The cytokines involved in the impairments of MSCs differentiation are TNFα and IFN-γ[461]. The Knock in of TNFs in MSCs mice model showed a decrease in osteogenic markers[462] .Conversely, the exhaustion of TNFα or IFN-γ lead to the reappearance of the osteogenic potential of MSCs[454]. Thus, the composition of each niche defines the fate of MSCs regenerative potential in aged population.

The biochemical composition of Young MSCs was shown to be more concentrated in saturated lipids, proteins, glycogen, and nucleic acids than aged MSCs [463]. The Collagen expression increased in fetal monkey MSCS relatively to the adult MSCs counterpart [410].

The secreted vesicles, called senescence-associated secretory phenotype (SASP), contain pro-inflammatory cytokines causing chronic inflammation and apoptosis via production of strong oxidants (Fig.22). The worst cases is when oxidative damages lead to suppression of immunity and promote cancer and malignant phenotypes [464, 465]. The SASP secreted by aged MSCs were shown to contain LEPTIN, INFγ, TNFα and a number of factors exacerbating inflammatory response [79, 466, 467]. Young MSCs were shown to produce expressively higher levels of neovascularization cytokines such as VEGF [403] , bFGF and IGF and HGF [405] compared to aged MSCs. Recently, senescence of MSCs showed to influence their therapeutical potential and their immunoregulatory activity *in vitro and in vivo*. Data demonstrated that graft of senescent MSCs secretes pro-inflammatory signals, INFγ and TNFα that can promote systemic lupus erythematosus , coronary artery disease, inflammatory bowel disease and several inflammatory diseases [468] . In addition, Pan et al. [469] showed that lower expression of TNF receptor superfamily in human liver cell line, in the presence of functional young MSCs , decreases the hepatocytes apoptosis in perfusion liver injury model. These observations indicate the impairing of paracrine activity with DA of MSCs. Recently, it was proposed that the regulation of the paracrine biology of aged MSCs , their exosomes and mitochondrial transfer for tissue repair, constitute a main issue for the future perspective of MSC-based therapy[65, 470]. The exosome composition is enriched in cholesterol, sphingomyelin, ceramide, lipid proteins and RNAs and varies within population and age. However, they conserved, no matter the MSCs source, a set of proteins including the tetraspanins (CD81, CD63 and CD9), heat-shock proteins (HSP60, HSP70 and HSP90), ALIX and tumor susceptibility gene 101 (TSG101) [471]. The inflammation atmosphere and ROS production raising in aged MSCs change the surface and intracellular content of exosomes and therefore decrease their regenerative potential[472].

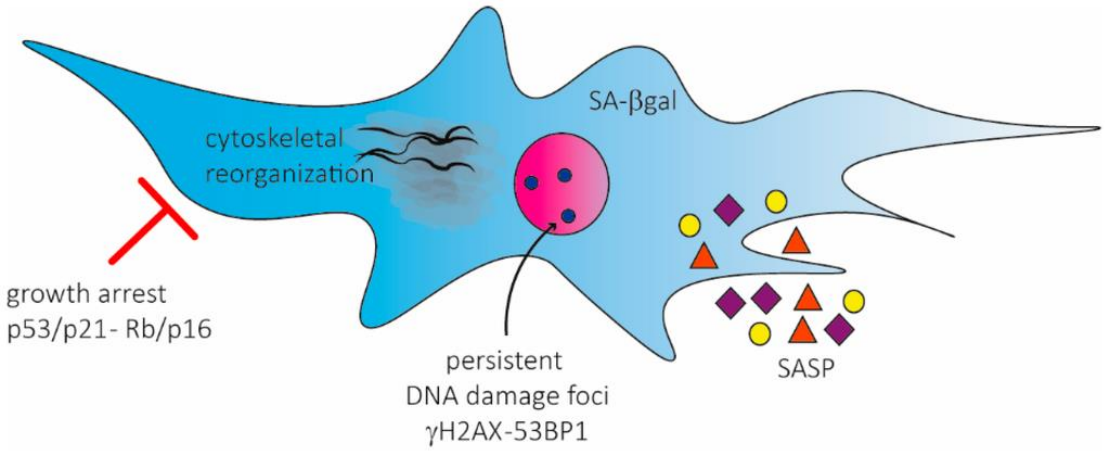


Figure 22: Age-Senescent Biomarkers in human MSCs. SA-βgal: senescence-associated-galactosidase, SASP: senescence-associated secretory phenotype, γH2AX-53BP1: Proteins resulting from epigenetic damage pf DNA [473]

Several data suggested that SASP secretions including exosomes deregulated cell-cell interaction in their niche and promote the neo-formation of malignant epithelium, then can enhance invasion and promote EMT transition and the growth of tumors in vivo[474-477]. More deeply, IL6 and IL8 are shown to be mediators for tumor activity of aged hMSCs promoting breast cancer *in vivo* within a co-transplant xenograft mouse model [478]. Moreover, aged MSCs exhibited a declined immunosuppressive function when CD3/28 and IL-2 were treated to induce the activation and proliferation of T cells [479]. Indeed, in aged MSCs, pro-inflammatory cytokines (IFNγ) and (TNFα) triggered the phosphorylation of the p38 MAPK and declined the immunomodulatory ability of MSCs gradually[480]. However, some mechanisms remained unclear in aged MSCs and the maintenance of their senescence *in vitro* was well exposed by Sui et al [453], recently as follow "Why do MSCs from an aged or inflammatory environment *in vivo* continue to demonstrate impairments ex vivo despite normal culture media? Does cellular memory exist? Is it attributed to the epigenetic changes provoked by pro-inflammatory cytokines or hyperglycemia *in vivo?*" They concluded that the establishment of 3D microenvironment for MSCs with the good immune atmosphere is other crucial key parameters for cell replacement liver therapy.

6.3.2. The deficiency of differentiation and regenerative potential

Ageing of MSCs was shown to negatively impact their osteogenic lineage. The Osteocalcin and RUNKX 2 expression were significantly affected with DA [402, 481] . Aged UCB derived MSCs showed a red retaining of Alizarin Red staining and less calcium deposits typical to bone compared to the young group [401]. In fact, data showed that the differentiation potential is affected with age due to a decrease in expression of genes involved in Wnt and TGF/BMP[482]. On the other hand, cystosolic ROS accumulation in aged hMSCs has been proposed as a cause for decrease in osteogenic potential [483]. However, human nasal MSCs didn't show the same osteogenic potential irrespectively to DA[400] . Furthermore, young MSCs were shown to have a significant ability to tube-forming and thus to have better proangiogenic ability than aged MSCs [403]. *In vivo* osteogenic assay using periodontal human MSCs confirmed that aged MSCs lose their osteogenic potential[412]. In rabbits, the chondrogenesis potential of MSCs was shown to decline with age [484]. Similarly, Bari et al. [485], observed that spontaneous chondrogenesis *in vitro* was only initiated in young MSCs patients. Moreover, even hMSCs from older patients retain their chondrogenic differentiation capacity and lose part of their synthetic capacity and early apoptosis [485]. Concerning the adipogenic potential of MSCs, several data showed no correlation with DA [401]while human periodontal MSCs showed an impairing in adipogenic potential with ageing of the donor [402, 412] . Accordingly, Al Raeis et al[408], showed that high proliferated (non- senescent) periodontal CD31-/CD90+/ $CD271^{LOW}$ MSCs exhibited a higher multipotency to the adipo, chondro and osteogenic lineages wheras CD34+/ $CD271^{high}$ showed neurogenic lineage commitment. On the other hand, the neutrotrophic ability of aged MSCs was closely correlated with the age niche of the donor [486] .

6.3.3. Migration and adhesion ability: Implication of Cdc42 in stem cell aging

The signaling pathways regulating the ECM homeostasis are deregulated with aging of Stem cells. To assess the impact of DA on MSCs migration in vivo, different studies showed that the long-term cultivation of MSCs (inducing senescence in vitro) promotes high decrease in migration ability and homing compared to their primary cultures[79, 466]. After 72h, young rat MSCs displayed higher migration and injury healing than old MSCs in culture [403]. Also, human periodontal derived MSCs from young donors showed a significant increase of migratory potential over elderly derived MSCs [412]. A lot of reports studied the negative impact of DA on MSCs migration capacities[76, 243, 487-489] but rare are the papers describing the molecular mechanisms involved in migration pathway. Moreover, other citations showed that loss of migration potential in aged MSCs can be related to a down-regulation of some cytokines receptors preventing the recruitment of MSCs to the lesion site [490]. Aged stem cell were reported to reduce adhesion to stroma cells in their niche and the ability to homing upon transplantation[84, 491, 492]. Data described the adhesion receptors such as N-cadherin, integrin, VCAM and CD44 are altered with aging of adult stem cells [493] . Moreover, deficiency in Ca leads to a failure in Collagen 1 adhesion of stem cells in the niche [494].

Recently, the loss of migration ability was correlated to a defect in cytoskeleton organization and actin formation, which is mainly regulated by the Rho GTPase and which was described to be strongly affected with MSC age [79, 85, 495]. In fact, the RhoGTPase proteins promote the fillopodia and focal complex formation for cellular migration[79]. These outside-inside interactions between the receptors and the family of small Rho GTPases are deregulated over the stem cell aging and affect the intracellular environment. A family member from RhoGTPase family, the Cell division cycle 42 (Cdc42) protein was shown to be activated in aged HSCs and never in young HSCs and to promote less adhesion [85]. Furthermore, the knock-out mice model presenting an elevated activity of Cdc42 in HSCs, demonstrate a remarkable decrease of adhesion of these stem cells [84]. Thus, we speculate that the reduced stromal adhesion of HPCs from aged animals might be a consequence of elevated activity of Cdc42 in HPCs in aged mice.

CHAPTER 7
Hepatogenic potential of Adipose Tissue Mesenchymal Stem cells (ADSCs)

7.1. General debate about Hepatogenic potential of MSCs and their molecular characteristics

Many approaches have highlighted a link between MSCs and hepatic fate by explaining and elaborating the importance of MSCs as a viable alternative to human adult hepatocytes. Therefore, this characteristic of MSCs has offered a new hope for autologous cell therapy with low ethical problems and a wide range of applications in chronic and acute liver diseases. Indeed, undifferentiated hMSCs express LPs markers involved in liver development and endodermic specification [57]. Cellular interaction with the mesenchyme is required for endodermal cells to differentiate into hepatocyte raising the question about the link between mesenchymal cells and the hepatocytes' formation[496] . Thus, this approach was raised again few months ago with Asai et al, 2017 [497], by inducing hepatic organoid from iPSs and showing that mesodermal paracrine signals promote hepatocytes maturation of endodermic progenitors. Petersen and coworkers [270] were the first to describe that resident liver stem cells termed "oval cells" in rodents and "ductal reactions" in human express markers previously believed to be hematopoietic stem cells (HSCs) and might be derived from the bone Marrow .

Although these intrahepatic progenitors proliferate in response to liver injury, they are not sufficient to repair liver failure. Furthermore, Herrera and coworkers, described a population of human pluripotent resident liver stem cells (HPLSCs) able to contribute to injured liver regeneration when injected to mice with acute liver failure induced by acetaminophen [498]. These cells, contrary to primary hepatocytes, are able to grow in culture for up to 30 passages. HPLSCs express CD29, CD90, CD73; CD44 MSCs (MSCs) markers associated to liver specific markers such as KRT8/ KRT18 and are negative for hematopoietic markers CD34 and CD45. Moreover, these MSCs like cells are capable to differentiate *in vitro* to mature hepatocytes when cultured in the presence of FGF4 and HGF (hepatocyte growth factor) and to repair liver injury in CCL4 induced liver fibrosis [499].

On the other hand, MSC exosomes were described to activate several signaling pathways important in liver regeneration (Akt, ERK, and STAT3) and to induce the expression of a number of growth factors such as HGF and insulin-like growth factor-1 (IGF1)[500]. Fascinatedly, exosomes isolated from hiPSCs -derived MSCs were shown recently to protect liver against liver injury and participate to hepatocytes regeneration[501]. These evidences support a strong link between MSCs and their implication in the hepatogenic and liver regeneration potential. The table 2 below reports the recent application of MSC-derived exosomes in liver diseases and thus supports a strong link between MSCs and their implication in the hepatogenic and liver regeneration potential. Interestingly, as stated previously, MSCs are considered to be a promising therapeutic tool for liver disease based on (i) their ability to differentiate or to fuse with hepatocytes (ii) their immunomodulatory and immunosuppressive effect providing a safe source and (iii) their paracrine potential via the secreted growth factors and cytokines which promote an endogenous regeneration potential and inhibit hepatocellular apoptosis and stimulate liver regeneration [60]

In the following sections, the evidences considering hMSCs as a promising tool for liver regeneration and drug screen testing will be discussed thoroughly

Table 2: Implication of MSCS derived exosomes in liver regeneration and nanovesicles therapy

Source of MSCs	***Animal strain***	***Disease model***	***Functions of MSC's derived exosomes***	***Ref***
human Umbilical cord	Kunming mouse	CCl_4-induced liver fibrosis	Inhibit hepatocytes EMT and collagen production through inactivating TGF-β1/Smad pathway	[502]
Chorionic plate MSCs	Sprague–Dawley rat	CCl_4-induced liver fibrosis	Impede Hh signaling activation in hepatic stem cells s by inhibiting Smo expression	[503]
hESC-derived HuES9.E1 MSCs	C57BL/6 mouse	CCl_4, APAP or H_2O_2-induced liver injury	Increase hepatocyte proliferation by upregulating proliferation proteins and anti-apoptosis gene	[504]
Adipose tissue	Fischer-344 rat	Rat N1S1 cell-bearing orthotopic HCC model	Suppress HCC by promoting NKT cell anti-tumor responses	[505]
miR-122/CCNG1, ADAM10, etc.	BALB/c nu/nu mouse	HepG2 cell xenograft nude mice	Sensitize HCC to chemo therapy	[506]

7.2. Mesenchymal stem cells: safe alternative for liver resident stem cells and induced hepatocytes from pluripotent stem cells

Evidence from several studies indicates the presence of resident stem cells in the adult liver [10, 507]. The human LPs cell activation has been observed during acute and chronic liver diseases but was insufficient to perform complete liver regeneration. Various alternatives of stem cells sources have been used in the recent era to produce a large number of functional hepatocytes in vitro intended to be used in drug toxicity screening or for therapeutic applications. Several studies have described the differentiation of the embryonic stem cells (ESC) into hepatocytes-like cells (HLCs)[11, 12]. Different contributions have been made for optimizing the combinations and sequences of inducers used for the process of ESCs differentiation into hepatocytes [12]. Despite the ESCs gold standard potential for cell lineages differentiation, their clinical application is associated with practical and ethical concerns. These cells when injected into mice generate teratoma and majority of strategies lead to uncontrolled processes of differentiation resulting in heterogeneous differentiated cell populations [13].

While Samira Asgari et al[14], described the induced pluripotent stem cells (iPS) as a new era of hepatology, Si Tayeb et al., explored for the first time the possibility of generating murine and human hepatocytes from iPSCs reprogrammed from foreskin fibroblasts by lentiviruses transduction for the expression of OCT3/4, SOX2, NANOG and Lin28. These hepatocytes – like derived from iPSCs showed an incomplete cyp450 repertoire compared to primary hepatocytes. IPSCs-like ESCs can proliferate indefinitely without loss of potency and give rise to cell line diseases model. However, for drug toxicity evaluation, the advantage of using iPSCs relies on their ability to provide a source of autologous hepatocytes[14, 15, 508]. In addition, a new approach generated vascularized and functional human liver buds (LB) containing immature specific hepatic cells organized in 3D structure between endothelial and MSCs[319]. These IPSC-LBs were capable to connect to animal host vessels within 48 hours and differentiate into functional tissue resembling the adult liver. The main aim of this trial was to highlight on the huge therapeutic potential of using generated liver buds for liver failure transplantation without using iPSCs as hepatocytes cell source.

Indeed, even iPSCs have, as previously mentioned, a high differentiation potential noting that (i) iPSCs cell lines are incompletely reprogrammed and maintain exogenous transgene expression which can interfere with differentiation protocols [16] and (ii) iPSCs-hepatocytes show fetal-like properties in the majority of the generated population. These facts would absolutely limit iPSCs therapeutic translation and therefore would demand new 3D approaches to generate efficient iPSCs-hepatocytes which remain excessively complex strategies and are restricted to highly specialized labs [321] Moreover, Sekiya and coworkers showed that the activation of hepatic gene keys, notably HNF4α and Foxa2 , in murine fibroblasts leads to the generation of Induced hepatocytes-like (iHep) , although the expression level of liver enzymes differed from those in adult mouse hepatocytes, the generated hepatocytes-like were capable to secrete Alb, synthetize triglycerides, produce urea, also possess Cyp450 activity upon drugs induction and were able to repopulate mice liver after transplantation [509]. In addition, several hepatic gene transfer strategies were described recently converting ESCs, Fetal hepatic progenitors, adult stem cells and somatic cells into hepatocyte-like cells[12] . However, all these hepatocytes-like are always limited by the conservation of some gene expression in the initial ESCs intended to be converted , by the association of genetic disorders due to viral infection used to overexpress the hepatic genes and by the fact that it's impossible to maintain all functions and gene expression levels similar to that of mature hepatocytes in vivo [189].

In summary, all these previously elaborated strategies introduce specific hepatic factors and genes by viral, chemical and DNA-mediated transfer, which have restricted clinical use due to safety and ethical issues. . Based on all these previous limitations, finding other hepatocytes alternatives with low ethical risks, ease of availability and safe plasticity or trans-differentiation was mandatory. hMSCs have been emerged as a promising tool and an attractive stem cell source for cell therapy [507]. MSCs were reported largely for their multipotency and their ability to differentiate into hepatocytes and liver failure repair [290]. The hepatic differentiation potential of MSCs may deliver an infinite source of cells for hepatocyte replacement therapies [510, 511]. Human Wharton Jelly derived MSCs were reported as an interesting stem cell population to develop human hepatocyte-like cells expressing before differentiation transcriptional factors involved in liver development such as GATA4, GATA6, SOX9 and SOX17 and LPs markers such as K19 were [57]. Moreover, although the proliferation rate is shown to be different within different types of MSCs, isolated MSCs from human BM , adipose tissue and amniotic fluid (fetal MSCs) were able to be differentiated *in vivo* and *in vitro* into functional committed hepatocytes [6, 507, 512]. Many studies were conducted to evaluate the best source of MSCs for stem cell therapy taking into consideration the invasive procedures and the frequency of stem cell content especially in aged patients [59]. Therefore, the following paragraph will focus on the importance of hADSCs as an appeal source for liver cell-based therapy and the effect of DA on their molecular behaviors.

7.3. ADSCs as a potent source of human hepatocytes generation : Contribution of exosomes and Wnt pathway

Under specific culture conditions, MSCs possess differentiation potential ranging from mesenchymal related multipotency to endodermal (hepatic fate) competency. Therefore, ADSCs, is derived from embryonic mesoderm and contains an attractive stem cell population within the stromal vascular fraction (SVF) [513]. Even though human ADSCs have similar phenotype and behaviors to BMSCs *in vitro* and *in vivo* [514-517], ADSCs represents a rich source of MSCs and is accessible with minimal patients' discomfort following a liposuction procedure [513]. More interestingly,

compared to ESC and iPSCs, ADSCs is showed to be more advantageous in regard to ethical and safety issues for hepatic regeneration and Hep-Dif [62].

Briefly, ADSCs were shown to be a better therapeutic implement for liver disorders, as well as an alternative hepatocytes source *in vitro* with potential use in preclinical drug testing based *on* several evidences as follow:

First, hepatocytes derived from ADSCs resisted to Hepatitis B infection during differentiation more than their BM derived counterparts. Thus, compared to BMSCs, ADSCs may be alternative source of stem cells' for hepatitis B treatment[518].

Furthermore, ADSCs derived exosomes are considered as a new challenge in exosome-based therapies for liver disease underlining the strong molecular mechanisms shared between ADSCs and liver environment [65]. human ADSCs exosomes were shown very recently to promote fibroblast migration and tissue healing [519] as well as yielding a benefit tool for liver fibrosis and ischemic liver injury in animal model[520] . Indeed, ADSCs derived exosomes were described to act by cell-cell micro-communication via containing paracrine factors improving angiogenesis and regenerative potential in liver diseases [65 521] (Table2).

Taléns-Visconti and coworkers showed that ADSCs derived hepatocytes have a higher proliferation capacity and a longer culture period compared to BMSCs differentiated in the same culture conditions [64]. Besides, several reports showed that hADSCs were rapidly induced into hepatocytes (iHep) *in vitro*; iHep showed a polygonal shape, co-expressed albumin and AFP, uptake LDL and stored glycogen; and also showed maturity and functionality with ability to repopulate and regenerate liver function of acute liver-injured in a mice model [522-526]. Moreover, a comparison between MSC-derived hepatocyte-like from adipose tissue, UCB and BM showed that ADSCs give rise to hepatocytes with more consistent gene expression and hepatogenic profile[524]. Interestingly, microarrays analysis showed a high similarity of gene clusters between ADSCs derived hepatocytes and whole liver. In addition, signaling pathways regulated by the common genes were relevant to liver functions such as cyp450 drug metabolism and a typical MET cascade [508, 527]. *In vivo* engraftment of ADSCs modulates kupffer cell (liver macrophages) activity to inhibit the TNF*α* secretion. This immunosuppression potential of ADSCs improve their therapeutic potential in liver injuries [528]. The transplantation of GFP-hADSCs derived hepatocytes in the periportal region of the lobule of mouse host liver showed Alb expression and similar landscapes of human hepatocytes and long term integration *in vivo* compared to non-differentiated grafted hMSCs [529]. In the same way, hADSCs derived-hepatocytes were able after 10 weeks of engraftment, to replace 10 % of the host hepatocytes where hBMSCs didn't repopulate more than 1%. [530]. Additionally, the hADSC-derived hepatocytes were discussed as a potential tool study drug toxicity expressing hepatogenic functional gene profile for drug metabolism and hepatic protein secretion [63]. Interestingly, Wnt signaling was reported to regulate the BMSCs and ADSCs differentiation into hepatocytes. More specifically in BMSCs, Wnt-1, Wnt-5a, Frizzled1, DSH (disheveled), GSK3β beta (glycogen synthase kinase 3 beta) and β-catenin where shown to be elevated up to day 11 of differentiation then they decreased at the end of differentiation. In a next step , the activation of Wnt pathway impaired the Hep-Dif of BMSCs while Wnt inhibition showed earlier albumin expression in the BMSCs derived hepatocytes[531]. These findings means that downregulating of Wnt pathway promote BMSCs differentiation into hepatocytes within the endodermal lineage. In order to explore the role of Wnt signaling in the differentiation of ADSCs into hepatocytes, Huang etal[532] showed recently that the inhibition of the GSK3 alone was sufficient to increase the β-catenin in a manner with activation of Wnt/β-catenin signaling. This mechanism triggers the expression of definitive endoderm

specific genes GATA4, FOXA2, and SOX17 significantly. These ADSCs derived- endodermic progenitors were directed to differentiate into hepatocyte-like cells after further combinations of soluble factors and were able to express important drug-metabolizing cytochrome P450 (CYP450) enzymes with great albumin secretion and LDL uptake activity. Similarly to BMSCs and UCMSCs [533], these findings propose that activation of Wnt/β-catenin signaling during the endoderm specification represent a crucial mechanism intermediating hADSCs differentiation process. However the Wnt/β-catenin OFF mode is crucial for hepatocytes specification from hADSCs.

7.4. Impact of DA on regeneration potential of ADSCs

In this section, some reports focusing on the effect of DA on human and animal ADSCs will be discussed in order to elucidate the impact of DA on their regenerative potential. Pulmonary inflammatory lesions treated in aged mice with young ADSCs showed a decrease in fibrosis, lower TNFα secretions' level and increase in collagen synthesis [76]. Other reports compared hADMCSs derived from young and eldered donors and observed that ROS and NO levels increase with aging in human ADSCs. Aged ADSCs demonstrated SA-β-gal activity, enlarged morphology, and p53/Rb protein upregulation [77, 78]. Moving to another immunomodulator characteristic of hADSCs in neurodegenerative disease and particularly in the multiple sclerosis associated with Encephalomyelitis (EAE), it was shown that ADSCs derived from aged donors failed to ameliorate the neuro-rejuvenation status. However, young ADSCs provided HGF secretion and neuroprotection and remyelination in EAE mice [487]. Similarly to human BM derived-MSCs, aged hADSCs revealed a progressive decrease in the angiogenic capacities in a mouse ischemic model. In a first step, the elderly hADSCs failed to differentiate in endothelial-like cells and to secrete angiogenic cytokine such as VEGF. In a second step, the increase of DA reduced the angiogenic abilities of hADSC in ischemic model; however hypoxic preconditioning overturned the negative effect of aging by modulating the ROS paracrine activity in aged ADSCs. The transplantation of hypoxia-ADSCs enhanced neovascularization after induced ischemic injury [534]. Vilaboa et al.[535], demonstrated that women donor age impacts dramatically the cell yield isolated from lipoaspirates and thereby further studies were suggested to be conducted for specific age-related factors to explain these results. Furthermore, the transplantation of Young ADSCs in lung fibrosis-induced old mice decreased fibrosis level, collagen deposition, ROS accumulation and apoptosis markers where old ADSCs didn't reduce fibrosis neither senescence markers [76]. Furthermore, hADSCs *in vitro* -induced exhibited mitochondrial dysfunction, endoplasmic reticulum stress and altered lipid profile with a higher ratio of mono unsaturated over polyunsaturated fatty acids[82].

7.5. Impact of DA on differentiation potential of ADSCs

Within basic differentiation potential investigations, several reports demonstrated that aged ADSCs have a great loss in osteogenic, adipogenic and chondrogenic potentials[78-80]. Although adipogenic differentiation of ADSCs was demonstrated in few reports to be independent from DA [78, 536], conversely some studies reported that adipogenic potential of ADSCs increased in incidence with age. Thus, the DA showed a negative impact in osteogenic and chondrogenic potential bone and joint diseases [394]. Several questions were raised about the utility of aged ADSCs intended to be differentiated *in vitro* since numerous studies considered strongly the negative impact of DA of ADSCs on their therapeutic potential in several diseases. Moreover, the Chondrogenic differentiation of ADSCs in a clinical and epidemiologic experience showed that young DA of ADSCs might be the key index to complete

cartilage regeneration in osteoarthritis patients [394]. In fact , The CFU-F, the proliferation rate, the population doubling time (PDT) and differentiation abilities decrease in aged ADSCs [81] . Also, the genes related to senescence such as cyclin-dependent kinase inhibitor $p16^{ink4a}$ increased with age of ADSCs. The microarray of aged ADSCs showed abnormal expression of mir-RNA. Also, a correlation was observed between the age increasing and the low quantity of ADSCs resident stem cells, with low capacity of self-renewal and differentiation [82]. In a Monkey model, ADSCs showed a decrease in stemness with the age, increase in p21 expression and apoptosis with IL6 expression. In addition, Young ADSCs population resisted to senescence *in vitro*[83].

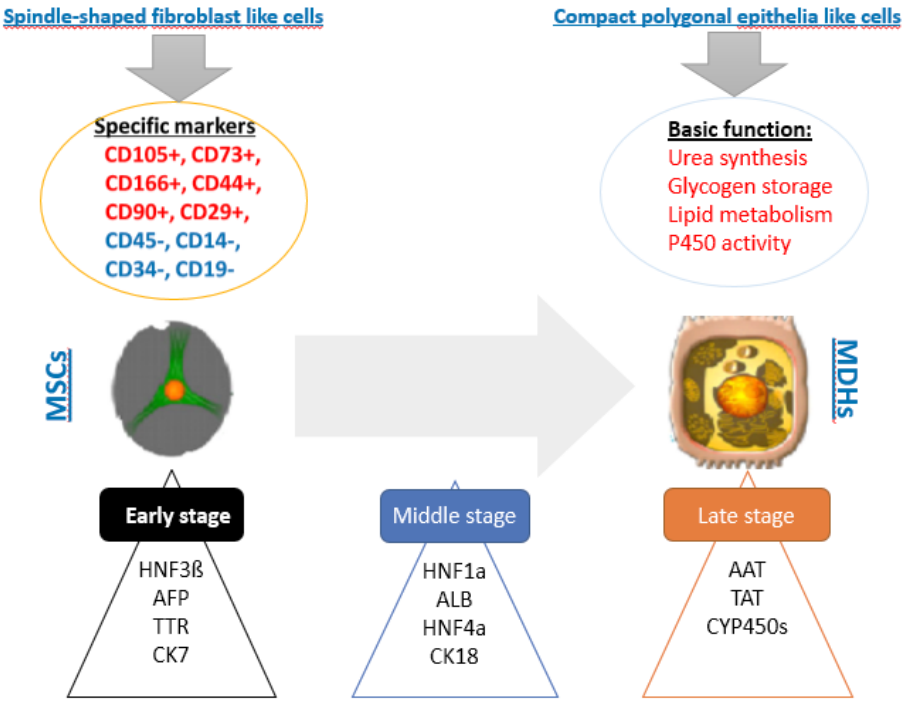


Figure 23: Morphological and molecular considerations within MSC differentiation towards hepatocytes

Nowadays, there is no report discussing the impact of DA on hepatogenic potential of hADSCs. However, few reports discussed the impact of DNA damage on hepatogenic potential of MSCs. Where UCMSCs expressing ROS and high oxidative damage ratio gave rise to hepatocytes like morphologically and biologically normal [537] , hADSCs with high methylation rate showed a weak hepatogenic potential compared to their counterparts treated with a methyltransferase inhibitor [538]. Indeed, demethylated generated hepatocytes showed high activity of the CYP450, AFP and Alb with urea metabolism comparable to freshly isolated human hepatocytes suggesting that hADSCs may be suitable for liver cell-based therapy after DNA damage repairing.

7.6. Human MSCs to hepatocytes: the *in vitro* differentiation cues

The shift from fibroblast-like to the polygonal is a key parameter for the transdifferentiation of MSCs into hepatocytes like cells**.** This phenomena was described in human BMSCs [539] and ADSCs[540] showing a remarkable transition from a bipolar fibroblast-like morphology to a round epithelial during the initiation step of hepatic differentiation (Fig.23) . However, the maturation step is associated with cytoplasm contraction**.** Clearly, double nuclei appear in the late differentiation stage**.** This morphology shift is associated in the initiation step to the differentiation towards hepatocyte with alpha-fetoprotein (AFP) and Sox17 , followed by the expression of mid and late markers such as HNF4a, Alb and CK 18 [541,542] . Furthermore, HNF4a plays a crucial role in the development of the liver-specific phenotype [147, 543]. Indeed, overexpression of HNF4a in MSCs induces better hepatocyte functionality [544]. In the late stage of maturation, MSCs express hepatocyte markers such as tryptophan 2,3-dioxygenase (TO), anti-trypsin (AAT), tyrosine amino transferase (TAT), CCAAT enhancer-binding protein (C/EBP) and cytochrome P450 (CYP450) (Fig.23). Conversely, MSCs embryonic markers are downregulated [542]**.**

Besides to these markers, other functionality parameters are evaluated within the MSCs and their derived hepatocytes such as glycogen storage, detoxification ,lipid metabolism, the synthesis of urea, the uptake of low-density lipoprotein and the inducing of the cytochrome P450 activity [542].

Recently, several hepatic differentiation protocols of MSCs have been conducted and developed four types of strategies to derive hepatocytes from MSCs: The addition of growth medium and cytokines, the genetic modification by overexpressing of hepatic key genes, adjustment of the microenvironment by ameliorating and mimick a 3D liver ECM or by alteration of the physical parameters used for culturing MSCs such as inhibiting senescence process *in vitro* (Fig.24).

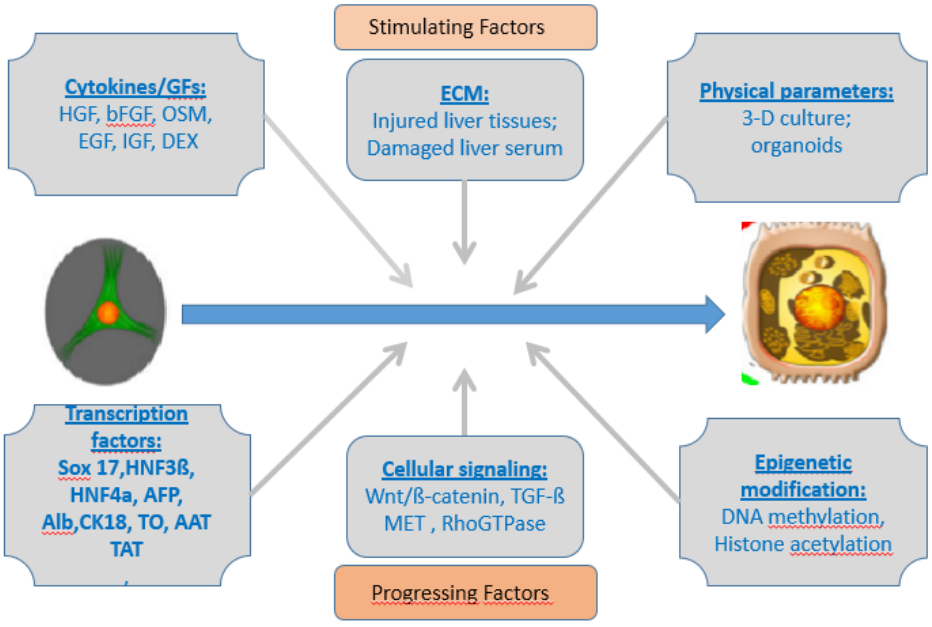


Figure 24: Main molecular mechanisms involved in MSCs trans-differentiation into hepatocytes

Some reports revealed that culture of MSCs in fetal liver-conditioned medium induced differentiation [545, 546]. Since no cell-cell interaction takes place in monolayer MSCs culture, cytokines are mandatory to induce MSCs

differentiation towards hepatocytes. A lot of strategies and reports showed that many cytokines are involved in liver cell proliferation and differentiation such as hepatocyte growth factor (HGF), oncostatin M (OSM), epidermal growth factor (EGF), transforming growth factor beta (βFGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), dexamethasone (DEX) and nicotinamide [58, 547, 548]. Indeed, βFGF plays an essential role in the liver regeneration and the MET transition cascade, βFGF is required to induce a hepatic fate from the foregut endoderm (Sox17+), and OSM increases hepatocyte size and hepatocyte maturation [59, 546]. Insulin growth factor with transferrin and DEX can promote the proliferation of hepatocytes [549, 550]. Successful MSCs differentiation *in vitro* was showed to be ameliorated with multistep process and sequential cytokine release *in vitro* and the ability of systems to reconstitute the *in vivo*-cell matrix [60, 64, 551, 552]. In sharp contrast to the critical role of serum in MSC expansion differentiation steps, serum free conditions have been successfully applied [60, 64, 498].

Other cues were discussed to ameliorate the efficacy of Hep-Dif from MSCs such as the spatial distribution between cells as it is an important factor where differentiation is initiated upon 60%-80% confluence. Highest cell density is in favor of differentiation [143]. Also, the presence of various ECM components (Collagen type I, II, IV, elastin, gelatin) is employed as coating material for better ECM conditioning. Several components were tested on ADSCs , however the decellularized liver matrix was shown to better coat plastic dishes for Hep-Dif [553, 554]. Thus, the choice of collagen and aminoglycanes turns out to be most effective [555] since culturing human ADSCs on top of HGF/Collagen on a glass of protein spots arrays induced greater differentiation into Hepatocytes [554]. Furthermore, some strategies used inhibitors of the main cellular signaling pathways such as p38, ERK1/2, JNK and improved better hepatic functionalities in presence of HGF, bFGF and OSM. Others used small molecules to repair DNA damage and histone acetylation [507, 523, 556-561]. Further epigenetic regulation in ADSCs such as the inhibition of some miRNA resulted in the increased expression of specific hepatocyte markers [562, 563]. Moreover, notably in ADSCs the supplementation of endodermal transcription factor such as Foxa1, Foxa2, and Gata4 induced and enhanced their differentiation into hepatocytes [564].

The hallmark of this trans-differentiation is considered to be a mesenchymal to epithelial transition (MET). In fact, by comparing the gene expression of undifferentiated ADSCS, hepatocyte like derived ADSCs and human primary hepatocytes, the expression of the mesenchymal markers Twist and Snail were reported to be remarkably downregulated, as well as the expression of N-Cadherin and the cytoskeletal marker vimentin. Furthermore, microarray-based integrated analysis of methylation by isoschizomers (MIAMI) showed that the promoter region of the Twist gene was hypermethylated in differentiated ADSCs, causing the decrease in the expression of this gene. Concurrently, the expression of the epithelial marker E cadherin was progressively increasing[540, 565]**.** These findings support the notion that MET plays an important regulator key in determining stem cell trans-differentiation[565] (Fig.24).Frequently used molecular markers for MET involve downregulated expression of N-cadherin and vimentin with reduced transcription of SNAIL1 and TWIST that regulate expression of E-cadherin. BMP, WNT and TGFβ pathways have been widely designated for their contribution in EMT/MET process [566]. Altogether, these data suggest that plasticity perceived in ADSCs is dependent on MET.

CHAPTER 8

Cdc42: A novel biomarker of stem cell aging

8.1. Regulation of cdc42 GTPase protein

The Rho-GTPases family involves a large number of proteins. The well-known members of this family are RhoA, Rac1, and Cdc42 which were reported in 1990 to be specific for actin assembly and reorganization by regulating formation of ruffles, filopodia, and stress fibers, endocytosis, phagocytosis and intracellular traffic [567]. Cdc42 is a member of the Rac subfamily. Cdc42 was first identified in *Saccharomyces cerevisiae* as a mediator of cell division [568]. The downstream proteins of Cdc42 called the Wiskott - Aldrich syndrome proteins were shown to strictly regulate cell migration and molecular adhesion mechanisms [86]. Furthermore, Cdc42 was reported to regulate different targets and to impact several cellular responses fundamental to cell growth, differentiation, apoptosis and PAK cellular stress pathways [86, 569]. Par3/6 regulating actin and tubulin cytoskeleton reorganization and cell polarity were reported as Cdc42 downstreams[92]. On the other side Cdc42 is implied in regulation of cell growth signaling and was found to crosstalk with the JAK-STAT pathway and to be central for triggering of Erk, JNK, p38 MAP kinases, and NF-κB . Several limitations avoided the determination of Cdc42 roles *in vitro such the* early lethality phenotype upon cdc42 gene deletion in mouse embryos[570], and the lack of specificity when using dominant negative or constitutively active mutant over-expression [571, 572] . Thus the role of Cdc42 was revealed within Cre/loxP mouse conditional knockout methodology very important in heart, liver, pancreas, nervous system, blood, bone, eye, immune system and the skin. However, the regulation of Cdc42 was shown to be tissue and cell type specific [8]. Thus, it was suggested that Cdc42 data cannot just be inferred on all cell type and the general principles of Cdc42 function well-defined by in vitro may not apply to another cell type in *in vivo*.

Fascinatedly, Genome-wide association studies of longevity in humans related elevated expression of Cdc42 in blood stem cells to increased morbidity and aging [573]. This interesting issue will be largely discussed in this chapter.

Being a GTP-binding protein, the Cdc42, act over a binary switching mechanism directed by the binding of GTP. Three classes of proteins regulate the Cdc42 status. (1) the guanine nucleotide exchange factors (GEFs) enhancing the swapping of GDP to GTP, (2) the GTPase-activating proteins (GAPs) promoting the hydrolysis of GTP to GDP noting that the Cdc42 is inactive when bound to GDP and (3) the Guanine nucleotide dissociation inhibitors (GDIs) sequestering Cdc42 in the inactive GDP-bound state [86-89] (Fig. 25).

8.2. Cdc42 activation and stem cell aging (2001-2017)

Initially in Cdc42 molecular mechanisms, Cdc42 was shown to be implicated in endocytosis and CARGO transport inquiring its involvement in cycle arrest and differentiation [86]. Therefore, the primary challenge was to understand if the guanine nucleotide exchange factor, the GTP binding, triggers the cellular growth regulation. The first approach suggested that the level of activation of Cdc42 (Cdc42-GTP) can probably influence cell adhesion, migration, division and polarity [574] .

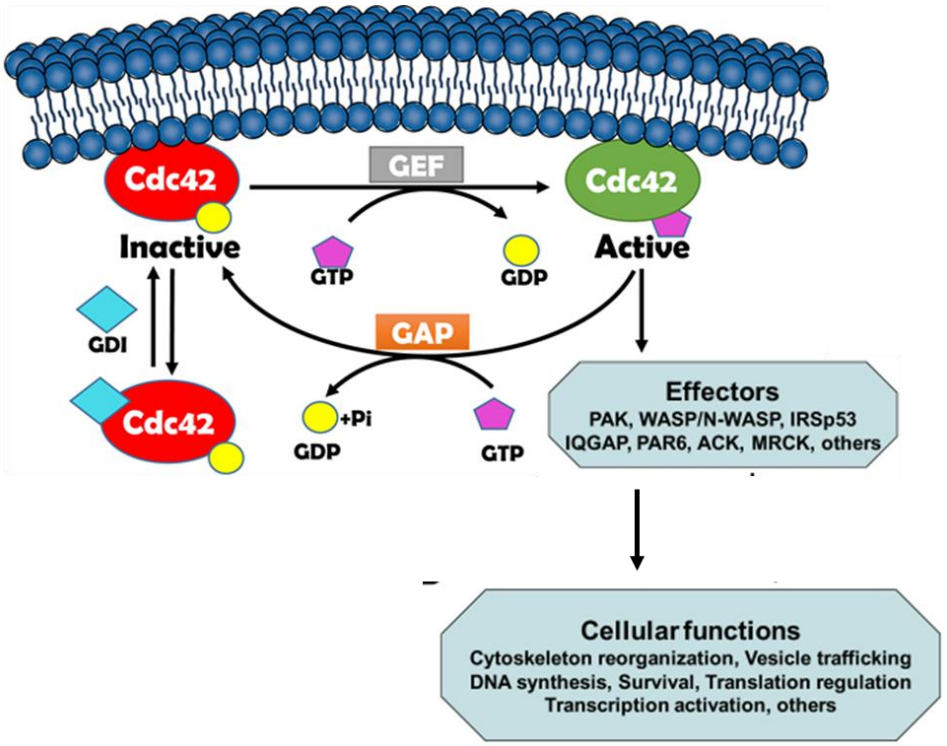


Figure 25: Schematic of Cdc42 activity mode. Cyclic activity between active (GTP-bound) Cdc42 and inactive (GDP-bound) Cdc42. Guanine exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine dissociation inhibitor (GDI) regulate Cdc42 cycle. Cdc42-GTP bound can transiently interact with multiple effector proteins to transduce signals that impact on cell cytoskeleton organization, exosomic trafficking, cell cycle progression, survival, translation, and transcription [575].

Afterward, Cdc42 was qualified to have the central role of polarity by regulating the membrane traffic and the formation of intercellular junctions for cells ranging from yeast to mammals. It was so vague for researchers to define mechanisms of this positive-feedback loops regulating pathways into upstream activation and downstream effectors of Cdc42 [576] (fig.26). Next, some targets of activated Cdc42-GTP complexes such as calveolin1- and Pak4 were shown to trigger cell senescence when overexpressed and to reverse age when Knocked out [577,578]. Evidently, the Cdc42-GDP binding complex and not the activated compound Cdc42-GTP was shown to be essential for regulating the actin fibers synthesis and cell proliferation [579].

Based on all these findings, the advanced approach appeared with Xing et al (2006) [84] showing that the high mobilization of HSCs in aged mice correlated with a high expression of GTP-bound Cdc42 binding complex compared to young mice derived progenitors cells. During the same year, Fortier et al [580] showed that chondrocytes derived from aged horses presented also a high activity of GTP-Cdc42 leading to loss of proliferation and regeneration potential. In 2007, Yang et al [85] extended their research in HSC aging applications as they observed in knockout aged mice Cdc42 $^{-/-}$ model that HSCs exhibited a shift from quiescence to an active cell cycle activity and high retention level in their BM niche . The same group reported later that elevated level of Cdc42 activity causes aging in HSCs via deregulating several cross matched molecular pathways; the evaluation of these pathways can lead to therapeutic strategies for hematopoiesis in aged individuals [491]. Wang et al[90], reproduced the same work but in another transgenic mice model by knockout a selective negative regulator of Cdc42-activity, the

GTPase Activating Protein (GAD). The clampdown of Cdc42-GTP expression resulted in significantly reducing the expansion time, enhancing the DNA damaging repair, and suppressing p53, $p16^{Ink4a}$, p21, and SA- β gal expressions in several aged tissues in mice, such as heart, brain, lung, liver, spleen, kidney, and BM. These results suggested a role of Cdc42 activity in regulating genomic stability and aging in stem cells. Interestingly, Human Genome-wide association studies identified *Cdc42* as the gene the most positively associated with aging in human lymphoblastoid cell lines where levels of expression correlated positively with aging and mortality [573]. Furthermore, at this period, researchers have found that the deregulation in activated form of Cdc42 resulted in other cellular aberrant polarity transformations by promoting tumorigenesis and tumor invasion as well as initiation of cardiovascular diseases, diabetes, and neuronal diseases and wound healing with aging. Thus, Cdc42 activity has clearly been correlated with age and degenerative human diseases[581-584]. Although the knockout mice Cdc42-GAD $^{-/-}$ have previously been shown to exhibit early aging phenotypes in several different tissues, the HSCs Cdc42-GAD $^{-/-}$ aged phenotype had not been observed [90]. Therefore, Florian et al[21], knocked out the Cdc42 GAP in Young HSCs and screened the effect on their aging and polarity. Indeed, they observed that young $Cdc42GAP^{-/-}$ HSCs presented functions similar to aged HSCs in competitive transplantation assays in male mice. In a second step, they were the first to inhibit pharmacologically Cdc42-GTP *in vitro* in aged mice derived-HSCs with a selective Cdc42 activity inhibitor (CASIN). Interestingly in this report, the term "rejuvenation "of stem cells by inhibiting the Cdc42-GTP compound in aged HSCs was adopted. Moreover, CASIN could reverse age-related biomarkers by increasing the cell polarity in aged HSC population and restoring the level of histone acetylation distribution like the level found in young HSCs. Thus, Cdc42 activity is considered a pharmacological target for encountering stem cell aging [21,585]. Furthermore, Cdc42 is showed to be activated in aged HSCs via a stroma derived- Wnt pathway in aged niche [586]. The Wnt-Cdc42 axis cascades will be discussed in the next sections.

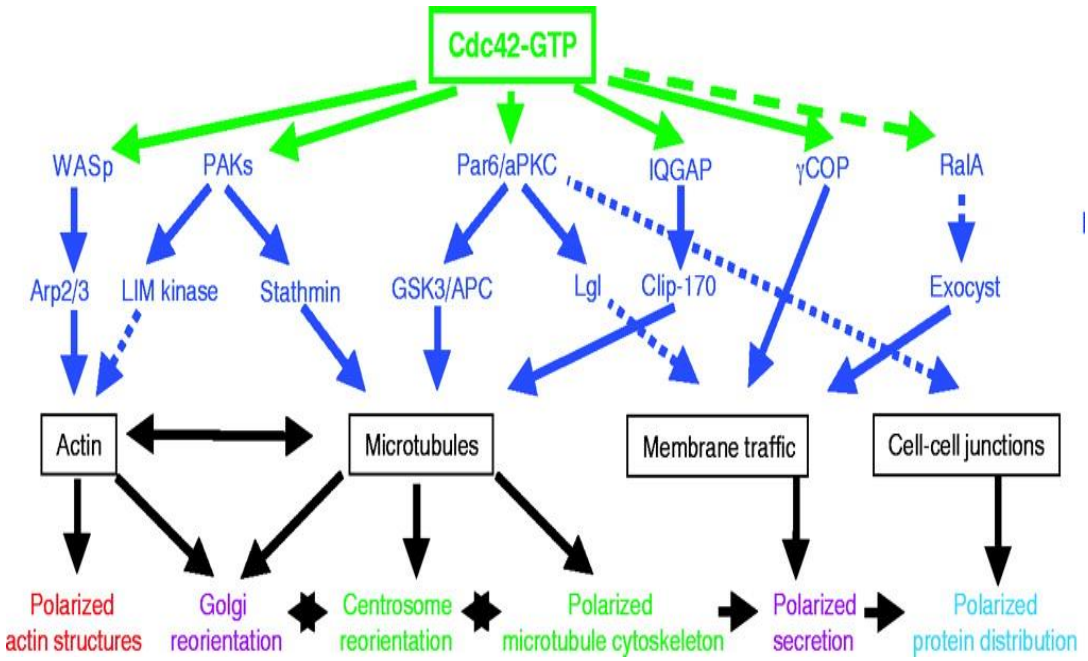


Figure 26: Multiple signaling pathways controlling the cellular polarity via Cdc42 activation. Cell polarization implies different cellular functions (black box). These signals are transduced by different Cdc42 direct (solid line) or indirect (dotted line) effectors (blue) and involve several intermediate proteins (blue) [576]

Based on Geiger's team reports, Carrillo-Garcia et al [91], considered in their review that inhibiting Cdc42-GTP in HSCs is their fountain of Youth . Although Cdc42-GTP was linked to tissue aging and rejuvenation in mice models, the Cdc42 activity and expression as biomarker for aging in humans was still missing. Thus, recently, Florian et al, 2017[93] developed an approach to validate Cdc42 activity in human aging. For this end, they established a cohort of 196 aged human donors of peripheral blood. The trial involved 40.0% of donors with hypertension, 21.9% of donors with cardiovascular diseases, 17.4% with cancer, 10.2% with diabetes, and 9.2% of osteoporosis patients. They showed a high correlation between the Cdc42-GTP expression in both biological and chronologically aged hematopoietic cells .The novel association between Cdc42 activities and the cardiovascular disease and myocardial infarction defined the Cdc42 activity as a novel biomarker of aging-associated heart diseases. In our study, the aim was to investigate the Cdc42 activity implication in human MSCs aging and regenerative potential.

8.3. Cdc42 and Wnt pathways in stem cell aging context

Under normal hematopoiesis conditions, canonical Wnt pathway is implicated in HSCs regulations (Fig.27a). This pathway implicates a translocation of β-catenin towards the nucleus and activation of transcription factors implicated in cell proliferation, self-renewing and cytoskeleton organization.

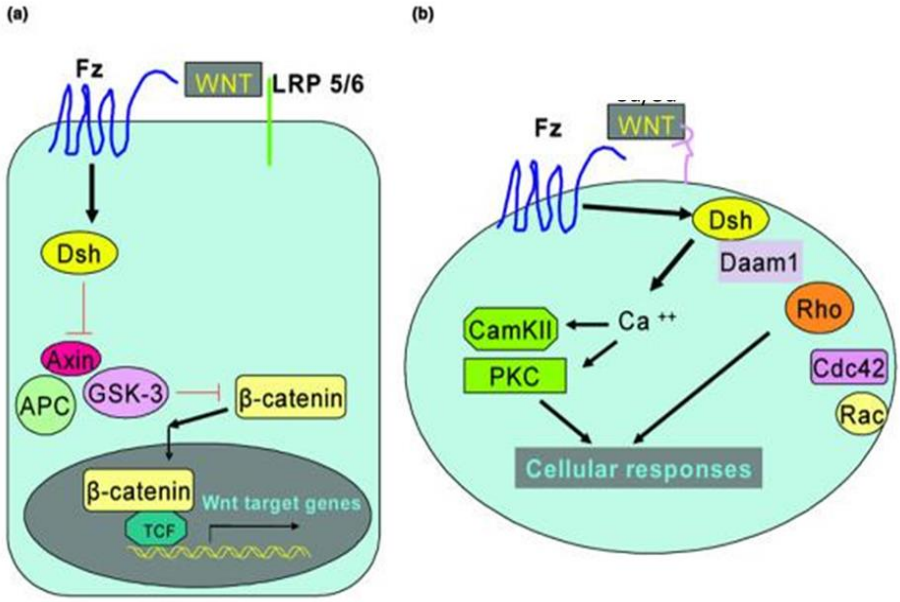


Figure 27: Wnt signaling pathways; (a) the canonical Wnt/β-catenin pathway implicating the β-Catenin translocation in the nucleus. (b) The noncanonical Wnt signaling pathway involving the Cdc42 activation pathway. Modified from Liu et al, 2005[587]

To study the aging process, Florian et al [586] , focused on the Wnt signaling pathway in HSCs, which plays a initial role in cell-cell communication, tissue regeneration, and is also involved in the pathology of various diseases. Actually, they were based on a work where Wnt5a-driven signaling pathway was described to regulate quiescence in HSCs via regulating the activity of the small Rho GTPase Cdc42. The onset of HSC aging was shown to be mediated by a shift from canonical to a distinct Wnt signal transduction pathways called "noncanonical" in result to epigenetic aberration[588] (Fig.27b). Indeed, they observed that aged HSCs presented a high expression of non-canonical Wnt component such as Wnt5a whereas young HSCs expressed canonical Wnt counterparts. In

the next step they showed that the treatment of young HSCs with non-canonical Wnt5a induced ageing and reduced the rejuvenation potential via Cdc42 activation manner. Briefly, in their strategy they overexpressed the Wnt5a in young HSCs and showed that these stem cells gain immediately signs of aging and polarity disruptions. Interestingly, Cdc42 was one of the Wnt5a target which impact cell polarity and actin polymerization. Hence, they showed that Cdc42–GTP bound is activated in the Wnt5a inducing-aging young HSCs. Inhibiting the Cdc42 with CASIN was able to reverse the aging process in HSCs. The Knockdown of Wnt5 in aged mice showed a rejuvenation of their HSCs. More interestingly, Knockdown Wnt5a in aged HSCs reverted to a high expression level and nuclear localization of β-catenin, and therefore to active canonical Wnt signaling [586]. Recently, the same group, reported the Wnt-Cdc42 axis implicated in the apolarity related to stem cell aging otherwise to the decrease of migration and adhesion cell abilities [92] (Fig.28). Based on these findings, this cross-talk between Wnt and Cdc42 brings additional challenge in stem cell aging.

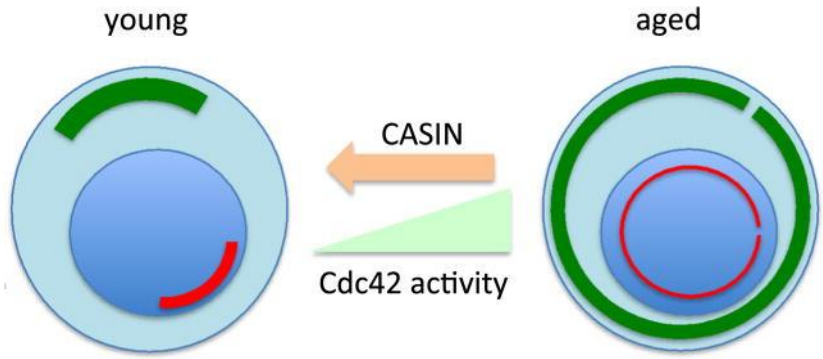


Figure 28: Impact of CASIN on the polarity and epigenetic behaviors of aged HSCs related to Cdc42 GTP activity. Young stem cells are polar with low protein like tubulin (green) and nuclear proteins like the acetylated form of histone 4 on lysine 16 (AcH4K16, red) expression. Aged HSCs are apolar for both tubulin and AcH4K16. Inhibition of Cdc42 activity via CASIN rejuvenates aged HSCs and reverses apolarity in chronologically aged HSCs into polarity. Modified from Geiger et al, 2014 [92]

8.4. Cdc42 and Wnt pathways in the liver regeneration context

Mouse Cdc42 targeting studies showed that Cdc42 downstream pathways are tissue and cell-type specific [571]. The unified function of all cell type is the actin-based morphogenesis and the polarity. Thus we will focus on the role of Cdc42 in hepatocytes regeneration and development. Mice in which Cdc42 was ablated in hepatocytes and bile duct cells were generated by Cre-*lox*P technology and showed chronic jaundice. They developed hepatomegaly quickly after birth associated with liver nodules and tumors. Furthermore, Hepatocellular carcinoma was observed eight months after birth. Interestingly, tumors grew gradually and didn't express of nuclear β-catenin the downstream of wnt pathway. In addition, enlarged biliary canaliculis were observed with a dramatically decreased expression of E-cadherin. Analysis of serum samples indicated cholestasis observed in hepatocarcinogenesis. This mouse model knock outing Cdc42 impairs the normal liver development and exhibits a delay in liver regeneration post partial hepatechtomy (PH)[19] . Eight years later, the same group, reported that phosphorylated cytoplasmic β-catenin (p120 catenin) was shown to regulate E/N cadherins expression via Cdc42 activity in hepatocytes and cholangiocytes. The Cre-*lox*P p120 KO mice model showed normal Hep-Dif ability however the hepatobiliary structures were reduced and associated to inflammation and fibrosis. These results suggested that the Wnt/β-

catenin , β-catenin/E-cadherin complexes and Cdc42 activation are implied in hepatocytes and cholangiocytes development and differentiation[589]. In rat liver failure model, after aggressive induced chemical liver injury, activated resident stem cells were shown to express Cdc42 suggesting that Cdc42 is playing a role in LPs differentiation into hepatocytes[31]. Moreover, a proteomic analysis post partial hepatectomy of rat liver proved that Cdc42 regulation is mandatory to the termination of liver regeneration[240]. More interestingly, Cdc42/JNK/PI3K activation pathway was essential to delay primary rat hepatocytes life span *in vitro* [255]. These evidences might support a crucial role of Cdc42 in stem cell ageing, stem cell differentiation into hepatocytes.

8.5. Main signaling pathways regulating Cdc42-GTPase activity

A use of dominant-negative mutant of Cdc42 by sequestering the upstream GEFs has been largely reviewed and elucidated multiple signaling pathways in cell regulation [571, 590]. However this approach is limited by the fact that more than 70 GEFs exist in the mammalian genome [591] . Cdc42 has been implicated as a key regulator of adhesion, wound healing, polarity and migration via PAK (p21-activated kinase) and GSK-3 (glycogen synthase kinase-3) pathways[532] (Fig.26). Another cell function implicates the Par6 atypical PKC (aPKC) complex important for the establishment of epithelial polarity [592] . We will focus below on the signaling pathways screened during my project and hypothesized to be regulated or to regulate Cdc42.

8.5.1. RAS pathway

Ras proteins regulate apoptosis, differentiation, senescence and cell aging via Cdc42 activation. Indeed, Ras effectors are suppressed in overexpressed dominant negative Cdc42 cell model[593]. This phenomena explains that Ras proteins act via Cdc42 activity to regulate cell activity [594]. Otherwise, it has been shown that activated Ras recruits GEF and thus activates Cdc42-GTP bounds leading to senescence of human primitive cell line. Furthermore, Ras GTPase transduces different signaling pathways. First, Ras transduces the tyrosine kinase-linked receptors, triggering the mitogen-activated protein kinase (MAPK) signalling pathway. Like other G proteins, Ras functions as a binary switch with Cdc42 that is activated by binding GTP. Second , Ras can activate the PI3-kinase signalling pathway.

8.5.2. Mitogen-activated protein kinase (MAPK) signalling pathway

MAPK controls several cellular processes such as migration, proliferation, apoptosis and differentiation and was seen to be implicated in cell aging and senescence aberrations [595, 596]. Three main pathways are involved in MAPK activation leading to three downstream effectors: Extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 pathways (Fig.29).

ERK pathway is activated post binding Ras and Raf protein kinases to activate the phosphorylation cascade and the cell proliferation. Interestingly, Cdc42 deletion downregulated the activation of ERK, JNK and proliferation gene transcription in the remained hepatocytes post resection[239]

Moreover ERK is shown to be implicated indirectly in cell aging. In fact, Pak4 gene expression is one of the downstream effectors of Cdc42-GTP activated bounds. However, Pak4 avoids cell proliferation and promotes cell aging *in vitro* via activation of the ERK [578]. Moreover, one of the major functions of the ERK pathway is to activate a

range of different transcription factors such as cyclic AMP response element-binding protein (CREB) and p53 phosphorylation [597-601].

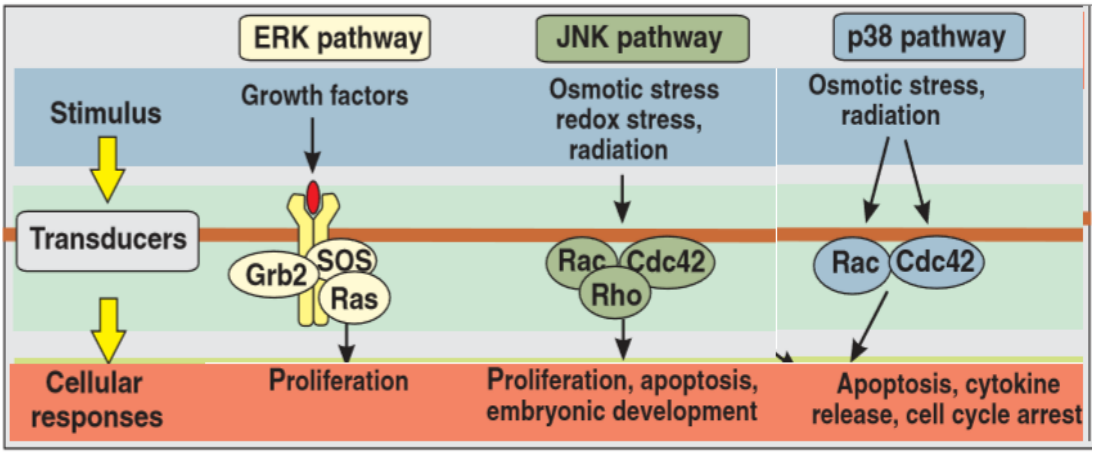


Figure 29: Regulation of Cdc42 by the MAPK signaling pathways. Implication of ERK, JNK and p38 cascades.

C-Jun N-terminal kinase (JNK) pathway controlled a number of cellular processes including, self-renewal, proliferation and apoptosis [602]. The inhibition of Cdc42-GTP was able to downregulate the JNK pathway. Besides, the inhibition of JNK pathway was able to accumulate ROS and toxicity in human cells[596, 602, 603]. No data about the regulation processes of JNK pathway in ADSCs.

The p38 regulates apoptosis and cytokine secretions. p38 is described to be the regulator of ROS intracellular accumulation and thus activation of p53 and cell aging [431, 432]. Particularly, in hMSCs, phosphorylation of p38 decreases with age and the osteogenic potential is impaired with p38 activation [479, 604].

8.5.3. Nuclear factor κB (NFκB) signalling pathway

The transcription nuclear factor κB (NFκB) is triggered by external stimuli such as the tumor necrosis factors (TNFs), interleukins, controlling processes of inflammatory modulation, cell proliferation and apoptosis. It is translocated into the nucleus upon activation and enhanced TF expression [605]. In BMSCs, TNFα and IFN-γ activate NFκB pathway [461]. The TNFα /NF-κB pathway activates oxidative damage in BMSCs. Simultaneously, ROS activates NFκB pathway, forming a loop inhibiting their osteogenic potential [454, 606]. NFκB is shown to be implicated in stem cell aging[607]. Wang et al [272], showed that NFκB activation suppresses β-catenin expression, a downstream gene of canonical Wnt signaling and thus interacts together to decrease differentiation potential in inflammatory senescent microenvironment [608, 609].

8.5.4. The Phosphoinositol 3 kinase (PI3k) and Insuline receptor substrate pathways

The PI3k signaling pathway controls the glycogen metabolism, lipid synthesis, protein synthesis, gene transcription and cell growth, inflammation, cytoskeletal rearrangement and apoptosis. JNK pathway can be activated by PI3K and activates Cdc42- GTP binding [610]. On the other hand, RAS activation triggers the direct activation of pI3K pathways and the phosphorylation of ERK [611]. In addition, HGF-induced cell spreading involves pI3K and activates

Cdc42. Moreover, Pi3K can be activated by the phosphorylation of the intramembrane Insulin receptor (pIR) and directly from Cdc42-GTP activated bound signaling cascade [611]. Binding of IGF (Insulin growth factor) to its receptor activates the PI3K pathway [612]. Furthermore, IGF binding to the tyrosine kinase receptor leads to Cdc42 activation [613]. IGF binding to the IRS-1 was shown to induce ERK signaling [614]. Insulin receptors pathways play a crucial role in inhibiting cell death. The actin organization is regulated by Cdc42 acting on profilin through insulin receptor substrate (IRS or pIR) [87, 493, 576, 615]. The transduction of these signals leads to intracellular phosphorylation and the activation of several signaling pathways[616]. These two pathways are very implicated in Cdc42 activation process and MSCs aging[617, 618]

8.5.5. Cyclic Camp protein kinase A (PKA)

PKA involves two regulatory (R) subunits and two catalytic (C) subunits and controls the phosphorylation of particular sites on downstream effectors. Deregulation of this pathway can cause apoptotic cell death and is implicated in a range of aged human disease. PKA activates the transcription factor c-CAMP response element-binding protein (CREB) leading to altered histone acetylation and methylation pattern similar to age-related biomarkers observed in aged stem cells [619, 620]. Besides, direct acetylation of p53 by CREB pathway mediates apoptosis and cell aging[621]. PKA phosphorylation leads to cell degeneration with age [622]. The Cdc42 GDP/GTP-bound state isn't regulated directly by PKA phosphorylation. The targets of PKA phosphorylation stimulate GTP binding on Cdc42 and thus cell senescence[623].

8.6. Pharmacological inhibition of activated Cdc42

8.6.1. CASIN : Unspecific Cdc42 inhibitor

Geiger et al, were the first, for two consecutive years 2012 and 2013, to inhibit pharmacologically in vitro Cdc42-GTP in aged mice derived-HSCs with a selective Cdc42 activity inhibitor (CASIN) referred to Peterson et al,[624]. CASIN was able to reverse age-related biomarkers by increasing the cell polarity (tubulin circulation) in aged HSC population and restoring the level of histone acetylation distribution to a status similar to that seen in young HSCs. Thus, they consider Cdc42 activity as a pharmacological target for ameliorating stem cell aging [21, 585] (Fig. 30). CASIN was able by repairing epigenetic alteration in aged HSCs to imply a non-reversible inhibition of Cdc42 activation and to reverse definitely the a-polar aged HSC to polar state identical to young HCS [92]. In addition, CASIN was used as a preparative regimen for HSCs transplantation in mice without myeloablation able to enhance mobilization of human xenograft HSC progenitors after serial transplantations of CASIN- mobilized peripheral blood cells (PBSCs) and UCB stem cells [625]. Conversely, CASIN promoted apoptosis in cancer cell line and suppressed tumorigenic migration of several colorectal stem cell lines [625] and Leukemic stem cells line [626]. These findings showed that inhibition of Cdc42-GTP with CASIN is crucial for self-renewal and rejuvenation of aged HSCs and homing in their niche as well as it represents a rational therapeutic principle to target cancer stem cells survival. CASIN was never been used as cdc42 inhibitor in MSCs. Thus, this protein use was crucial to be applied within our project on ADSCs as one of the selected Cdc42 inhibitors.

8.6.2. ML141 A novel spatial selective non-competitive specific Cdc42 inhibitors

To identify the molecular effectors and stimulators regulating Cdc42 with aging, it was evident to classify the small molecules targeting Cdc42 activity. Secramine was the first inhibitor for Cdc42 to be recognized. This compound blocks the GTP bound exchanging via the GTP dissociating inhibitor (GDI) [627] and blocks guanine nucleotide exchange on Cdc42 and any subsequent interaction with downstream effectors .

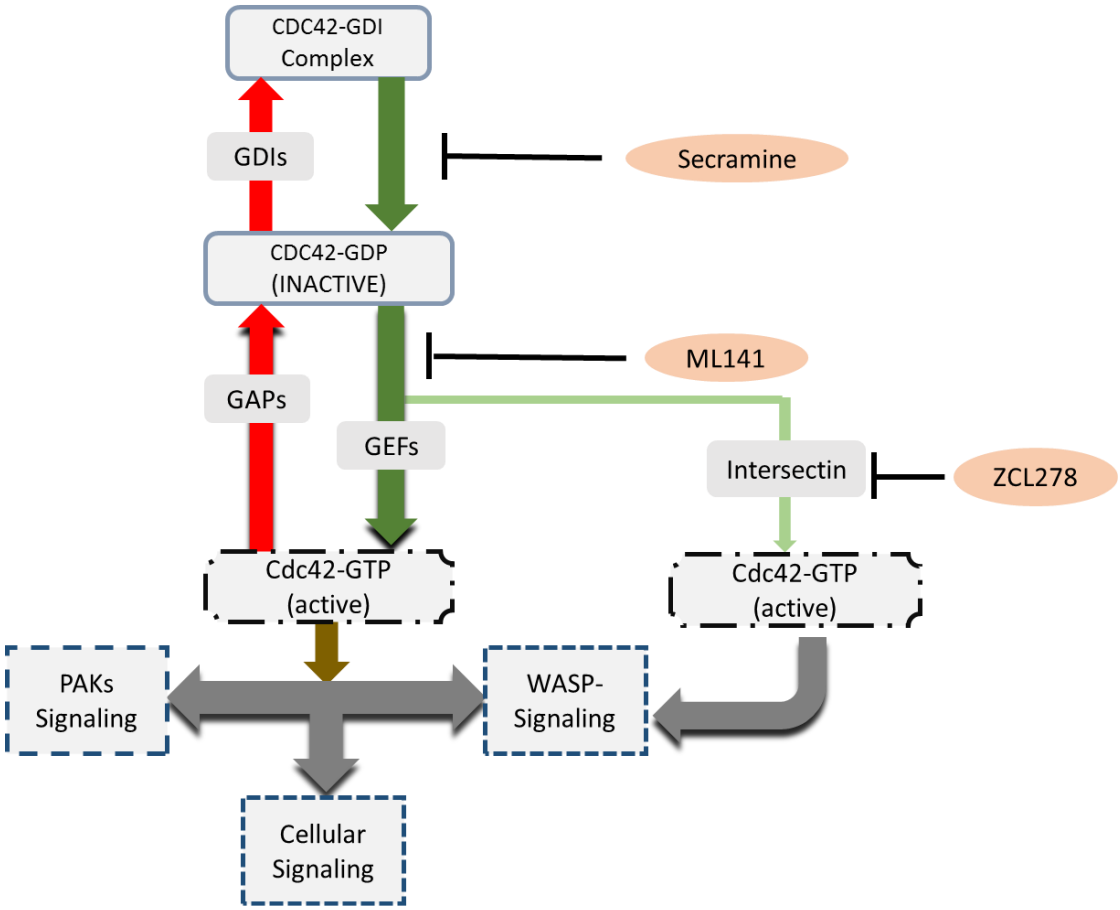


Figure 30: Mechanisms of small molecule inhibition of Cdc42 signaling. Guanine exchange factors (GEFs) such as intersectin, promote the exchange of GDP for GTP and activate Cdc42. Secramine inhibits the activation of Cdc42/GDI complexes, blocking guanine nucleotide exchange on Cdc42 and any following interface with downstream effectors. ML141 is a selective reversible non-competitive allosteric inhibitor of Cdc42, preventing GTP binding to the active site of Cdc42 without targeting any Cdc42-regulating molecules. ZCL278 acts by selectively blocking intersectin preventing WASP and PAKs signaling.

However, this inhibitor, in addition to its toxicity, affects the activation status of other GTPases proteins subfamilies such as RhoA and Rac, and thus it didn't serve the understanding of the specific roles of Cdc42 in cellular pathway. Recently, ZCL278 is identified to target chemically the interaction of Cdc42 with the Intersectin – GEFs specifically and thereby disrupts the GTP binding ability and prevents any further Cdc42 activation. This inhibitor suppresses actin

formation and migration ability but unfortunately can interact unselectively with all intersectin-GEFs regulating other G protein activation. A second Cdc42 small-molecule inhibitor, CID2950007, termed ML141, the first potent reversible non-competitive inhibitor selectively binds the guanine nucleotide associated Cdc42 and induces ligand dissociation. This compound blocks the nucleotide binding site and thus it does not impact the GEF binding regions against other members of the Rho family of GTPases[89, 628, 629] (Fig.30) . ML141 was recently used to inhibit Cdc42 activity in different cancer types and to downregulate some signaling pathways like EGFR , JNK and p21-activated kinases pathways (PAK) regulating cell migration, adhesion, and invasion[596 630, 631]. In addition, it regulates MET in melanoma cell line [632].

Furthermore, ML141 inhibited Cdc42 activity in chondrocytes by modulating the TGFβ1 signaling pathway and regulating the cartilage proteins accumulation [633]. Besides, ML141 was able to positively regulate the G-CSF HSC mobilization from BM by decreasing the GTP binding sites on Cdc42-GDP complex [634]. In vascular smooth muscle cells, ML141 was able to decrease, by Cdc42-GTP inhibition, arteriosclerotic calcification in absence of LDL receptor in mice models[635]. In a 3D angiogenesis cell culture model *in vitro*, ML141 reduces migration of ECs by inhibiting the Cdc42 and promotes vascular branching [636]. Several months ago, some studies described that ML141 inhibits Cdc42 activity in hBM derived MSC and is able to prevent their migration ability[637] and to decrease actin distribution in the cortex associated failure of polar body extrusion from porcine oocyte [638]. By searching deeply for ML141 applications in fundamental applications, we never found a report using this Cdc42 selective inhibitor to reverse the age-related aberrations in aged stem cells.

8.6.3. Trichostatin A (TSA) : Non-specific Cdc42 inhibitiors

Long span culture time increases the histone deacetylase aberrations in MSCs DNA which promotes GTP binding on Cdc42. Consecutively, the proliferation rate of MSCs decreases and thereby restricts their sufficient number for therapy and tissue engineering. TSA is a histone deacetylase (HDAC) inhibitor described to repress Cdc42 activity in aged hMSCs by reducing the acetylation ability. TSA was able to prevent appearance of aging signs in UC and placenta derived- MSCs by increasing their proliferation rate, their pluripotency genes expression (Oct4 and Nanog) and by suppressing spontaneous osteogenic differentiation [639, 640]. Furthermore, The TSA was described in several reports to enhance cardiogenic and neurogenic potential in chronological aging of BMSCs (induced senescence *in vitro*) [641-643] . Attentively, TSA increased the telomere elongation which is a crucial biomarker for stem cell self-renewal [644, 645]. TSA- treated hMSCs showed a better osteogenic, chondrogenic and neurogenic differentiation compared to the non-treated group [646, 647] .

Conversely, in cancer glioblastoma cell line, TSA inhibits proliferation and survival of the tumor and promotes the differentiation of glioblastoma cells into neuronal like cells [648, 649]. Otherwise, TSA constituted a regenerative potential being able to reverse stem cell aging and by having therapeutical potential in some cancer cell lines.

On the other side, TSA was shown to rescue Ciliogenesis in Kidney cell line by inhibiting the HDAC resulted from the Cdc42 complexes [502]. In aging context, TSA enhanced the binding of dystrophic kinase to the Cdc42 and thus caused a decreasing of the GTP binding competitively [650]. Furthermore TSA-HDAC inhibition was essential to repair epigenetic aberrations of hBMSCs acquired during aging or long term expansion *in vitro*. In addition, TSA promoted a better MET within the Hep-Dif [651]. TSA treatment during both the induction and the maturation differentiation steps, enhanced in hADSCs a potent hepatogenic profile exhibiting a low AFP expression , a high synthesis levels of

Alb, Urea and great HNF4α expression compared to non-treated aged group of ADSCs [558]. Another report, showed that hADSCs TSA-treatment during the maturation step of Hep-Dif protocol (From day 14 to day 28) gives rise to functional Hepatocytes like cells with high capacity of LDL uptake, Alb secretion and liver repopulation of CCl4-injured nude mice [523]. On the same way, the TSA treatment of UCMSCs during the maturation differentiation step, induces K18, TAT, AFP, ALB, HNF4A ,KRT8 expression in UCMSCs-derived endodermic progenitors oriented their fate to hepatocyte like cells [557,559].

8.6.4. Other pharmacological indirect Cdc42 inhibitors

Several strategies are using signaling modulators molecules to rescue differentiation and genetic aberrations of aged and senescent stem cells *in vitro* such as anti-inflammatory agents, antioxidants, and low oxygen tension. Some competitors for signaling growth factors were used to reduce the Cdc42 GTP binding in aged HSCs and increased mobilization rate[652] . In addition, these molecules contribute indirectly to regulate the cell activity, MET, polarity and G protein activation in aged stem cells. I will review only in this section the inhibitors used through my work.

8.6.4.1. PI3K phosphorylation inhibitor : Wortmanin

Cdc42 activation has been shown to be a downstream of PI3-kinase pathway [653]. Wortmanin, a PI3K inhibitor was able to reverse the Cdc42 activation in cholangiocytes [654]. Furthermore, inhibition of PI3K leads to a decrease in the MSCs functionality such as survival, proliferation, migration, angiogenesis, cytokine production, and differentiation [655]. However, Wortmanin inhibits in the same time JNK pathway [656]

8.6.4.2. MAPK specific inhibitor : PD98059

EGFR inhibitor such as Erlotinib was used to reduce the Cdc42 activity in aged HSCs and increased mobilization rate[652]. Indeed, the inhibition of EGF signaling pathway blocks the MAPK family cassette phosphorylation. On the other side, several studies have shown that PD98059, a MAPK cascade antagonist inhibits cell migration, apoptosis proliferation and osteogenic potential of MSCs and other stem cells by inhibiting the main three downstream effector pathways, Erk/JNK and p38[657-659].

8.6.4.3. c-Jun NH2-terminal kinase (JNK) inhibitor: SP600125

Activation of c-JNKs cassette called also stress-activated protein kinases is an early response of cells upon exposure to DNA-damaging agents [656]. The inhibition of this pathway leads to block the proliferation rate in stem cells [660]. SP600125 causes a decrease in BM MSCs- osteogenesis differentiation potential with a concurrent increase in adipogenesis demonstrating the importance of JNK cascades in osteogenic differentiation [661, 662]. Moreover, SP600125 promotes apoptosis of ADSCs and was shown to play a key role in the PDGF-induced proliferation pathway and migration of ADSCs [560, 663]. JNK activation correlates with Cdc42-GTP bound since in Cdc42GAP-/- mice Progenitors cells SP600125 -JNK inhibition reverses stem cell apoptosis [664].

8.6.4.4. PKA inhibitor : H-89

H-89 reversed the activation of Cdc42 in epithelial [623] and ECs [665]. Furthermore the inhibition of the PKA pathway by H-89 was shown to reversing the age-related hormone level of adrenal cortex [666] . Moreover, H89 blocked the TNFα/PKA age-associated in human aged adipocytes[667] . The inhibition of PKA phosphorylation is supposed to inhibit indirectly the histone acetylation and methylation via c-CAMP /CREB complex [283, 284] which are implicated in age related p53 activation [621]. Altogether, these evidence support our choice to study the impact of PKA inhibition on the Cdc42 age-related activation.

SCIENTIFIC PAPER 2

Chapter 9 -: Introduction to the scientific paper 2

Human adipose tissue has emerged as a promising tool for liver stem cell-based therapy and drug screening *in vitro*. However, the donor age constitutes a serious limitation for long term expansion and high throughput of hMSCs for preclinical therapeutical application and liver microengineering strategies. Cdc42 a RhoGTPase G-protein was shown to be activated in aged Hematopoietic stem cells. The inhibition of Cdc42 was able to reverse senescence hallmarks in aged -derived HSCs aged donors towards a young HSCs profile. Based on these evidence, our aim was to assess the Cdc42 activity in hADSCs isolated from different age breaks. Also, we were interested to evaluate the impact of its pharmacology selective inhibition on age-related biomarkers attenuation and the hepatogenic potential within the aged-derived hADSCs population

TITLE:

"ML141 reverses the negative impact of the RhoGTPase Cdc42-dependent donor's age on hepatogenic differentiation of hADSCs"

OBJECTIVES

- Screening the Cdc42 activity within different breaks of age in human ADSCs
- Correlating the activity of Cdc42, otherwise the donor age, with the activation of the main signaling pathways involving transcription factors, cytokines, growth factors, membrane receptors, tumor suppressive markers, all together implicated in cell cycle, senescence and differentiation of hMSCs
- Evaluating the impact of Cdc42 inhibition on the reversibility of age-related biomarkers and the hepatogenic potential of hADSCs

STRATEGY

Our strategy involved two main parts. First, Cdc42 activity (GTP versus GAP) was studied in 61 ADSCs samples derived from healthy women donors with different intervals of age (Young, Middle-aged and Aged). In parallel, the expression of MAP kinases (RAS, ERK, JNK and p38) and other kinases (PI3K, PKA), transcription factors (CREB, SREBP, NFkB, PPARgamma), growth factors (PEDF, VEGF, HGF and IGF), components of the Wnt pathways canonical and non-canonical (beta-catenin, different isoforms of Wnt(s)), regulating the main cell activity functions were assessed within the different ADSCs age breaks. Furthermore, the immunomodulatory paracrine activity was evaluated within the different groups of ADSCs donors charting the inflammatory cytokines. Next, results were correlated to the Cdc42-GTP. To identify the superlative Cdc42 inhibitor able to reverse the age of ADSCs derived from aged donors and enhance their proliferation and differentiation abilities, it was essential to inhibit pharmacologically the Cdc42 activity. Consequently, we treated different ADSCs groups with CASIN, TSA and ML141 and with indirect inhibitors targeting respectively PI3K, p-JNK, p-ERK and PKA regulatory pathways (Wortmanin, SP600125, PD98059 and H-89, respectively). The exosomes release was also assessed.

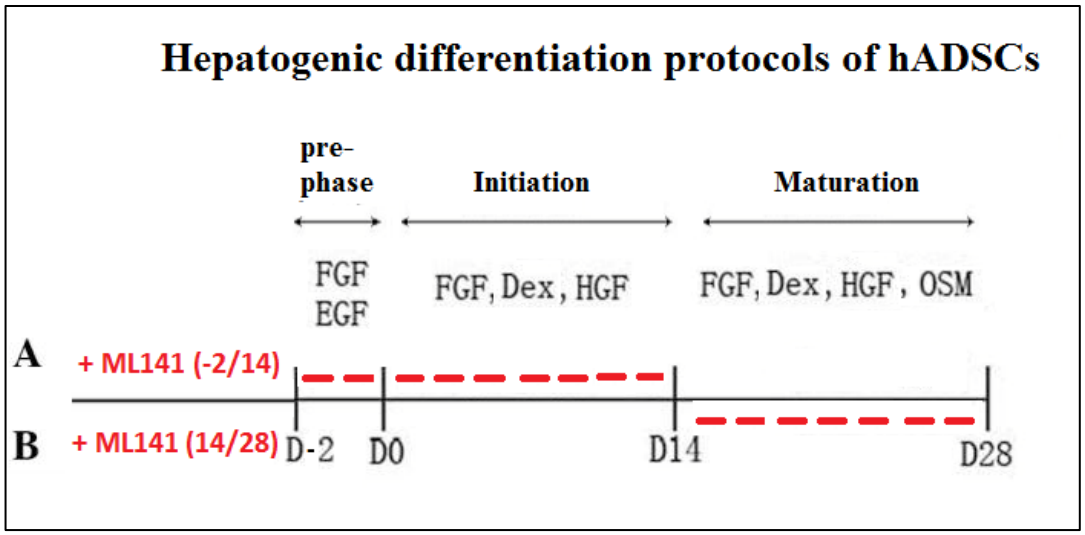
In the second part of the study, we validated whether exists any significant correlation between the high Cdc42 activity and the decrease of the hepatogenic potential in aged ADSCs. Two approaches of pharmacological treatment by ML141 were used by treating cells: (1) hADSCs for a period of ~16 days (Early phase: incubation approach from D-2 to D14 of the hepatic differentiation, reaching the hepatoblast stage) and (2) hepatoblast-like

cells for a period of ~14 days (Late phase: incubation approach from D14 to D28 of the hepatocyte differentiation, reaching the hepatocyte stage) (see below Shema1). Thus, an efficient *in vitro* culture method was crucial to provide key insights into the understanding of the molecular mechanisms controlling the Cdc42 inhibition within the aged differentiated ADSCs. Our considerations involved a human extracellular matrix (ECM) coating step and a sequentially multistep differentiation protocol over 28 days of culture. The expression of a panel of genes involved in the hepatogenic process was evaluated. To test whether hepatocyte-like cells derived from ADSCs were functional, periodic acid-Schiff (PAS) staining and specific hepatic markers expression were analyzed. Detoxification of ammonia through the synthesis of urea, secretion of Albumin (Alb), uptake of low-density lipoprotein (LDL) and Rifampicin inducible cytochrome P450 activity were also evaluated in young, aged and ML141-treated age groups at days 0, 14 and 28 of differentiation. The EMT was also considered and screened within our novel strategy by evaluating E/N cadherin ratio, SNAIL1, Twist and vimentin expression. Finally, the MSCs surface markers with a panel of 12 paracrine cytokines were assessed by flow cytometry overall the differentiation steps and different treatments of ML141 in aged ADSCs group. Similarly, Young ADSCs were differentiated following the same protocol and served as a control of rejuvenation.

RESULTS

Aged hADSCs showed high levels of Cdc42-GTP concomitant with decreases in the levels of Cdc42-GAP, low expression of IGF-1, VEGF, a decrease of anti-inflammatory cytokines and an increase in NF-κB upregulating the $p53/p16^{INK\alpha}$. Furthermore, Cdc42-GTP binding in aged hASCs group correlate positively with β-catenin and Wnt-3a expression and negatively with Wnt-5. The high expression of Cdc42-GTP bounds in aged hADSCs is associated to an activation of RAS, PKA catalytic unit, p-JNK, p-ERK and p-PI3K. The mechanism of action seems to be dependent of MAPK and Wnts signaling, evolving exosomes release.

The inhibition of the above mentioned pathways via specific small molecules inhibitors showed that Cdc42 activation in aged hADSCs involved p-ERK, p-JNK and PKA signaling pathways but not PI3K. The inhibition of Cdc42 in hADSCs didn't succeed with CASIN, the classical inhibitor of Cdc42 reported to have significant effects in hematopoietic stem cells. However, ML141 and TSA were able to reverse age-related behavior of hADSCs by decreasing the Cdc42-GTP binding complexes, the level of pro-inflammatory cytokines (TNFα and INFγ) and increasing their proliferation rate to the levels seen in younger donors. Although both of ML141 and TSA showed better improvement on yield, growth and adherence characteristics of hADSCs, ML141 proved less toxicity than TSA. The treatment of hADSCs with ML141 over their differentiation into hepatocytes showed an increasing of hepatic specific markers expression such as CK18 (KRT18), Alb, HNF4α and high CYP450 activity compared to non-treated aged group. Furthermore, a mesenchymal to epithelial transition occurred intensively in the ML141-aged treated group undergoing hepatocytes differentiation. Indeed, the ratio of E/N-cadherins increased markedly over the differentiation time compared to the aged group non-exposed to ML141. The ML141-aged group derived hepatocytes were able to produce urea, Alb and to uptake easily the LDL (highest expression level even surpassing that of HLCs from the young control group). Finally, ML141 was able to reduce markedly the MSCs markers in the treated aged group and to reverse their paracrine secretion panel to a profile of young ADSCs.



Schema 1: Hepatic differentiation protocols. All cells were pre-cultured in expansion medium until 80 % confluence was achieved (A) ADSCs were exposed to EGF and FGF-4 for 2 days followed by hepatic inducing agents at day 0. ML141 was added up to day 14 (B) ADSCs were treated with ML141 from day 14 to day 28. The media was changed every two days; hepatic differentiation was then assessed at different time-points. F/E/HGF, fibroblast/epidermal/hepatocyte growth factor; D, day; OSM, oncostatin M; Dex, dexamethasone

Title Page

Title

ML141 reverses the negative impact of the RhoGTPase Cdc42-dependent donor's age on hepatogenic differentiation of hADSCs.

Running Title

Impact of ML141 on Cdc42 in aged MSCs

Authors

Diana Chaker[1,2,3] (PhD student), Charbel Mouawad[4] (PhD), Albert Azar[2] (MD), Didier Quilliot[5] (MD, PhD), Ibrahim El Achkar[6] (MD), Ziad Fajloun[1,7] (PhD), Nehman Makdissy[1,7*] (PhD)

1 Lebanese University, Doctoral School for Sciences and Technology, Laboratory of Applied Biotechnology, Azm Center for Research in Biotechnology and its Applications, Tripoli, Lebanon
2 Reviva Regenerative Medicine Center, Human genetic Center, Middle East Institute of Health Hospital, Bsalim, Lebanon
3 Paris Saclay University, Doctoral School, Therapeutical Innovation, Inserm UMR935, Villejuif 94700, France,
4 Independent researcher
5 Service de Diabétologie-Endocrinologie et Nutrition, CHRU de Nancy and INSERM 954, Faculty of Medicine, University Henri Poincaré - Nancy 1, France
6 Achkar Clinics, St. Elie Center, Antelias
7 Lebanese University, Faculty of Sciences III, Department of Biology, Tripoli, Lebanon

*To whom correspondence should be addressed: almakdissy@hotmail.com

Abstract

Human adipose derived-mesenchymal stem cells (hADSCs) are promising cells that may promote hepatocyte differentiation and improve liver function, but the underlying mechanisms are still not well defined particularly the involvement of age. In mouse models, hematopoietic stem cell (HSC) aging was shown to be regulated by the small RhoGTPase Cdc42. We hypothesized that the inhibition of Cdc42 may rejuvenate hADSCs derived from aged donors (AD) and thus stimulate their hepatogenic potential. In hADSCs isolated from 61 women belonging to different age intervals, we showed that Cdc42 activity was positively correlated with growth factors, cytokines, transcriptional factors, Wnt signaling, exosomes releases, MAPK and PKA but not PI3K pathways in AD-hADSCs. The cells were less functional and showed decreased potential of hepatocyte differentiation as compared to cells derived from the younger group. Pharmacological inhibition of Cdc42 activity by ML141 and TSA but not CASIN, rejuvenated AD-hADSCs into functionally younger-like cells; cell proliferation, doubling, adherence and apoptotic index were significantly reversed. In parallel to a mesenchymal-epithelial transition, ML141 gave rise to mature and functional hepatocytes-like cells as assessed by hepatic genes' expression, cytochrome activity, urea and albumin production, LDL uptake, glycogen storage and anti-inflammatory cytokines release. Thus, selective inhibition of Cdc42 could be a potential target of drug therapy for aging and may give new insights on the improvement of hepatocyte differentiation. **Keywords**

Aging, Cdc42, ML141, Adipose tissue, Mesenchymal stem cells, Hepatocytes, Hepatogenic differentiation, Liver failure

Introduction

Aging is a process that results from an increased failure in a system normally designed for growth and reproduction[1]. Stem cell aging could drive to the collapse in tissue function and regeneration in older humans[2]. Although specialized niches usually protect stem cells from age-dependent stress mechanisms, data showed that these cells can also grow old [3]. Moreover, the senescence in stem cells may be passed to their differentiated progeny leading to the exacerbation of this process. Current research is focusing on deviating any feature that could possibly slow down the progression of aging [4]. Mesenchymal stem cells (MSCs) have emerged as a promising tool and an attractive stem cell source for this purpose. While the differentiation potential of MSCs is comparatively limited to that of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), they are a much safer source regarding the risk of inducing tetratoma and raise less ethical debates when it comes to clinical applications [5, 6]. Recent strategies in tissue engineering and cell therapy have shown that human Adipose-derived MSCs (hADSCs) hold numerous benefits over Bone Marrow-derived MSCs with higher potentials of proliferation and differentiation capacities *in vitro* [4, 7]. Indeed, once induced into functional hepatocyte-like cells, hADSCs provided a promising non-transgenic tool for autologous, hepatocyte-based therapies by displaying *in vitro* functions of mature hepatocytes and showing efficient repopulation properties in mice models of liver injury. [7-10]. nevertheless, hADSCs donor's age presents a negative impact on the properties of ADSCs differentiation, doubling population, homing, and immune modulation[11-16]. Agedependent MSCs senescence has been linked to a decrease in mitochondrial activity, high expression of p16INK4a, p53 and p21 and an increase of β-galactosidase and ROS accumulation [4, 12, 17-19]. Recent findings showed that the functional decline in the elderly HSCs was associated with an upregulation of the activity of the Cell division cycle 42 (Cdc42) protein. Cdc42 is a small G-protein that belongs to the RhoGTPase family of the Ras superfamily and depends on the GTPases-Activating Proteins (GAPs) in order to undergo switch cycles between the active and inactive form [20, 21]. Cdc42 regulates actin cytoskeleton, cell polarization and adhesion, migration of stem cells and tissue regeneration [22, 23]. Cdc42 was described to crosstalk with the JAKSTAT pathway, and is crucial for the activation of ERK, JNK and p38 MAP kinases [24, 25]. Mice HSCs showed elevated Cdc42-GTP expression after genetic deletion of a negative regulator of Cdc42 and exhibited a severe early aging pattern [26, 27].

Several reports discussed the role of Cdc42 in human MSC proliferation, migration and differentiation potential [28, 29]. The histone deacetylase inhibitor Trichostatin A (TSA) was shown to be a non-specific inhibitor of Cdc42 activity as well as being a key factor for hepatocytes differentiation of MSCs [30, 31]. Other studies indicated that the pharmacological and irreversible inhibition of the Cdc42 activity by CASIN (Cdc42 activityspecific inhibitor) was able to overturn aging and restore the spatial distribution of histone H4 lysine 16 acetylation of aged HSCs to a status similar to that seen in young HSCs [32, 33]. Thereby, upon transplantation, CASIN-treated HSCs were shown to be potentially identical to HSCs isolated from young donors [26]. More recently, ML141 (CID-2950007) was referred to as a novel selective and non-competitive allosteric inhibitor of Cdc42; it promotes migration and regulation of mice HSCs polarity with a notably low potency of inhibition against other members of the Rho family of GTPases [34, 35]. TSA was able to enhance hepatic differentiation from human Bone Marrow and adipose tissue MSCs[36, 37]. Since for this moment there is no published data showing the impact of the Cdc42 inhibition on reversing the age of elderly hADSCs and reducing their senescence aberrations, our strategy was based on identifying the superlative Cdc42 inhibitor able to rejuvenate hADSCs from aging donors and then stimulate their healthy hepatocytes differentiation potential. Here we described that ML141 succeeded in decreasing the Cdc42-GTP levels in hADSCs and promoted hepatocytes-like cells generation from older donors in a manner that is functionally equivalent to hADSCsderived from younger patients.

Results

Ccd42 activity increases with age: correlations with growth factors, cytokines, transcription factors, kinases, Wnt signaling and exosomes

First, the screening of Cdc42 activity in hADSCs showed an age-dependent significant increase of Cdc42GTP (and a decrease in Cdc42 GAP) (Fig.1A-B). We also noted that the expression of a number of growth factors and cytokines was altered in hADSCs from the aged group as compared to the other two groups. The Insulin growth factor (IGF-1) regulating survival, self-renewal, and differentiation of hADSCs decreased significantly in the aged population while the age had no or little effect on the anti-inflammatory, anti-fibrotic and anti-apoptotic hepatocyte growth factor (HGF) (Fig.1C). Our result showed that VEGF decreased considerably in hADSCs isolated from aged donors compared to young and Middle-aged counterparts (Fig.1D). The positive ratio of VEGF over PEDF (Pigment epithelial-derived factor) has been used in bone marrow MSCs (BMSCs) as an indicator of the angiogenic potential and here, we showed that the PEDF increases with age thus potentially exerting anti- angiogenic effects. Since hADSCs have immunomodulatory properties *in vitro*, we were interested in evaluating the inflammatory paracrine potential of hADSCs from different age groups. In terms of anti-inflammatory cytokines, IL-10 but not IL-4 decreased significantly in the aged group (Fig.1E). On the other side, these same cells secreted significantly more pro-inflammatory cytokines such TNF-α and IFN-γ (Fig.1F).

Furthermore, we evaluated the expression of certain transcription factors Sterol regulating element binding protein1 SREBP-1 expression which is an aging-associated biomarker in *in vivo* aging MSCs. The SREBP-1 subtype increased significantly with age and adequately to Cdc42-GTP expression. However the subtype 2 didn't show significant change (Fig.1G). Although the phosphorylation of the c-AMP regulatory element binding CREB was discussed in BMMSCs, this pathway had never been reported in human ADSCs. Indeed, our results showed for the first time, that CREB phosphorylation is increased in aged-ADSCs (Fig.1H).

Simultaneously, the phosphorylated NF-κB subunits were shown to be significantly increased in the aged ADSCs group (Fig.1H).

The mechanisms of regulating Cdc42 activation are very complex. To better understand which signaling pathways may be involved in the observed increased Cdc42-GTP expression, we investigated the main mitogen-activated protein kinases (MAPKs) signaling pathways. JNK pathway can be activated by PI3K and activates Cdc42-GTP binding. On the other hand RAS activation triggers the direct activation of PI3K pathways and the phosphorylation of ERK and our results showed an up-regulation of RAS, p-JNK, p-ERK and p-PI3K in the aged group but not of the p-p38 (Fig.1I-K). The binding of the PKA enzyme to a catalytic subunit is suggested to activate the Cdc42; correspondently, our results exhibited an up regulation of the PKA catalytic subunits with the increase of the donor's age (Fig.1K). On the other hand, no significant changes were observed in the levels of Insulin receptor (IR) and its substrate IRS (Fig.1L), neither PKB or glucose transporters GLUT-1/-4 (data not shown) indicating no involvement of the insulin pathway.

The release of exosomes from MSCs may play an important role in several physiological pathways. Impaired exosomes release may affect aging and have crucial impacts in cell differentiation. Several markers of the tetraspanin family including CD9, CD63 and CD81 are highly enriched in exosomal membranes. hADSCs showed significantly impaired release of exosomes in hADSCs-derived from the aged group as observed by a significant reduction in the expression of CD9/CD63/CD81 (Fig.1M) recovered in the exosomes fraction. Finally, to whether or not the Wnt-signaling pathway may be affected, we investigated whether any correlation occurred between Cdc42-GTP and β-catenin/Wnts. Significant positive correlations were found with β-catenin and Wnt-3a contrary to Wnt-5a (Fig.1N), whereas no correlations were found with Wnt-4/7a/11. In addition, this study shows for the first time correlations between Cdc42-GTP and several screened factors in hADSCs derived from donors of different ages. In summary, Cdc42-GTP was significantly correlated and positively to PEDF, TNF-α, IFN-γ, p-CREB, p-NFkB, Ras, p-JNK, p-ERK, PKA and p-PI3K, and negatively to Cdc42GAP, IGF, VEGF and IL-10. No significant correlations were observed with p-p38, p-IR, p-IRS, IL4, HGF, SREBP-1 and -2 (Figure 1).

Reversibility by inhibitors of PKA, JNK, ERK and Cdc42 activity but not PI3K

To investigate the signaling mechanisms that may be involved in the increase of Cdc42-GTP observed in the older donors group, we inhibited the main signaling pathways screened previously and evaluated the activity of Cdc42-GTP: Wortmanin (PI3K inhibitor) didn't show any impact on the CdC42-GTP expression (Fig.2A) whereas SP600125, PD98059 and H-89, the inhibitors of JNK, ERK and PKA pathways respectively, significantly reduced the level of Cdc42-GTP in the aged group to levels similar to that observed with the middle-aged or young ones. Next, we proceeded to inhibit pharmacologically the Cdc42 activity. Since for the moment there is no data showing the impact of Cdc42 GTPase inhibition in hADSCs, we chose inhibitors known to inhibit Cdc42 activity in HSC, cell progenitors and some other cell lines: CASIN, ML141 and TSA. CASIN, shown to reverse cell aging in HSCs, had no impact in our cells on Cdc42 GTPases inhibition. However, both the direct selective Cdc42 GTPases inhibitor ML141 and the indirect inhibitor TSA (HDAC inhibitor) were able to reverse

significantly the age-related behavior of hADSCs by decreasing the Cdc42GTP binding complexes to the levels seen in younger donors (Fig.2B). To evaluate the impact of TSA and ML141 on the immunomodulatory behavior of hADSCs from aged donors, we showed, using ELISA assays, that ML141 and TSA stimulated the anti-inflammatory cytokine IL-10 (but not IL4) while they decreased significantly the levels of the pro-inflammatory cytokines TNFα and INFγ (Fig.2C-D). CASIN did not have significant effects on regulating the secretion of the inflammatory studied cytokines in hADSCs-derived from the three studied groups (data not shown); more prolonged time of incubation of CASIN and higher doses were toxic. These results showed that Cdc42 inhibition reversed the effect of age observed in hADSCs isolated from older donors.

Pharmacological targeting of Cdc42 activity by ML141 and TSA on the yield, growth and adherence characteristics of hADSCs

Based on our previous findings, the study was pursued using the ML141 and the TSA in the young and aged groups to screen for the cell cycle behavior of hADSCs. First, cells derived from the aged group showedsignificant decreases in cell proliferation, doubling, adherence, and viability with concomitant increase in the apoptotic index (Fig.3). Compared to the non-treated control aged hADSCs group, both of the TSA and ML141 treated cells showed significant increases of cell numbers and doubling time in aged-group treated hADSCs (Fig.3A-B). Distinctly, ML141 more than TSA, promoted higher cell proliferation rate (Fig.3A) and improved cell doubling time (Fig.3B). The adhesion assay highlighted a very marked effect of the ML141 which was more pronounced after 48 h (Fig.3C-D). Toxicity studies proved ML141 to be less toxic then TSA in terms of apoptotic index and cell viability (Fig.3E-F). Moreover, ML141 was able to repress the expression of the apoptotic and senescence genes p16, p53 and p21 more than TSA within the aged-group treated hADSCs; the non-treated control aged group showed high senescence gene profile expression (Table 2) compared to the young one. These results prompted us to continue using solely ML141 in the rest of the work.

Pharmacological Cdc42 inhibition rescued the hepatogenic potential of Aged ADSCs and induced an MET transition

We explored multiple protocols to induce hepatic differentiation within the hADSCs isolated from the young and aged groups. Six hADSCs groups were subjected to hepatocyte differentiation divided into: young, aged, and ML141-treated aged groups [ML141 kept in contact with the cells for: 48 h (D-2 to D0), 16 days (D-2 to D14), 14 days (D14 to D28) or 30 days (D-2 to D28)]. Progressively, we ruled out two groups where cells were treated with ML141 during the pre-differentiation phase (D-2/0) or treated continuously over the whole period (D-2/28): the group -2/0 didn't show significant improvements, however an aggressive cell apoptosis was observed during the maturation step of the group -2/28 starting from D19/D20 where it was impossible to maintain the cells adherence and to achieve the differentiation (data not shown). Thus, we proceeded to treat the cells with ML141 during the early differentiation (D-2/14) and maturation (D14/28) phases. Adherence and viability (7AAD staining *vs* Annexin V/PI) were significant and above 85% in these two groups. The multipotency of hADSCs was confirmed in parallel the hepatocyte differentiation by inducing an adipocyte and neural differentiation (data not shown).

First, we examined the cell immunophenotyping at days 0, 14 and 28: all hADSCs were negative for the hematopoietic stem cell markers CD14, CD34, CD45 and for HLA-DRs but positive for the specific mesenchymal stem cell markers CD90, CD105 and CD73 (Fig.S1). Interestingly, a decline in the mesenchymal phenotype was observed in the young group indicating a transition of the undifferentiated phenotype to a differentiated one. In addition, at day 28, cells from the non-treated aged group always showed a higher expression of MSCs markers compared to the young group. Conversely, ML141 reduced markedly the MSCs markers (Fig.S1H).

Second, the expression of several genes that play key role in the hepatocyte differentiation was evaluated (Fig.4). Compared to the young group, our results showed that at day 14 post-induction, differentiating cells have already lost their Ki67 expression indicating a full commitment into hepatogenic differentiation; Sox17, the specific marker of the endodermic lineage, was shown to be highly expressed in aged-hADSCs and ML141 significantly reduced its expression. HNF4α, the master gene of the hepatogenic lineage, increased significantly in the young group contrary to the aged group and ML141 reversed its expression; however, HNF6 expression didn't show any remarkable variation. AFP, the hepatoblast fetal gene, showed a drop in expression independently of Cdc42 inhibition contrary to the albumin expression which was significantly increased by ML141. Cyp3A4 (premature form) and Cyp3A7 (natal hepatocyte form) increased during differentiation with marked increases in the young group and ML141 restored their expressions. The obtained gene's profile indicate a differentiation of hepatocyte-like cells (HLCs) which led us to check the expression of genes responsible of the metabolic activity: TAT and G6P expressions were significantly reduced in the aged compared to the young group, and reversibility was obtained by ML141 treatment. In addition, ML141 significantly induced the expression of hepatic functional genes, the epithelial cytokeratin CK8 and CK18, but not CK7. The mesenchymal-epithelial transition (MET) genes levels reflected a variable and age-dependent pattern in the different groups: SNAIL, Vimentin, and N-cadherin decreased during the

differentiation with higher levels in the aged-untreated group, contrary to E-cadherin which increased; ML141 reversed the situation. SLUG and TWIST didn't show a significant variation in all differentiated groups.
Next, we assessed the expression of Vimentin, Ki67, and AFP proteins by immunohistochemistry (Fig.5): the results confirmed the high expression of Vimentin in the aged-untreated cells compared to young group, and ML141 treatment was in favor of an epithelial transition and rejuvenation of aged-treated hADSCs. Ki67 protein expression disappeared during differentiation and ML141 maintained its absence in HLCs. Concomitantly, the expression of AFP protein persisted in non-treated aged-hADSCs and disappeared after ML141 treatment. These results are in favor of hepatogenic commitment of treated ADSCs and a maturation index by loss of the AFP expression.

ML141 restores hADSCs function during hepatocyte differentiation
First, to assess the responsiveness of the differentiated HLCs, cells at day 27 were treated with Rifampicin for 24 h, and the mRNAs expression of Cyp450 enzymes, albumin, TAT and G6P were determined (Fig.6A-C). HLCs, post-rifampicin induction, displayed significant increases in the expression of Cyp3A4 and Cyp3A7 as well as albumin in ML141-treated groups. However, no significant inductions were seen for TAT and G6P (data not shown).
To test the functionality of the obtained HLCs, we quantified the levels of secreted albumin, urea production and LDL uptake (Fig.6D-F). During differentiation, we observed progressive increases in the levels of production of albumin and urea, as well as the LDL uptake. However, aged-untreated cells exhibited low levels in comparison to young group and a significant reversibility was observed after ML141 treatments. Interestingly, the storage of glycogen as evaluated by PAS staining (Fig.6G) confirmed the HLCs functionality and that young-derived HLCs stored high levels of glycogen contrary to the aged-untreated cells; the morphology of the cells changed from being long-spindled shaped at D0 to short-spindled shaped at D14 in all groups, and to large-sized, polygonal shaped HLCs at D28. ML141-treated aged cells retained high levels of purple stain similar to the intensity seen in cells derived from young donors and in HepG2 cell line.

ML141 reverts the profile of secreted cytokines
The analyses of cytokines profile determined by flow cytometry on the collected supernatants of undifferentiated-ADSCs (D0), differentiated-hepatoblast-like cells (D14) and differentiated-hepatocyte-like cells (D28) (Fig.7) showed that the main pro-inflammatory cytokines TNF-α/IFN-γ/IL-17A/IL-12p70/IL6/IL-2 significantly decreased during the differentiation of the young-derived cells and were significantly elevated in the aged untreated group. Besides and during differentiation, anti-inflammatory cytokines, IL10/IL-5/IL-4 significantly increased in the young group contrary to the aged group. On the other hand, no significant changes were observed in the levels of INF/GM-CSF/IL-9. Treatment of aged-group derived cells with ML141 significantly reversed the situation especially when ML141 was incubated during D-2/14 and less importantly in case of D14/28. ML141 (D-2/0) didn't show significant variations (data not shown). These results demonstrate the evident impact of ML141 on diminishing the inflammatory levels of cytokines produced during differentiation of cells derived from aged subjects, thus making the cell phenotype closer to the young one.

Discussion
In this study, we investigated the potential of pharmacological inhibition of Cdc42 to reverse the aged phenotype of adipose-derived mesenchymal stem cells (ADSCs) into a younger phenotype, which may remove the blockade or inhibition of the hepatocyte differentiation. ADSCs have recently emerged as a great tool for clinical application in regenerative medicine [7]. At this moment, 71 studies using human ADSCs for cell based therapies are registered on Clinical Trials webpage. A high yield of these cells can be isolated with slight patient discomfort[38-40]. However, several studies suggested that the donor's age may strongly impact various hADSCs properties[12, 13, 41]. In fact, although the cells isolated from elderly subjects didn't completely lose their proliferative potential they, nonetheless showed lower rates of expansion *in vitro*, which makes them a weak tool for clinical autologous use. Even though ADSCs yield was shown to decline in aged collected adipose tissues [40, 42], within our population this yield didn't show a significant variability within the different age groups.
Cdc42 was shown to be closely involved in human BMSCs cell proliferation, polarity, migration and differentiation [28], and Cdc42 activity exhibited an increase of the GTP binding complex in HSCs derived from aged subjects [33, 43, 44]. Several strategies aim to reverse the ageing in human adult stem cells by inhibiting the Cdc42 activity. Furthermore, the inhibition of the Cdc42 signaling pathway was used to prevent pro-oncogenic mutations and cell senescence [43]. Based on these evidences, our strategy was to screen for the first time the activity of Cd42 in hADSCs isolated from healthy donors than to investigate the effects of selectively inhibiting its activity. A positive correlation was found to exist between the donor age and the ratio of Cdc42GTP/total Cdc42 in HSCs and progenitors blood cells[26, 45]. Similarly, our results showed that in ADSCs, the activity of Cdc42-GTP increased with age. On the other hand, many reports indicate a proinflammatory cytokines' profile increased with aged MSCs; TNF-α and IFN-γ secretion is increased in bone marrow and umbilical cord aged patients derived MSCs [46-51]. Equally, our findings showed that the paracrine cytokine panel within aged derived ADSCs is typically pro-inflammatory

since TNF-α and IFN-γ are markedly increased with aged and correlate closely to the Cdc42-GTP bindings. Previous observations explained how TNF-α / IFN-γ upregulated the p38-MAPK kinase phosphorylation pathway and negatively altered the immunomodulatory potential of hMSCs. We also noted that the levels of growth factors were affected in ADSCs from aging donors.

While the positive ratio of VEGF over PEDF was used in BMMSCs as an indicator of the angiogenic potential [52] and the Cdc42 was shown to regulate VEGF-mediated angiogenesis in human and mice endothelial progenitors cells [53,54], there is no data reporting a loop of regulation between VEGF expression and Cdc42 activation during MSCs aging. Our results revealed that the increased activity of Cdc42 in ADSCs correlated with a negative transcription of VEGF potentially reducing their angiogenic and regeneration abilities. The MAPK pathways was shown to be implicated in MSCs aging; indeed p38, JNK and ERK pathways trigger the tumor suppressor gene p53 transcriptional program, ROS accumulation and apoptosis in fibroblasts [55,56], HSC [45,57] and human umbilical cord MSCs (UCMSCs) [51]. Also, PI3K can be triggered by IRS ligand binding to IGF [58], thus activating JNK pathway and subsequently Cdc42-GTP binding [59]. In parallel, it was described that the targets of PKA phosphorylation stimulate GTP binding on Cdc42 and thus cell aging in human mast cells [46]. Similarly, in our study, Cdc42-GTP binding levels in aged ADSCs correlated with p-ERK, p-JNK and PKA activation but not with PI3K pathway. Since it was reported that IGF binding to the tyrosine kinase receptor can directly lead to Cdc42 activating without passing by the upstream PI3K [60], this result indicates that activation of Cdc42 in aged ADSCs is regulated by different cassettes of signals independently of PI3K. Interestingly, the use of Cdc42 inhibitors on hADSCs had never been studied before. First, CASIN-treated aged HSCs exhibited a level of Cdc42-GTP similar to that found in HSCs from young donors [33]. Contrariwise, CASIN impact was revealed to be loudly different in cancer stem cells by promoting their growth arrest and migration [61,62]. Moreover in our study, CASIN didn't inhibit Cdc42 activation in aged ADSCs. These controvert mechanisms remain unclear since CASIN has never been used before as Cdc42 inhibitor in other subtypes of MSCs. Therefore, the CASIN inhibition appeared to be cell-type dependent and doesn't inhibit the GEF activity in aged ADSC. Thereby, CASIN impact on other age-related proteins within the aged ADSCs could be a remarkable field of study in the future. Second, TSA, an HDAC inhibitor, which showed significant inhibitory effects of Cdc42 activity in hADSCs; In other studies, it was able to maintain the pluripotency expression in placenta derived hMSCs, delay the appearance of aging signs [63], and promoted the proliferation and self-renewal of hUCMSCs [64]. Third, ML141 was recently used as a potent Cdc42 inhibitor in cancer stem cells to downregulate EGFR and JNK signaling pathways[65-67]. We are the first to inhibit the Cdc42 activity in aged hADSCs by both ML141 and TSA, and correlate its activity with the expression of biomarkers in aged hADSCs. In our study, ML141, more than TSA, promoted better population doubling and less toxicity, better proliferation rate and adhesion potential in a way that was similar and sometimes better than the non-treated young ADSCs. Furthermore, ML141 was able to repress the expression of the apoptotic and senescence genes p16, p53 and p21 within the treated ADSCs. In addition, we showed that both TSA and ML141 promoted antiinflammatory potential in the treated ADSC subpopulation by decreasing the TNF-α/IFN-γ/IL-17A/IL12p70/IL-6/IL-2 secretion levels.

These results correlate closely with recent findings where ML141 was shown to inhibit Cdc42 activity in hBMSCs, endothelial cells and cancer stem cells by downregulating EGFR/JNK/PAK pathways implicated in aging and polarity of stem cell processes[66-70]. Other studies reported that TSA may reverse the age impact within other human MSCs subtypes such as UCMSCs and BMMSCs[63,64,71-73].

Our second goal was to study the impact of Cdc42 inhibition on the rejuvenation of ADSCs and their hepatogenic differentiation potential. TSA was described to enhance cardiogenic and neurogenic potential in aged BMMSCs and to induce albumin and CK8 expression in UCMSCs derived hepatic progenitors[71,72,74-76]. Since human ADSCs were shown to be a promising tool for hepatocytes generation *in vitro* [77,78], we conducted hepatocyte differentiation protocols adapted by Yin *et al*, 2015. In this report, TSA was shown to be essential to promote *in vitro* differentiation of human ADSC towards functional hepatocyte-like cells between days 14 and 28 [79]. However, the derived HLCs secreted albumin and were able to uptake LDL, they continued to express AFP which is a fetal hepatocytes marker. Distinctly, our novel strategy consisted on replacing the TSA treatment by a specific and direct Cdc42 GTPase inhibitor, the ML141. Remarkably, our strategy was a new and innovated step in terms of (i) following a two-step protocol of ML141 treatment within the hepatogenic differentiation protocol and (ii) evaluating an extensive range of cytokines, growth factors, hepatocytes maturation markers and the MET process within young, aged and ML141-treated aged ADSCs. Indeed, our results showed that young and ML141-treated aged ADSCs groups showed a decrease in endodermic genes (Sox17) , fetal liver genes (AFP, HNF6) and an increase in hepatic master gene HNF4α and hepatic functional genes (Alb, Cyp3A7, TAT,G6P, CK8 and CK18). Conversely, at day 28, HLC derived from non-treated aged ADSCs showed an incomplete fetal hepatic profile similar to the profile seen in TSA treatedHLC generated by Yin and coworkers [79]. Our HLCs were functional and expressed high levels of albumin and Cyp450 upon Rifampicin induction. They were also able to stock glycogen, to uptake LDL and produce urea better than to the HLCs derived from young donors, and efficiently reversed the levels of secreted cytokines. These results

lead us to consider the ML141-treated ADSCs derived from aged donors as a promising tool for new therapeutic strategies that use MSCs exosomes in liver diseases [80].

The MET was shown to be a crucial process when we transdifferentiate MSCs into hepatocytes [81]. An epithelial transition was reported in cancer cell line treated with ML141 [82]. Based on these evidences, it was interesting to study the MET process in our differentiation protocol. Our results showed that ML141 treatment of aged ADSCs gave rise to HLCs presenting a typical epithelial phenotype and high Ecad/Ncad expression ratio especially in the D14/28 treated group. Previous data showed that HLCs derived from ADSCs presented a downregulation of Twist and Snail expression [83]. This MET correlates closely with the appearance of functional hepatic markers hardly expressed in non-treated aged ADSCs such as albumin, TAT, G6P and CYP450. Moreover, the positive co-expression of CK18 and E-Cadherin was reported to be a crucial parameter for MET in murine hepatic and embryonic stem cells [84] and human iPSCs [85] differentiation into hepatocytes. This evidence support our findings, where HLCs generated from treated ML141 showed high expression of CK18. Our novelty in this study resides in the fact that currently there is no previous report using ML141 to reverse the age-related aberrations in aged stem cells and promote greater hepatogenic potential than young differentiated ADSCs counterparts.

These data suggest that the inhibition of Cdc42-GTP activity might represent a novel target to rejuvenate not only HSCs but also aged ADSCs by altering their immunomodulatory effects, decreasing apoptosis and improving the cell activity potential. Cdc42 activity can be pharmacologically targeted to rejuvenate aged ADSCs for MSC_based therapies and tissue engineering. Clinical liver diseases applications in the future will need new approaches for *ex vivo* safe manipulations including ADSCs cultivation and direct hepatic fate within a short time. Thus, Cdc42 inhibition may also be important in converting the weak hepatogenic potential of aged ADSCs into functional and mature derived-ADSCs hepatocytes.

Methods

Population design

61 healthy subjects were enrolled in this study and classified into three age groups: Young (23.8 years ± 0.4), middle-aged (40.8 years ± 0.6) and old/aged (57.6 years ± 0.9 years). Adipose tissue harvests (<600 mg) collected from the abdominal area were processed manually for the SVF isolation. The cell yield (total nucleated cells and MSCs numbers) showed no significant differences between the different age groups (Table 1). Subjects were included in the study only if they had no notable pathological history, in particular liver diseases and were excluded based on a list of criteria as shown in table 1. Investigations on human samples have been conducted according to the principles expressed in the Declaration of Helsinki, as revised in 2008 (http://www.wma.net/e/policy/b3.htm). All donors gave written informed consent and samples were procured by the Nancy University Hospital Clinical Investigation Center (ethical approval number, CPP3.07.01, France).

Isolation and expansion of hADSCs

Human adipose tissue (200 ml) was obtained by lipoaspiration of 61 healthy female donors (belonging to different age groups) who underwent abdominal subcutaneous fat liposuction. Stromal vascular fraction (SVF) cells were manually isolated by enzymatic digestion in standard grade collagenase. Total fat was dispensed into 50 ml tubes to be washed twice with phosphate-buffered saline (PBS) and 5% penicillin-streptomycin-amphotericin B (PSA), and then centrifuged at 300xg for 3 min with elimination of the pellet containing red blood cells. Washing and centrifugation were repeated 3 times. Collagenase type I was added to the fat (1v/1v) and the mixture was incubated 30 min at 37°C with gentle agitation. A volume of 5 ml fetal bovine serum (FBS) was added to stop the activity of collagenase. After incubation, sample is centrifuged at 600x g for 10 min. The cell pellet was resuspended in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. ADSCs were selected by plastic adherence. Cell concentration was adjusted to a density of 10^5 cells/cm^2. At 80% confluence cells were harvested, and serial subcultures were performed at a density of 10^4 cells/cm^2. Medium was changed every 3 days. The hepatic differentiation was induced at the 3rd ADSCs passage.

ADSCs were treated in some experiments for 24 hours with the following pharmacological inhibitors 0.8 µM of TSA, 10 µM of CASIN and 5 µM of ML141. All reagents were purchased from Sigma-Aldrich.

Hepatogenic induction of hADSCs hADSCs were seeded into MaxGel™ ECM-coated plates and divided into four groups: young, aged, aged treated with ML141 (5 µM) from day -2 to 14 (–2/14) or 14 to 28 (14/28). All groups underwent the same hepatic differentiation protocol : (1) pre-induction at 80% of confluence (day -2) where hADSCs were cultured in serumfree medium for 48 h with 20 ng/ml b-FGF and 20 ng/ml EGF (2) induction from day 0 to 14 of the differentiation using media free of serum and supplemented with 30 ng/ml HGF, 1x iTS and 10^{-8}M dexamethasone and (3) maturation from day 14 to 28 of the differentiation using media free of serum supplemented with 50 ng/ml OSM, 20 ng/ml HGF, 1x iTS and 1µM dexamethasone. Media were changed every 3 days. All growth factors and supplements were purchased from Sigma Aldrich. Cell morphology and cytotoxicity were controlled daily.

RT-qPCR

RNA was extracted from the cell pellets using RNAspin Mini kit (GE Healthcare), according to the manufacturer's instructions. The mRNA was reverse transcribed to complementary DNA. Complementary DNA was amplified using VeriQuest Fast SYBR Green qPCR Master Mix (75690 500 RXN, Affymetrix). Thermal cycling was performed on LightCycler 2.0 (Roche) with the following protocol: 1 cycle (50°C/2 min), 1 cycle (95°C/5 min), 45 cycles (95□C/3s and 60□C/30s). The analysis of the melting curve was performed to exclude non-specific amplification products. Relative changes in expression were calculated after normalization to GAPDH. Primers used are listed in Table S1.

Protein Pull-down and Western blot analysis

Proteins lysates were prepared using a lysis buffer provided by the Active Cdc42 Pull-Down and Detection Kit (#16119-Thermo Scientific). 700 µl of each lysate were used for every assay. Cdc42-GTP was precipitated by fusion to p21-PBD-Pak1 with glutathione agarose resin. The reaction mixture was incubated at 4°C for 1 hour in spin cups. The resin was removed and the protein separated on 12% of acrylamide gel following the manufacturer's instructions. GTPγS and GDP were used to generate positive and negative control lysates, respectively. Protein concentrations were determined using the Bradford Reagent (Sigma Aldrich). 20µg of proteins were loaded in all experiments. SDS-PAGE analysis was performed as previously described by Makdissy *et al.*[86] on a 10 % resolving gels. The proteins were transferred onto a PVDF membrane using a Bio-Rad Mini Trans-Blot apparatus and detected using a Protein Detector LumiGLO Western Blot Kit (54-12-50, KPL Laboratories, Gaithersburg, MD, USA) according to the manufacturer's instructions. The membranes were probed with diluted primary human antibodies, Anti Cdc42-GTP, Cdc42-GAP, HGF, IGF-1, VEGF, PEDF, IL4, IL10, TNF4α, INFγ, p68-SREBP-1, p68-SREBP-2, p-CREB, p-NKFβ, RAS, P-JNK , p-ERK, p-p38, PKA, pPI3K, p-IR and p-IRS1 overnight at 4°C (all phosphorylated proteins were controlled relatively to total targeted protein). Bound antibodies were detected by incubation with a with horseradish peroxidase-conjugated goat antimouse IgG antibody at Room temperature. The detection was performed with SuperSignal West Pico Chemiluminescent Substrate (#34080-Thermo scientific) and followed by exposure to X-ray film. The exposure time was 2 seconds. The images were captured and quantified using a Gel Doc 2000 imaging system and Quantity One software (Bio-Rad). B-actin was used as an internal control.

Cell Proliferation assay

To estimate the proliferation rate of the cells under different cell culture conditions, doubling time assays were performed. A total of 10^3 cells/cm^2 were plated in 12-well tissue culture plates (in triplicate) with 0.5 ml/well of culture medium and incubated at 37°C, 5% CO_2, for 0-96 hours. At each endpoint, cells were washed once with 1 ml PBS 1X, detached with 0.2 ml trypsin/EDTA, resuspended in 0.5 ml in complete culture medium and counted on a hemocytometer. Results are validated by MTT colorimetric assay (Sigma-Aldrich). Cell doubling (n) was determined as following: $Cf/C0 = 2^n$ [n = (logCf-logC0)/log2], where C0 and Cf are the number of cells at time T0 and Tfinal, respectively.

Adhesion assay

24-well tissue culture plates coated with Fibronectin (Millipore PIFB24P05) were used for adhesion assays. 10^5 cells were plated in each well, and allowed to adhere between10 min and 96h at 37 °C. Subsequently, the nonadherent cells were carefully removed at each time point and counted. The percentage of adhesion was calculated as a ratio of the number of adherent cells of each sample to the total number of cells added in the coated wells.

Immunohistochemistry (IHC) staining

10^4 cells were adhered to a slide by Cytospin® centrifugation and probed with primary antibodies (anti-vimentin, anti -Ki67 and anti-AFP (1/250; Abcam)). IHC was performed on NexEs (benchmark, ROCHES) automate.
Images were captured using Zeiss microscope.

Dosage of Albumin by ELISA

The level of Albumin was measured in cell supernatants using ELISA quantification kit (#ab108788, Abcam) in accordance to manufacturer's instructions. Briefly, supernatants were first incubated with biotinylated albumin antibody, then the conjugate and later with the chromogen substratein. The optical absorbance was measured on the Varioskan™ Multimode Microplate Reader (Thermofisher scientific) at 450 nm.

Low-density lipoprotein (LDL) uptake assay

Fluorometric LDL Uptake Assay Kit (#ab204716, Abcam) was used to assess the LDL uptake capacity. $3x10^4$ of hADSCs and hepatocyte-like cells were incubated with fluorescent labeled-LDL overnight at 37°C; after incubation cells were washed and fluorescence measured on the Varioskan™ LUX Multimode Microplate Reader. Cell culture media alone and HepG2 cell line were used as negative and positive controls, respectively.
RFU values were calculated from a standard curve following the manufacturer's instructions.

Urea production assay

The amount of urea present in the cell lysate was assessed using the colorimetric urea assay kit (#MAK006, Sigma-Aldrich) according to the manufacturer's instructions. The optical absorbance was measured on
Varioskan™ LUX Multimode Microplate Reader (Thermofisher scientific) at 570 nm. Human urine served as the positive control.

Periodic Acid-Shiff (PAS) staining

Glycogen storage was evaluated using the PAS staining kit (#395B, Sigma-Aldrich). Cells were fixed with 4% formaldehyde following the manufacturer's instructions and HepG2 cell line was used as positive control; Samples were assessed under a light microscope (Nikon).

Exosomes purification

Exosomes releases were collected from the culture media of 10^6 cells and isolated by ultracentrifugation according to the method previously described by Thery *et al.*[87]. Purified exosomes were fixed and incubated with the specific tetraspanins exosome biomarkers: human anti-CD9 (PE), CD63 (APC), CD81 (PerCP) and isotype control antibodies from MACS (Miltenyi-Biotec). Released exosomes were quantitated by measuring the activity of their specific enzyme: the acetylcholinesterase (AChE). Exosomes fractions were suspended to PBS (1v:4v) and incubated with 5,5′-dithiobis(2-nitrobenzoic acid) (100 □M) and acetylthiocholine (125 □M) in a final volume of 1 ml at 37 °C, and the change in absorbance at 412 nm was determined continuously up to 240 min. The data represents the enzymatic activity at 30 min of incubation at maximum saturation.

Flow cytometry analysis

For surface markers immunophenotyping, cells were stained with the following conjugated antibodies; antiCD45-FITC, anti-CD34-PE, anti-CD14-PE, anti-CD73-APC, anti-CD90-FITC, anti-CD105-Vioblue and relevant isotypes (Miltenyi-Biotec). 7AAD and Annexin V/PI were used to assess cell viability and apoptosis. At least 20,000 events for test samples were acquired. The MACSplex Cytokin12 kit was used for the cytokines analysis. Supernatants were mixed to capture specific beads for each cytokines: GM-CSF, IFN-α, IFN-γ, lL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A, and TNF-α. PE-conjugated antibodies were added and incubated for 2 h at room temperature, away from light. After centrifugation, the pellets containing beads were resuspended; flow cytometric acquisition and data analysis were performed by the MACSQuant® Express Mode Background signals were determined by analyzing beads incubated with the cell culture media alone. The background signals were subtracted from the signals obtained for beads incubated with supernatants.

Statistical analysis

All experiments were performed in triplicates. Results are presented as the means ± SEM and have been analyzed for statistical significance (on absolute values) using Student's t-test. For all statistical tests, *P* values were twotailed and the level of significance was set at 0.05.

References

1. Goodell, M.A. & Rando, T.A. Stem cells and healthy aging. *Science* **350**, 1199-1204 (2015).
2. Brunet, A. & Rando, T.A. Interaction between epigenetic and metabolism in aging stem cells. *Current Opinion in Cell Biology* **45**, 1-7 (2017).
3. Liu, L. & Rando, T.A. Manifestations and mechanisms of stem cell aging. *The Journal of cell biology* **193**, 257-266 (2011).
4. Beane, O.S., Fonseca, V.C., Cooper, L.L., Koren, G. & Darling, E.M. Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells. *PloS one* **9**, e115963 (2014).
5. Flohr, T.R., Bonatti, H.J., Brayman, K.L. & Pruett, T.L. The use of stem cells in liver disease. *Current opinion in organ transplantation* **14**, 64-71 (2009).
6. Charif, N. *et al.* Aging of bone marrow mesenchymal stromal/stem cells: Implications on autologous regenerative medicine. *Bio-Medical Materials and Engineering* **28**, S57-S63 (2017).
7. Frese, L., Dijkman, P.E. & Hoerstrup, S.P. Adipose tissue-derived stem cells in regenerative medicine. *Transfusion Medicine and Hemotherapy* **43**, 268-274 (2016).
8. Taléns-Visconti, R. *et al.* Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. *World Journal of Gastroenterology* **12**, 5834 (2006).
9. Zuk, P.A. *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue engineering* **7**, 211-228 (2001).
10. Fu, Y. *et al.* Rapid generation of functional hepatocyte-like cells from human adipose-derived stem cells. *Stem Cell Research & Therapy* **7**, 105 (2016).
11. Yu, K.-R. & Kang, K.-S. Aging-related genes in mesenchymal stem cells: a mini-review. *Gerontology* **59**, 557-563 (2013).
12. Choudhery, M.S., Badowski, M., Muise, A., Pierce, J. & Harris, D.T. Donor age negatively impacts adipose tissuederived mesenchymal stem cell expansion and differentiation. *Journal of translational medicine* **12**, 8 (2014).
13. Marędziak, M., Marycz, K., Tomaszewski, K.A., Kornicka, K. & Henry, B.M. The influence of aging on the regenerative potential of human adipose derived mesenchymal stem cells. *Stem cells international* **2016** (2016).
14. Legzdina, D., Romanauska, A., Nikulshin, S., Kozlovska, T. & Berzins, U. Characterization of Senescence of Culture-expanded Human Adipose-derived Mesenchymal Stem Cells. *International Journal of Stem Cells* **9**, 124136 (2016).
15. Khan, H., Mafi, P., Mafi, R. & Khan, W. The effects of ageing on differentiation and characterisation of human mesenchymal stem cells. *Current stem cell research & therapy* (2016).
16. Stolzing, A., Jones, E., McGonagle, D. & Scutt, A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mechanisms of ageing and development* **129**, 163-173 (2008).
17. Estrada, J. *et al.* Human mesenchymal stem cell-replicative senescence and oxidative stress are closely linked to aneuploidy. *Cell death & disease* **4**, e691 (2013).
18. Yu, J.M. *et al.* Age_related changes in mesenchymal stem cells derived from rhesus macaque bone marrow. *Aging cell* **10**, 66-79 (2011).
19. Higuchi, M., Honda, T., Proske, R.J. & Yeh, E.T. Regulation of reactive oxygen species-induced apoptosis and necrosis by caspase 3-like proteases. *Oncogene* **17**, 2753-2760 (1998).
20. Chi, X., Wang, S., Huang, Y., Stamnes, M. & Chen, J.-L. Roles of rho GTPases in intracellular transport and cellular transformation. *International journal of molecular sciences* **14**, 7089-7108 (2013).
21. Smithers, C.C. & Overduin, M. Structural mechanisms and drug discovery prospects of Rho GTPases. *Cells* **5**, 26 (2016).
22. Nobes, C.D. & Hall, A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62 (1995).
23. Kesavan, G. *et al.* Cdc42-mediated tubulogenesis controls cell specification. *Cell* **139**, 791-801 (2009).
24. Lamarche, N. *et al.* Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65 PAK and the JNK/SAPK MAP kinase cascade. *Cell* **87**, 519-529 (1996).
25. Coso, O.A. *et al.* The small GTP-binding proteins Rac1 and Cdc42regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137-1146 (1995).
26. Florian, M.C. *et al.* Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. *Cell stem cell* **10**, 520530 (2012).
27. Wang, L., Yang, L., Debidda, M., Witte, D. & Zheng, Y. Cdc42 GTPase-activating protein deficiency promotes genomic instability and premature aging-like phenotypes. *Proceedings of the National Academy of Sciences* **104**, 1248-1253 (2007).
28. Xu, X.-p. *et al.* Ang II-AT2R increases mesenchymal stem cell migration by signaling through the FAK and RhoA/Cdc42 pathways in vitro. *Stem cell research & therapy* **8**, 164 (2017).
29. Lu, J. *et al.* Correlation of CDC42 Activity with Cell Proliferation and Palmitate-mediated Cell Death in Human umbilical cord Wharton's jelly derived Mesenchymal Stromal cells. *Stem Cells and Development* (2017).
30. He, Q. *et al.* Characterization of an apical ceramide-enriched compartment regulating ciliogenesis. *Molecular biology of the cell* **23**, 3156-3166 (2012).
31. Snykers, S. *et al.* Chromatin remodeling agent trichostatin A: a key-factor in the hepatic differentiation of human mesenchymal stem cells derived of adult bone marrow. *BMC developmental biology* **7**, 24 (2007).
32. Geiger, H., de Haan, G. & Florian, M.C. The ageing haematopoietic stem cell compartment. *Nature Reviews Immunology* **13**, 376-389 (2013).

33. Geiger, H. & Zheng, Y. Regulation of hematopoietic stem cell aging by the small RhoGTPase Cdc42. *Experimental cell research* **329**, 214-219 (2014).
34. Surviladze, Z. *et al.* A potent and selective inhibitor of Cdc42 GTPase. (2010).
35. Chen, C. *et al.* Cdc42 inhibitor ML141 enhances G-CSF-induced hematopoietic stem and progenitor cell mobilization. *International journal of hematology* **101**, 5-12 (2015).
36. Alizadeh, E. *et al.* Upregulation of MiR_122 via Trichostatin A Treatments in Hepatocyte_ like Cells Derived from Mesenchymal Stem Cells. *Chemical biology & drug design* **87**, 296-305 (2016).
37. Snykers, S. *et al.* Sequential exposure to cytokines reflecting embryogenesis: the key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells. *Toxicological Sciences* **94**, 330-341 (2006).
38. Kokai, L.E. *et al.* Adipose stem cell function maintained with age: an intra-subject study of long-term cryopreserved cells. *Aesthetic Surgery Journal* **37**, 454-463 (2017).
39. Hanke, A. *et al.* Semi-automated extraction and characterization of Stromal Vascular Fraction using a new medical device. *Clinical hemorheology and microcirculation* **64**, 403-412 (2016).
40. Vilaboa, S.D.-A., Navarro-Palou, M. & Llull, R. Age influence on stromal vascular fraction cell yield obtained from human lipoaspirates. *Cytotherapy* **16**, 1092-1097 (2014).
41. Kornicka, K., Marycz, K., Tomaszewski, K.A., Marędziak, M. & Śmieszek, A. The effect of age on osteogenic and adipogenic differentiation potential of human adipose derived stromal stem cells (hASCs) and the impact of stress factors in the course of the differentiation process. *Oxidative medicine and cellular longevity* **2015** (2015).
42. Liu, M. *et al.* Adipose-derived mesenchymal stem cells from the elderly exhibit decreased migration and differentiation abilities with senescent properties. *Cell transplantation* (2017).
43. Aguilar, B., Zhou, H. & Lu, Q. Cdc42 Signaling Pathway Inhibition as a Therapeutic Target in Ras-Related Cancers. *Current medicinal chemistry* (2017).
44. Geiger, H., Koehler, A. & Gunzer, M. Stem cells, aging, niche, adhesion and Cdc42: a model for changes in cellcell interactions and hematopoietic stem cell aging. *Cell Cycle* **6**, 884-887 (2007).
45. Florian, M.C. *et al.* Expression and activity of the small RhoGTPase Cdc42 in blood cells of older adults are associated with age and cardiovascular disease. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences* **72**, 1196-1200 (2017).
46. Wagner, W. *et al.* Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PloS one* **3**, e2213 (2008).
47. Geißler, S. *et al.* Functional comparison of chronological and in vitro aging: differential role of the cytoskeleton and mitochondria in mesenchymal stromal cells. *PLoS One* **7**, e52700 (2012).
48. Kode, J.A., Mukherjee, S., Joglekar, M.V. & Hardikar, A.A. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. *Cytotherapy* **11**, 377-391 (2009).
49. Wang, L. *et al.* IFN_γ and TNF_ α synergistically induce mesenchymal stem cell impairment and tumorigenesis via NFκB signaling. *Stem cells* **31**, 1383-1395 (2013).
50. Kim, H.S. *et al.* Human umbilical cord blood mesenchymal stem cells reduce colitis in mice by activating NOD2 signaling to COX2. *Gastroenterology* **145**, 1392-1403. e1398 (2013).
51. Yu, K.-R. *et al.* A p38 MAPK-mediated alteration of COX-2/PGE2 regulates immunomodulatory properties in human mesenchymal stem cell aging. *PloS one* **9**, e102426 (2014).
52. Yang, L. *et al.* Mesenchymal stem cells engineered to secrete pigment epithelium-derived factor inhibit tumor metastasis and the formation of malignant ascites in a murine colorectal peritoneal carcinomatosis model. *Human gene therapy* **27**, 267-277 (2016).
53. Nakamura, N. *et al.* Adiponectin promotes migration activities of endothelial progenitor cells via Cdc42/Rac1. *FEBS letters* **583**, 2457-2463 (2009).
54. Kusuhara, S. *et al.* Arhgef15 promotes retinal angiogenesis by mediating VEGF-induced Cdc42 activation and potentiating RhoJ inactivation in endothelial cells. *PLoS One* **7**, e45858 (2012).
55. Pouysségur, J., Volmat, V. & Lenormand, P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Biochemical pharmacology* **64**, 755-763 (2002).
56. Cammarano, M.S., Nekrasova, T., Noel, B. & Minden, A. Pak4 induces premature senescence via a pathway requiring p16INK4/p19ARF and mitogen-activated protein kinase signaling. *Molecular and cellular biology* **25**, 9532-9542 (2005).
57. Carrillo-García, C. & Janzen, V. Restoring cell polarity: an HSC fountain of youth. *Cell stem cell* **10**, 481-482 (2012).
58. Zoncu, R., Efeyan, A. & Sabatini, D.M. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews Molecular cell biology* **12**, 21-35 (2010).
59. Golub, T. & Pico, C. in Biochemical Society Symposia, Vol. 72 119-127 (Portland Press Limited, 2005).
60. Berfield, A.K., Andress, D.L. & Abrass, C.K. IGF-1–induced lipid accumulation impairs mesangial cell migration and contractile function. *Kidney international* **62**, 1229-1237 (2002).
61. Sakamori, R. *et al.* CDC42 inhibition suppresses progression of incipient intestinal tumors. *Cancer research* **74**, 5480-5492 (2014).
62. O'Brien, E. *et al.* (Am Soc Hematology, 2013).

63. Han, B. *et al.* Trichostatin A stabilizes the expression of pluripotent genes in human mesenchymal stem cells during ex vivo expansion. *PloS one* **8**, e81781 (2013).
64. Wang, Y. *et al.* Role of histone deacetylase inhibitors in the aging of human umbilical cord mesenchymal stem cells. *Journal of cellular biochemistry* **114**, 2231-2239 (2013).
65. Ryan, M.A. *et al.* Pharmacological inhibition of EGFR signaling enhances G-CSF-induced hematopoietic stem cell mobilization. *Nature medicine* **16**, 1141-1146 (2010).
66. Chen, H.Y., Yang, Y.M., Stevens, B.M. & Noble, M. Inhibition of redox/Fyn/c_Cbl pathway function by Cdc42 controls tumour initiation capacity and tamoxifen sensitivity in basal_like breast cancer cells. *EMBO molecular medicine* **5**, 723-736 (2013).
67. Kumar, A., Al-Sammarraie, N., DiPette, D.J. & Singh, U.S. Metformin impairs Rho GTPase signaling to induce apoptosis in neuroblastoma cells and inhibits growth of tumors in the xenograft mouse model of neuroblastoma. *Oncotarget* **5**, 11709 (2014).
68. Guo, Y. *et al.* R-ketorolac targets Cdc42 and Rac1 and alters ovarian cancer cell behaviors critical for invasion and metastasis. *Molecular cancer therapeutics* **14**, 2215-2227 (2015).
69. Nguyen, D.H.T., Gao, L., Wong, A. & Chen, C.S. Cdc42 regulates branching in angiogenic sprouting in vitro. *Microcirculation* (2017).
70. ZHANG, Y. *et al.* The small GTPase CDC42 regulates actin dynamics during porcine oocyte maturation. *Journal of Reproduction and Development*, 2017-2034 (2017).
71. Fila-Danilow, A., Borkowska, P., Paul-Samojedny, M., Kowalczyk, M. & Kowalski, J. The influence of TSA and VPA on the in vitro differentiation of bone marrow mesenchymal stem cells into neuronal lineage cells: Gene expression studies. *Postepy higieny i medycyny doswiadczalnej (Online)* **71**, 236 (2017).
72. Mezentseva, N.V. *et al.* The histone methyltransferase inhibitor BIX01294 enhances the cardiac potential of bone marrow cells. *Stem cells and development* **22**, 654-667 (2012).
73. Yang, J., Kaur, K., Edwards, J.G., Eisenberg, C.A. & Eisenberg, L.M. Inhibition of Histone Methyltransferase, Histone Deacetylase, and β-Catenin Synergistically Enhance the Cardiac Potential of Bone Marrow Cells. *Stem cells international* **2017** (2017).
74. Li, X., Kato, Y., Tsuji, Y. & Tsunoda, Y. The effects of trichostatin A on mRNA expression of chromatin structure, DNA methylation-, and development-related genes in cloned mouse blastocysts. *Cloning and stem cells* **10**, 133142 (2008).
75. Cipriano, M. *et al.* The role of epigenetic modifiers in extended cultures of functional hepatocyte-like cells derived from human neonatal mesenchymal stem cells. *Archives of toxicology* **91**, 2469-2489 (2017).
76. Azandeh, S., Gharravi, A.M., Orazizadeh, M., Khodadi, A. & Tabar, M.H. Improvement of mesenchymal stem cell differentiation into the endoderm lineage by four step sequential method in biocompatible biomaterial. *BioImpacts: BI* **6**, 9 (2016).
77. Banas, A. *et al.* Adipose tissue_derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* **46**, 219-228 (2007).
78. Lee, S.W., Min, S.O., Bak, S.Y., Hwang, H.K. & Kim, K.S. Efficient endodermal induction of human adipose stem cells using various concentrations of Activin A for hepatic differentiation. *Biochemical and biophysical research communications* **464**, 1178-1184 (2015).
79. Yin, L. *et al.* Adipose tissue-derived mesenchymal stem cells differentiated into hepatocyte-like cells in vivo and in vitro. *Molecular medicine reports* **11**, 1722-1732 (2015).
80. Lou, G., Chen, Z., Zheng, M. & Liu, Y. Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. *Experimental & molecular medicine* **49**, e346 (2017).
81. Yamamoto, Y. *et al.* A comparative analysis of the transcriptome and signal pathways in hepatic differentiation of human adipose mesenchymal stem cells. *The FEBS journal* **275**, 1260-1273 (2008).
82. Lee, J.G. & Heur, M. Interleukin-1β-induced Wnt5a enhances human corneal endothelial cell migration through regulation of Cdc42 and RhoA. *Molecular and cellular biology* **34**, 3535-3545 (2014).
83. Teng, N.-Y. *et al.* Promotion of mesenchymal-to-epithelial transition by Rac1 inhibition with small molecules accelerates hepatic differentiation of mesenchymal stromal cells. *Tissue Engineering Part A* **21**, 1444-1454 (2015).
84. Li, B., Zheng, Y.-W., Sano, Y. & Taniguchi, H. Evidence for mesenchymal− epithelial transition associated with mouse hepatic stem cell differentiation. *PloS one* **6**, e17092 (2011).
85. Chen, Y.F. *et al.* Rapid generation of mature hepatocyte_like cells from human induced pluripotent stem cells by an efficient three_step protocol. *Hepatology* **55**, 1193-1203 (2012).
86. AlBacha, J.d.A. *et al.* High incidence of ACE/PAI-1 in association to a spectrum of other polymorphic cardiovascular genes involving PBMCs proinflammatory cytokines in hypertensive hypercholesterolemic patients: reversibility with a combination of ACE inhibitor and statin. *PloS one* **10**, e0127266 (2015).
87. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* Ch **3**: Unit 3.22 (2006).

Acknowledgments

This work has been funded with support from the Lebanese University and the CEDRE from the French MENESR. We sincerely thank, Mrs Ghada Oreibi, Mr Charbel Khalil, Mrs Amira Bekdach and Mr Rania Boueiz for their assistance. We are thankful for Mrs Sandy Hage for her scientific and administrative support. We thank, Dr Roy Khalaf for the fluorescence reading, Dr Georges Aftimos and Dr Saad Khairalla for IHC assays at INP center.

Conflict of interest disclosure

The authors declare no conflicts of interest related to this study.

Figure legends

Figure 1. Ccd42 activity increases with age: correlations with growth factors, cytokines, transcription factors, and kinases. hADSCs were isolated from lipoaspirates derived from young, middle and aged subjects. Cell lysates were used for the assessment of the activity of Cdc42 as determined by Pull-down of Cdc42-GTP (500µg) and Cdc42GAP (100µg) proteins (A,B), or were separated by SDS-PAGE (1000µg of proteins) and immunoblotted with antibodies raised against: growth factors (HGF and IGF-1) (C), pro- and anti-angiogenic factors (VEGF and PEDF) (D), anti- and pro-inflammatory cytokines (IL-4 and IL-10, TNFα and INFγ) (E,F), transcription factors (p68 SREBP-1 and SREBP-2, p-CREB and p-NFkB) (G,H), G proteins and MAPK (RAS and p-JNK, p-ERK and p-p38) (I,J), kinases (PKA catalytic and p-PI3K), p-IR and p-IRS-1 (K,L). Exosomes release (M) and Wnts signaling (N) were analyzed by flow cytometry and mRNAs expression by RT-qPCR. Results are expressed as mean±SEM of two independent experiments realized on 19 (young), 22 (middle) and 20 (aged) subjects after normalization to GAPDH. **P*<0.05, ***P*<0.01, ****P*<0.001; Aged/Middle *versus* Young.

Figure 2. Reversibility by inhibitors of PKA, JNK, ERK and Cdc42 activity. hADSCs derived from young, middle and aged subjects were cultured for 96 h and then treated 24 h with inhibitors of PI3K (Wortmannin, 10µM), PKA (H-89, 5µM), JNK (SP600125, 10µM), ERK (PD98059, 50µM), Cdc42-GTPase (Casin, 10µM), Cdc42/Rac1 GTPase (ML141, 10µM), and HDAC (TSA, 0.8µM). 500 µg of cell lysates were used for the assessment of the activity of Cdc42 as determined by Pull-down of Cdc42GTP proteins (A,B). The impact of pharmacological targeting of Cdc42 activity by ML141 and TSA was assessed on the production of pro- (TNF-α (C), INF-γ (D)) and anti- (IL-4 (E), IL-10 (F)) inflammatory cytokines as secreted in the culture supernatants. Results are expressed as mean±SEM of two independent experiments realized on 19 (young), 22 (middle) and 20 (aged, aged+ML141, aged+TSA) subjects. §*P*<0.05, §§*P*<0.01; Aged/Middle *versus* Young. **P*<0.05, ***P*<0.01, ****P*<0.001; Aged treated with ML141/TSA *versus* untreated.

Figure 3. Pharmacological targeting of Cdc42 activity by ML141 and TSA on the yield, growth and adherence characteristics of hADSCs. Cells derived from young and aged subjects were cultured for 96 h. Proliferative potential as indicated by cells number (A) and time for population doublings (B). Kinetics of hADSCs adherence as evaluated by the number of adhered cells and expressed as the percentage of applied cells (C,D). Apoptotic index (E) and cell viability (F) as assessed by labeled cells with Annexin V / 7AAD / propidium iodide (PI): the apoptotic index was expressed as the percentage of Annexin V-positive cells divided by total cells; the cell viability was expressed as the percentage of Annexin $V^{(-)}/PI^{(-)}/7AAD^{(-)}$ divided by total cells. Early from late-apoptotic cells were identified as Annexin $V^{(+)}/PI^{(-)}/7AAD^{(-)}$ versus Annexin $V^{(+)}/PI^{(+)}/7AAD^{(+)}$, respectively. Results are expressed as mean±SEM of two independent experiments realized on 19 (young) and 20 (aged, aged+ML141, aged+TSA) subjects. §*P*<0.05, §§*P*<0.01; Aged *versus* Young. **P*<0.05, ***P*<0.01, ****P*<0.001; Aged treated with ML141/TSA *versus* untreated.

Figure 4. : Impact of pharmacological targeting of Cdc42 activity by ML141 on the gene expression profile during hepatocyte differentiation. hADSCs were isolated from young and aged subjects and were induced to hepatocyte differentiation for 28 days with or without ML141 (5 µM) for the indicated time of incubation. RNAs were collected at the D0/14/28 and mRNAs levels of the studied genes were determined by RT-qPCR. The results are expressed as fold variation relative to Young at D0 after normalization to GAPDH, are the mean±SEM of two independent experiments performed in

duplicate. D0 = day of induction of the differentiation. d-2/0, d-2/14, d14/28: cells were treated with ML141 from day(-2) to day(0), day(-2) to day(14), or day(14) to day(28), respectively. $^{\S}P<0.05$, $^{\S\S}P<0.01$; Aged *versus* Young. $*P<0.05$, $**P<0.01$, $***P<0.001$; Aged treated with ML141 *versus* untreated.

Figure 5. Extinction of AFP, Vimentin and Ki67 from hepatocyte-like cells derived from aged-ADSCs treated with ML141. Cells derived from young and aged groups were differentiated and treated with or without ML141 as indicated in figure 4 and cells were evaluated for AFP (fetal hepatoblasts marker), Vimentin (EMT marker) and Ki67 (proliferation marker) by immunohistochemistry (IHC) as described in Methods. Cells were examined microscopically and phase-contrast images were captured. Cultures of primary human fetal and adult hepatocytes were assessed as positive controls and negative controls, respectively.

Figure 6. Pharmacological targeting of Cdc42 activity by ML141 induces rifampicin responsiveness and restores cell function. hADSCs derived from young and aged subjects were differentiated and treated with or without ML141 as indicated in figure 4. ***A,B,C***, rifampicin responsiveness: RNAs were collected at day 28 of the differentiation after cells' treatment with rifampicin (20µM, 24h) and mRNAs expression were determined by RT-qPCR and normalized to GAPDH as described in methods. Expression levels of CYP3A4, CYP3A7 and Albumin genes are shown. The results are expressed as fold variation relative to controls (without rifampicin) after normalization to GAPDH, are the mean±SEM and the experiments were performed twice each in duplicate. ***D,E,F,*** Albumin, urea production and LDL uptake: supernatants of cultured cells and the lysates were collected at D0/D14/D28 of the differentiation for the quantification of albumin, urea and LDL-uptake as indicated in methods. Results are expressed per ng/ml (for the production of albumin and urea) and RFU (fluorescent LDL-uptake) and are presented as fold of variation relative to Young at day D0, are the mean±SEM of several measures (5, 6 and 10, respectively). ***D,*** hepatocyte-like cells derived from aged-ADSCs treated with ML141 exhibit hepatic-specific function of glycogen storage. Cells were evaluated for glycogen storage capacity (pink color) using periodic acid-schiff (PAS) staining as described in Methods. Cells were examined microscopically and phase-contrast images were captured. Cultures of HepG2 cells were assessed as positive controls. $^{\S}P<0.05$, $^{\S\S}P<0.01$; Rifampicin *versus* controls or Aged *versus* Young. $*P<0.05$, $**P<0.01$; Aged treated with ML141 *versus* untreated.

Figure 7. Pharmacological targeting of Cdc42 activity by ML141 reverts the profile of cytokines secreted during the differentiation of hADSCs derived from aged subjects. Cells derived from young and aged groups were differentiated and treated with or without ML141 as indicated in figure 4 and cell culture supernatants were collected and undiluted samples were analyzed for the detection of cytokines as indicated in methods. The concentrations of 12 cytokines were determined using MACSQuant analyzer at days D0/D14/D28 of the differentiation. 3-5 measures were realized by group and results are the mean±SEM presented in fold of variation relative to day D0. The cytokines limit of detection (pg/ml) were: TNF-α (2.17), IFN-γ 6.26, IFN-α (10.4), GM-CSF (0.20), IL-2 (1.52), IL-4 (34.2), IL-5 (0.27), IL-6 (0.077), IL-9 (32.7), IL-10 (2.76), IL-12 (3.44), and IL-17A (0.7). $^{\S}P<0.05$, $^{\S\S}P<0.01$; Aged *versus* Young. $*P<0.05$, $**P<0.01$; Aged treated with ML141 *versus* untreated.

Table 1: Characteristics of the studied population (results are expressed as the mean ± SEM)

Healthy Group (N = 61)	**YOUNG**	**MIDDLE**	**AGED**
Number of subjects	19	22	20
Sex	Female 100%	Female 100%	Female 100%
Mean Age (years)	23.8 ± 0.4	40.8 ± 0.6	57.6 ± 0.9
Adipose tissue (mg)	342.1 ± 78.4	299.4 ± 63.6	390.0 ± 87.4
Number of isolated SVF cells	$254.3 \pm 23.7 \times 10^6$	$210.2 \pm 18.5 \times 10^6$	$288.6 \pm 21.4 \times 10^6$
Number of MSCs / mg tissue	$743.5 \pm 102.7 \times 10^3$	$702.1 \pm 91.4 \times 10^3$	$738.3 \pm 45.3 \times 10^3$
Never smoking	12.1 ± 0.9 %	41.9 ± 3.3 %	18.2 ± 4.5 %
Ex-smoking	68.4 ± 3.7 %	47.2 ± 6.5 %	61.6 ± 7.1 %
Current smoker	19.5 ± 4.5 %	10.9 ± 4.2 %	20.3 ± 3.0 %
Physically active [1]	42.0 ± 6.6 %	49.8 ± 5.7 %	40.5 ± 8.6 %
CAD or Stroke	0	0	0
Diabetic	0	0	0
Hypertension	0	0	0
Hypercholesterolemia	0	0	0
Liver, GIT, metabolic, neurological, cardiac, renal disorders	0	0	0
Healthy subjects	19 out 19	22 out of 22	20 out of 20
Exclusion criteria			
Total Cholesterol (mg/dL)	> 250		
Triglycerides (mg/dL)	> 200		
LDL[2] cholesterol (mg/dL)	> 150		
HDL cholesterol (mg/dL)	< 35		
Fasting glucose (mg/dL)	> 120		
Fasting plasma insulin (µU/mL)	> 10		
Systolic blood pressure	> 140 mm Hg		
Diastolic blood pressure	> 90 mm Hg		
Diabetic	Previously- or currently- treated or untreated		
Stroke	Previously- or currently- treated or untreated		
Body mass index (kg/m^2)	> 30		

[1] *Physically active: walking or doing other kinds of exercise at least once per week or self-report of moderate/intense level of activity in daily life.*

[2] *Abbreviations: LDL: low density lipoprotein; HDL: high density lipoprotein; CAD: coronary artery disease; SVF: Stromal Vascular Fraction*

Table 2. Real-time quantitative RT-PCR determination of $p16^{INK4a}$, p53 and p21 mRNA levels in ADSCs derived from young *versus* aged groups.

	$p16^{INK4a}$		**p53**		**p21**	
	R	*P value*	**R**	*P value*	**R**	*P value*
Young	1[a]		1		1	
Aged	4.72 ± 0.99	0.002[b]	10.15 ± 2.07	<0.0001[b]	7.32 ± 1.74	0.002[b]
Aged+ML141	2.17 ± 0.66	0.042[c]	3.98 ± 0.95	0.001[c]	2.78 ± 0.77	0.024[c]
Aged+TSA	2.08 ± 0.31	0.026[c]	6.12 ± 1.23	0.040[b]	5.44 ± 0.98	0.051[b]

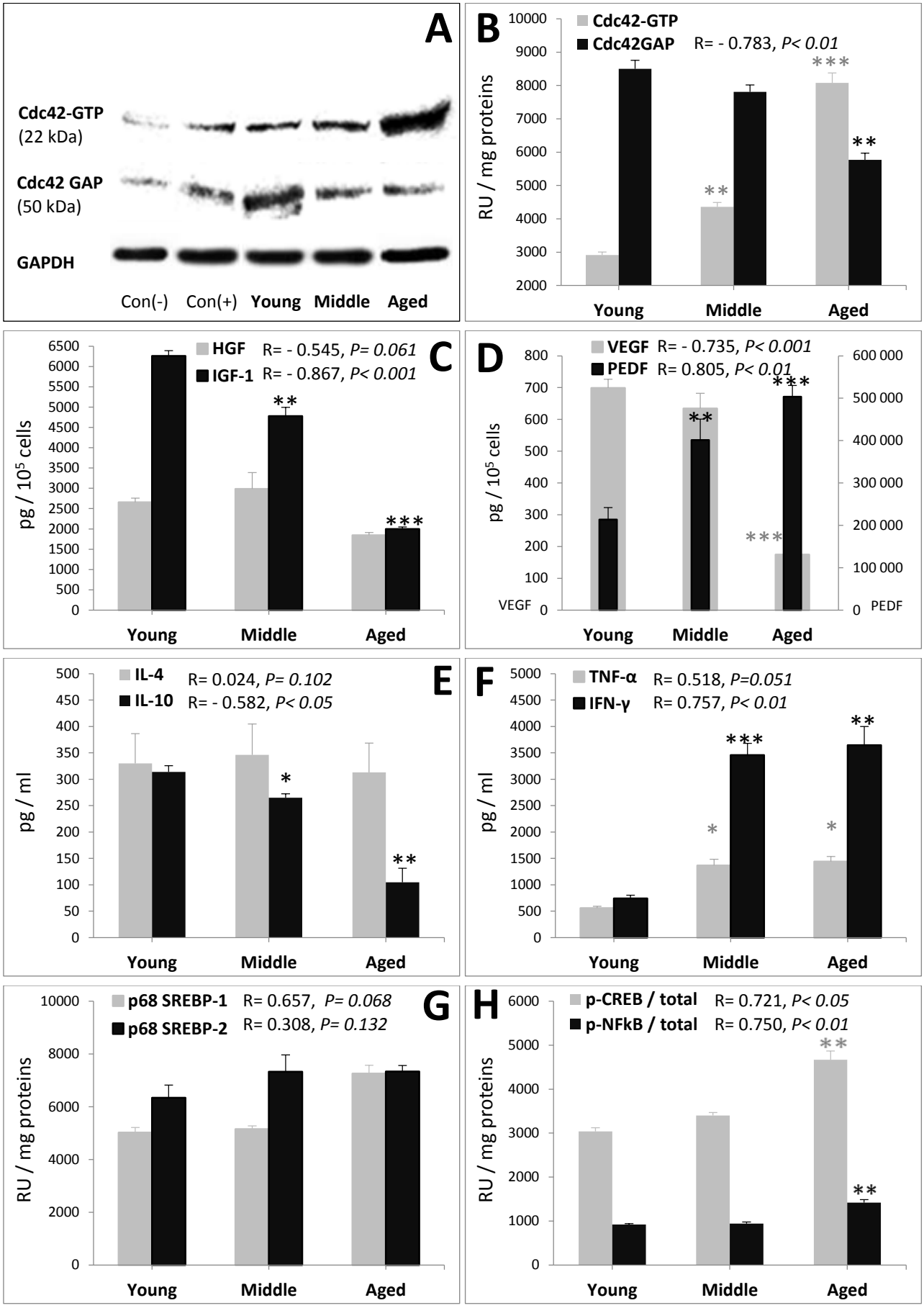
ADSCs were isolated from young and aged groups and cultured for 96 h as described in methods. Cells from the indicated group were treated or not with 10µM (24h) ML141 or 0.8µM (24h) TSA. The mRNA levels of the studied genes were determined. The results are expressed as -fold variations ± SEM over young group (R) after normalization to β-actin. The results represented individual expression per subject: young (n = 19), aged (n=20), aged+ML141 (n=20) and aged+TSA (n=20) are the means of 2 independent experiments performed in triplicate.

[a] The young group was expressed as 1.

[b] Compared to young group.

[c] Compared to aged group.

Figure 1


A
Cdc42-GTP
(22 kDa)
Cdc42 GAP
(50 kDa)
GAPDH
Con(-) Con(+) Young Middle Aged
B
Cdc42-GTP
Cdc42GAP
R= - 0.783, P< 0.01
RU / mg proteins
Young Middle Aged
C
HGF R= - 0.545, P= 0.061
IGF-1 R= - 0.867, P< 0.001
pg / 10^5 cells
Young Middle Aged
D
VEGF R= - 0.735, P< 0.001
PEDF R= 0.805, P< 0.01
pg / 10^5 cells
VEGF
PEDF
Young Middle Aged
E
IL-4 R= 0.024, P= 0.102
IL-10 R= - 0.582, P< 0.05
pg / ml
Young Middle Aged
F
TNF-α R= 0.518, P=0.051
IFN-γ R= 0.757, P< 0.01
pg / ml
Young Middle Aged
G
p68 SREBP-1 R= 0.657, P= 0.068
p68 SREBP-2 R= 0.308, P= 0.132
RU / mg proteins
Young Middle Aged
H
p-CREB / total R= 0.721, P< 0.05
p-NFkB / total R= 0.750, P< 0.01
RU / mg proteins
Young Middle Aged

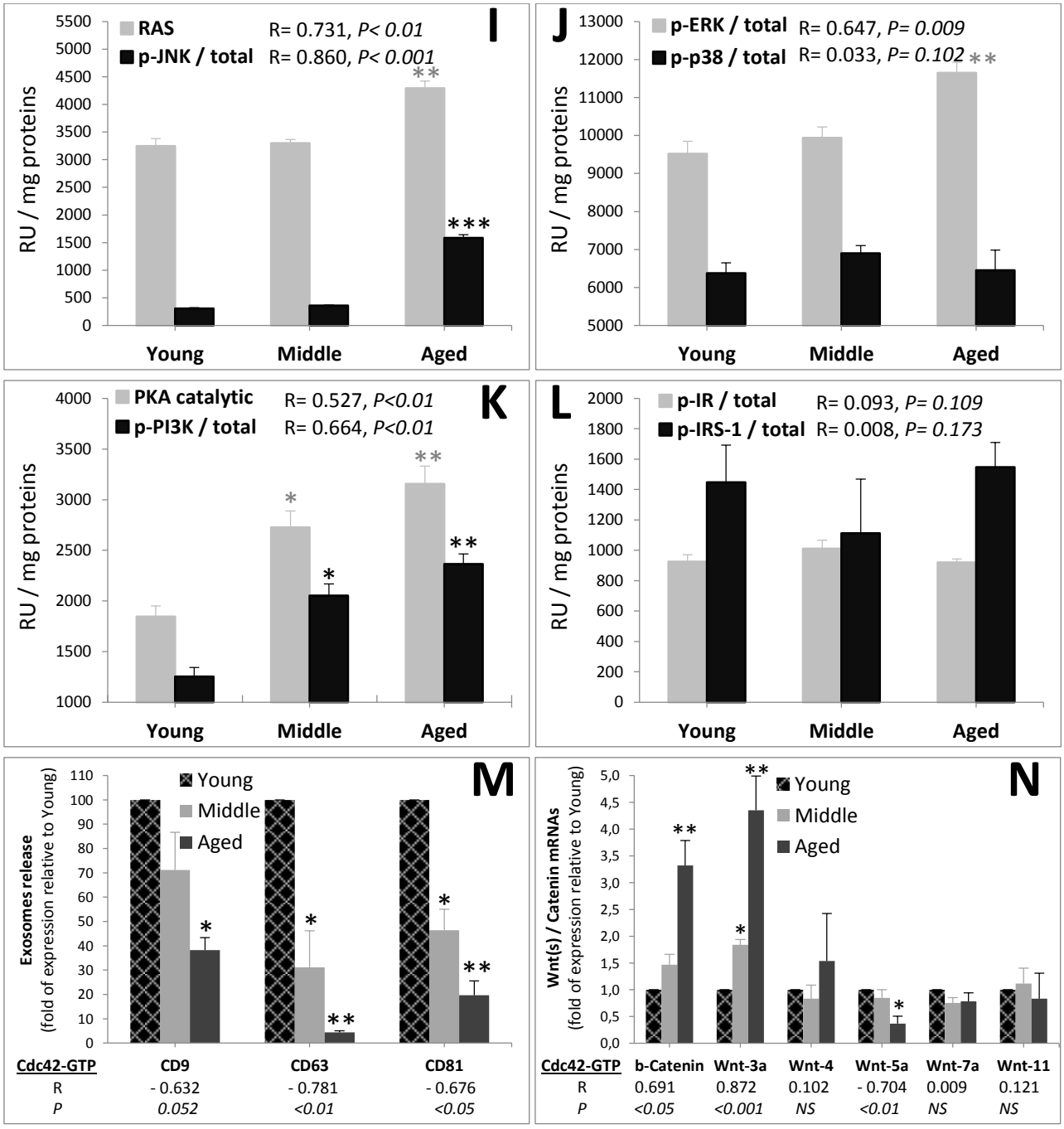

I
RAS
p-JNK / total
R= 0.731, P< 0.01
R= 0.860, P< 0.001
RU / mg proteins
Young
Middle
Aged
J
p-ERK / total
p-p38 / total
R= 0.647, P= 0.009
R= 0.033, P= 0.102
K
PKA catalytic
p-PI3K / total
R= 0.527, P<0.01
R= 0.664, P<0.01
L
p-IR / total
p-IRS-1 / total
R= 0.093, P= 0.109
R= 0.008, P= 0.173
M
Exosomes release
(fold of expression relative to Young)
Cdc42-GTP
CD9
CD63
CD81
R
- 0.632
- 0.781
- 0.676
P
0.052
<0.01
<0.05
N
Wnt(s) / Catenin mRNAs
(fold of expression relative to Young)
Cdc42-GTP
b-Catenin
Wnt-3a
Wnt-4
Wnt-5a
Wnt-7a
Wnt-11
R
0.691
0.872
0.102
- 0.704
0.009
0.121
P
<0.05
<0.001
NS
<0.01
NS
NS

Figure 2

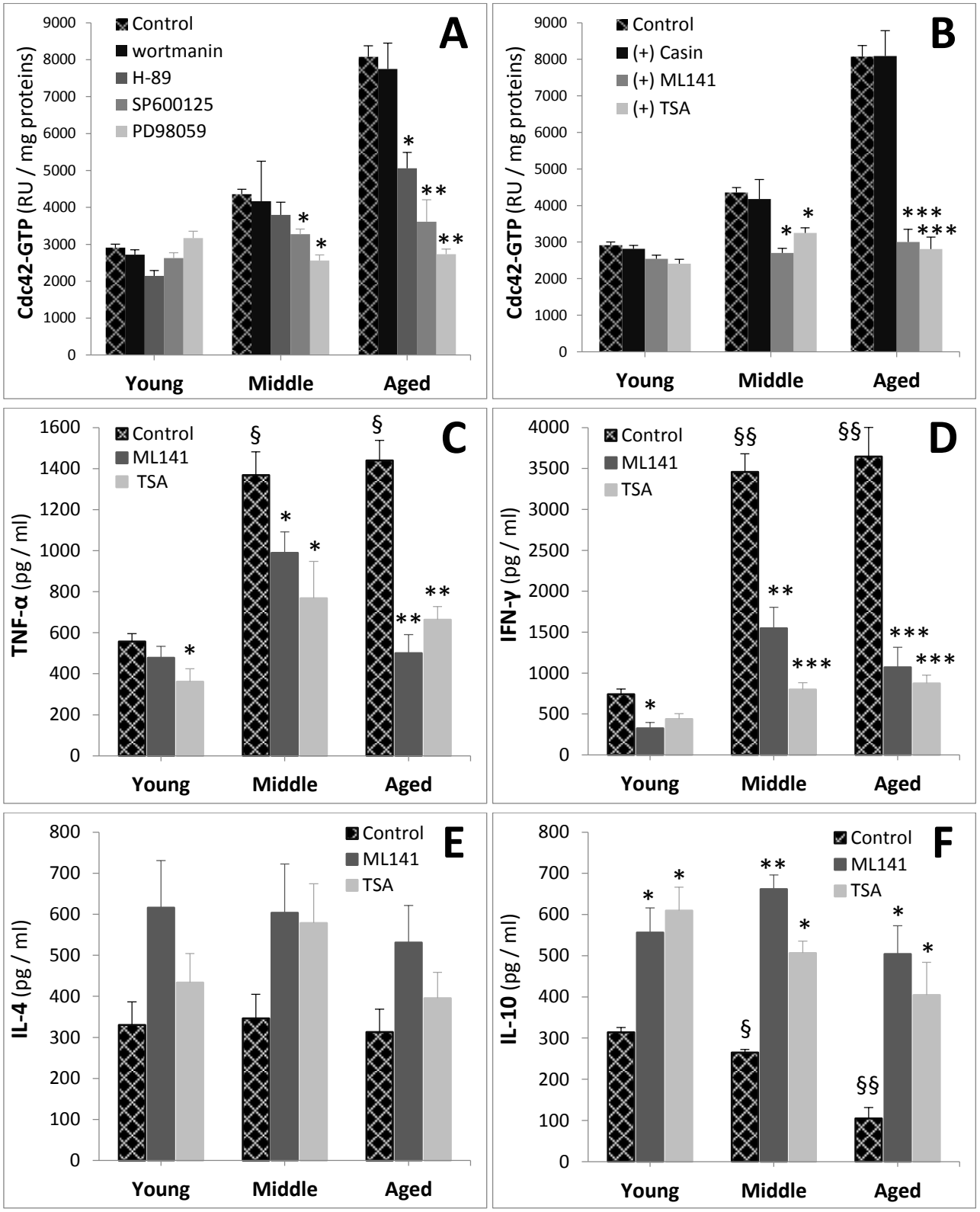

A
Control
wortmanin
H-89
SP600125
PD98059
Cdc42-GTP (RU / mg proteins)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Young
Middle
Aged
B
Control
(+) Casin
(+) ML141
(+) TSA
Cdc42-GTP (RU / mg proteins)
C
Control
ML141
TSA
TNF-α (pg / ml)
1600
1400
1200
1000
800
600
400
200
0
D
Control
ML141
TSA
IFN-γ (pg / ml)
4000
3500
3000
2500
2000
1500
1000
500
0
E
Control
ML141
TSA
IL-4 (pg / ml)
800
700
600
500
400
300
200
100
0
F
Control
ML141
TSA
IL-10 (pg / ml)

Figure 3

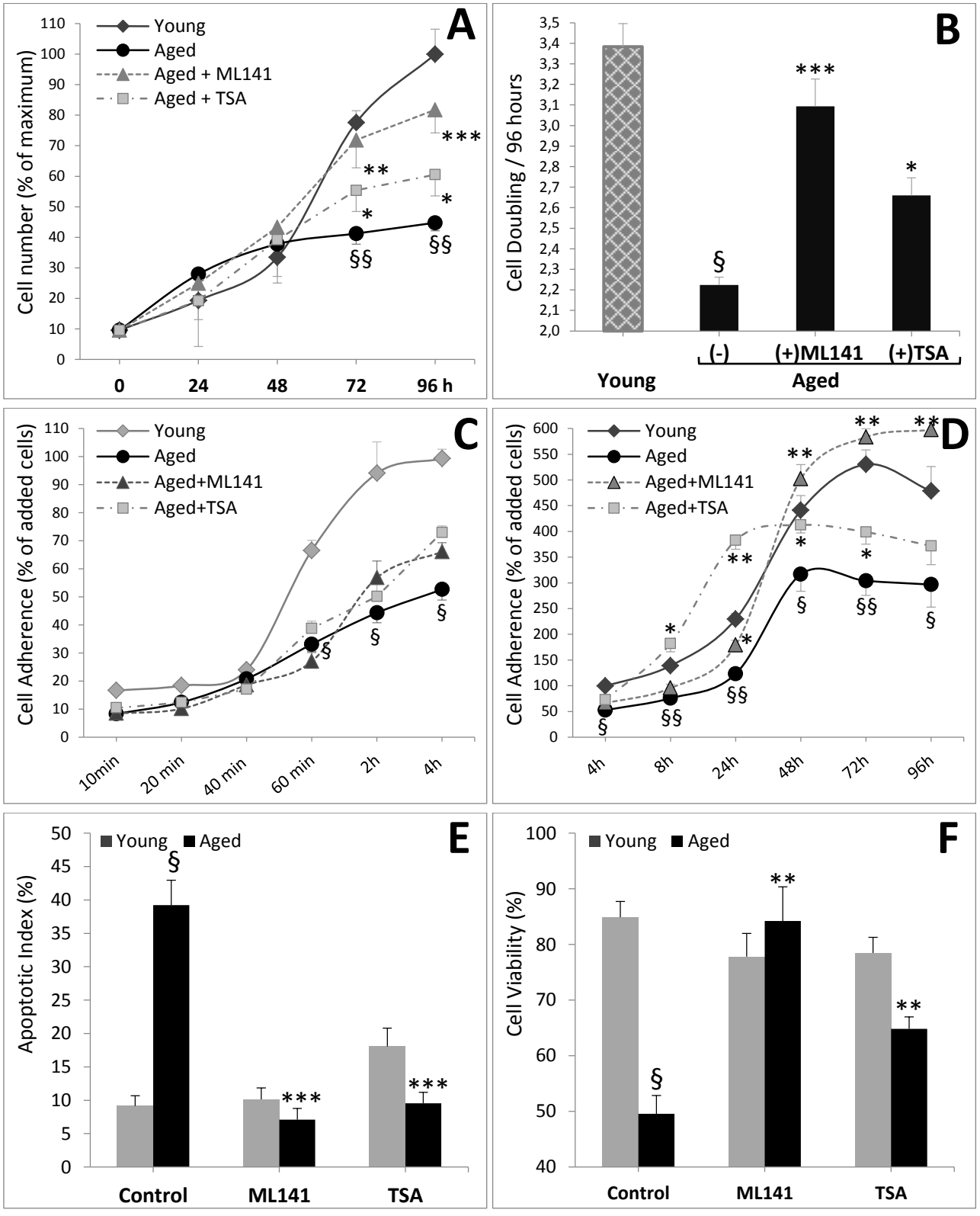

A
Young
Aged
Aged + ML141
Aged + TSA
Cell number (% of maximum)
0
24
48
72
96 h
B
Cell Doubling / 96 hours
Young
(-)
(+)ML141
(+)TSA
Aged
C
Cell Adherence (% of added cells)
Aged+ML141
Aged+TSA
10min
20 min
40 min
60 min
2h
4h
D
4h
8h
24h
48h
72h
96h
E
Apoptotic Index (%)
Control
ML141
TSA
F
Cell Viability (%)

Figure 4

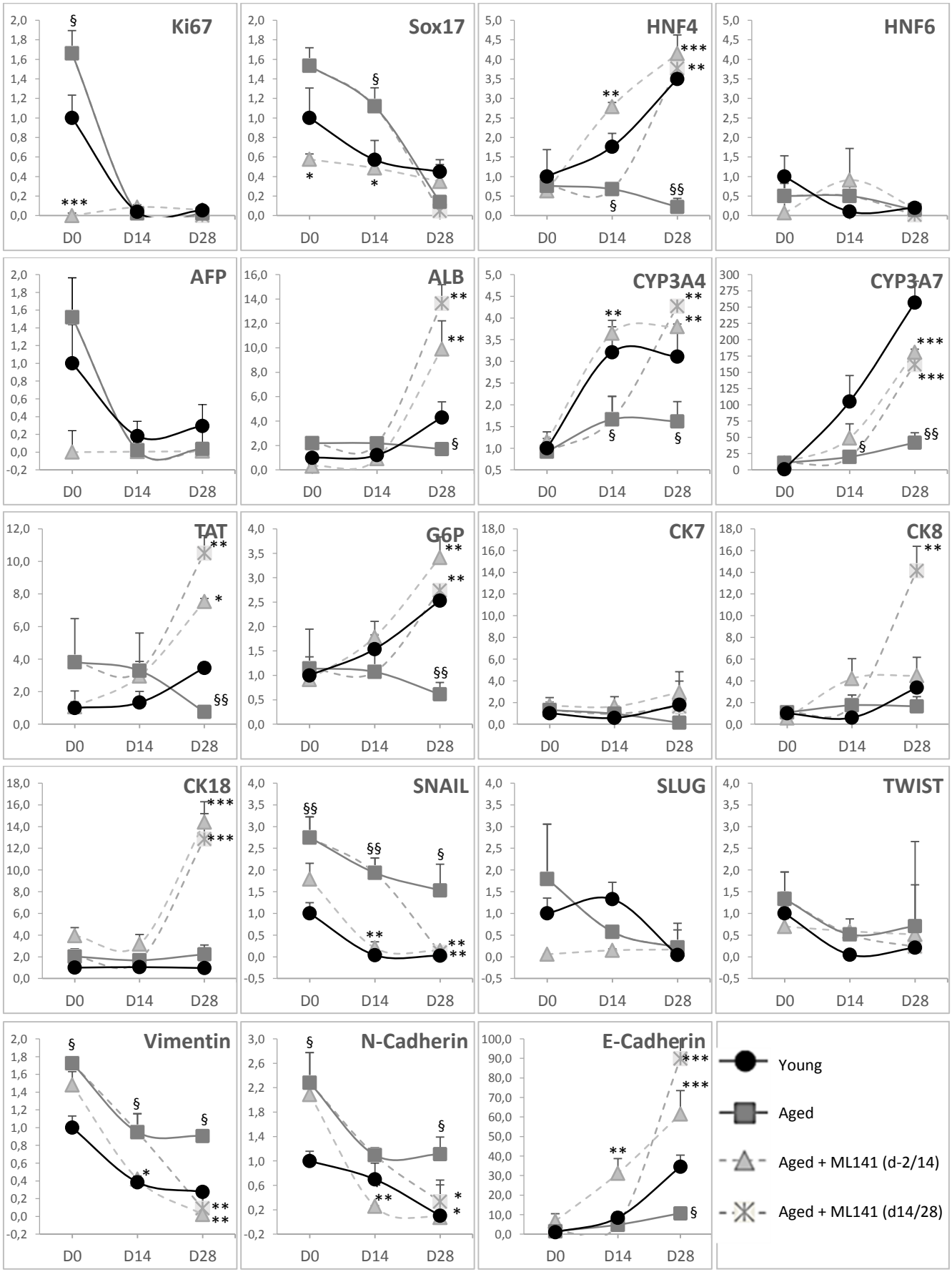

Ki67
Sox17
HNF4
HNF6
AFP
ALB
CYP3A4
CYP3A7
TAT
G6P
CK7
CK8
CK18
SNAIL
SLUG
TWIST
Vimentin
N-Cadherin
E-Cadherin
D0
D14
D28
Young
Aged
Aged + ML141 (d-2/14)
Aged + ML141 (d14/28)

Figure 5

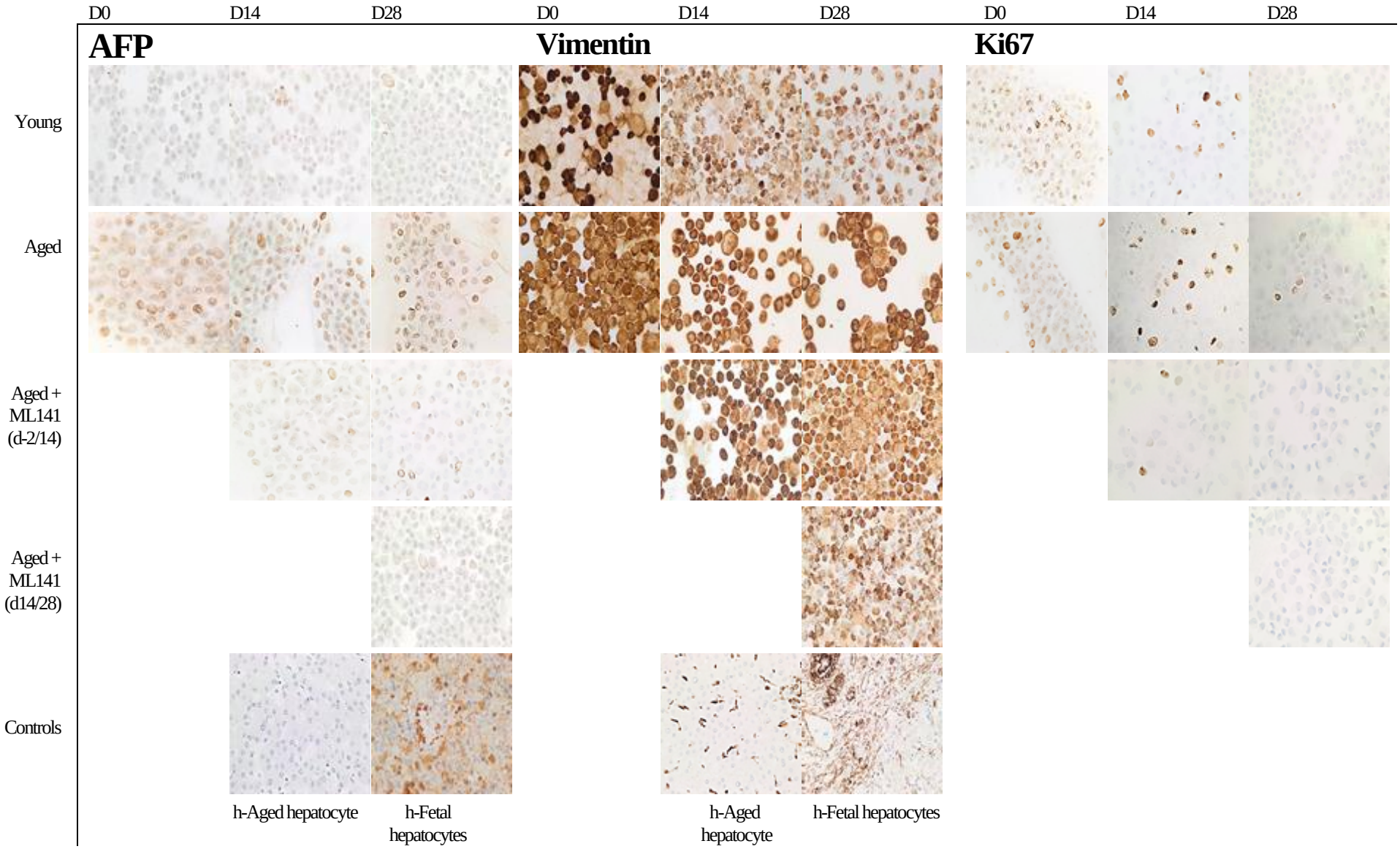
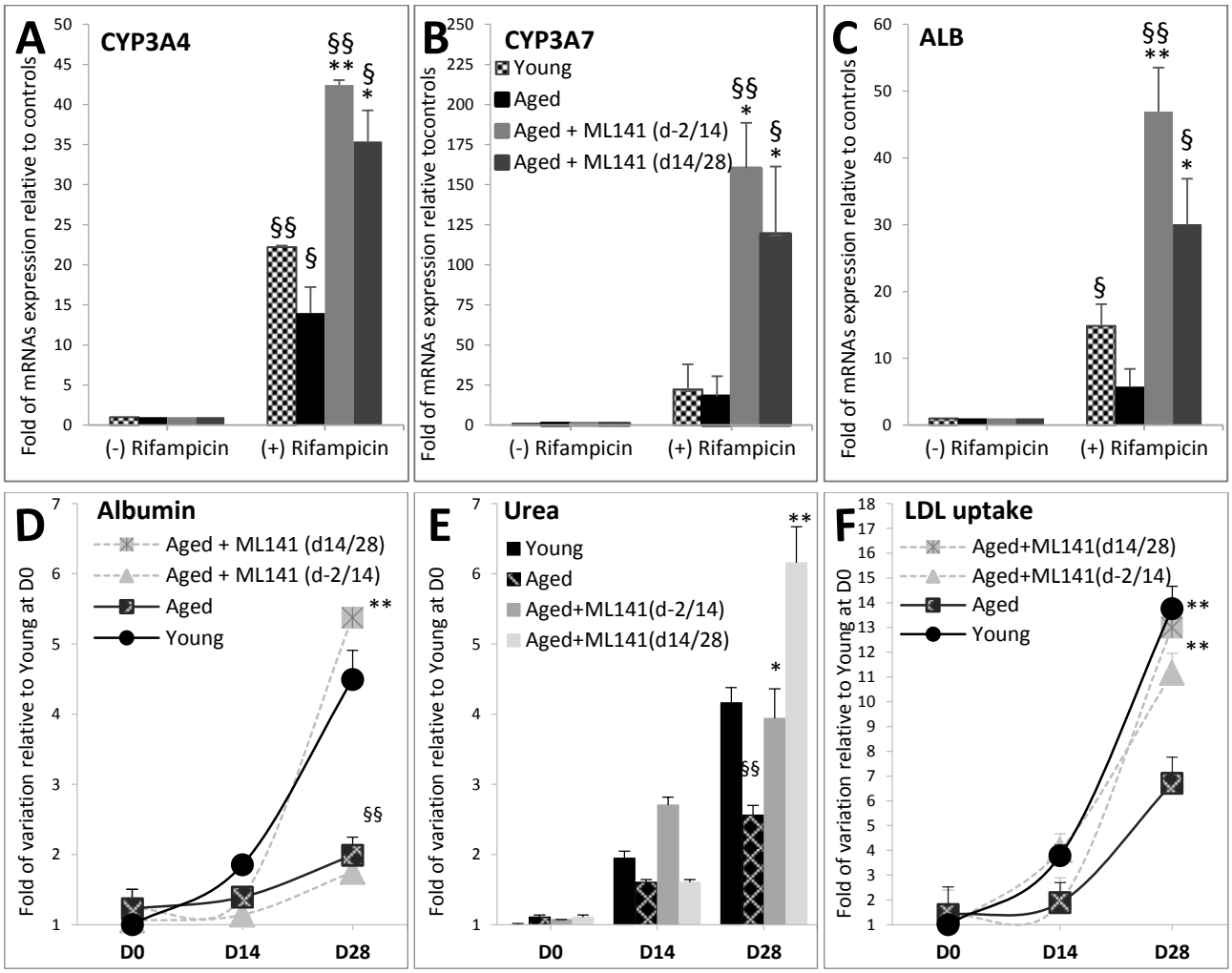

D0
D14
D28
D0
D14
D28
D0
D14
D28
AFP
Vimentin
Ki67
Young
Aged
Aged + ML141 (d-2/14)
Aged + ML141 (d14/28)
Controls
h-Aged hepatocyte
h-Fetal hepatocytes
h-Aged hepatocyte
h-Fetal hepatocytes

Figure 6


A
CYP3A4
Fold of mRNAs expression relative to controls
(-) Rifampicin
(+) Rifampicin
B
CYP3A7
Young
Aged
Aged + ML141 (d-2/14)
Aged + ML141 (d14/28)
Fold of mRNAs expression relative tocontrols
C
ALB
D
Albumin
Aged + ML141 (d14/28)
Aged + ML141 (d-2/14)
Aged
Young
Fold of variation relative to Young at D0
D0
D14
D28
E
Urea
Aged+ML141(d-2/14)
Aged+ML141(d14/28)
F
LDL uptake

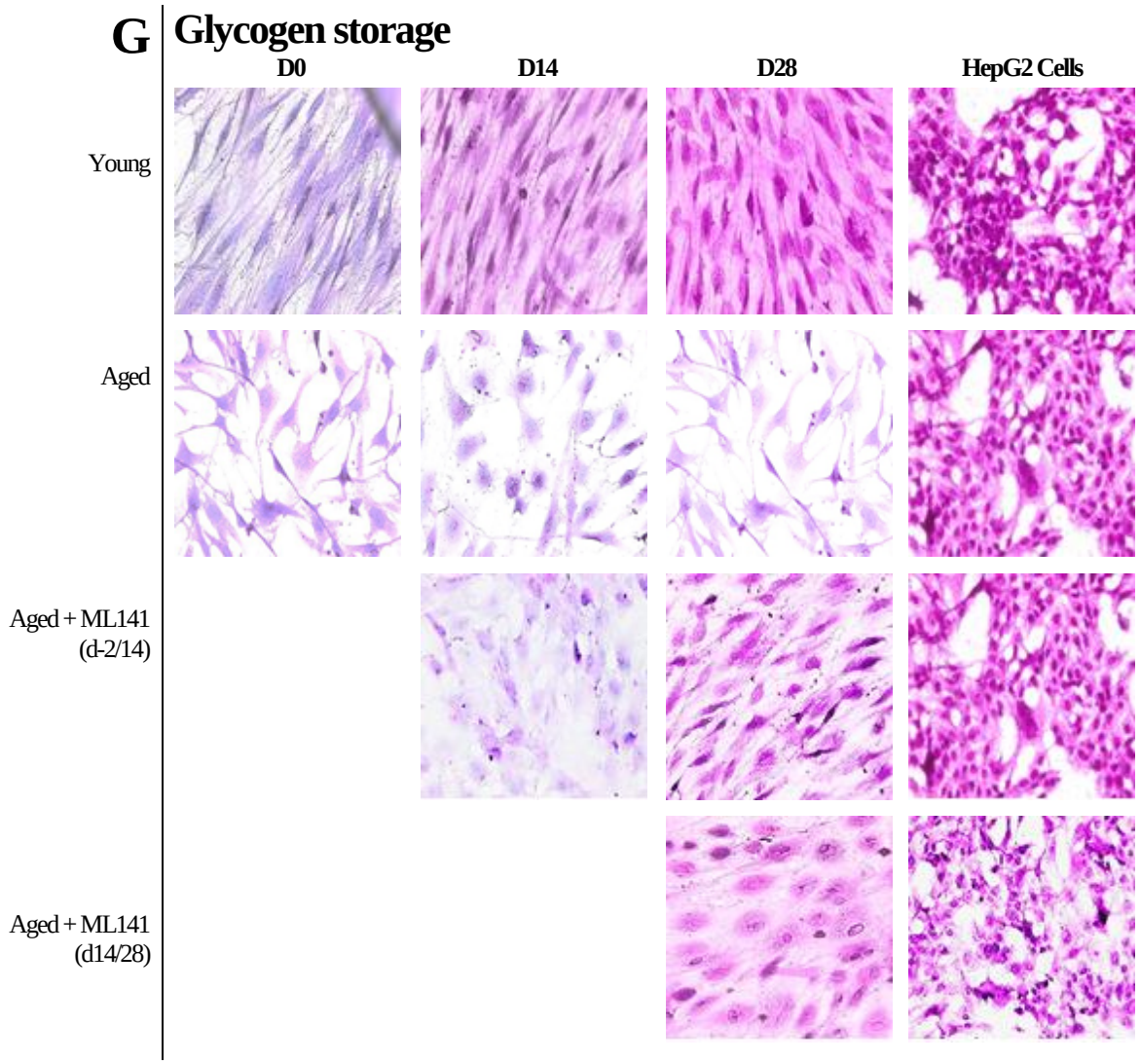
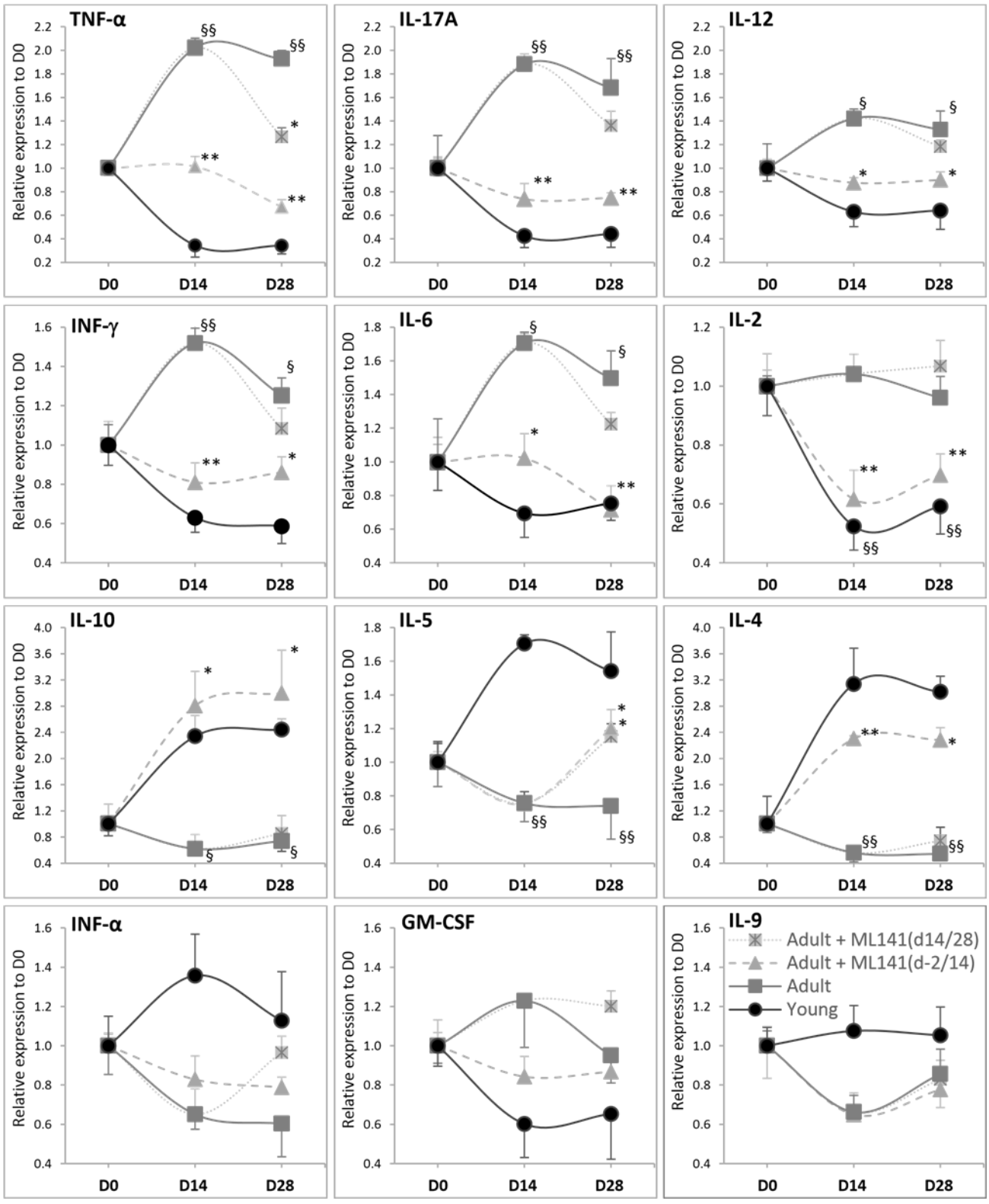

G
Glycogen storage
D0
D14
D28
HepG2 Cells
Young
Aged
Aged + ML141 (d-2/14)
Aged + ML141 (d14/28)

Figure 7


TNF-α
IL-17A
IL-12
INF-γ
IL-6
IL-2
IL-10
IL-5
IL-4
INF-α
GM-CSF
IL-9
Relative expression to D0
D0
D14
D28
Adult + ML141(d14/28)
Adult + ML141(d-2/14)
Adult
Young

Supplementary data – Figure S1 and Table S1

Table S1. Primer sequences used for quantitative RT–PCR

Gene	Forward Primer 5'- 3'	Reverse Primer 5'- 3'
hAFP	CTGCTGCAGCCAAAGTGAAG	CCAGCACATCTCCTCTGCAA
hALB	AAGCAGATGTTCCCAAAGCG	GCAAAGCAGGCCTCCTTATC
hCK18	ATCTTGGTGATGCCTTGGAC	CCTGCTTCTGCTGGCTTAAT
hCK7	ATAAAAGGCGCGGAGTGTCC	TCCAGAAACCGCACCTTGTC
hCK8	TGTTCCCAGTGCTACCCTG	GCCGTGGTTGTGAAGAAGAT
hCXCR4	CTGGCCTTCATCAGTCTGGA	TCATCTGCCTCACTGACGTT
hcyp3A4	GAGCTGAGATTGCACCACTG	GGGTGTTGAGGATGGAATGC
hcyp3A7	GGCTATCACAGATCCCGACA	TTTCCGCTGGTGAATGTTGG
hFoxa1	AAGACTCCAGCCTCCTCAAC	CGTATGCCTTGAAGTCCAGC
hFoxa2	ATTGCTGGTCGTTTGTTGTGG	GTACATGGGGCTCATGGAGTT
hFoxa3	AGTGGAGCTACTACCCGGAG	ACCTTGACGAAGCAGTCGTT
hG6Pc	ATTGACACCACACCCTTTGC	GACGTAGAAGACCAGCTCGA
hGATA 4	CGACACCCCAATCTCGATATGT	TTTGGATCCCCTCTTTCCGC
hHNF1 α	CAGAGTGTGCCGGTCATCAA	GAGGTGAAGACCTGCTTGGT
hHNF1 β	CATACTCTCACCAACGGCCA	ACTGTCTGGTTGAATTGTCGG
hHNF4 α	GGTGTTGACGATGGGCAATG	CTCATTCTGGACGGCTTCCTA
hHNF6	GCTTAGCAGCATGCAAAAGGAA	ACACCTTCGTGGCATGGTAG
hSox17	TTCATGGTGTGGGCTAAGGA	CCGGTACTTGTAGTTGGGGT
hTAT	GAGTCAGCGCATTTTGGGAC	TCGGGTACTCAAAGCACGTT
hp16INKa	TGACTCCCTCCCCATTTTCC	TTTTGGAGAGTCGGACTGCT
hp53	TGGCCATCTACAAGCAGTCA	GGTACAGTCAGAGCCAACCT
hp21	CCCAAGCTCTACCTTCCCAC	CTGAGAGTCTCCAGGTCCAC
Wnt-3a	CCACACCGTCAGGTACTCCT	TGTAGCTGGATGGAGTGCAG
Wnt-4	ACAGCTGGAAGGCTGACAGT	TGCATGTCCTTCTCACAAGC
Wnt-5a	ATACTGGCTGACCACCTTGG	GACAAAGGCCTCAGAAGCAC
Wnt-7a	CCCTGAAGCTTACTGCTTGG	GCTACGATGTATGGGGCACT
Wnt-11	CACCCCCAGATAGTTGTGCT	GAGGAGGAAAGCGACACAAG
β-catenin	GAAACGGCTTTCAGTTGAGC	CTGGCCATATCCACCAGAGT
hGADPH	AGCTCATTTCCTGGTATGACAAC	GTGGTCCAGGGGTCTTACTC

Figure S1.

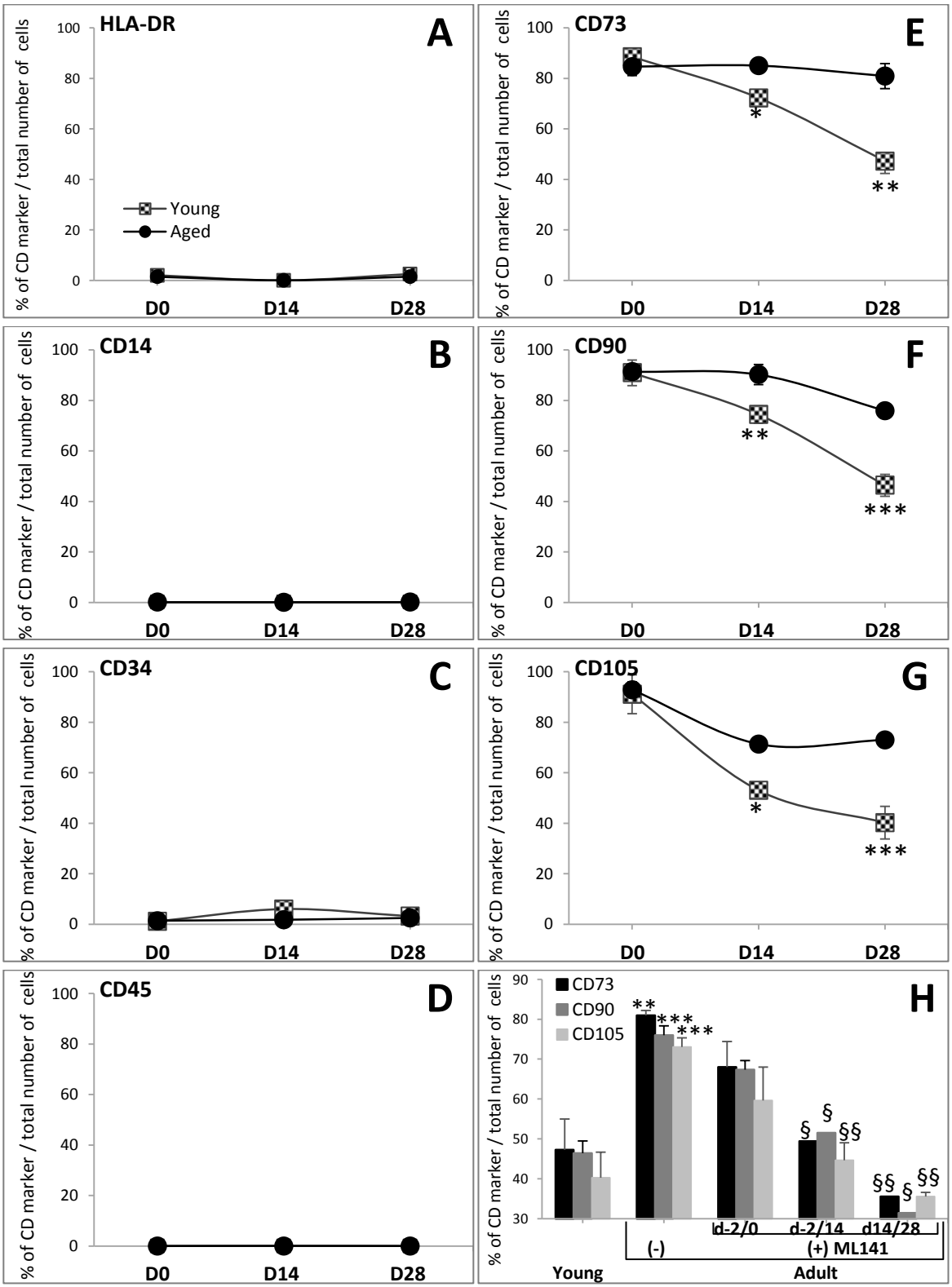

HLA-DR
A
% of CD marker / total number of cells
Young
Aged
D0
D14
D28
CD14
B
CD34
C
CD45
D
CD73
E
CD90
F
CD105
G
CD73
CD90
CD105
H
d-2/0
d-2/14
d14/28
(-)
(+) ML141
Young
Adult

Figure S1. Immunophenotype of ADSCs derived from young and aged subjects, and differentiated into hepatocyte-like cells. Cells without (A-G) or with ML141 (H) were labeled with fluorescence-coupled antibodies against HLA-DR, CD14, CD34, CD45, CD73, CD90 and CD105, and analyzed using a MACSQuant flow analyzer as indicated in materials and methods. The results are expressed as the mean±SEM and presented as percentage of CD marker per total number of cells. Cells were collected at day 0 at the moment of induction of the differentiation, day 14 and 28 of the differentiation (hepatoblast-like cells and hepatocyte-like cells, respectively). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$; Aged *versus* Young. Effect of ML141 was evaluated at day 28 (F). $^{\S}P<0.05$, $^{\S\S}P<0.01$; Aged treated with ML141 *versus* untreated.

CHAPTER 10

GENERAL DISCUSSION

Our study includes two important outcomes. *First*, taking in account of the difficulty to keep primary hepatocyte in culture and to overcome the high need of functional hepatocyte we proposed a new tool to reprogram mouse mature hepatocytes into endodermic progenitors (EndoPCs) satisfying most of the phenotypic criterias of bipotent liver stem cells that are activated after liver injury. *Second*, in front of the limited proliferation and differentiation *in vitro* of aged stem cell senescence, we reinforce the knowledge about the impact of Cdc42 activation on stem cell aging which has never reported on hADSCs. The results of both studies are developed and analyzed in order to show the implication of our findings as a novel support for liver regeneration and bioengineering development strategies.

Debate 1

The new technology of EndoPCs allowing the activation of Wnt/Lgr5 axis pathway in primary hepatocytes in absence of Wnt activators

Like specific LPs[341], the EndoPCs were shown in our study to have unlimited self-renewal capacity *in vitro*, to respond to LIFR ligands and to present bipotency with the capacity to differentiate into both mature hepatocytes and cholangiocytes. *In vivo* recent lineage tracing strategies have been conducted by several groups who have interestingly found that mature hepatocytes can be reprogrammed *in vivo* into active divided bipotent epithelial LPs through a cascade similar to that followed to induce iPSCs from fibroblasts[361] or by requiring modulation of Notch/Hippo pathway[359]. Indeed, these strategies relied on isolation of $EpCAM^+$ cells[352] or $EpCAM^-/Sox9^+$ cells [353] from liver biopsies followed by their transplantation to evaluate their bipotentiality and regenerative potential *in vivo*[45]. Consecutively, these hypothesis of *in vivo* adult liver hepatocytes reprogramming was requiring *in vitro* good evidence. Our study and strategy filled this need perfectly. Like Lgr5 induced injury liver stem cells, EndoPCs expressed Sox 9 and were able to give rise to new cholangiocytes and hepatocytes *in vitro* and to recover liver cell loss in hepatectomized liver animal model with high efficiency. However, oppositely to isolated induced injury Lgr5+, EndoPCs generation skipped any pre-step isolation and cell contamination relying on nonspecific markers such as EpCAM, Sox9 and OPN common with different epithelial liver cell type. In addition, our experimental strategy provides a new indication for *in vitro* primary

hepatocyte reprogramming. Our study induced liver specific progenitors following non-replicative reprogramming of adult hepatocytes without reversion to a pluripotent stem cell state. A transient expression of embryonic makers Oct-4, Sox2, klf4 and c-Myc was sufficient to expand infinitely these liver specific progenitors in a LIF dependent manner. As a proof-of-concept, this *in vitro* strategy identified that the Wnt target gene Lgr5 is readily expressed by the EndoPCs and is regulated by the gp130/JAK/STAT3 pathway under Rspo/Wnt activated pathway in ex-vivo culture. Recently, the strong crosstalk of IL6/STAT3 phosphorylation and Wnt /Lgr5 was studied on freshly isolated liver stem cells[370]. These findings suggest that our reprograming protocol could be also conducted by adult hepatocytes *in vivo* upon injury even though more examinations are mandatory to test this purpose.

Distinctly from similar strategy trying to generate endodermal lineage from ESCs[343,344] and iPSCs[37,348,121], EndoPCs being readily endodermic progenitors didn't require Activin A , BMP4 and Wnt3a to express markedly the endodermal lineage markers such as GATA, SOX17, and FOXA2. Also, EndoPCs are more interesting than PSC- derived endoderm lineage cells since they are clonally multipotent and already express hepatocytes-lineage markers (*KRT18, Hnf4α Hnf1α*, *AFP, and ALB*) and bile duct lineages markers (*KRT19, Hnf6*, *Hnf1α*,) before any differentiation process.

Furthermore, EndoPCs possess a special molecular signature discriminating their stemness hallmarks from mouse oval, embryonic, mesenchymal and hematopoietic stem cells. Indeed, they are negative for CD117, Oct-4/SSEA1, and CD90/105 and CD34/CD45 respectively.

On the other hand, EndoPCs are different from iPSCs and ESCs since they didn't require multistep protocols with sequential release of growth factors to induce hepatocyte or cholangiocytes differentiation *in vitro*[668]. Although EndoPCs give rise spontaneously to biliary duct structures *in vivo*, the *in vitro* cholangiocytes differentiation seemed more complicated by requiring 5-Step differentiation protocol. Moreover, hepatocytes derived from EndoPCs showed more functionality than hepatocytes derived from PSCs [316]. They were able to produce Alb and to be negative for AFP expression. However Alb expression was found to be less important in the EndoPCs derived hepatocytes compared to adult hepatocytes. Thus we proceeded to optimize the differentiation protocol by modifying the matrix dimensional structure. Thereby, we aimed to compare the EndoPCs hepatogenic potential between 2D and 3D scaffold structures. Our findings showed that 3D scaffolds were crucial to increase functionality of EndoPCs- derived hepatocytes_in OSM/Dex medium. Remarkably, where 3D- culture suspensions for iPSCs didn't reach to generate functional hepatocytes[319], 3D-EndoPCs derived hepatocytes showed dropped downregulation of LIFR, high level of Alb expression and uptaked more easily the LDL than their 2D-counterparts. In this context, it's important to note that Hep-Dif on 3D- scaffold was performed within an OFF state of Rspo/Wnt axis where no Wnt activators were added to the cell culture differentiation media.

Conversely to specific-liver induced Lgr5+, long term expansion of EndoPCs and their organoids formation were shown to be independent from the matrix 3D-structure. EndoPCs were able to express Lgr5 even in monolayer cell culture condition. Furthermore, EndoPCs generation *in vitro* didn't require the non-canonical Wnt, Notch and BMP chemical inhibition steps such as YAC cocktail used recently by Katsuda et al [54] to reprogram mice primary hepatocytes. Although both of YAC–induced liver stem cells and EndoPCs permitted a stable and long-term culturing in 3D structure and gave rise to functional hepatocytes, however our reprogramming strategy is chemical-free and could today serve as a novel source of LPs and overcome the limitations of cell source lack for use in liver regenerative medicine.

The Wnt target gene Lgr5 is highly expressed in mitotic stem cells of high rate auto-renewing tissues such as intestine and colon[46], stomach [47] and hair follicles [48]. Distinctly in liver, Lgr5+ progenitors range to damage-induced stem cells and dependent mainly on Wnt pathway activation within the bile duct. This evidence was observed after *in vivo* lineage tracing by creating liver injury in FAH-/- mice [320, 323] and more recently by inducing injury in specific liver Lgr5 knockout mice [52]. These Lgr5 stem cells act as a switch between on and off states depending on the levels of Wnt in the environment in a manner to order the cells whether to proliferate or not. Thus, isolated induced Lgr5 + progenitors upon injury were able to proliferate in a Wnt activating cell culture media. Here, we proposed EndoPCs as LPs deriving from hepatocytes programming authentically expressing Lgr5 independently from Wnt pathway activators in a monolayer cell culture system.

Moreover 3D culture system was shown to be required for long-term clonal expansion of Lgr5+ progenitors. Also, small molecules of Wnt activators proteins were essential for tissue-specific organoids generation[47, 669]. Nevertheless, given the readily expression of Wnt-dependent Lgr5, EndoPCs were not able to generate organoids in absence of Wnt activator such as Rspo-1 and Wnt3a. Thus our results revealed that the clonal EndoPCs - derived organoids expressed Wnt downstream effectors such as Axin2, Lgr5 and TCF4. These findings suggest that in absence of LIF the Lgr5 /Wnt loop is switched to OFF and EndoPCs require Wnt activators to Switch ON the LGR5 expression which is crucial for self-renewal and organoids formation.

Suitably, the gp130/JAK/STAT3 pathway is shown to activate LGR5 expression in EndoPCs via Wnt/β-catenin driven pathway. Indeed, our GSEA analysis shows enrichment of JAK/STAT3 signaling pathway within EndoPCs. We can understand that EndoPCs expressing LIFR used LIF/gp130/STAT3 or IL6/gp130/STAT3 to activate the transcriptional program of JAK/STAT3 to insure self-renewal via continuous reactivation of the Wnt/β-catenin loop. By these means our results confirmed the latest study showing that STAT3 phosphorylation inhibition leaded to attenuation of the self-renewal of liver stem cell and blocked the Wnt-Lgr5+ activation axis [370] . Besides, our findings support also the LIF/IL6-induced WNT signaling loop observed previously in ESCs self-renewal and pluripotency pathways[670]. Thus the regulators of this crosstalk constitute new targets in our further investigations.

Interestingly, Huch et al [17] performed a microarray analysis for mice liver Lgr5- derived organoids and revealed that these progenitors shared same profile of adult liver Lgr5 progenitors and some genes with hepatocyte-lineage markers as well as bile duct markers. Identically, to evaluate the lineage link of Lgr5 cells, we performed gene expression profiling of EndoPCs compared to adult hepatocytes. Up-regulated genes in EndoPCs as compared to primary hepatocytes where crossed by the upregulated genes LGR5+ liver stem compared to primary hepatocytes(Huch et al) ; the results show that the majority of these genes are common and belong to Wnt pathway , to TNFα/NF-KB pathway and other pathway of cellular PluriNetWork. These interesting findings reflect that EndoPCss are so much closed genetically to injury induced-LGR5 liver stem cells upon injury and are hepatocyte lineage specific progenitors. Thus EndoPCs might be a promising tool for liver stem cell based regenerative medicine although further investigations are required to test this safety and efficacy of EndoPCs derived organoids transplantation in chronic injury animal liver model.

Considering the bile duct formation in mice spleen after intra splenic transplantation of EndoPCs, possible risks must be carefully assessed. In probable upcoming transplantation applications of EndoPCs technology, it might be better to pick out Ck19 positive clones. Nevertheless, the fact that we didn't observe any oncogenic symptoms and chromosome aberrations, our transplantation assay is promising.

The Wnt/ STAT3 axis implies several molecular mechanisms instructing the EndoPCs either to proliferate indefinitely or to differentiate. Recently PEDF gene was shown to regulate Wnt/ STAT3 axis in LGR5 stem cells[370]. In addition the mi-RNAs regulating these mechanisms constitute a primordial end for our further investigations.

Although this technology must be also proven in humans, the EndoPCs technology provide a promising tool for producing Lgr5 organoid system obtained directly from adult hepatocyte without the need for genetic modifications . In addition human origin of EndoPCs can be usefull in the future for the production of patient-specific cholangiocytes and hepatocytes in a spontaneous manner and thus serving as a cell source for the liver regenerative medicine.

Debate 2

Novel strategy to convert the age of hADSCs and their derived hepatocytes through ML141-Cdc42 inhibition

We have shown here how to limit age-related hallmarks in aged derived hADSCs by inhibiting the Cdc42 activity by ML141 treatment and not by epigenetic modifiers such as TSA and CASIN. ADSCs have recently emerged as a great tool for clinical application in regenerative medicine [671] offering a biological reservoir of MSCs and being isolated with less donor discomfort[535, 672, 673]. The negative impact of donor age on hADSCs molecular mechanisms have been previously reported[77, 78, 394]. In fact, aged ADSCs showed lower rates of expansion *in vitro*, which makes them a weak

tool for clinical autologous use in elderly's diseases. Interestigly, even though ADSCs yield was shown to decline when isolated from aged adipose tissues biopsies[535, 674], our results showed no significant variability within the different age groups which probably due to the healthy recruited subjects.

Recently, Cdc42 was shown to be closely involved in human MSCs cell proliferation, polarity, migration and differentiation [637] . Based on Geiger[93] and Florian[21] observations on HSCs derived from mice and human subjects, Cdc42 activity exhibited an increase of the GTP binding complex correlated with age[92, 491, 675]. Several strategies aimed to reverse aging in human adult stem cells by inhibiting the Cdc42 activity[675]. Our strategy aimed to screen for the first time the activity of Cd42 in hADSCs isolated from healthy donors then to investigate the effects of selective ihnibition on their molecular mechanisms.

Similarly to HSC[21, 93], we have shown that the Cdc42-GTP bounds level increased with aging of hADSCs. Furthermore, like aged-bone marrow[676] and UC- MSCs[79, 461, 466, 467, 479, 480], aged hADSCs revealed high levels of TNFα and IFN-γ , low levels of VEGF correlating closely with high levels of Cdc42-GTP bindings potentially reducing their angiogenic, regeneration and immunomodulatory abilities.

Interestingly, we observed a significant positive correlation between Cdc42-GTP and β-catenin and Wnt-3a expression in aged ADSCs. Indeed, age related-Cdc42 was reported to be triggered by Wnt pathway in aged niche [586]. In addition, the inhibition of Wnt pathway, was able to reverse the age of adult multipotent stem cells via Cdc42 activity modulation and inversing the Cdc42-related apolarity[92] . Our results are in favor of this cross-talk between Wnt and Cdc42 in stem cell aging.

The activation of Cdc42 in stem cells is associated with a deregulation of the main signaling pathways implicated in the cell division and apoptosis such as MAPK, PI3K, IGF, PKA pathways [91, 93, 578, 601 479] . Similarly, in our study, Cdc42-GTP binding levels in aged hADSCs correlated with p-ERK, p-JNK and PKA activation but not with PI3K pathway. Our result support the fact that IGF binding to the tyrosine kinase receptor can directly lead to Cdc42 activating without passing by the upstream PI3K [613]. No correlations between Cdc42 and insulin pathway were found, indicating the non-involvment of the PI3K pathway: the results were supported by the obsevations of non-signficant variations on the levels of insulin receptor and its substrate, insulin sensitive transcription factors. In fact, the mechanism involved RAS / ERK / JNK MAPK pathway, CREB / NFkB / C/EBPα /PPARγ transcription factors, IGF/VEGF grow factors and Wnts signaling.

Interestingly, CASIN-treated aged HSCs exhibited a level of Cdc42-GTP similar to that found in HSCs from young donors [92]. In our study, CASIN didn't inhibit Cdc42 activation in aged ADSCs. These controvert mechanisms remain unclear since CASIN has never been used before as Cdc42 inhibitor in MSCs. Therefore, the CASIN inhibition appears to be cell-

type dependent and doesn't inhibit the GEF activity in aged ADSC. However, CASIN could have regulatory impact on other age-related hallmark other than Cdc42 in hADSCs which can be considered in future steps.

Interestingly, TSA was able to maintain the pluripotency expression in placenta [640], UC [639] and BM[60,62] derived hMSCs and to delay the appearance of cellaging signs. On the other hand, the ML141 was recently used as a potent Cdc42 inhibitor[596, 630, 652]. Thus we are the first to inhibit the Cdc42 activity in aged hADSCs via ML141 and TSA. Both of them reversed the aging state of ADSCs. Indeed, ML141, more than TSA, promoted better the population doubling and less toxicity to a level similar to young ADSCs. Furthermore, ML141 was able to repress the expression of the apoptotic and senescence genes and to upregulate the pro-inflammatory potential of aged-treated ADSC. These results correlate closely with recent findings where ML141 was shown to inhibit Cdc42 activity in hBMSCs, ECs and cancer stem cells by downregulating some signaling pathways like EGFR, JNK and p21-activated kinases pathways (PAK) which are closely implicated in aging and polarity stem cell processes[596, 630, 631, 636, 638].

Recently, ADSCs have been reported as a promising source of MSCs used in regenerative medicine treatment for hepatic failure [62]. Thus our aim was to study the impact of Cdc42 inhibition on the hepatogenic potential of aged hADSCs. We conducted Hep-Difprotocol adapted from Yin et al, 2015 [523] where TSA was shown to differentiate partially the hADSCs towards hepatocytes. They observed that although the derived hepatocytes secrete albumin and uptake LDL, they continue to express the hepatoblast fetal marker AFP. Thus, our novel strategy consists on replacing the TSA treatment by ML141, a specific and direct Cdc42 GTPase inhibitor during the hepatic differentiation of hADSCs. ML141-treatment efficiently reversed markedly the age-related hallmarks in the hepatocytes derived from ML141-treated aged hADSCs compared to their counterparts derived from non-treated ADSCs. Indeed, the ML141 treated groups showed expression of pro-inflammatory cytokines, produced urea, secreted Alb, uptaked LDL and dropped down the AFP expression to levels found to be similar to hepatocytes derived from Young hADSCs.

Recently, new therapeutic strategies used MSCs exosomes in liver diseases [65]. Our results showed impairement in the release of exosomes in hADSCs-derived from the aged group. These results lead us to consider that ML141 can be a promising tool to isolate younger functional exosomes from aged derived hADSCs for liver nanomolecules- based therapy.

Considering the MET , this transition was shown to be a crucial process when transdifferentiating MSCs into hepatocytes[527]. Previous data showed that hepatocytes derived from ADSCs presented a downregulation of Twist and Snail expression [677]. However, in our work, Twist didn't show any significant change in expression with or without ML141 treatment. ML141 treatment enhanced the MET in hADSCs which was highly correlated with the upregulation of specific hepatic markers such as albumin, TAT, G6P and CYP450. Moreover, the positive co-expression of CK18 (KRT18) and E-Cadherin was reported to be a crucial parameter for MET within hepatic differentiation of hiPSCs [317].

Similarly, we observed that hepatocytes generated by ML141 treatment showed higher expression of CK18 (KRT18) and E-cadherin. Overall, our novelty exist in the fact that ML141 was able to reverse the age-related aberrations in aged hADSCs and to promote greater hepatogenic potential similar to young differentiated ADSCs counterparts.

Debate 3: Reverse-aged hADSCs and EndoPCs technologies in the liver's engineering and regenerative context

Crosstalk between Wnt/LGR5 and Cdc42 in Lgr5+ stem cells

Interestingly, the Knock Out of Lgr5 in hepatic cancer cell line (FLAG-tagged LGR5) lead in absence of Rspo-1/Wnt activation to serious changes in actin cytoskeleton and cell-cell adhesion independently of Rspo/LGR5 Wnt activation[678] . More interestingly, Rho-GTPase was shown, for the first time, in 2013 to be a downstream target of LGR5 even in absence of RSPO[679]. Recently, LGR5 was shown to interact with GAPs (GTPase activated protein) which are the positive regulator for GTP binding to Cdc42. LGR5/GAP cross talking leads to Cdc42-GTP binding and thereby to cell-cell adhesion, actin crosslinking and increases the bonding of β-catenin to the cell membrane's E-Cadherins. Conversely, the ablation of LGR5 from stem cells impaired severely the actin organization which is the crucial function of Cdc42 GTPase pathway. This process was proposed to be crucial in maintaining intestinal Lgr5 stem cells retained in their niche [20].

Although this novel discoveries should be validated in liver Lgr5+ stem cells, this novelty explain to us 12 years later the mechanisms underlying the expression of Cdc42 by induced-injury oval cells during their Hep-Dif which was reported clearly by Cimica et al in 2005 [31] .

Crosstalk between Wnt/LGR5 and Cdc42 in Hep-Dif

It was previously clearly reported that Wnt signaling pathways meet Rho-GTPases[680]. Also, Cdc42 was shown to regulate hepatocyte development *in vivo* by regulating the Wnt/β-catenin pathway. The accumulated phosphorylated β-catenin was shown to form a complex with E-cadherin and to trigger oval cells differentiation during liver regeneration [19]. Moreover, Cdc42 was shown to be crucial in the terminal phase of hepatocyte proliferation during liver injury [240]. To explain this mechanism, it was observed that after chemical rat liver injury, the activated liver stem cells, discovered to express Lgr5+ few years later, express clearly Cdc42. These results suggested in 2005 that Cdc42 is playing a role in LP's differentiation into hepatocytes. Following this report, Cdc42 was considered to be "a novel identified gene expressed in oval cells only during regeneration which could be important in regulating and triggering

the hepatic proliferation" [31]. Although this study didn't define if the GTP or GDP component of Cdc42 was assessed, this evidence suggests that Wnt/Lgr5 axis and Cdc42 are cross-talking somewhere in LGR5-bipotent stem cells undergoing Hep-Dif.

While, the mechanisms underlying this link between Lgr5 and Cdc42 were never been studied in the context of Hep-Dif, we can suggest some relatives based on the following indications.

Before Hep-Dif, to maintain self-renewal and the LGR5 expression in liver stem cell, it was crucial to inhibit the non-canonical and BMP/SMAD pathways and activate the canonical WNT /β-catenin via GSK-3 inhibition [54]. In a next step, BMP/Smad pathway was essential for inducing and maintaining the endodermal differentiation with nonstop Wnt activation conditions[336]. *In vivo* and *in vitro* LP's hepatic differentiation was requiring down-regulation of the Wnt signaling pathway via β-catenin/ E-cadherin bounding complex [24]. In this context, interestingly, LGR5 was shown to be implicated in the Cdc42 activation process regulating the β-catenin bound to the membrane[20]. These suggestions might reflect a possible coordination between Lgr5 and Cdc42 for the Hep-Dif from EndoPCs.

In order to explore the role of Wnt signaling in the differentiation of ADSCs into hepatocytes, Huang et al (2017) [532] showed that the inhibition of the GSK3 alone was sufficient to increase the β-catenin in a manner to activate the Wnt/β-catenin signaling. This mechanism triggers significantly the expression of definitive endoderm specific genes GATA4, FOXA2, and SOX17 significantly. These ADSCs derived-endodermic progenitors were directed to differentiate into hepatocyte-like cells after further combinations of soluble factors, and able to express important CYP450 enzymes with great albumin secretion and LDL uptake activity.These findings propose that activation of Wnt/β-cateninsignaling during the endoderm specification represent a crucial mechanism intermediating hADSCs Hep-Dif.

This fact was also reported previously on other human MSCs subtypes undergoing hepatocytes differentiation[533]. On the other side, our results showed that Cdc42 inhibition by ML141 was able to boost the hepatocytes functionality in hADSCs. Thereby, the ON/OFF switch of the Wnt signaling pathway might regulate in the same way the Hep-Dif either from EndoPCs and hADSCs. Noticeably, although Cdc42 is shown to be crucial for liver stem cell differentiation into hepatocytes, its inhibition is required for ADSCs hepatic differentiation process.

Future Request-for-information to reinforce the suggestion that LGR5/Wnt pathway meets Cdc42 in EndoPCs

Basing on above-mentioned evidence, coordination between Lgr5 and Cdc42 could exist in liver stem cells such as for the EndoPCs during their differentiation into hepatocytes *in vivo and in vitro.*

Thus, further investigation might be essential to validate the cross talk of LGR5 and Cdc42 in stem cell regulation mechanisms. Notably, the Cdc42 activation state should be studied in EndoPCs Lgr5+ before and after Hep-Dif.

In addition, it will be important to inquire if hADSCs express Lgr5 before or within differentiation. If not, searching to activate this receptor by Rspo-1 would be an interest. Also, Lgr5 variation within the age could be a new concept that has never been debated before.

Furthermore, while hMSCs were reprogrammed into iPCS then differentiated into hepatocytes, it will be exciting to test if the Cdc42 inhibitor ML141 could impact the LGR5 expression in EndoPCs and their hepatogenic and cholangiogenic differentiation. Moreover it has never been discussed whether OSKM factors can confer young features for aged hADSCs after inducing their pluripotency.

More interestingly, despite the fact that Cdc42 activation in primary hepatocyte was able to increase their DNA synthesis and their life span *in vitro*[255], Cdc42 inhibition by ML141 constitute an attentive inquiry for reprogramming or not the process of primary hepatocytes.

What is the best cell model to use for liver organoid reconstruction?

Recently, decellularized liver matrix has been emerged as a novel tool for tissue-engineered liver graft by preserving the structural and functional features of the natural microvascular system of the liver. The recellularization of the liver matrix by primary rat hepatocytes upregulates the liver-specific functions such as albumin secretion, urea synthesis and cytochrome P450 expression at analogous amounts to normal liver. These hepatocytes grafts were able to regenerate injured liver of rat model [681]. Recently, a complete decellularization of the whole human liver and lobes was performed and served to prepare cubic scaffolds mimicking the liver architecture. These scaffolds were repopulated for up to 21 days using different healthy and cancer liver cell line [682]. Although this technology by far the most suitable for liver transplantation, it constitutes an attractive basis for hepatic regenerative medicine. The optimization of the recellularization protocol and the accuracy of the cell types used within perfusion bio-reactors are crucial hints to progress towards possible clinical applications.

In this novel context, we propose both EndoPCs and ADSCs singularly or together as good candidates for Hep-Dif on decellularized liver matrix. Distinctly, EndoPCs present higher proliferation capacity than hADSCs and then can constitute a better platform for high cells throughput production which is an important element to get good repopulation on a 3D structure. The resulting 3D-structures can be central for liver disease modelling as well as for drug toxicity.

On the other hand, the in situ-recellularization approach such as repopulating the hepatic sinusoids by ECs could represent a new challenge with hADSCs which showed a better hepatogenic potential when co-cultured with human umbilical cord blood endothelial cells (HUVECs) during my Master 2 work (data not shown)

CONCLUSION

In summary, a reprogramming approach that can convert human hepatocytes to patient-specific liver stem cells may be an ideal option for evading immunological rejection during liver cell based transplantation. The abundant expansion of these Lgr5+ EndoPCs derived from small liver biopsy would make it relatively an easy start of EndoPCs-derived cell library from patient presenting liver diseases. Moreover, human EndoPCs could be differentiated towards functional hepatocytes in 3D-setups from biocompatible matrix.

By 2050 the number of aged people is estimated to triple globally [70]. Thus, our findings propose ML141 as a new pharmacological tool targeting the aging of hADSCs and able to rejuvenate their paracrine and molecular age-related mechanisms before their use in MSC-based therapies and tissue engineering associated to aging diseases. The inhibition of Cdc42 by ML141 proposes a new *ex vivo* approach which is safe and efficacy, and directs the hepatic differentiation of hADSCs to be used in clinical stem cell liver therapy.

REFERENCES

1. Cobb, M., Puchalski, C.M. & Rumbold, B. *Oxford textbook of spirituality in healthcare*. (Oxford University Press, 2012).
2. Liu, T., Zhang, S., Chen, X., Li, G. & Wang, Y. Hepatic differentiation of mouse embryonic stem cells in three-dimensional polymer scaffolds. *Tissue engineering Part A* **16**, 1115-1122 (2009).
3. Paine, A.J. & Andreakos, E. Activation of signalling pathways during hepatocyte isolation: relevance to toxicology in vitro. *Toxicology in Vitro* **18**, 187-193 (2004).
4. Elaut, G. *et al.* Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Current drug metabolism* **7**, 629-660 (2006).
5. Cheng, J. *et al.* The impact of miR-34a on protein output in hepatocellular carcinoma HepG2 cells. *Proteomics* **10**, 1557-1572 (2010).
6. Li, Q. *et al.* In vivo tracking and comparison of the therapeutic effects of MSCs and HSCs for liver injury. *PloS one* **8**, e62363 (2013).
7. Berger, D.R., Ware, B.R., Davidson, M.D., Allsup, S.R. & Khetani, S.R. Enhancing the functional maturity of induced pluripotent stem cell–derived human hepatocytes by controlled presentation of cell–cell interactions in vitro. *Hepatology* **61**, 1370-1381 (2015).
8. Muraca, M. Evolving concepts in cell therapy of liver disease and current clinical perspectives. *Digestive and Liver Disease* **43**, 180-187 (2011).
9. Puppi, J. *et al.* Improving the techniques for human hepatocyte transplantation: report from a consensus meeting in London. *Cell transplantation* **21**, 1-10 (2012).
10. Fausto, N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* **39**, 1477-1487 (2004).
11. Agarwal, S., Holton, K.L. & Lanza, R. Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem cells* **26**, 1117-1127 (2008).
12. Liu, T., Zhang, S., Xiang, D. & Wang, Y. Induction of hepatocyte-like cells from mouse embryonic stem cells by lentivirus-mediated constitutive expression of Foxa2/Hnf4a. *Journal of cellular biochemistry* **114**, 2531-2541 (2013).
13. Przyborski, S.A. Differentiation of human embryonic stem cells after transplantation in immune-deficient mice. *Stem Cells* **23**, 1242-1250 (2005).
14. Asgari, S. *et al.* Induced pluripotent stem cells: a new era for hepatology. *Journal of hepatology* **53**, 738-751 (2010).
15. Si-Tayeb, K. *et al.* Highly efficient generation of human hepatocyte–like cells from induced pluripotent stem cells. *Hepatology* **51**, 297-305 (2010).
16. Mikkelsen, T.S. *et al.* Dissecting direct reprogramming through integrative genomic analysis. *Nature* **454**, 49 (2008).
17. Huch, M. *et al.* In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* **494**, 247-250 (2013).
18. Lien, W.-H. & Fuchs, E. Wnt some lose some: transcriptional governance of stem cells by Wnt/β-catenin signaling. *Genes & development* **28**, 1517-1532 (2014).
19. Van Hengel, J. *et al.* Continuous cell injury promotes hepatic tumorigenesis in cdc42-deficient mouse liver. *Gastroenterology* **134**, 781-792 (2008).
20. Carmon, K.S. *et al.* LGR5 receptor promotes cell–cell adhesion in stem cells and colon cancer cells via the IQGAP1–Rac1 pathway. *Journal of Biological Chemistry* **292**, 14989-15001 (2017).
21. Florian, M.C. *et al.* Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. *Cell stem cell* **10**, 520-530 (2012).
22. Karp, S. Clinical implications of advances in the basic science of liver repair and regeneration. *American Journal of Transplantation* **9**, 1973-1980 (2009).
23. Wang, X. *et al.* The origin and liver repopulating capacity of murine oval cells. *Proceedings of the National Academy of Sciences* **100**, 11881-11888 (2003).
24. Monga, S.P., Pediaditakis, P., Mule, K., Stolz, D.B. & Michalopoulos, G.K. Changes in WNT/β-catenin pathway during regulated growth in rat liver regeneration. *Hepatology* **33**, 1098-1109 (2001).

25. Monga, S.P. *et al.* Hepatocyte growth factor induces Wnt-independent nuclear translocation of β-catenin after Met-β-catenin dissociation in hepatocytes. *Cancer research* **62**, 2064-2071 (2002).
26. Terui, K. & Ozaki, M. The role of STAT3 in liver regeneration. *Drugs of Today* **41**, 461-470 (2005).
27. Cressman, D.E., Diamond, R.H. & Taub, R. Rapid activation of the Stat3 transcription complex in liver regeneration. *Hepatology* **21**, 1443-1449 (1995).
28. Mangnall, D., Bird, N.C. & Majeed, A.W. The molecular physiology of liver regeneration following partial hepatectomy. *Liver international* **23**, 124-138 (2003).
29. Kim, R.D., Kim, J.S., Watanabe, G., Mohuczy, D. & Behrns, K.E. in Seminars in interventional radiology, Vol. 25 092-103 (© Thieme Medical Publishers, 2008).
30. J. Vessey, P.d.l.M.H., Carina Hepatic stem cells: a review. *Pathology* **33**, 130-141 (2001).
31. Cimica, V. *et al.* Transcriptome analysis of rat liver regeneration in a model of oval hepatic stem cells. *Genomics* **86**, 352-364 (2005).
32. Dorrell, C. *et al.* Surface markers for the murine oval cell response. *Hepatology* **48**, 1282-1291 (2008).
33. Ramboer, E. *et al.* Strategies for immortalization of primary hepatocytes. *Journal of hepatology* **61**, 925-943 (2014).
34. Shinozuka, H., Lombardi, B., Sell, S. & Iammarino, R.M. Early histological and functional alterations of ethionine liver carcinogenesis in rats fed a choline-deficient diet. *Cancer research* **38**, 1092-1098 (1978).
35. Baumann, U., Crosby, H.A., Ramani, P., Kelly, D.A. & Strain, A.J. Expression of the stem cell factor receptor c-kit in normal and diseased pediatric liver: Identification of a human hepatic progenitor cell? *Hepatology* **30**, 112-117 (1999).
36. Jin, C. *et al.* Blocking SCF/c-Kit signal did not inhibit the proliferation of cultured liver progenitor cells. *IUBMB life* **59**, 458-464 (2007).
37. Tsuchiya, A. *et al.* Sca-1+ endothelial cells (SPECs) reside in the portal area of the liver and contribute to rapid recovery from acute liver disease. *Biochemical and biophysical research communications* **365**, 595-601 (2008).
38. Fujio, K., Evarts, R.P., Hu, Z., Marsden, E.R. & Thorgeirsson, S.S. Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in adult rat. *Laboratory investigation; a journal of technical methods and pathology* **70**, 511-516 (1994).
39. Zhang, L., Theise, N., Chua, M. & Reid, L.M. The stem cell niche of human livers: symmetry between development and regeneration. *Hepatology* **48**, 1598-1607 (2008).
40. Schmelzer, E., Wauthier, E. & Reid, L.M. The phenotypes of pluripotent human hepatic progenitors. *Stem cells* **24**, 1852-1858 (2006).
41. Furuyama, K. *et al.* Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. *Nature genetics* **43**, 34-41 (2011).
42. Carpentier, R. *et al.* Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. *Gastroenterology* **141**, 1432-1438. e1434 (2011).
43. Español–Suñer, R. *et al.* Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. *Gastroenterology* **143**, 1564-1575. e1567 (2012).
44. Sell, S. Heterogeneity and plasticity of hepatocyte lineage cells. *Hepatology* **33**, 738-750 (2001).
45. Tarlow, B.D. *et al.* Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. *Cell stem cell* **15**, 605-618 (2014).
46. Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003-1007 (2007).
47. Barker, N. *et al.* Lgr5+ ve stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell stem cell* **6**, 25-36 (2010).
48. Jaks, V. *et al.* Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nature genetics* **40**, 1291-1299 (2008).
49. Xu, A.T., Tong, J.L. & Ran, Z.H. Organoids derived from digestive tract, liver, and pancreas. *Journal of digestive diseases* **17**, 3-10 (2016).
50. Lindemans, C.A. *et al.* Interleukin-22 promotes intestinal stem cell-mediated epithelial regeneration. *Nature* **528**, 560 (2015).

51. Tian, Y. *et al.* Stress responsive miR-31 is a major modulator of mouse intestinal stem cells during regeneration and tumorigenesis. *eLife* **6** (2017).
52. Huch, M. *et al.* In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* **494**, 247 (2013).
53. Huch, M. *et al.* Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell* **160**, 299-312 (2015).
54. Katsuda, T. *et al.* Conversion of terminally committed hepatocytes to culturable bipotent progenitor cells with regenerative capacity. *Cell stem cell* **20**, 41-55 (2017).
55. Crouzet, J. *et al.* Recombinational construction in Escherichia coli of infectious adenoviral genomes. *Proceedings of the National Academy of Sciences* **94**, 1414-1419 (1997).
56. Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262 (2009).
57. Buyl, K. *et al.* Characterization of hepatic markers in human Wharton's Jelly-derived mesenchymal stem cells. *Toxicology in vitro* **28**, 113-119 (2014).
58. Li, C.-y. *et al.* Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. *Stem cell research & therapy* **6**, 55 (2015).
59. Lee, H.-J. *et al.* Comparison of in vitro hepatogenic differentiation potential between various placenta-derived stem cells and other adult stem cells as an alternative source of functional hepatocytes. *Differentiation* **84**, 223-231 (2012).
60. Liu, W.h. *et al.* The multiple functional roles of mesenchymal stem cells in participating in treating liver diseases. *Journal of cellular and molecular medicine* **19**, 511-520 (2015).
61. Parker, A.M. & Katz, A.J. Adipose-derived stem cells for the regeneration of damaged tissues. *Expert opinion on biological therapy* **6**, 567-578 (2006).
62. Ishikawa, T., Banas, A., Hagiwara, K., Iwaguro, H. & Ochiya, T. Stem cells for hepatic regeneration: the role of adipose tissue derived mesenchymal stem cells. *Current stem cell research & therapy* **5**, 182-189 (2010).
63. Al Battah, F., De Kock, J., Vanhaecke, T. & Rogiers, V. Current status of human adipose–derived stem cells: differentiation into hepatocyte-like cells. *The Scientific World Journal* **11**, 1568-1581 (2011).
64. Taléns-Visconti, R. *et al.* Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. *World journal of gastroenterology: WJG* **12**, 5834 (2006).
65. Lou, G., Chen, Z., Zheng, M. & Liu, Y. Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. *Experimental & molecular medicine* **49**, e346 (2017).
66. Hayflick, L. How and why we age. *Experimental gerontology* **33**, 639-653 (1998).
67. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *cell* **100**, 57-70 (2000).
68. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *cell* **144**, 646-674 (2011).
69. López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* **153**, 1194-1217 (2013).
70. Florian, M. *et al.* Expression and activity of the small RhoGTPase Cdc42 in blood cells of older adults are associated with age and cardiovascular disease. *The Journals of Gerontology: Series A* (2017).
71. Ono, Y. *et al.* The influence of donor age on liver regeneration and hepatic progenitor cell populations. *Surgery* **150**, 154-161 (2011).
72. Feng, S. *et al.* Characteristics associated with liver graft failure: the concept of a donor risk index. *American Journal of Transplantation* **6**, 783-790 (2006).
73. Kfoury, Y. & Scadden, D.T. Mesenchymal cell contributions to the stem cell niche. *Cell stem cell* **16**, 239-253 (2015).
74. Oh, J., Lee, Y.D. & Wagers, A.J. Stem cell aging: mechanisms, regulators and therapeutic opportunities. *Nature medicine* **20**, 870-880 (2014).
75. Li, C.-J. *et al.* MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation. *The Journal of clinical investigation* **125**, 1509 (2015).
76. Tashiro, J. *et al.* Therapeutic benefits of young, but not old, adipose-derived mesenchymal stem cells in a chronic mouse model of bleomycin-induced pulmonary fibrosis. *Translational Research* **166**, 554-567 (2015).

77. Kornicka, K., Marycz, K., Tomaszewski, K.A., Marędziak, M. & Śmieszek, A. The effect of age on osteogenic and adipogenic differentiation potential of human adipose derived stromal stem cells (hASCs) and the impact of stress factors in the course of the differentiation process. *Oxidative medicine and cellular longevity* **2015** (2015).
78. Choudhery, M.S., Badowski, M., Muise, A., Pierce, J. & Harris, D.T. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. *Journal of translational medicine* **12**, 8 (2014).
79. Geißler, S. *et al.* Functional comparison of chronological and in vitro aging: differential role of the cytoskeleton and mitochondria in mesenchymal stromal cells. *PLoS One* **7**, e52700 (2012).
80. Wu, W., Niklason, L. & Steinbacher, D.M. The effect of age on human adipose-derived stem cells. *Plastic and reconstructive surgery* **131**, 27-37 (2013).
81. Zhang, Y. & Herman, B. Ageing and apoptosis. *Mechanisms of ageing and development* **123**, 245-260 (2002).
82. Alt, E.U. *et al.* Aging alters tissue resident mesenchymal stem cell properties. *Stem cell research* **8**, 215-225 (2012).
83. Ock, S.-A. *et al.* Evaluation of phenotypic, functional and molecular characteristics of porcine mesenchymal stromal/stem cells depending on donor age, gender and tissue source. *Journal of Veterinary Medical Science* **78**, 987-995 (2016).
84. Xing, Z. *et al.* Increased hematopoietic stem cell mobilization in aged mice. *Blood* **108**, 2190-2197 (2006).
85. Yang, L. *et al.* Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the bone marrow. *Proceedings of the National Academy of Sciences* **104**, 5091-5096 (2007).
86. Erickson, J.W. & Cerione, R.A. Multiple roles for Cdc42 in cell regulation. *Current opinion in cell biology* **13**, 153-157 (2001).
87. Johnson, D.I. Cdc42: an essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiology and Molecular Biology Reviews* **63**, 54-105 (1999).
88. Rojas, R., Ruiz, W.G., Leung, S.-M., Jou, T.-S. & Apodaca, G. Cdc42-dependent modulation of tight junctions and membrane protein traffic in polarized Madin-Darby canine kidney cells. *Molecular biology of the cell* **12**, 2257-2274 (2001).
89. Hong, L. *et al.* Characterization of a Cdc42 protein inhibitor and its use as a molecular probe. *Journal of Biological Chemistry* **288**, 8531-8543 (2013).
90. Wang, L., Yang, L., Debidda, M., Witte, D. & Zheng, Y. Cdc42 GTPase-activating protein deficiency promotes genomic instability and premature aging-like phenotypes. *Proceedings of the National Academy of Sciences* **104**, 1248-1253 (2007).
91. Carrillo-García, C. & Janzen, V. Restoring cell polarity: an HSC fountain of youth. *Cell stem cell* **10**, 481-482 (2012).
92. Geiger, H. & Zheng, Y. Regulation of hematopoietic stem cell aging by the small RhoGTPase Cdc42. *Experimental cell research* **329**, 214-219 (2014).
93. Florian, M.C. *et al.* Expression and activity of the small RhoGTPase Cdc42 in blood cells of older adults are associated with age and cardiovascular disease. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences* **72**, 1196-1200 (2017).
94. Theise, N.D. & Krause, D.S. in Seminars in cell & developmental biology, Vol. 13 411-417 (Elsevier, 2002).
95. Soto-Gutierrez, A., Gough, A., Vernetti, L.A., Taylor, D. & Monga, S.P. Pre-clinical and clinical investigations of metabolic zonation in liver diseases: The potential of microphysiology systems. *Experimental Biology and Medicine*, 1535370217707731 (2017).
96. Guo, X., Li, W., Ma, M., Lu, X. & Zhang, H. Endothelial cell-derived matrix promotes the metabolic functional maturation of hepatocyte via integrin-Src signalling. *Journal of Cellular and Molecular Medicine* (2017).
97. Kmieć, Z. Cooperation of Liver Cells in the Synthesis and Degradation of Eicosanoids, in *Cooperation of Liver Cells in Health and Disease* 51-59 (Springer, 2001).
98. Li, R. *et al.* Role of liver sinusoidal endothelial cells and stabilins in elimination of oxidized low-density lipoproteins. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **300**, G71-G81 (2011).
99. Roberts, R.A. *et al.* Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. *Toxicological Sciences* **96**, 2-15 (2006).
100. Michalopoulos, G.K. Liver regeneration. *Journal of cellular physiology* **213**, 286-300 (2007).
101. TAKEISHI, T. *et al.* The role of Kupffer cells in liver regeneration. *Archives of histology and cytology* **62**, 413-422 (1999).
102. Bilzer, M., Roggel, F. & Gerbes, A.L. Role of Kupffer cells in host defense and liver disease. *Liver International* **26**, 1175-1186 (2006).
103. Senoo, H. Structure and function of hepatic stellate cells. *Medical Electron Microscopy* **37**, 3-15 (2004).

104. Saito, Y., Morine, Y. & Shimada, M. Mechanism of impairment on liver regeneration in elderly patients: the role of hepatic stellate cell function. *Hepatology Research* (2017).
105. Peng, H., Wisse, E. & Tian, Z. Liver natural killer cells: subsets and roles in liver immunity. *Cellular & molecular immunology* **13**, 328-336 (2016).
106. Tian, Z., Chen, Y. & Gao, B. Natural killer cells in liver disease. *Hepatology* **57**, 1654-1662 (2013).
107. Sekiya, S. & Suzuki, A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature* **475**, 390-393 (2011).
108. Fausto, N., Campbell, J. & Riehle, K. Liver regeneration Hepatology 43. *S45-S53* (2006).
109. Turner, R. *et al.* Human hepatic stem cell and maturational liver lineage biology. *Hepatology* **53**, 1035-1045 (2011).
110. DiPaola, F. *et al.* Identification of intramural epithelial networks linked to peribiliary glands that express progenitor cell markers and proliferate after injury in mice. *Hepatology* **58**, 1486-1496 (2013).
111. Kallis, Y.N. *et al.* Remodelling of extracellular matrix is a requirement for the hepatic progenitor cell response. *Gut* **60**, 525-533 (2011).
112. Wang, Y. *et al.* Paracrine signals from mesenchymal cell populations govern the expansion and differentiation of human hepatic stem cells to adult liver fates. *Hepatology* **52**, 1443-1454 (2010).
113. McClelland, R., Wauthier, E., Uronis, J. & Reid, L. Gradients in the liver's extracellular matrix chemistry from periportal to pericentral zones: influence on human hepatic progenitors. *Tissue Engineering Part A* **14**, 59-70 (2008).
114. Arriazu, E. *et al.* Extracellular matrix and liver disease. *Antioxidants & redox signaling* **21**, 1078-1097 (2014).
115. Baiocchini, A. *et al.* Extracellular matrix molecular remodeling in human liver fibrosis evolution. *PloS one* **11**, e0151736 (2016).
116. Roskams, T.A. *et al.* Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* **39**, 1739-1745 (2004).
117. Cardinale, V. *et al.* Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets. *Hepatology* **54**, 2159-2172 (2011).
118. Carpino, G. *et al.* Biliary tree stem/progenitor cells in glands of extrahepatic and intraheptic bile ducts: an anatomical in situ study yielding evidence of maturational lineages. *Journal of anatomy* **220**, 186-199 (2012).
119. Wang, Y. *et al.* Biliary tree stem cells, precursors to pancreatic committed progenitors: Evidence for possible life-long pancreatic organogenesis. *Stem cells* **31**, 1966-1979 (2013).
120. Maraldi, T. *et al.* Human biliary tree stem/progenitor cells immunomodulation: role of hepatocyte growth factor. *Hepatology Research* **47**, 465-479 (2017).
121. Touboul, T. *et al.* Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* **51**, 1754-1765 (2010).
122. Cai, J. *et al.* Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* **45**, 1229-1239 (2007).
123. Asahina, K., Zhou, B., Pu, W.T. & Tsukamoto, H. Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology* **53**, 983-995 (2011).
124. Schwartz, R.E. *et al.* Defined conditions for development of functional hepatic cells from human embryonic stem cells. *Stem cells and development* **14**, 643-655 (2005).
125. Khetani, S.R. *et al.* Microengineered liver tissues for drug testing. *Journal of laboratory automation* **20**, 216-250 (2015).
126. Conigliaro, A. *et al.* Evidence for a common progenitor of epithelial and mesenchymal components of the liver. *Cell Death & Differentiation* **20**, 1116-1123 (2013).
127. Li, Y., Wang, J. & Asahina, K. Mesothelial cells give rise to hepatic stellate cells and myofibroblasts via mesothelial–mesenchymal transition in liver injury. *Proceedings of the National Academy of Sciences* **110**, 2324-2329 (2013).
128. Burke, Z. & Oliver, G. Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. *Mechanisms of development* **118**, 147-155 (2002).
129. Margagliotti, S. *et al.* Role of metalloproteinases at the onset of liver development. *Development, growth & differentiation* **50**, 331-338 (2008).

130. Medico, E. *et al.* Osteopontin is an autocrine mediator of hepatocyte growth factor-induced invasive growth. *Cancer research* **61**, 5861-5868 (2001).
131. Papoutsi, M. *et al.* Gene regulation by homeobox transcription factor Prox1 in murine hepatoblasts. *Cell and tissue research* **330**, 209-220 (2007).
132. Lin, X. *et al.* Prenylcysteine carboxylmethyltransferase is essential for the earliest stages of liver development in mice. *Gastroenterology* **123**, 345-351 (2002).
133. Matsumoto, K., Yoshitomi, H., Rossant, J. & Zaret, K.S. Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**, 559-563 (2001).
134. Ding, B.-S. *et al.* Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature* **468**, 310 (2010).
135. Nahmias, Y., Schwartz, R.E., Hu, W.-S., Verfaillie, C.M. & Odde, D.J. Endothelium-mediated hepatocyte recruitment in the establishment of liver-like tissue in vitro. *Tissue engineering* **12**, 1627-1638 (2006).
136. Hayward, C.J. *et al.* Harvesting the potential of the human umbilical cord: isolation and characterisation of four cell types for tissue engineering applications. *Cells Tissues Organs* **197**, 37-54 (2013).
137. Zaret, K.S. Regulatory phases of early liver development: paradigms of organogenesis. *Nature reviews. Genetics* **3**, 499 (2002).
138. Zong, Y. *et al.* Notch signaling controls liver development by regulating biliary differentiation. *Development* **136**, 1727-1739 (2009).
139. Kamiya, A., Kinoshita, T. & Miyajima, A. Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. *FEBS letters* **492**, 90-94 (2001).
140. Suzuki, A., Iwama, A., Miyashita, H., Nakauchi, H. & Taniguchi, H. Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. *Development* **130**, 2513-2524 (2003).
141. Michalopoulos, G.K., Bowen, W.C., Mulé, K. & Luo, J. HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures. *Gene expression* **11**, 55-75 (2003).
142. Matsui, T., Kinoshita, T., Hirano, T., Yokota, T. & Miyajima, A. STAT3 down-regulates the expression of cyclin D during liver development. *Journal of Biological Chemistry* **277**, 36167-36173 (2002).
143. Snykers, S., De Kock, J., Rogiers, V. & Vanhaecke, T. In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem cells* **27**, 577-605 (2009).
144. Ito, Y., Matsui, T., Kamiya, A., Kinoshita, T. & Miyajima, A. Retroviral gene transfer of signaling molecules into murine fetal hepatocytes defines distinct roles for the STAT3 and ras pathways during hepatic development. *Hepatology* **32**, 1370-1376 (2000).
145. Imamura, M. *et al.* Oncostatin M induces upregulation of claudin-2 in rodent hepatocytes coinciding with changes in morphology and function of tight junctions. *Experimental cell research* **313**, 1951-1962 (2007).
146. Kamiya, A. *et al.* Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *The EMBO journal* **18**, 2127-2136 (1999).
147. Odom, D.T. *et al.* Control of pancreas and liver gene expression by HNF transcription factors. *Science* **303**, 1378-1381 (2004).
148. Cheng, W. *et al.* HNF factors form a network to regulate liver-enriched genes in zebrafish. *Developmental biology* **294**, 482-496 (2006).
149. Li, J., Ning, G. & Duncan, S.A. Mammalian hepatocyte differentiation requires the transcription factor HNF-4α. *Genes & Development* **14**, 464-474 (2000).
150. Parviz, F. *et al.* Hepatocyte nuclear factor 4α controls the development of a hepatic epithelium and liver morphogenesis. *Nature genetics* **34**, 292-296 (2003).
151. Konopka, G., Tekiela, J., Iverson, M., Wells, C. & Duncan, S.A. Junctional adhesion molecule-A is critical for the formation of pseudocanaliculi and modulates E-cadherin expression in hepatic cells. *Journal of Biological Chemistry* **282**, 28137-28148 (2007).
152. Battle, M.A. *et al.* Hepatocyte nuclear factor 4α orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver. *Proceedings of the National Academy of Sciences* **103**, 8419-8424 (2006).

153. Pontoglio, M. *et al.* Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell* **84**, 575-585 (1996).
154. Wang, N.-d., Finegold, M.J., Bradley, A. & Ou, C.N. Impaired energy homeostasis in C/EBPalpha knockout mice. *Science* **269**, 1108 (1995).
155. Yamasaki, H. *et al.* Suppression of C/EBPα expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression. *Development* **133**, 4233-4243 (2006).
156. Tomizawa, M., Garfield, S., Factor, V. & Xanthopoulos, K.G. Hepatocytes deficient in CCAAT/enhancer binding protein α (C/EBPα) exhibit both hepatocyte and biliary epithelial cell character. *Biochemical and biophysical research communications* **249**, 1-5 (1998).
157. Thiery, J.P. & Sleeman, J.P. Complex networks orchestrate epithelial-mesenchymal transitions. *Nature reviews. Molecular cell biology* **7**, 131-142 (2006).
158. Hay, E.D. An overview of epithelio-mesenchymal transformation. *Acta anatomica* **154**, 8-20 (1995).
159. Kalluri, R. & Neilson, E.G. Epithelial-mesenchymal transition and its implications for fibrosis. *The Journal of Clinical Investigation* **112**, 1776-1784 (2003).
160. Lee, K. & Nelson, C.M. New insights into the regulation of epithelial-mesenchymal transition and tissue fibrosis. *International review of cell and molecular biology* **294**, 171-221 (2012).
161. Lemaigre, F.P. Development of the biliary tract. *Mechanisms of development* **120**, 81-87 (2003).
162. Nitou, M., Sugiyama, Y., Ishikawa, K. & Shiojiri, N. Purification of fetal mouse hepatoblasts by magnetic beads coated with monoclonal anti-e-cadherin antibodies and their in vitro culture. *Experimental cell research* **279**, 330-343 (2002).
163. Nierhoff, D., Ogawa, A., Oertel, M., Chen, Y.Q. & Shafritz, D.A. Purification and characterization of mouse fetal liver epithelial cells with high in vivo repopulation capacity. *Hepatology* **42**, 130-139 (2005).
164. Watanabe, T. *et al.* SEK1/MKK4-Mediated SAPK/JNK Signaling Participates in Embryonic Hepatoblast Proliferation via a Pathway Different from NF-κB-Induced Anti-Apoptosis. *Developmental biology* **250**, 332-347 (2002).
165. Tanaka, M. *et al.* Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. *Mechanisms of development* **126**, 665-676 (2009).
166. Wauthier, E. *et al.* Hepatic Stem Cells and Hepatoblasts: Identification, Isolation, and Ex Vivo Maintenance. *Methods in Cell Biology* **86**, 137-225 (2008).
167. Terrace, J.D. *et al.* Progenitor cell characterization and location in the developing human liver. *Stem cells and development* **16**, 771-778 (2007).
168. McLin, V.A., Rankin, S.A. & Zorn, A.M. Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. *Development* **134**, 2207-2217 (2007).
169. Ober, E.A., Verkade, H., Field, H.A. & Stainier, D.Y. Mesodermal Wnt2b signalling positively regulates liver specification. *Nature* **442**, 688-691 (2006).
170. Bort, R., Signore, M., Tremblay, K., Martinez Barbera, J.P. & Zaret, K.S. Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Developmental biology* **290**, 44-56 (2006).
171. Lemaigre, F.P. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. *Gastroenterology* **137**, 62-79 (2009).
172. Margagliotti, S. *et al.* Role of metalloproteinases at the onset of liver development. *Development, growth & differentiation* **50**, 331-338 (2008).
173. Su, J. *et al.* The existence of multipotent stem cells with epithelial-mesenchymal transition features in the human liver bud. *The international journal of biochemistry & cell biology* **42**, 2047-2055 (2010).
174. Goldman, O., Valdes, V.J., Ezhkova, E. & Gouon-Evans, V. The mesenchymal transcription factor SNAI-1 instructs human liver specification. *Stem cell research* **17**, 62-68 (2016).
175. Tanimizu, N. & Miyajima, A. Molecular mechanism of liver development and regeneration. *International review of cytology* **259**, 1-48 (2007).
176. Weinstein, M. *et al.* Smad Proteins and Hepatocyte Growth Factor Control Parallel Regulatory Pathways That Converge on β1-Integrin To Promote Normal Liver Development. *Molecular and Cellular Biology* **21**, 5122-5131 (2001).

177. Zhao, R. & Duncan, S.A. Embryonic development of the liver. *Hepatology* **41**, 956-967 (2005).
178. Imamura, M. *et al.* Oncostatin M induces upregulation of claudin-2 in rodent hepatocytes coinciding with changes in morphology and function of tight junctions. *Experimental cell research* **313**, 1951-1962 (2007).
179. Matsui, T. *et al.* K-Ras mediates cytokine-induced formation of E-cadherin-based adherens junctions during liver development. *The EMBO journal* **21**, 1021-1030 (2002).
180. McCright, B., Lozier, J. & Gridley, T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development* **129**, 1075-1082 (2002).
181. Tanimizu, N. & Miyajima, A. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. *Journal of cell science* **117**, 3165-3174 (2004).
182. Suzuki, A., Iwama, A., Miyashita, H., Nakauchi, H. & Taniguchi, H. Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. *Development* **130**, 2513-2524 (2003).
183. Si-Tayeb, K., Lemaigre, F.P. & Duncan, S.A. Organogenesis and development of the liver. *Developmental cell* **18**, 175-189 (2010).
184. Asahina, K., Zhou, B., Pu, W.T. & Tsukamoto, H. Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology* **53**, 983-995 (2011).
185. Yin, C., Evason, K.J., Asahina, K. & Stainier, D.Y. Hepatic stellate cells in liver development, regeneration, and cancer. *J Clin Invest* **123**, 1902-1910 (2013).
186. Guyton, A. Textbook of Medical Physiology, 8th Edition'(WB Saunders Company: Philadelphia, USA). (1991).
187. Zhao, L. *et al.* Berberine improves glucogenesis and lipid metabolism in nonalcoholic fatty liver disease. *BMC endocrine disorders* **17**, 13 (2017).
188. Klover, P.J. & Mooney, R.A. Hepatocytes: critical for glucose homeostasis. *The international journal of biochemistry & cell biology* **36**, 753-758 (2004).
189. Godoy, P. *et al.* Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of toxicology* **87**, 1315-1530 (2013).
190. Yoshida, T. *et al.* Pigment Epithelium-Derived Factor (PEDF) Prevents Hepatic Fat Storage, Inflammation, and Fibrosis in Dietary Steatohepatitis of Mice. *Digestive Diseases and Sciences*, 1-10 (2017).
191. Charlton, M.R. Protein metabolism and liver disease. *Bailliere's clinical endocrinology and metabolism* **10**, 617-635 (1996).
192. Mizejewski, G. Alpha-fetoprotein (AFP) and inflammation: is AFP an acute and/or chronic phase reactant? *Journal of Hematology & Thromboembolic Diseases* (2015).
193. Nikolaou, N., Green, C.J., Gunn, P.J., Hodson, L. & Tomlinson, J.W. Optimizing human hepatocyte models for metabolic phenotype and function: effects of treatment with dimethyl sulfoxide (DMSO). *Physiological reports* **4**, e12944 (2016).
194. Bouwens, L., De Bleser, P., Vanderkerken, K., Geerts, B. & Wisse, E. Liver cell heterogeneity: functions of non-parenchymal cells. *Enzyme* **46**, 155-168 (1992).
195. Saito, H. Metabolism of iron stores. *Nagoya journal of medical science* **76**, 235 (2014).
196. Villar-Palasi, C. & Guinovart, J. The role of glucose 6-phosphate in the control of glycogen synthase. *The FASEB journal* **11**, 544-558 (1997).
197. Iyanagi, T. Molecular mechanism of phase I and phase Ii drug-metabolizing enzymes: implications for detoxification. *International review of cytology* **260**, 35-112 (2007).
198. Lu, C., Berg, C., Prakash, S.R., Lee, F.W. & Balani, S.K. Prediction of pharmacokinetic drug-drug interactions using human hepatocyte suspension in plasma and cytochrome P450 phenotypic data. III. In vitro-in vivo correlation with fluconazole. *Drug metabolism and disposition* **36**, 1261-1266 (2008).
199. Peters, W., Kock, L., Nagengast, F. & Kremers, P. Biotransformation enzymes in human intestine: critical low levels in the colon? *Gut* **32**, 408-412 (1991).
200. Martignoni, M., Groothuis, G.M. & de Kanter, R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert opinion on drug metabolism & toxicology* **2**, 875-894 (2006).
201. Guengerich, F.P. Update information on human P450s. *Drug metabolism reviews* **34**, 7-15 (2002).

202. Preissner, S. *et al.* SuperCYP: a comprehensive database on Cytochrome P450 enzymes including a tool for analysis of CYP-drug interactions. *Nucleic acids research* **38**, D237-D243 (2009).
203. Jover, R., Moya, M. & Gómez-Lechón, M.J. Transcriptional regulation of cytochrome p450 genes by the nuclear receptor hepatocyte nuclear factor 4-alpha. *Current drug metabolism* **10**, 508-519 (2009).
204. Han, X.M. *et al.* Inducibility of CYP1A2 by omeprazole in vivo related to the genetic polymorphism of CYP1A2. *British journal of clinical pharmacology* **54**, 540-543 (2002).
205. Williams, R. Global challenges in liver disease. *Hepatology* **44**, 521-526 (2006).
206. Murray, C.J. & Lopez, A.D. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *The Lancet* **349**, 1498-1504 (1997).
207. Blachier, M., Leleu, H., Peck-Radosavljevic, M., Valla, D.-C. & Roudot-Thoraval, F. The burden of liver disease in Europe: a review of available epidemiological data. *Journal of hepatology* **58**, 593-608 (2013).
208. Bruzzone, P. *et al.* in Transplantation proceedings, Vol. 45 2613-2615 (Elsevier, 2013).
209. Sarin, S. & Maiwall, R. Global burden of liver disease: a true burden on health sciences and economies. *World Gastroenterol Organ* **17** (2012).
210. Niro, G.A., Fontana, R., Ippolito, A.M. & Andriulli, A. Epidemiology and diagnosis of hepatitis D virus. *Future Virology* **7**, 709-717 (2012).
211. Trivedi, P.J. *et al.* Stratification of hepatocellular carcinoma risk in primary biliary cirrhosis: a multicentre international study. *Gut* **65**, 321-329 (2016).
212. Kanwal, F. *et al.* Increasing prevalence of HCC and cirrhosis in patients with chronic hepatitis C virus infection. *Gastroenterology* **140**, 1182-1188. e1181 (2011).
213. Townsend, S. & Newsome, P.N. Non-alcoholic fatty liver disease in 2016. *British medical bulletin* **119**, 143-156 (2016).
214. Preiss, D. & Sattar, N. Non-alcoholic fatty liver disease: an overview of prevalence, diagnosis, pathogenesis and treatment considerations. *Clinical science* **115**, 141-150 (2008).
215. Zajicek, G., Oren, R. & Weinreb, M. The streaming liver. *Liver International* **5**, 293-300 (1985).
216. Fellous, T.G. *et al.* Locating the stem cell niche and tracing hepatocyte lineages in human liver. *Hepatology* **49**, 1655-1663 (2009).
217. Ponder, K.P. Analysis of liver development, regeneration, and carcinogenesis by genetic marking studies. *The FASEB journal* **10**, 673-682 (1996).
218. Bucher, N.L. & Swaffield, M.N. The rate of incorporation of labeled thymidine into the deoxyribonucleic acid of regenerating rat liver in relation to the amount of liver excised. *Cancer research* **24**, 1611-1625 (1964).
219. Stöcker, E. & Pfeifer, U. On the manner of proliferation of the liver parenchyma after partial hepatectomy. Autoradiography studies using 3H-thymidine. *Die Naturwissenschaften* **52**, 663-663 (1965).
220. Duncan, A.W., Dorrell, C. & Grompe, M. Stem cells and liver regeneration. *Gastroenterology* **137**, 466-481 (2009).
221. Michalopoulos, G.K. & DeFrances, M.C. Liver regeneration. *Science* **276**, 60-66 (1997).
222. Fausto, N., Campbell, J.S. & Riehle, K.J. Liver regeneration. *Journal of hepatology* **57**, 692-694 (2012).
223. Wu, Y. *et al.* Triple labeling with three thymidine analogs reveals a well-orchestrated regulation of hepatocyte proliferation during liver regeneration. *Hepatology Research* **41**, 1230-1239 (2011).
224. Greenbaum, L.E. & Wells, R.G. The role of stem cells in liver repair and fibrosis. *The international journal of biochemistry & cell biology* **43**, 222-229 (2011).
225. Lambotte, L. *et al.* Effect of sialoadenectomy and epidermal growth factor administration on liver regeneration after partial hepatectomy. *Hepatology* **25**, 607-612 (1997).
226. Cressman, D.E., Greenbaum, L.E., Haber, B.A. & Taub, R. Rapid activation of post-hepatectomy factor/nuclear factor kappa B in hepatocytes, a primary response in the regenerating liver. *Journal of Biological Chemistry* **269**, 30429-30435 (1994).
227. Soriano, H.E., Bilyeu, T.A., Juan, T.S.-C., Zhao, W. & Darlington, G.J. DNA binding by C/EBP proteins correlates with hepatocyte proliferation. *In Vitro Cellular & Developmental Biology-Animal* **31**, 703-709 (1995).
228. Stolz, D.B., Mars, W.M., Petersen, B.E., Kim, T.-H. & Michalopoulos, G.K. Growth factor signal transduction immediately after two-thirds partial hepatectomy in the rat. *Cancer Research* **59**, 3954-3960 (1999).

229. Diehl, A.M. Roles of CCAAT/enhancer-binding proteins in regulation of liver regenerative growth. *Journal of Biological Chemistry* **273**, 30843-30846 (1998).
230. Bhanumathy, C. *et al.* Itih-4, a serine protease inhibitor regulated in interleukin-6–dependent liver formation: role in liver development and regeneration. *Developmental dynamics* **223**, 59-69 (2002).
231. Mohn, K., Melby, A., Tewari, D., Laz, T. & Taub, R. The gene encoding rat insulinlike growth factor-binding protein 1 is rapidly and highly induced in regenerating liver. *Molecular and cellular biology* **11**, 1393-1401 (1991).
232. Akerman, P. *et al.* Antibodies to tumor necrosis factor-alpha inhibit liver regeneration after partial hepatectomy. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **263**, G579-G585 (1992).
233. Matsunami, H. *et al.* in Transplantation proceedings, Vol. 24 1971-19721992).
234. Albrecht, J.H. *et al.* Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver. *Oncogene* **16** (1998).
235. Cressman, D.E. *et al.* Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science*, 1379-1383 (1996).
236. Sakuda, S. *et al.* Activation of signal transducer and activator transcription 3 and expression of suppressor of cytokine signal 1 during liver regeneration in rats. *Journal of hepatology* **36**, 378-384 (2002).
237. Fausto, N., Campbell, J.S. & Riehle, K.J. Liver regeneration. *Hepatology* **43** (2006).
238. Westwick, J., Weitzel, C., Leffert, H. & Brenner, D. Activation of Jun kinase is an early event in hepatic regeneration. *Journal of Clinical Investigation* **95**, 803 (1995).
239. Yuan, H. *et al.* Hepatocyte-specific deletion of Cdc42 results in delayed liver regeneration after partial hepatectomy in mice. *Hepatology* **49**, 240-249 (2009).
240. Chen, X.-G. & Xu, C.-S. Proteomic analysis of the regenerating liver following 2/3 partial hepatectomy in rats. *Biological research* **47**, 59 (2014).
241. Wiemann, S.U. *et al.* Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *The FASEB journal* **16**, 935-942 (2002).
242. Wang, G.-L., Iakova, P., Wilde, M., Awad, S. & Timchenko, N.A. Liver tumors escape negative control of proliferation via PI3K/Akt-mediated block of C/EBPα growth inhibitory activity. *Genes & development* **18**, 912-925 (2004).
243. Aikata, H. *et al.* Telomere reduction in human liver tissues with age and chronic inflammation. *Experimental cell research* **256**, 578-582 (2000).
244. Wang, G.-L. *et al.* HDAC1 cooperates with C/EBPα in the inhibition of liver proliferation in old mice. *Journal of Biological Chemistry* **283**, 26169-26178 (2008).
245. Sedivy, J.M., Banumathy, G. & Adams, P.D. Aging by epigenetics—a consequence of chromatin damage? *Experimental cell research* **314**, 1909-1917 (2008).
246. Sánchez, P. *et al.* Age-Related Lipid Metabolic Signature in Human LMNA-Lipodystrophic Stem Cell-Derived Adipocytes. *The Journal of Clinical Endocrinology & Metabolism* **100**, E964-E973 (2015).
247. Bucher, N.L. Regeneration of mammalian liver. *International review of cytology* **15**, 245-300 (1963).
248. Lewan, L., Yngner, T. & Engelbrecht, C. The biochemistry of the regenerating liver. *International Journal of Biochemistry* **8**, 477-487 (1977).
249. Timchenko, N.A. Aging and liver regeneration. *Trends in Endocrinology & Metabolism* **20**, 171-176 (2009).
250. Etienne, P. *et al.* Transient expression of c-fos and constant expression of c-myc in freshly isolated and cultured normal adult rat hepatocytes. *Oncogene Research* **3**, 255-262 (1988).
251. Loyer, P. *et al.* Growth Factor Dependence of Progression through G and S Phases of Adult Rat Hepatocytes in Vitro EVIDENCE OF A MITOGEN RESTRICTION POINT IN MID-LATE G. *Journal of Biological Chemistry* **271**, 11484-11492 (1996).
252. Mayhew, C.N. *et al.* Liver-specific pRB loss results in ectopic cell cycle entry and aberrant ploidy. *Cancer research* **65**, 4568-4577 (2005).
253. Ilyin, G. *et al.* Growth control and cell cycle progression in cultured hepatocytes, in *The Hepatocyte Review* 263-280 (Springer, 2000).
254. Hayflick, L. The limited in vitro lifetime of human diploid cell strains. *Experimental cell research* **37**, 614-636 (1965).

255. Auer, K.L. *et al.* The Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes. *Molecular Biology of the Cell* **9**, 561-573 (1998).
256. Szabo, M., Veres, Z., Baranyai, Z., Jakab, F. & Jemnitz, K. Comparison of human hepatoma HepaRG cells with human and rat hepatocytes in uptake transport assays in order to predict a risk of drug induced hepatotoxicity. *PloS one* **8**, e59432 (2013).
257. Yamaguchi, Y. *et al.* Change in telomerase activity of rat organs during growth and aging. *Experimental cell research* **242**, 120-127 (1998).
258. Frémin, C. *et al.* Multiple division cycles and long-term survival of hepatocytes are distinctly regulated by extracellular signal-regulated kinases ERK1 and ERK2. *Hepatology* **49**, 930-939 (2009).
259. Pan, X.-P. & Li, L.-J. Advances in cell sources of hepatocytes for bioartificial liver. *Hepatobiliary & pancreatic diseases international* **11**, 594-605 (2012).
260. Hewitt, N.J. *et al.* Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug metabolism reviews* **39**, 159-234 (2007).
261. Crosby, H.A. *et al.* Immunolocalization of putative human liver progenitor cells in livers from patients with end-stage primary biliary cirrhosis and sclerosing cholangitis using the monoclonal antibody OV-6. *The American journal of pathology* **152**, 771 (1998).
262. Yang, S. *et al.* Oval cells compensate for damage and replicative senescence of mature hepatocytes in mice with fatty liver disease. *Hepatology* **39**, 403-411 (2004).
263. Alison, M.R. Liver stem cells. *Stem cell reviews* **1**, 253-260 (2005).
264. Farber, E. Similarities in the Sequence of Early Histological Changes Induced in the Liver of the Rat by Ethionine, 2-Acetylamino-fluorene, and 3'-Methyl-4dimethylaminoazobenzene. *Cancer research* **16**, 142 (1956).
265. Fausto, N. & Campbell, J.S. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mechanisms of development* **120**, 117-130 (2003).
266. Alison, M.R., Golding, M., Sarraf, C.E., Edwards, R.J. & Lalani, E.-N. Liver damage in the rat induces hepatocyte stem cells from biliary epithelial cells. *Gastroenterology* **110**, 1182-1190 (1996).
267. Yang, L. *et al.* In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. *Proceedings of the National Academy of Sciences* **99**, 8078-8083 (2002).
268. Theise, N.D. *et al.* The canals of Hering and hepatic stem cells in humans. *Hepatology* **30**, 1425-1433 (1999).
269. Paku, S., Schnur, J., Nagy, P. & Thorgeirsson, S.S. Origin and structural evolution of the early proliferating oval cells in rat liver. *The American journal of pathology* **158**, 1313-1323 (2001).
270. Petersen, B. *et al.* Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168-1170 (1999).
271. Petersen, B.E. *et al.* Mouse A6–positive hepatic oval cells also express several hematopoietic stem cell markers. *Hepatology* **37**, 632-640 (2003).
272. Dunsford, H., Karnasuta, C., Hunt, J. & Sell, S. Different lineages of chemically induced hepatocellular carcinoma in rats defined by monoclonal antibodies. *Cancer research* **49**, 4894-4900 (1989).
273. Dunsford, H.A. & Sell, S. Production of monoclonal antibodies to preneoplastic liver cell populations induced by chemical carcinogens in rats and to transplantable Morris hepatomas. *Cancer research* **49**, 4887-4893 (1989).
274. Preisegger, K.H. *et al.* Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine mouse model for chronic alcoholic liver disease. *Laboratory investigation; a journal of technical methods and pathology* **79**, 103-109 (1999).
275. Jung, Y. *et al.* Accumulation of hedgehog-responsive progenitors parallels alcoholic liver disease severity in mice and humans. *Gastroenterology* **134**, 1532-1543. e1533 (2008).
276. Sackett, S.D. *et al.* Foxl1 promotes liver repair following cholestatic injury in mice. *Laboratory investigation* **89**, 1387 (2009).
277. Yui, S. *et al.* Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5+ stem cell. *Nature medicine* **18**, 618-623 (2012).
278. Soldatow, V.Y., LeCluyse, E.L., Griffith, L.G. & Rusyn, I. In vitro models for liver toxicity testing. *Toxicology research* **2**, 23-39 (2013).

279. Hattoum, A., Rubin, E., Orr, A. & Michalopoulos, G.K. Expression of hepatocyte epidermal growth factor receptor, FAS and glypican 3 in EpCAM-positive regenerative clusters of hepatocytes, cholangiocytes, and progenitor cells in human liver failure. *Human pathology* **44**, 743-749 (2013).
280. Huch, M. & Dollé, L. The plastic cellular states of liver cells: Are EpCAM and Lgr5 fit for purpose? *Hepatology* **64**, 652-662 (2016).
281. Wang, H. *et al.* Post-isolation inducible nitric oxide synthase gene expression due to collagenase buffer perfusion and characterization of the gene regulation in primary cultured murine hepatocytes. *The Journal of Biochemistry* **124**, 892-899 (1998).
282. Hariparsad, N., Carr, B.A., Evers, R. & Chu, X. Comparison of immortalized Fa2N-4 cells and human hepatocytes as in vitro models for cytochrome P450 induction. *Drug Metabolism and Disposition* **36**, 1046-1055 (2008).
283. Guguen-Guillouzo, C. & Guillouzo, A. General review on in vitro hepatocyte models and their applications. *Hepatocytes: Methods and Protocols*, 1-40 (2010).
284. Božina, N., Bradamante, V. & Lovrić, M. Genetic polymorphism of metabolic enzymes P450 (CYP) as a susceptibility factor for drug response, toxicity, and cancer risk. *Archives of Industrial Hygiene and Toxicology* **60**, 217-242 (2009).
285. Bianco, P., Robey, P.G. & Simmons, P.J. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell stem cell* **2**, 313-319 (2008).
286. Erices, A., Conget, P. & Minguell, J.J. Mesenchymal progenitor cells in human umbilical cord blood. *British journal of haematology* **109**, 235-242 (2000).
287. Igura, K. *et al.* Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. *Cytotherapy* **6**, 543-553 (2004).
288. Cho, K.A. *et al.* Mesenchymal stem cells showed the highest potential for the regeneration of injured liver tissue compared with other subpopulations of the bone marrow. *Cell Biology International* **33**, 772-777 (2009).
289. Shi, M., Liu, Z.W. & Wang, F.S. Immunomodulatory properties and therapeutic application of mesenchymal stem cells. *Clinical & Experimental Immunology* **164**, 1-8 (2011).
290. Li, M. & Ikehara, S. Bone-marrow-derived mesenchymal stem cells for organ repair. *Stem cells international* **2013** (2013).
291. Bhartiya, D., Nagvenkar, P., Sriraman, K. & Shaikh, A. An overview of pluripotent stem cells, in *Pluripotent Stem Cells* (InTech, 2013).
292. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *science* **282**, 1145-1147 (1998).
293. Dahéron, L. *et al.* LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* **22**, 770-778 (2004).
294. Zhao, H. & Jin, Y. Signaling networks in the control of pluripotency. *Current Opinion in Genetics & Development* **46**, 141-148 (2017).
295. Kristensen, D.M., Kalisz, M. & Nielsen, J.H. Cytokine signalling in embryonic stem cells. *Apmis* **113**, 756-772 (2005).
296. del Valle, I. *et al.* E-cadherin is required for the proper activation of the Lifr/Gp130 signaling pathway in mouse embryonic stem cells. *Development* **140**, 1684-1692 (2013).
297. Kim, H. *et al.* Modulation of β-catenin function maintains mouse epiblast stem cell and human embryonic stem cell self-renewal. *Nature communications* **4**, 2403 (2013).
298. Burridge, P.W. *et al.* Chemically defined generation of human cardiomyocytes. *Nature methods* **11**, 855-860 (2014).
299. Vosough, M. *et al.* Generation of functional hepatocyte-like cells from human pluripotent stem cells in a scalable suspension culture. *Stem cells and development* **22**, 2693-2705 (2013).
300. Paşca, A.M. *et al.* Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nature methods* **12**, 671-678 (2015).
301. Zaret, K.S. & Grompe, M. Generation and regeneration of cells of the liver and pancreas. *Science* **322**, 1490-1494 (2008).
302. McLaren, A. Ethical and social considerations of stem cell research. *Nature* **414**, 129 (2001).
303. Swijnenburg, R.-J. *et al.* Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. *Proceedings of the National Academy of Sciences* **105**, 12991-12996 (2008).
304. Takahashi, K., Okita, K., Nakagawa, M. & Yamanaka, S. Induction of pluripotent stem cells from fibroblast cultures. *Nature protocols* **2**, 3081-3089 (2007).

305. Zhao, X.-y. *et al.* iPS cells produce viable mice through tetraploid complementation. *Nature* **461**, 86 (2009).
306. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *science* **318**, 1917-1920 (2007).
307. Siller, R., Greenhough, S., Park, I.-H. & J Sullivan, G. Modelling human disease with pluripotent stem cells. *Current gene therapy* **13**, 99-110 (2013).
308. Nicolas, C., Wang, Y., Luebke-Wheeler, J. & Nyberg, S.L. Stem cell therapies for treatment of liver disease. *Biomedicines* **4**, 2 (2016).
309. Toivonen, S. *et al.* Comparative analysis of targeted differentiation of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells reveals variability associated with incomplete transgene silencing in retrovirally derived hiPSC lines. *Stem cells translational medicine* **2**, 83-93 (2013).
310. Lund, R.J., Närvä, E. & Lahesmaa, R. Genetic and epigenetic stability of human pluripotent stem cells. *Nature Reviews Genetics* **13**, 732-744 (2012).
311. Hinson, J.A., Roberts, D.W. & James, L.P. Mechanisms of acetaminophen-induced liver necrosis, in *Adverse Drug Reactions* 369-405 (Springer, 2010).
312. Liu, H., Ye, Z., Kim, Y., Sharkis, S. & Jang, Y.Y. Generation of endoderm-derived human induced pluripotent stem cells from primary hepatocytes. *Hepatology* **51**, 1810-1819 (2010).
313. Choi, S.M. *et al.* Liver engraftment potential of hepatic cells derived from patient-specific induced pluripotent stem cells. *Cell Cycle* **10**, 2423-2427 (2011).
314. Warren, L. *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell stem cell* **7**, 618-630 (2010).
315. Hou, P. *et al.* Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. *Science* **341**, 651-654 (2013).
316. Yu, Y. *et al.* Hepatocyte-like cells differentiated from human induced pluripotent stem cells: relevance to cellular therapies. *Stem cell research* **9**, 196-207 (2012).
317. Chen, Y.F. *et al.* Rapid generation of mature hepatocyte-like cells from human induced pluripotent stem cells by an efficient three-step protocol. *Hepatology* **55**, 1193-1203 (2012).
318. Asgari, S. *et al.* Differentiation and transplantation of human induced pluripotent stem cell-derived hepatocyte-like cells. *Stem Cell Reviews and Reports* **9**, 493-504 (2013).
319. Takebe, T. *et al.* Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **499**, 481-484 (2013).
320. Lei, Y., Jeong, D., Xiao, J. & Schaffer, D.V. Developing defined and scalable 3D culture systems for culturing human pluripotent stem cells at high densities. *Cellular and molecular bioengineering* **7**, 172-183 (2014).
321. Gieseck III, R.L. *et al.* Maturation of induced pluripotent stem cell derived hepatocytes by 3D-culture. *PLoS One* **9**, e86372 (2014).
322. Huang, P. *et al.* Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. *Cell stem cell* **14**, 370-384 (2014).
323. Du, Y. *et al.* Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. *Cell stem cell* **14**, 394-403 (2014).
324. Willenbring, H., Benet, L., Ding, S. & Mattis, A. Mouse liver repopulation with hepatocytes generated from human fibroblasts. (2014).
325. Liu, Z. *et al.* The tumourigenicity of iPS cells and their differentiated derivates. *Journal of cellular and molecular medicine* **17**, 782-791 (2013).
326. Dhodapkar, K.M. *et al.* Natural immunity to pluripotency antigen OCT4 in humans. *Proceedings of the National Academy of Sciences* **107**, 8718-8723 (2010).
327. Tan, Y., Ooi, S. & Wang, L. Immunogenicity and tumorigenicity of pluripotent stem cells and their derivatives: genetic and epigenetic perspectives. *Current stem cell research & therapy* **9**, 63-72 (2014).
328. Wilson, A.A. *et al.* Emergence of a stage-dependent human liver disease signature with directed differentiation of alpha-1 antitrypsin-deficient iPS cells. *Stem cell reports* **4**, 873-885 (2015).

329. Sampaziotis, F., Segeritz, C.P. & Vallier, L. Potential of human induced pluripotent stem cells in studies of liver disease. *Hepatology* **62**, 303-311 (2015).
330. Conlon, F.L. *et al.* A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120**, 1919-1928 (1994).
331. Vallier, L., Alexander, M. & Pedersen, R.A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *Journal of cell science* **118**, 4495-4509 (2005).
332. Göke, J., Chan, Y.-S., Yan, J., Vingron, M. & Ng, H.-H. Genome-wide kinase-chromatin interactions reveal the regulatory network of ERK signaling in human embryonic stem cells. *Molecular cell* **50**, 844-855 (2013).
333. Kubo, A. *et al.* Development of definitive endoderm from embryonic stem cells in culture. *Development* **131**, 1651-1662 (2004).
334. Brown, S. *et al.* Activin/Nodal signaling controls divergent transcriptional networks in human embryonic stem cells and in endoderm progenitors. *Stem cells* **29**, 1176-1185 (2011).
335. Pauklin, S. & Vallier, L. Activin/Nodal signalling in stem cells. *Development* **142**, 607-619 (2015).
336. Engert, S. *et al.* Wnt/β-catenin signalling regulates Sox17 expression and is essential for organizer and endoderm formation in the mouse. *Development* **140**, 3128-3138 (2013).
337. Tahamtani, Y. *et al.* Stauprimide priming of human embryonic stem cells toward definitive endoderm. *Cell Journal (Yakhteh)* **16**, 63 (2014).
338. Kaestner, K.H. The making of the liver: competence in the foregut endoderm and induction of liver-specific genes. *Cell Cycle* **4**, 1146-1148 (2005).
339. Ogawa, M. *et al.* Directed differentiation of cholangiocytes from human pluripotent stem cells. *Nature biotechnology* **33**, 853-861 (2015).
340. Cerec, V. *et al.* Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. *Hepatology* **45**, 957-967 (2007).
341. Guguen-Guillouzo, C., Corlu, A. & Guillouzo, A. Stem cell-derived hepatocytes and their use in toxicology. *Toxicology* **270**, 3-9 (2010).
342. Bar-Nur, O., Russ, H.A., Efrat, S. & Benvenisty, N. Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. *Cell stem cell* **9**, 17-23 (2011).
343. Hay, D.C. *et al.* Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities. *Cloning and stem cells* **9**, 51-62 (2007).
344. Hay, D.C. *et al.* Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. *Stem cells* **26**, 894-902 (2008).
345. Hay, D.C. *et al.* Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. *Proceedings of the National Academy of Sciences* **105**, 12301-12306 (2008).
346. Han, S. *et al.* Generation of functional hepatic cells from pluripotent stem cells. *Journal of stem cell research & therapy*, 1 (2012).
347. Sullivan, G.J. *et al.* Generation of functional human hepatic endoderm from human induced pluripotent stem cells. *Hepatology* **51**, 329-335 (2010).
348. Brolén, G. *et al.* Hepatocyte-like cells derived from human embryonic stem cells specifically via definitive endoderm and a progenitor stage. *Journal of biotechnology* **145**, 284-294 (2010).
349. Kubota, H. & Reid, L.M. Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. *Proceedings of the National Academy of Sciences* **97**, 12132-12137 (2000).
350. Haridass, D. *et al.* Repopulation efficiencies of adult hepatocytes, fetal liver progenitor cells, and embryonic stem cell-derived hepatic cells in albumin-promoter-enhancer urokinase-type plasminogen activator mice. *The American journal of pathology* **175**, 1483-1492 (2009).
351. Mahieu-Caputo, D. *et al.* Repopulation of athymic mouse liver by cryopreserved early human fetal hepatoblasts. *Human gene therapy* **15**, 1219-1228 (2004).

352. Schmelzer, E. *et al.* Human hepatic stem cells from fetal and postnatal donors. *Journal of Experimental Medicine* **204**, 1973-1987 (2007).
353. Tanimizu, N., Nishikawa, Y., Ichinohe, N., Akiyama, H. & Mitaka, T. Sry HMG box protein 9-positive (Sox9+) epithelial cell adhesion molecule-negative (EpCAM−) biphenotypic cells derived from hepatocytes are involved in mouse liver regeneration. *Journal of Biological Chemistry* **289**, 7589-7598 (2014).
354. Hayner, N.T., Braun, L., Yaswen, P., Brooks, M. & Fausto, N. Isozyme profiles of oval cells, parenchymal cells, and biliary cells isolated by centrifugal elutriation from normal and preneoplastic livers. *Cancer research* **44**, 332-338 (1984).
355. Michalopoulos, G.K. The liver is a peculiar organ when it comes to stem cells. *The American journal of pathology* **184**, 1263 (2014).
356. Michalopoulos, G.K., Barua, L. & Bowen, W.C. Transdifferentiation of rat hepatocytes into biliary cells after bile duct ligation and toxic biliary injury. *Hepatology* **41**, 535-544 (2005).
357. Sekiya, S. & Suzuki, A. Hepatocytes, rather than cholangiocytes, can be the major source of primitive ductules in the chronically injured mouse liver. *The American journal of pathology* **184**, 1468-1478 (2014).
358. Jeliazkova, P. *et al.* Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. *Hepatology* **57**, 2469-2479 (2013).
359. Yimlamai, D. *et al.* Hippo pathway activity influences liver cell fate. *Cell* **157**, 1324-1338 (2014).
360. Sekiya, S. & Suzuki, A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. *The Journal of clinical investigation* **122**, 3914 (2012).
361. Yanger, K. *et al.* Robust cellular reprogramming occurs spontaneously during liver regeneration. *Genes & development* **27**, 719-724 (2013).
362. Forbes, S.J., Gupta, S. & Dhawan, A. Cell therapy for liver disease: From liver transplantation to cell factory. *Journal of hepatology* **62**, S157-S169 (2015).
363. Vinson, C.R., Conover, S. & Adler, P.N. A Drosophila tissue polarity locus encodes a protein containing seven potential transmembrane domains. *Nature* **338**, 263-264 (1989).
364. Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J. & Skarnes, W.C. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535 (2000).
365. Peifer, M., Pai, L.-M. & Casey, M. Phosphorylation of the Drosophila adherens junction protein Armadillo: roles for wingless signal and zeste-white 3 kinase. *Developmental biology* **166**, 543-556 (1994).
366. Aberle, H., Bauer, A., Stappert, J., Kispert, A. & Kemler, R. β-catenin is a target for the ubiquitin–proteasome pathway. *The EMBO journal* **16**, 3797-3804 (1997).
367. Koo, B.-K. & Clevers, H. Stem cells marked by the R-spondin receptor LGR5. *Gastroenterology* **147**, 289-302 (2014).
368. Matthews, J., Sansom, O. & Clarke, A. Absolute requirement for STAT3 function in small-intestine crypt stem cell survival. *Cell death and differentiation* **18**, 1934 (2011).
369. Chen, Q. *et al.* Inactivation of STAT3 Signaling Impairs Hair Cell Differentiation in the Developing Mouse Cochlea. *Stem Cell Reports* **9**, 231-246 (2017).
370. Shih, S.-C., Ho, T.-C., Chen, S.-L. & Tsao, Y.-P. Pigment epithelium-derived factor (PEDF) peptide promotes the expansion of hepatic stem/progenitor cells via ERK and STAT3-dependent signaling. *American journal of translational research* **9**, 1114 (2017).
371. Pramanik, K.C. *et al.* Inhibition of β-catenin signaling suppresses pancreatic tumor growth by disrupting nuclear β-catenin/TCF-1 complex: critical role of STAT-3. *Oncotarget* **6**, 11561 (2015).
372. Ho, T.C. *et al.* PEDF promotes self-renewal of limbal stem cell and accelerates corneal epithelial wound healing. *Stem Cells* **31**, 1775-1784 (2013).
373. Van Andel, H. *et al.* Aberrantly expressed LGR4 empowers Wnt signaling in multiple myeloma by hijacking osteoblast-derived R-spondins. *Proceedings of the National Academy of Sciences*, 201618650 (2016).
374. Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772 (2011).
375. Huch, M. *et al.* Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *The EMBO journal* **32**, 2708-2721 (2013).

376. Jung, P. *et al.* Isolation and in vitro expansion of human colonic stem cells. *Nature medicine* **17**, 1225-1227 (2011).
377. Tojo, M. *et al.* The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-β. *Cancer science* **96**, 791-800 (2005).
378. Young, P.R. *et al.* Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *Journal of Biological Chemistry* **272**, 12116-12121 (1997).
379. Nikolaou, K.C. *et al.* Spontaneous development of hepatocellular carcinoma with cancer stem cell properties in PR-SET7-deficient livers. *The EMBO journal*, e201489279 (2014).
380. Benhamouche, S. *et al.* Apc tumor suppressor gene is the "zonation-keeper" of mouse liver. *Developmental cell* **10**, 759-770 (2006).
381. Hu, M. *et al.* Wnt/β-catenin signaling in murine hepatic transit amplifying progenitor cells. *Gastroenterology* **133**, 1579-1591. e1571 (2007).
382. Cao, W. *et al.* Dynamics of Proliferative and Quiescent Stem Cells in Liver Homeostasis and Injury. *Gastroenterology* **153**, 1133-1147 (2017).
383. Carmon, K.S., Gong, X., Lin, Q., Thomas, A. & Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. *Proceedings of the National Academy of Sciences* **108**, 11452-11457 (2011).
384. Huch, M., Boj, S.F. & Clevers, H. Lgr5+ liver stem cells, hepatic organoids and regenerative medicine. *Regenerative medicine* **8**, 385-387 (2013).
385. Bigorgne, A.E. *et al.* TTC7A mutations disrupt intestinal epithelial apicobasal polarity. *The Journal of clinical investigation* **124**, 328 (2014).
386. Dekkers, J.F. *et al.* A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nature medicine* **19**, 939-945 (2013).
387. Wiegerinck, C.L. *et al.* Loss of syntaxin 3 causes variant microvillus inclusion disease. *Gastroenterology* **147**, 65-68. e10 (2014).
388. Shin, S. *et al.* Foxl1-Cre-marked adult hepatic progenitors have clonogenic and bilineage differentiation potential. *Genes & development* **25**, 1185-1192 (2011).
389. Dorrell, C. *et al.* Prospective isolation of a bipotential clonogenic liver progenitor cell in adult mice. *Genes & development* **25**, 1193-1203 (2011).
390. Lué, A. *et al.* How important is donor age in liver transplantation? *World journal of gastroenterology* **22**, 4966 (2016).
391. Adam, R. *et al.* Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). *Journal of hepatology* **57**, 675-688 (2012).
392. Zhu, C. *et al.* Senescence-related genes possibly responsible for poor liver regeneration after hepatectomy in elderly patients. *Journal of gastroenterology and hepatology* **29**, 1102-1108 (2014).
393. Rodier, F. & Campisi, J. Four faces of cellular senescence. *The Journal of cell biology*, jcb. 201009094 (2011).
394. Marędziak, M., Marycz, K., Tomaszewski, K.A., Kornicka, K. & Henry, B.M. The influence of aging on the regenerative potential of human adipose derived mesenchymal stem cells. *Stem cells international* **2016** (2016).
395. Yu, K.-R. & Kang, K.-S. Aging-related genes in mesenchymal stem cells: a mini-review. *Gerontology* **59**, 557-563 (2013).
396. Sotiropoulou, P.A., Perez, S.A., Salagianni, M., Baxevanis, C.N. & Papamichail, M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem cells* **24**, 462-471 (2006).
397. Duggal, S. & Brinchmann, J.E. Importance of serum source for the in vitro replicative senescence of human bone marrow derived mesenchymal stem cells. *Journal of cellular physiology* **226**, 2908-2915 (2011).
398. Mareschi, K. *et al.* Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. *Journal of cellular biochemistry* **97**, 744-754 (2006).
399. Tokalov, S.V. *et al.* A number of bone marrow mesenchymal stem cells but neither phenotype nor differentiation capacities changes with age of rats. *Molecules & Cells (Springer Science & Business Media BV)* **24** (2007).
400. Hwang, S.H. *et al.* Age-related characteristics of multipotent human nasal inferior turbinate-derived mesenchymal stem cells. *PloS one* **8**, e74330 (2013).
401. Huang, S. *et al.* Dissimilar characteristics of umbilical cord mesenchymal stem cells from donors of different ages. *Cell and tissue banking* **14**, 707-713 (2013).

402. Yi, Q. *et al.* Analysis of Senescence-Related Differentiation Potentials and Gene Expression Profiles in Human Dental Pulp Stem Cells. *Cells Tissues Organs* **203**, 1-11 (2017).
403. Choudhery, M.S. *et al.* Bone marrow derived mesenchymal stem cells from aged mice have reduced wound healing, angiogenesis, proliferation and anti-apoptosis capabilities. *Cell biology international* **36**, 747-753 (2012).
404. Artandi, S.E. & Attardi, L.D. Pathways connecting telomeres and p53 in senescence, apoptosis, and cancer. *Biochemical and biophysical research communications* **331**, 881-890 (2005).
405. Zhang, Z. *et al.* Inositol pyrophosphates mediate the effects of aging on bone marrow mesenchymal stem cells by inhibiting Akt signaling. *Stem cell research & therapy* **5**, 33 (2014).
406. Greco, S.J., Liu, K. & Rameshwar, P. Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells. *Stem cells* **25**, 3143-3154 (2007).
407. Lansdorp, P.M. Telomeres, stem cells, and hematology. *Blood* **111**, 1759-1766 (2008).
408. Alraies, A., Alaidaroos, N.Y., Waddington, R.J., Moseley, R. & Sloan, A.J. Variation in human dental pulp stem cell ageing profiles reflect contrasting proliferative and regenerative capabilities. *BMC cell biology* **18**, 12 (2017).
409. Tokalov, S.V. *et al.* Age-related changes in the frequency of mesenchymal stem cells in the bone marrow of rats. *Stem cells and development* **16**, 439-446 (2007).
410. Hacia, J.G. *et al.* Age-related gene expression profiles of rhesus monkey bone marrow-derived mesenchymal stem cells. *Journal of cellular biochemistry* **103**, 1198-1210 (2008).
411. Bajek, A. *et al.* High Quality Independent From a Donor: Human Amniotic Fluid Derived Stem Cells—A Practical Analysis Based on 165 Clinical Cases. *Journal of cellular biochemistry* **118**, 116-126 (2017).
412. Zhang, J. *et al.* The effect of aging on the pluripotential capacity and regenerative potential of human periodontal ligament stem cells. *Biomaterials* **33**, 6974-6986 (2012).
413. Yue, R., Zhou, B.O., Shimada, I.S., Zhao, Z. & Morrison, S.J. Leptin receptor promotes adipogenesis and reduces osteogenesis by regulating mesenchymal stromal cells in adult bone marrow. *Cell Stem Cell* **18**, 782-796 (2016).
414. Campisi, J. & di Fagagna, F.d.A. Cellular senescence: when bad things happen to good cells. *Nature reviews. Molecular cell biology* **8**, 729 (2007).
415. Wright, W.E. & Shay, J.W. Historical claims and current interpretations of replicative aging. *Nature biotechnology* **20**, 682-688 (2002).
416. Blackburn, E.H. Switching and signaling at the telomere. *Cell* **106**, 661-673 (2001).
417. Harley, C.B., Futcher, A.B. & Greider, C.W. Telomeres shorten during ageing of human fibroblasts. *Nature* **345**, 458 (1990).
418. Serakinci, N., Graakjaer, J. & Kolvraa, S. Telomere stability and telomerase in mesenchymal stem cells. *Biochimie* **90**, 33-40 (2008).
419. Huang, G. *et al.* Proteomic analysis of human bone marrow mesenchymal stem cells transduced with human telomerase reverse transcriptase gene during proliferation. *Cell proliferation* **41**, 625-644 (2008).
420. Baxter, M.A. *et al.* Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. *Stem cells* **22**, 675-682 (2004).
421. Parsch, D., Fellenberg, J., Brümmendorf, T.H., Eschlbeck, A.-M. & Richter, W. Telomere length and telomerase activity during expansion and differentiation of human mesenchymal stem cells and chondrocytes. *Journal of molecular medicine* **82**, 49-55 (2004).
422. Bentzon, J.F. *et al.* Tissue distribution and engraftment of human mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene. *Biochemical and biophysical research communications* **330**, 633-640 (2005).
423. Aubert, G. & Lansdorp, P.M. Telomeres and aging. *Physiological reviews* **88**, 557-579 (2008).
424. Blasco, M.A. Telomere length, stem cells and aging. *Nature chemical biology* **3**, 640-649 (2007).
425. Vinagre, J., Pestana, A. & Sobrinho, M. Telomeres in Cancer. *Mechanisms Linking Aging, Diseases and Biological Age Estimation*, 161 (2017).
426. Sohal, R.S. & Weindruch, R. Oxidative stress, caloric restriction, and aging. *Science (New York, NY)* **273**, 59 (1996).
427. Estrada, J. *et al.* Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis. *Cell death and differentiation* **19**, 743 (2012).

428. Passos, J.F. *et al.* Mitochondrial dysfunction accounts for the stochastic heterogeneity in telomere-dependent senescence. *PLoS biology* **5**, e110 (2007).
429. Stolzing, A., Jones, E., McGonagle, D. & Scutt, A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mechanisms of ageing and development* **129**, 163-173 (2008).
430. Yang, S.-R., Park, J.-R. & Kang, K.-S. Reactive oxygen species in mesenchymal stem cell aging: implication to lung diseases. *Oxidative medicine and cellular longevity* **2015** (2015).
431. Cuadrado, A. & Nebreda, A.R. Mechanisms and functions of p38 MAPK signalling. *Biochemical Journal* **429**, 403-417 (2010).
432. Tormos, A., Taléns-Visconti, R., Nebreda, A. & Sastre, J. p38 MAPK: a dual role in hepatocyte proliferation through reactive oxygen species. *Free radical research* **47**, 905-916 (2013).
433. Velu, C.S., Niture, S.K., Doneanu, C.E., Pattabiraman, N. & Srivenugopal, K.S. Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. *Biochemistry* **46**, 7765-7780 (2007).
434. Prigione, A., Fauler, B., Lurz, R., Lehrach, H. & Adjaye, J. The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. *Stem cells* **28**, 721-733 (2010).
435. Han, J. *et al.* Nanog reverses the effects of organismal aging on mesenchymal stem cell proliferation and myogenic differentiation potential. *Stem Cells* **30**, 2746-2759 (2012).
436. Tsai, C.-C., Su, P.-F., Huang, Y.-F., Yew, T.-L. & Hung, S.-C. Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Molecular cell* **47**, 169-182 (2012).
437. Karantzali, E. *et al.* Histone deacetylase inhibition accelerates the early events of stem cell differentiation: transcriptomic and epigenetic analysis. *Genome biology* **9**, R65 (2008).
438. Rayess, H., Wang, M.B. & Srivatsan, E.S. Cellular senescence and tumor suppressor gene p16. *International journal of cancer* **130**, 1715-1725 (2012).
439. Chau, B.N. & Wang, J.Y. Coordinated regulation of life and death by RB. *Nature reviews. Cancer* **3**, 130 (2003).
440. Bargonetti, J. & Manfredi, J.J. Multiple roles of the tumor suppressor p53. *Current opinion in oncology* **14**, 86-91 (2002).
441. Beauséjour, C.M. *et al.* Reversal of human cellular senescence: roles of the p53 and p16 pathways. *The EMBO journal* **22**, 4212-4222 (2003).
442. Wang, D. & Jang, D.-J. Protein Kinase CK2 Regulates Cytoskeletal Reorganization during Ionizing Radiation–Induced Senescence of Human Mesenchymal Stem Cells. *Cancer research* **69**, 8200-8207 (2009).
443. So, A.-Y., Jung, J.-W., Lee, S., Kim, H.-S. & Kang, K.-S. DNA methyltransferase controls stem cell aging by regulating BMI1 and EZH2 through microRNAs. *PloS one* **6**, e19503 (2011).
444. Shibata, K.R. *et al.* Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. *Stem Cells* **25**, 2371-2382 (2007).
445. Rodier, F. *et al.* DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci* **124**, 68-81 (2011).
446. Muthna, D. *et al.* Irradiation of adult human dental pulp stem cells provokes activation of p53, cell cycle arrest, and senescence but not apoptosis. *Stem cells and development* **19**, 1855-1862 (2010).
447. He, L. *et al.* A microRNA component of the p53 tumour suppressor network. *Nature* **447**, 1130 (2007).
448. di Fagagna, F.d.A., Reaper, P.M., Clay6-Farrace, L. & Flegler, H. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426**, 194 (2003).
449. Herbig, U., Jobling, W.A., Chen, B.P., Chen, D.J. & Sedivy, J.M. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21 CIP1, but not p16 INK4a. *Molecular cell* **14**, 501-513 (2004).
450. Rodier, F. *et al.* Persistent DNA damage signaling triggers senescence-associated inflammatory cytokine secretion. *Nature cell biology* **11**, 973 (2009).
451. Wang, Y., Han, Z.-b., Song, Y.-p. & Han, Z.C. Safety of mesenchymal stem cells for clinical application. *Stem cells international* **2012** (2012).
452. Carlson, M.E. & Conboy, I.M. Loss of stem cell regenerative capacity within aged niches. *Aging cell* **6**, 371-382 (2007).
453. Sui, B., Hu, C., Zheng, C. & Jin, Y. Microenvironmental views on mesenchymal stem cell differentiation in aging. *Journal of dental research* **95**, 1333-1340 (2016).

454. Yang, N. *et al.* Tumor necrosis factor α suppresses the mesenchymal stem cell osteogenesis promoter miR-21 in estrogen deficiency–induced osteoporosis. *Journal of Bone and Mineral Research* **28**, 559-573 (2013).
455. Abdelmagid, S.M., Barbe, M.F. & Safadi, F.F. Role of inflammation in the aging bones. *Life sciences* **123**, 25-34 (2015).
456. Pawelec, G., Goldeck, D. & Derhovanessian, E. Inflammation, ageing and chronic disease. *Current opinion in immunology* **29**, 23-28 (2014).
457. Graves, D., Li, J. & Cochran, D. Inflammation and uncoupling as mechanisms of periodontal bone loss. *Journal of dental research* **90**, 143-153 (2011).
458. Gamaletsou, M.N. *et al.* Candida osteomyelitis: analysis of 207 pediatric and adult cases (1970–2011). *Clinical infectious diseases* **55**, 1338-1351 (2012).
459. Singh, L. *et al.* Aging alters bone-fat reciprocity by shifting in vivo mesenchymal precursor cell fate towards an adipogenic lineage. *Bone* **85**, 29-36 (2016).
460. Liu, W. *et al.* Canonical Wnt signaling differently modulates osteogenic differentiation of mesenchymal stem cells derived from bone marrow and from periodontal ligament under inflammatory conditions. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1840**, 1125-1134 (2014).
461. Wang, L. *et al.* IFN-γ and TNF-α synergistically induce mesenchymal stem cell impairment and tumorigenesis via NFκB signaling. *Stem cells* **31**, 1383-1395 (2013).
462. Zhao, L. *et al.* Tumor necrosis factor inhibits mesenchymal stem cell differentiation into osteoblasts via the ubiquitin E3 ligase Wwp1. *Stem Cells* **29**, 1601-1610 (2011).
463. Aksoy, C., Kaya, F.A., Kuşkonmaz, B.B., Uçkan, D. & Severcan, F. Structural investigation of donor age effect on human bone marrow mesenchymal stem cells: FTIR spectroscopy and imaging. *Age* **36**, 9691 (2014).
464. Ovadya, Y. & Krizhanovsky, V. Senescent cells: SASPected drivers of age-related pathologies. *Biogerontology* **15**, 627-642 (2014).
465. Velarde, M.C., Demaria, M. & Campisi, J. Senescent cells and their secretory phenotype as targets for cancer therapy, in *Cancer and Aging*, Vol. 38 17-27 (Karger Publishers, 2013).
466. Wagner, W. *et al.* Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PloS one* **3**, e2213 (2008).
467. Kode, J.A., Mukherjee, S., Joglekar, M.V. & Hardikar, A.A. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. *Cytotherapy* **11**, 377-391 (2009).
468. Carlos Sepúlveda, J. *et al.* Cell senescence abrogates the therapeutic potential of human mesenchymal stem cells in the lethal endotoxemia model. *Stem Cells* **32**, 1865-1877 (2014).
469. Pan, G.-z. *et al.* Bone marrow mesenchymal stem cells ameliorate hepatic ischemia/reperfusion injuries via inactivation of the MEK/ERK signaling pathway in rats. *journal of surgical research* **178**, 935-948 (2012).
470. Liang, X., Ding, Y., Zhang, Y., Tse, H.-F. & Lian, Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell transplantation* **23**, 1045-1059 (2014).
471. Schey, K.L., Luther, J.M. & Rose, K.L. Proteomics characterization of exosome cargo. *Methods* **87**, 75-82 (2015).
472. Patel, D.B. *et al.* Impact of Cell Culture Parameters on Production and Vascularization Bioactivity of Mesenchymal Stem Cell-Derived Extracellular Vesicles. *Bioengineering & Translational Medicine*.
473. Turinetto, V., Vitale, E. & Giachino, C. Senescence in human mesenchymal stem cells: functional changes and implications in stem cell-based therapy. *International journal of molecular sciences* **17**, 1164 (2016).
474. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.-Y. & Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proceedings of the National Academy of Sciences* **98**, 12072-12077 (2001).
475. Liu, D. & Hornsby, P.J. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer research* **67**, 3117-3126 (2007).
476. Li, Y. *et al.* Senescent mesenchymal stem cells promote colorectal cancer cells growth via galectin-3 expression. *Cell & bioscience* **5**, 21 (2015).
477. Zhu, W. *et al.* Mesenchymal stem cell-secreted soluble signaling molecules potentiate tumor growth. *Cell Cycle* **10**, 3198-3207 (2011).

478. Di, G.-h. *et al.* IL-6 secreted from senescent mesenchymal stem cells promotes proliferation and migration of breast cancer cells. *PLoS One* **9**, e113572 (2014).
479. Yu, K.-R. *et al.* A p38 MAPK-mediated alteration of COX-2/PGE2 regulates immunomodulatory properties in human mesenchymal stem cell aging. *PloS one* **9**, e102426 (2014).
480. Kim, H.S. *et al.* Human umbilical cord blood mesenchymal stem cells reduce colitis in mice by activating NOD2 signaling to COX2. *Gastroenterology* **145**, 1392-1403. e1398 (2013).
481. Puts, R., Albers, J., Kadow-Romacker, A., Geissler, S. & Raum, K. Influence of Donor Age and Stimulation Intensity on Osteogenic Differentiation of Rat Mesenchymal Stromal Cells in Response to Focused Low-Intensity Pulsed Ultrasound. *Ultrasound in medicine & biology* **42**, 2965-2974 (2016).
482. Gaur, T. *et al.* Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *Journal of Biological Chemistry* **280**, 33132-33140 (2005).
483. Gharibi, B., Farzadi, S., Ghuman, M. & Hughes, F.J. Inhibition of Akt/mTOR attenuates age-related changes in mesenchymal stem cells. *Stem cells* **32**, 2256-2266 (2014).
484. O'Driscoll, S.W., Saris, D.B., Ito, Y. & Fitzimmons, J.S. The chondrogenic potential of periosteum decreases with age. *Journal of Orthopaedic Research* **19**, 95-103 (2001).
485. De Bari, C., Dell'Accio, F., Tylzanowski, P. & Luyten, F.P. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis & Rheumatology* **44**, 1928-1942 (2001).
486. Brohlin, M., Kingham, P.J., Novikova, L.N., Novikov, L.N. & Wiberg, M. Aging effect on neurotrophic activity of human mesenchymal stem cells. *PLoS One* **7**, e45052 (2012).
487. Scruggs, B.A. *et al.* Age of the Donor Reduces the Ability of Human Adipose-Derived Stem Cells to Alleviate Symptoms in the Experimental Autoimmune Encephalomyelitis Mouse Model. *Stem cells translational medicine* **2**, 797-807 (2013).
488. Cei, S. *et al.* Bone marrow stromal cells of young and adult rats respond similarly to platelet-released supernatant and bone morphogenetic protein-6 in vitro. *Journal of periodontology* **77**, 699-706 (2006).
489. Sharp, A., Kukulansky, T. & Globerson, A. In vitro analysis of age-related changes in the developmental potential of bone marrow thymocyte progenitors. *European journal of immunology* **20**, 2541-2546 (1990).
490. Kitaori, T. *et al.* Stromal cell–derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis & Rheumatology* **60**, 813-823 (2009).
491. Geiger, H., Koehler, A. & Gunzer, M. Stem cells, aging, niche, adhesion and Cdc42: a model for changes in cell-cell interactions and hematopoietic stem cell aging. *Cell Cycle* **6**, 884-887 (2007).
492. Liang, Y., Van Zant, G. & Szilvassy, S.J. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. *Blood* **106**, 1479-1487 (2005).
493. Price, L.S., Leng, J., Schwartz, M.A. & Bokoch, G.M. Activation of Rac and Cdc42 by integrins mediates cell spreading. *Molecular biology of the cell* **9**, 1863-1871 (1998).
494. Adams, G.B. *et al.* Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* **439**, 599 (2006).
495. Le Clainche, C. & Carlier, M.-F. Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiological reviews* **88**, 489-513 (2008).
496. Cascio, S. & Zaret, K.S. Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. *Development* **113**, 217-225 (1991).
497. Asai, A. *et al.* Paracrine signals regulate human liver organoid maturation from induced pluripotent stem cells. *Development* **144**, 1056-1064 (2017).
498. Herrera, M.B. *et al.* Isolation and characterization of a stem cell population from adult human liver. *Stem cells* **24**, 2840-2850 (2006).
499. Ewida, S.F., Abdou, A.G., El-Rasol, E.A. & El-Ghane, M.S. Hepatocyte-like Versus Mesenchymal Stem Cells in CCl4-induced Liver Fibrosis. *Applied immunohistochemistry & molecular morphology: AIMM* (2016).
500. Shabbir, A., Cox, A., Rodriguez-Menocal, L., Salgado, M. & Badiavas, E.V. Mesenchymal stem cell exosomes induce proliferation and migration of normal and chronic wound fibroblasts, and enhance angiogenesis in vitro. *Stem cells and development* **24**, 1635-1647 (2015).

501. Du, Y. *et al.* Exosomes from Human-Induced Pluripotent Stem Cell–Derived Mesenchymal Stromal Cells (hiPSC-MSCs) Protect Liver against Hepatic Ischemia/Reperfusion Injury via Activating Sphingosine Kinase and Sphingosine-1-Phosphate Signaling Pathway. *Cellular Physiology and Biochemistry* **43**, 611-625 (2017).
502. Li, T. *et al.* Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem cells and development* **22**, 845-854 (2012).
503. Hyun, J., Wang, S., Kim, J., Kim, G.J. & Jung, Y. MicroRNA125b-mediated Hedgehog signaling influences liver regeneration by chorionic plate-derived mesenchymal stem cells. *Scientific reports* **5**, 14135 (2015).
504. Tan, C.Y. *et al.* Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem cell research & therapy* **5**, 76 (2014).
505. Ko, S.-F. *et al.* Adipose-derived mesenchymal stem cell exosomes suppress hepatocellular carcinoma growth in a rat model: apparent diffusion coefficient, natural killer T-cell responses, and histopathological features. *Stem cells international* **2015** (2015).
506. Lou, G. *et al.* Exosomes derived from miR-122-modified adipose tissue-derived MSCs increase chemosensitivity of hepatocellular carcinoma. *Journal of hematology & oncology* **8**, 122 (2015).
507. Hu, C. & Li, L. In vitro and in vivo hepatic differentiation of adult somatic stem cells and extraembryonic stem cells for treating end stage liver diseases. *Stem cells international* **2015** (2015).
508. Ochiya, T., Yamamoto, Y. & Banas, A. Commitment of stem cells into functional hepatocytes. *Differentiation* **79**, 65-73 (2010).
509. Sekiya, S. & Suzuki, A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *nature* **475**, 390 (2011).
510. Aurich, I. *et al.* Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers. *Gut* **56**, 405-415 (2007).
511. Chamberlain, J. *et al.* Efficient generation of human hepatocytes by the intrahepatic delivery of clonal human mesenchymal stem cells in fetal sheep. *Hepatology* **46**, 1935-1945 (2007).
512. Saulnier, N. *et al.* Mesenchymal stromal cells multipotency and plasticity: induction toward the hepatic lineage. *Eur Rev Med Pharmacol Sci* **13**, 71-78 (2009).
513. Nunes, S. *et al.* Generation of a functional liver tissue mimic using adipose stromal vascular fraction cell-derived vasculatures. *Scientific reports* **3** (2013).
514. Zuk, P.A. *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue engineering* **7**, 211-228 (2001).
515. Kern, S., Eichler, H., Stoeve, J., Klüter, H. & Bieback, K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem cells* **24**, 1294-1301 (2006).
516. Seo, M.J., Suh, S.Y., Bae, Y.C. & Jung, J.S. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochemical and biophysical research communications* **328**, 258-264 (2005).
517. Papanikolaou, I.G. *et al.* Mesenchymal Stem Cells Transplantation following Partial Hepatectomy: A New Concept to Promote Liver Regeneration—Systematic Review of the Literature Focused on Experimental Studies in Rodent Models. *Stem cells international* **2017** (2017).
518. Wang, Y. *et al.* Human adipose-derived mesenchymal stem cells are resistant to HBV infection during differentiation into hepatocytes in vitro. *International journal of molecular sciences* **15**, 6096-6110 (2014).
519. Choi, E.W. *et al.* Exosomes from human adipose derived stem cells promote proliferation and migration of skin fibroblasts. *Experimental Dermatology* (2017).
520. Lee, S.C., Kim, J.O. & Kim, S.-J. Secretome from human adipose-derived stem cells protects mouse liver from hepatic ischemia–reperfusion injury. *Surgery* **157**, 934-943 (2015).
521. Lin, K.-C. *et al.* Combination of adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes for protecting kidney from acute ischemia–reperfusion injury. *International journal of cardiology* **216**, 173-185 (2016).
522. Fu, Y. *et al.* Rapid generation of functional hepatocyte-like cells from human adipose-derived stem cells. *Stem Cell Research & Therapy* **7**, 105 (2016).

523. Yin, L. *et al.* Adipose tissue-derived mesenchymal stem cells differentiated into hepatocyte-like cells in vivo and in vitro. *Molecular medicine reports* **11**, 1722-1732 (2015).
524. Manzini, B.M. *et al.* Useful properties of undifferentiated mesenchymal stromal cells and adipose tissue as the source in liver-regenerative therapy studied in an animal model of severe acute fulminant hepatitis. *Cytotherapy* **17**, 1052-1065 (2015).
525. Banas, A. *et al.* Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* **46**, 219-228 (2007).
526. Banas, A. Purification of adipose tissue mesenchymal stem cells and differentiation toward hepatic-like cells. *Liver Stem Cells: Methods and Protocols*, 61-72 (2012).
527. Yamamoto, Y. *et al.* A comparative analysis of the transcriptome and signal pathways in hepatic differentiation of human adipose mesenchymal stem cells. *The FEBS journal* **275**, 1260-1273 (2008).
528. Hong, I.-H. *et al.* Inhibition of kupffer cell activity improves transplantation of human adipose-derived stem cells and liver functions. *Cell transplantation* **22**, 447-459 (2013).
529. Aurich, H. *et al.* Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue in vitro promotes hepatic integration in vivo. *Gut* **58**, 570-581 (2009).
530. Puglisi, M.A. *et al.* Adipose tissue-derived mesenchymal stem cells and hepatic differentiation: old concepts and future perspectives. *Eur Rev Med Pharmacol Sci* **15**, 355-364 (2011).
531. Ke, Z. *et al.* Down-regulation of Wnt signaling could promote bone marrow-derived mesenchymal stem cells to differentiate into hepatocytes. *Biochemical and biophysical research communications* **367**, 342-348 (2008).
532. Huang, J., Guo, X., Li, W. & Zhang, H. Activation of Wnt/β-catenin signalling via GSK3 inhibitors direct differentiation of human adipose stem cells into functional hepatocytes. *Scientific reports* **7** (2017).
533. Yoshida, Y. *et al.* A role of Wnt/β-catenin signals in hepatic fate specification of human umbilical cord blood-derived mesenchymal stem cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **293**, G1089-G1098 (2007).
534. De Barros, S. *et al.* Aging-related decrease of human ASC angiogenic potential is reversed by hypoxia preconditioning through ROS production. *Molecular Therapy* **21**, 399-408 (2013).
535. Vilaboa, S.D.-A., Navarro-Palou, M. & Llull, R. Age influence on stromal vascular fraction cell yield obtained from human lipoaspirates. *Cytotherapy* **16**, 1092-1097 (2014).
536. Zhu, M. *et al.* The effect of age on osteogenic, adipogenic and proliferative potential of female adipose-derived stem cells. *Journal of tissue engineering and regenerative medicine* **3**, 290-301 (2009).
537. Esmaeli, S., Allameh, A., Adelipour, M., Soleimani, M. & Allameh, M. The impact of oxidative DNA changes and ATM expression on morphological and functional activities on hepatocytes obtained from mesenchymal stem cells. *Biologicals* **47**, 52-58 (2017).
538. Seeliger, C. *et al.* Decrease of global methylation improves significantly hepatic differentiation of Ad-MSCs: possible future application for urea detoxification. *Cell transplantation* **22**, 119-131 (2013).
539. Mohamadnejad, M., Bagheri, M. & Malekzadeh, R. In vitro differentiation of human bone marrow mesenchymal stem cells into hepatocyte-like cells. *Archives of Iranian medicine* **14**, 244 (2011).
540. Yamamoto, Y. *et al.* A comparative analysis of the transcriptome and signal pathways in hepatic differentiation of human adipose mesenchymal stem cells. *The FEBS journal* **275**, 1260-1273 (2008).
541. Paganelli, M. *et al.* Downregulation of Sox9 expression associates with hepatogenic differentiation of human liver mesenchymal stem/progenitor cells. *Stem cells and development* **23**, 1377-1391 (2014).
542. Wu, X.-B. & Tao, R. Hepatocyte differentiation of mesenchymal stem cells. *Hepatobiliary & Pancreatic Diseases International* **11**, 360-371 (2012).
543. Watt, A.J., Garrison, W.D. & Duncan, S.A. HNF4: a central regulator of hepatocyte differentiation and function. *Hepatology* **37**, 1249-1253 (2003).
544. Hang, H. *et al.* Induction of highly functional hepatocytes from human umbilical cord mesenchymal stem cells by HNF4α transduction. *PloS one* **9**, e104133 (2014).

545. Chen, Y., Dong, X.J., Zhang, G.R., Shao, J.Z. & Xiang, L.X. In vitro differentiation of mouse bone marrow stromal stem cells into hepatocytes induced by conditioned culture medium of hepatocytes. *Journal of cellular biochemistry* **102**, 52-63 (2007).
546. Mohsin, S. *et al.* Enhanced hepatic differentiation of mesenchymal stem cells after pretreatment with injured liver tissue. *Differentiation* **81**, 42-48 (2011).
547. (!!! INVALID CITATION !!!).
548. Schwartz, R.E. *et al.* Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *The Journal of clinical investigation* **109**, 1291 (2002).
549. Ayatollahi, M., Soleimani, M., Geramizadeh, B. & Imanieh, M.H. Insulin-like growth factor 1 (IGF-I) improves hepatic differentiation of human bone marrow-derived mesenchymal stem cells. *Cell biology international* **35**, 1169-1176 (2011).
550. Chivu, M. *et al.* In vitro hepatic differentiation of human bone marrow mesenchymal stem cells under differential exposure to liver-specific factors. *Translational Research* **154**, 122-132 (2009).
551. Lee, K.D. *et al.* In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology* **40**, 1275-1284 (2004).
552. Banas, A. *et al.* Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure. *Journal of gastroenterology and hepatology* **24**, 70-77 (2009).
553. Debnath, T. *et al.* Proliferation and differentiation potential of human adipose-derived stem cells grown on chitosan hydrogel. *PloS one* **10**, e0120803 (2015).
554. Ghaedi, M., Tuleuova, N., Zern, M.A., Wu, J. & Revzin, A. Bottom-up signaling from HGF-containing surfaces promotes hepatic differentiation of mesenchymal stem cells. *Biochemical and biophysical research communications* **407**, 295-300 (2011).
555. Snykers, S. *et al.* Sequential exposure to cytokines reflecting embryogenesis: the key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells. *Toxicological Sciences* **94**, 330-341 (2006).
556. Lu, T. *et al.* FGF4 and HGF promote differentiation of mouse bone marrow mesenchymal stem cells into hepatocytes via the MAPK pathway. *Genet Mol Res* **13**, 415-424 (2014).
557. Cipriano, M. *et al.* The role of epigenetic modifiers in extended cultures of functional hepatocyte-like cells derived from human neonatal mesenchymal stem cells. *Archives of toxicology* **91**, 2469-2489 (2017).
558. Alizadeh, E. *et al.* Upregulation of MiR-122 via Trichostatin A Treatments in Hepatocyte-like Cells Derived from Mesenchymal Stem Cells. *Chemical Biology & Drug Design* **87**, 296-305 (2016).
559. Azandeh, S., Gharravi, A.M., Orazizadeh, M., Khodadi, A. & Tabar, M.H. Improvement of mesenchymal stem cell differentiation into the endoderm lineage by four step sequential method in biocompatible biomaterial. *BioImpacts: BI* **6**, 9 (2016).
560. Kang, Y.J. *et al.* Role of c-Jun N-terminal kinase in the PDGF-induced proliferation and migration of human adipose tissue-derived mesenchymal stem cells. *Journal of cellular biochemistry* **95**, 1135-1145 (2005).
561. Raut, A. & Khanna, A. Enhanced expression of hepatocyte-specific microRNAs in valproic acid mediated hepatic trans-differentiation of human umbilical cord derived mesenchymal stem cells. *Experimental cell research* **343**, 237-247 (2016).
562. Alizadeh, E. *et al.* Up Regulation of Liver-enriched Transcription Factors HNF4a and HNF6 and Liver-Specific MicroRNA (miR-122) by Inhibition of Let-7b in Mesenchymal Stem Cells. *Chemical biology & drug design* **85**, 268-279 (2015).
563. Davoodian, N., Lotfi, A.S., Soleimani, M. & Mowla, S.J. MicroRNA-122 Overexpression Promotes Hepatic Differentiation of Human Adipose Tissue-Derived Stem Cells. *Journal of cellular biochemistry* **115**, 1582-1593 (2014).
564. Lue, J. *et al.* Transdifferentiation of adipose-derived stem cells into hepatocytes: a new approach. *Liver International* **30**, 913-922 (2010).
565. Saulnier, N. *et al.* Molecular mechanisms underlying human adipose tissue-derived stromal cells differentiation into a hepatocyte-like phenotype. *Digestive and Liver Disease* **42**, 895-901 (2010).
566. Lee, J.M., Dedhar, S., Kalluri, R. & Thompson, E.W. The epithelial–mesenchymal transition: new insights in signaling, development, and disease. *J Cell biol* **172**, 973-981 (2006).
567. Nobes, C.D. & Hall, A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62 (1995).

568. Bender, A. & Pringle, J.R. Multicopy suppression of the cdc24 budding defect in yeast by CDC42 and three newly identified genes including the ras-related gene RSR1. *Proceedings of the National Academy of Sciences* **86**, 9976-9980 (1989).
569. Moon, S.Y. & Zheng, Y. Rho GTPase-activating proteins in cell regulation. *Trends in cell biology* **13**, 13-22 (2003).
570. Mulloy, J.C. *et al.* Rho GTPases in hematopoiesis and hemopathies. *Blood* **115**, 936-947 (2010).
571. Melendez, J., Grogg, M. & Zheng, Y. Signaling role of Cdc42 in regulating mammalian physiology. *Journal of Biological Chemistry* **286**, 2375-2381 (2011).
572. Wang, L. & Zheng, Y. Cell type-specific functions of Rho GTPases revealed by gene targeting in mice. *Trends in cell biology* **17**, 58-64 (2007).
573. Kerber, R.A., O'Brien, E. & Cawthon, R.M. Gene expression profiles associated with aging and mortality in humans. *Aging Cell* **8**, 239-250 (2009).
574. Etienne-Manneville, S. & Hall, A. 2002Rho GTPases in cell biology. *Nature* **420629635** (2002).
575. Muhoza, D. & Adams, P.D. Two Small Molecules, ZCL278 and AZA197 Show Promise in Influencing Protein Interactions Involving the Ras-Related Protein Cell division cycle 42 [Cdc42] to Modulate Its Oncogenic Potential. *Open Journal of Biophysics* **7**, 71 (2017).
576. Etienne-Manneville, S. Cdc42-the centre of polarity. *Journal of cell science* **117**, 1291-1300 (2004).
577. Cho, K.A. *et al.* Morphological adjustment of senescent cells by modulating caveolin-1 status. *Journal of Biological Chemistry* **279**, 42270-42278 (2004).
578. Cammarano, M.S., Nekrasova, T., Noel, B. & Minden, A. Pak4 induces premature senescence via a pathway requiring p16INK4/p19ARF and mitogen-activated protein kinase signaling. *Molecular and cellular biology* **25**, 9532-9542 (2005).
579. Lavelin, I. & Geiger, B. Characterization of a novel GTPase-activating protein associated with focal adhesions and the actin cytoskeleton. *Journal of Biological Chemistry* **280**, 7178-7185 (2005).
580. Fortier, L.A. & Miller, B.J. Signaling through the small G-protein Cdc42 is involved in insulin-like growth factor-I resistance in aging articular chondrocytes. *Journal of orthopaedic research* **24**, 1765-1772 (2006).
581. Sinha, S. & Yang, W. Cellular signaling for activation of Rho GTPase Cdc42. *Cellular signalling* **20**, 1927-1934 (2008).
582. Carolina Florian, M. & Geiger, H. Concise review: polarity in stem cells, disease, and aging. *Stem Cells* **28**, 1623-1629 (2010).
583. Qian, L. *et al.* Tinman/Nkx2-5 acts via miR-1 and upstream of Cdc42 to regulate heart function across species. *The Journal of cell biology*, jcb. 201006114 (2011).
584. Anttonen, T. *et al.* Cdc42-dependent structural development of auditory supporting cells is required for wound healing at adulthood. *Scientific reports* **2**, 978 (2012).
585. Geiger, H. & Zheng, Y. Cdc42 and aging of hematopoietic stem cells. *Current opinion in hematology* **20**, 295 (2013).
586. Florian, M.C. *et al.* A canonical to non-canonical Wnt signalling switch in haematopoietic stem-cell ageing. *Nature* **503**, 392 (2013).
587. Liu, S., Dontu, G. & Wicha, M.S. Mammary stem cells, self-renewal pathways, and carcinogenesis. *Breast cancer research* **7**, 86 (2005).
588. Li, J. *et al.* WNT5A antagonizes WNT/β-catenin signaling and is frequently silenced by promoter CpG methylation in esophageal squamous cell carcinoma. *Cancer biology & therapy* **10**, 617-624 (2010).
589. van Hengel, J. *et al.* Inactivation of p120 catenin in mice disturbs intrahepatic bile duct development and aggravates liver carcinogenesis. *European journal of cell biology* **95**, 574-584 (2016).
590. Heasman, S.J. & Ridley, A.J. Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nature reviews. Molecular cell biology* **9**, 690 (2008).
591. Etienne-Manneville, S. & Hall, A. Rho GTPases in cell biology. *Nature* **420**, 629 (2002).
592. Melendez, J. *et al.* Cdc42 coordinates proliferation, polarity, migration, and differentiation of small intestinal epithelial cells in mice. *Gastroenterology* **145**, 808-819 (2013).
593. Cheng, C.-M. *et al.* Compartmentalized Ras proteins transform NIH 3T3 cells with different efficiencies. *Molecular and cellular biology* **31**, 983-997 (2011).
594. Qiu, R.-G., Abo, A., McCormick, F. & Symons, M. Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. *Molecular and cellular biology* **17**, 3449-3458 (1997).
595. Ferbeyre, G. Aberrant signaling and senescence associated protein degradation. *Experimental Gerontology* (2017).

596. Kumar, A., Al-Sammarraie, N., DiPette, D.J. & Singh, U.S. Metformin impairs Rho GTPase signaling to induce apoptosis in neuroblastoma cells and inhibits growth of tumors in the xenograft mouse model of neuroblastoma. *Oncotarget* **5**, 11709 (2014).
597. Davis, R.J. Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239-252 (2000).
598. Hattori, K., Naguro, I., Runchel, C. & Ichijo, H. The roles of ASK family proteins in stress responses and diseases. *Cell Communication and Signaling* **7**, 9 (2009).
599. Johnson, G.L. & Lapadat, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**, 1911-1912 (2002).
600. Morrison, D.K. & Davis, R.J. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annual review of cell and developmental biology* **19**, 91-118 (2003).
601. Pouysségur, J., Volmat, V. & Lenormand, P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Biochemical pharmacology* **64**, 755-763 (2002).
602. Coso, O.A. *et al.* The small GTP-binding proteins Rac1 and Cdc42regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137-1146 (1995).
603. Courtial, L. *et al.* The c-Jun N-terminal kinase prevents oxidative stress induced by UV and thermal stresses in corals and human cells. *Scientific Reports* **7** (2017).
604. Cong, Q. *et al.* p38α MAPK regulates proliferation and differentiation of osteoclast progenitors and bone remodeling in an aging-dependent manner. *Scientific Reports* **7** (2017).
605. Dolcet, X., Llobet, D., Pallares, J. & Matias-Guiu, X. NF-kB in development and progression of human cancer. *Virchows archiv* **446**, 475-482 (2005).
606. Liao, L. *et al.* TNF-α Inhibits FoxO1 by Upregulating miR-705 to Aggravate Oxidative Damage in Bone Marrow-Derived Mesenchymal Stem Cells during Osteoporosis. *Stem cells* **34**, 1054-1067 (2016).
607. Boehm, A.M., Rosenstiel, P. & Bosch, T.C. Stem cells and aging from a quasi-immortal point of view. *BioEssays* **35**, 994-1003 (2013).
608. Wang, N. *et al.* TNF-α-induced NF-κB activation upregulates microRNA-150-3p and inhibits osteogenesis of mesenchymal stem cells by targeting β-catenin. *Open biology* **6**, 150258 (2016).
609. Pandey, A.C. *et al.* MicroRNA profiling reveals age-dependent differential expression of nuclear factor κB and mitogen-activated protein kinase in adipose and bone marrow-derived human mesenchymal stem cells. *Stem cell research & therapy* **2**, 49 (2011).
610. Golub, T. & Pico, C. in Biochemical Society Symposia, Vol. 72 119-127 (Portland Press Limited, 2005).
611. Hawkins, P., Anderson, K., Davidson, K. & Stephens, L. Signalling through Class I PI3Ks in mammalian cells. *Biochemical Society Transactions* **34**, 647-662 (2006).
612. Zoncu, R., Efeyan, A. & Sabatini, D.M. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews Molecular cell biology* **12**, 21-35 (2010).
613. Berfield, A.K., Andress, D.L. & Abrass, C.K. IGF-1–induced lipid accumulation impairs mesangial cell migration and contractile function. *Kidney international* **62**, 1229-1237 (2002).
614. Bueno, O.F. & Molkentin, J.D. Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. *Circulation Research* **91**, 776-781 (2002).
615. Coso, O.A. *et al.* The small GTP-binding proteins Rac1 and Cdc42regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137-1146 (1995).
616. Vincent, A.M. & Feldman, E.L. Control of cell survival by IGF signaling pathways. *Growth hormone & IGF research* **12**, 193-197 (2002).
617. Chen, H. *et al.* Leptin and Neutrophil-Activating Peptide 2 Promote Mesenchymal Stem Cell Senescence Through Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway in Patients With Systemic Lupus Erythematosus. *Arthritis & rheumatology* **67**, 2383-2393 (2015).
618. Xian, L. *et al.* Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. *Nature medicine* **18**, 1095-1101 (2012).

619. Lee, Y. *et al.* CREB-binding protein (CBP) regulates β-adrenoceptor (β-AR)– mediated apoptosis. *Cell death and differentiation* **20**, 941 (2013).
620. Xie, S. *et al.* O-GlcNAcylation of protein kinase A catalytic subunits enhances its activity: a mechanism linked to learning and memory deficits in Alzheimer's disease. *Aging cell* **15**, 455-464 (2016).
621. Rahimi, A. *et al.* Role of p53 in cAMP/PKA pathway mediated apoptosis. *Apoptosis* **18**, 1492-1499 (2013).
622. Carlyle, B.C. *et al.* cAMP-PKA phosphorylation of tau confers risk for degeneration in aging association cortex. *Proceedings of the National Academy of Sciences* **111**, 5036-5041 (2014).
623. Feoktistov, I., Goldstein, A.E. & Biaggioni, I. Cyclic AMP and protein kinase A stimulate Cdc42: role of A2 adenosine receptors in human mast cells. *Molecular pharmacology* **58**, 903-910 (2000).
624. Peterson, J.R., Lebensohn, A.M., Pelish, H.E. & Kirschner, M.W. Biochemical suppression of small-molecule inhibitors: a strategy to identify inhibitor targets and signaling pathway components. *Chemistry & biology* **13**, 443-452 (2006).
625. Sakamori, R. *et al.* CDC42 inhibition suppresses progression of incipient intestinal tumors. *Cancer research* **74**, 5480-5492 (2014).
626. O'Brien, E. *et al.* (Am Soc Hematology, 2013).
627. Pelish, H.E. *et al.* Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation in vitro. *Nature chemical biology* **2**, 39 (2006).
628. Surviladze, Z., Young, S.M. & Sklar, L.A. High-throughput flow cytometry bead-based multiplex assay for identification of Rho GTPase inhibitors. *Rho GTPases: Methods and Protocols*, 253-270 (2012).
629. Surviladze, Z. *et al.* A potent and selective inhibitor of Cdc42 GTPase. (2010).
630. Chen, H.Y., Yang, Y.M., Stevens, B.M. & Noble, M. Inhibition of redox/Fyn/c-Cbl pathway function by Cdc42 controls tumour initiation capacity and tamoxifen sensitivity in basal-like breast cancer cells. *EMBO molecular medicine* **5**, 723-736 (2013).
631. Guo, Y. *et al.* R-ketorolac targets Cdc42 and Rac1 and alters ovarian cancer cell behaviors critical for invasion and metastasis. *Molecular cancer therapeutics* **14**, 2215-2227 (2015).
632. Lee, J.G. & Heur, M. Interleukin-1β-induced Wnt5a enhances human corneal endothelial cell migration through regulation of Cdc42 and RhoA. *Molecular and cellular biology* **34**, 3535-3545 (2014).
633. McNary, S.M., Athanasiou, K.A. & Reddi, A.H. Transforming growth factor β-induced superficial zone protein accumulation in the surface zone of articular cartilage is dependent on the cytoskeleton. *Tissue Engineering Part A* **20**, 921-929 (2013).
634. Chen, C. *et al.* Cdc42 inhibitor ML141 enhances G-CSF-induced hematopoietic stem and progenitor cell mobilization. *International journal of hematology* **101**, 5-12 (2015).
635. Cheng, S.-L. *et al.* Vascular smooth muscle LRP6 limits arteriosclerotic calcification in diabetic LDLR-/-mice by restraining noncanonical Wnt signals. *Circulation research*, CIRCRESAHA. 115.306712 (2015).
636. Nguyen, D.H.T., Gao, L., Wong, A. & Chen, C.S. Cdc42 regulates branching in angiogenic sprouting in vitro. *Microcirculation* (2017).
637. Xu, X.-p. *et al.* Ang II-AT2R increases mesenchymal stem cell migration by signaling through the FAK and RhoA/Cdc42 pathways in vitro. *Stem cell research & therapy* **8**, 164 (2017).
638. ZHANG, Y. *et al.* The small GTPase CDC42 regulates actin dynamics during porcine oocyte maturation. *Journal of Reproduction and Development*, 2017-2034 (2017).
639. Wang, Y. *et al.* Role of histone deacetylase inhibitors in the aging of human umbilical cord mesenchymal stem cells. *Journal of cellular biochemistry* **114**, 2231-2239 (2013).
640. Han, B. *et al.* Trichostatin A stabilizes the expression of pluripotent genes in human mesenchymal stem cells during ex vivo expansion. *PloS one* **8**, e81781 (2013).
641. Fila-Danilow, A., Borkowska, P., Paul-Samojedny, M., Kowalczyk, M. & Kowalski, J. The influence of TSA and VPA on the in vitro differentiation of bone marrow mesenchymal stem cells into neuronal lineage cells: Gene expression studies. *Postepy higieny i medycyny doswiadczalnej (Online)* **71**, 236 (2017).
642. Mezentseva, N.V. *et al.* The histone methyltransferase inhibitor BIX01294 enhances the cardiac potential of bone marrow cells. *Stem cells and development* **22**, 654-667 (2012).

643. Yang, J., Kaur, K., Edwards, J.G., Eisenberg, C.A. & Eisenberg, L.M. Inhibition of Histone Methyltransferase, Histone Deacetylase, and β-Catenin Synergistically Enhance the Cardiac Potential of Bone Marrow Cells. *Stem cells international* **2017** (2017).
644. Kong, Q. *et al.* Telomere elongation facilitated by trichostatin a in cloned embryos and pigs by somatic cell nuclear transfer. *Stem Cell Reviews and Reports* **10**, 399-407 (2014).
645. Abdallah, B. & Kassem, M. Human mesenchymal stem cells: from basic biology to clinical applications. *Gene therapy* **15**, 109 (2008).
646. Kim, H.J., Kwon, Y.-R., Bae, Y.-J. & Kim, Y.-J. Enhancement of human mesenchymal stem cell differentiation by combination treatment with 5-azacytidine and trichostatin A. *Biotechnology letters* **38**, 167-174 (2016).
647. Cho, H.H. *et al.* Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. *Journal of cellular biochemistry* **96**, 533-542 (2005).
648. de Almeida Sassi, F. *et al.* Inhibitory activities of trichostatin A in U87 glioblastoma cells and tumorsphere-derived cells. *Journal of Molecular Neuroscience* **54**, 27-40 (2014).
649. Alvarez, A.A., Field, M., Bushnev, S., Longo, M.S. & Sugaya, K. The effects of histone deacetylase inhibitors on glioblastoma-derived stem cells. *Journal of Molecular Neuroscience* **55**, 7-20 (2015).
650. Ng, Y., Tan, I., Lim, L. & Leung, T. Expression of the human myotonic dystrophy kinase-related Cdc42-binding kinase γ is regulated by promoter DNA methylation and Sp1 binding. *Journal of Biological Chemistry* **279**, 34156-34164 (2004).
651. Snykers, S. *et al.* Chromatin remodeling agent trichostatin A: a key-factor in the hepatic differentiation of human mesenchymal stem cells derived of adult bone marrow. *BMC developmental biology* **7**, 24 (2007).
652. Ryan, M.A. *et al.* Pharmacological inhibition of EGFR signaling enhances G-CSF-induced hematopoietic stem cell mobilization. *Nature medicine* **16**, 1141-1146 (2010).
653. Liliental, J. *et al.* Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. *Current Biology* **10**, 401-404 (2000).
654. Fukushima, K., Ueno, Y., Inoue, J., Kanno, N. & Shimosegawa, T. Filopodia formation via a specific Eph family member and PI3K in immortalized cholangiocytes. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **291**, G812-G819 (2006).
655. Chen, J., Crawford, R., Chen, C. & Xiao, Y. The key regulatory roles of the PI3K/Akt signaling pathway in the functionalities of mesenchymal stem cells and applications in tissue regeneration. *Tissue Engineering Part B: Reviews* **19**, 516-528 (2013).
656. Fritz, G. & Kaina, B. Activation of c-Jun N-terminal kinase 1 by UV irradiation is inhibited by wortmannin without affecting c-jun expression. *Molecular and cellular biology* **19**, 1768-1774 (1999).
657. Kim, B.S., Kang, H.-J., Park, J.-Y. & Lee, J. Fucoidan promotes osteoblast differentiation via JNK-and ERK-dependent BMP2–Smad 1/5/8 signaling in human mesenchymal stem cells. *Experimental & molecular medicine* **47**, e128 (2015).
658. Yin, Y. *et al.* Collagen nanofibers facilitated presynaptic maturation in differentiated neurons from spinal-cord-derived neural stem cells through MAPK/ERK1/2-Synapsin I signaling pathway. *Biomacromolecules* **15**, 2449-2460 (2014).
659. Wang, Y.-G. *et al.* Effect of intercellular adhesion molecule-1 on the migration in vitro of murine mesenchymal stem cells and its related mechanism. *Zhongguo shi yan xue ye xue za zhi* **22**, 479-484 (2014).
660. Du, L. *et al.* Inhibition of Cell Proliferation and Cell Cycle Progression by Specific Inhibition of Basal JNK Activity EVIDENCE THAT MITOTIC Bcl-2 PHOSPHORYLATION IS JNK-INDEPENDENT. *Journal of Biological Chemistry* **279**, 11957-11966 (2004).
661. Kilian, K.A., Bugarija, B., Lahn, B.T. & Mrksich, M. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proceedings of the National Academy of Sciences* **107**, 4872-4877 (2010).
662. Wu, Y., Xia, L., Zhou, Y., Xu, Y. & Jiang, X. Icariin induces osteogenic differentiation of bone mesenchymal stem cells in a MAPK-dependent manner. *Cell proliferation* (2015).
663. Hosogai, N. *et al.* Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* **56**, 901-911 (2007).
664. Wang, L., Yang, L., Filippi, M.-D., Williams, D.A. & Zheng, Y. Genetic deletion of Cdc42GAP reveals a role of Cdc42 in erythropoiesis and hematopoietic stem/progenitor cell survival, adhesion, and engraftment. *Blood* **107**, 98-105 (2006).
665. Chahdi, A. & Sorokin, A. Endothelin 1 stimulates β1Pix-dependent activation of Cdc42 through the Gsα pathway. *Experimental Biology and Medicine* **231**, 761-765 (2006).

666. Lo, M.J. & Wang, P.S. Involvement of cAMP but not PKA in the increase of corticosterone secretion in rat zona fasciculata-reticularis cells by aging. *Journal of cellular biochemistry* **85**, 35-41 (2002).
667. Zhang, H.H., Halbleib, M., Ahmad, F., Manganiello, V.C. & Greenberg, A.S. Tumor necrosis factor-α stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP. *Diabetes* **51**, 2929-2935 (2002).
668. Si-Tayeb, K., Lemaigre, F.P. & Duncan, S.A. Organogenesis and development of the liver. *Developmental cell* **18**, 175-189 (2010).
669. Sato, T. *et al.* Single Lgr5 stem cells build crypt villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-265 (2009).
670. Katoh, M. & Katoh, M. STAT3-induced WNT5A signaling loop in embryonic stem cells, adult normal tissues, chronic persistent inflammation, rheumatoid arthritis and cancer. *International journal of molecular medicine* **19**, 273-278 (2007).
671. Frese, L., Dijkman, P.E. & Hoerstrup, S.P. Adipose tissue-derived stem cells in regenerative medicine. *Transfusion Medicine and Hemotherapy* **43**, 268-274 (2016).
672. Kokai, L.E. *et al.* Adipose stem cell function maintained with age: an intra-subject study of long-term cryopreserved cells. *Aesthetic Surgery Journal* **37**, 454-463 (2017).
673. Hanke, A. *et al.* Semi-automated extraction and characterization of Stromal Vascular Fraction using a new medical device. *Clinical hemorheology and microcirculation* **64**, 403-412 (2016).
674. Liu, M. *et al.* Adipose-derived mesenchymal stem cells from the elderly exhibit decreased migration and differentiation abilities with senescent properties. *Cell transplantation* (2017).
675. Aguilar, B., Zhou, H. & Lu, Q. Cdc42 Signaling Pathway Inhibition as a Therapeutic Target in Ras-Related Cancers. *Current medicinal chemistry* (2017).
676. Yang, L. *et al.* Mesenchymal stem cells engineered to secrete pigment epithelium-derived factor inhibit tumor metastasis and the formation of malignant ascites in a murine colorectal peritoneal carcinomatosis model. *Human gene therapy* **27**, 267-277 (2016).
677. Teng, N.-Y. *et al.* Promotion of mesenchymal-to-epithelial transition by Rac1 inhibition with small molecules accelerates hepatic differentiation of mesenchymal stromal cells. *Tissue Engineering Part A* **21**, 1444-1454 (2015).
678. Fukuma, M. *et al.* Leucine-rich repeat-containing G protein-coupled receptor 5 regulates epithelial cell phenotype and survival of hepatocellular carcinoma cells. *Experimental cell research* **319**, 113-121 (2013).
679. Kwon, M.S., Park, B.-o., Kim, H.M. & Kim, S. Leucine-rich repeat-containing G-protein coupled receptor 5/GPR49 activates G12/13-Rho GTPase pathway. *Molecules and cells* **36**, 267-272 (2013).
680. Schlessinger, K., Hall, A. & Tolwinski, N. Wnt signaling pathways meet Rho GTPases. *Genes & development* **23**, 265-277 (2009).
681. Uygun, B.E. *et al.* Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nature medicine* **16**, 814-820 (2010).
682. Mazza, G. *et al.* Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. *Scientific reports* **5**, 13079 (2015).

Keywords : Liver; Cell-based therapy; Hepatocyte; Hepatic differentiation; Stem Cells; Wnt; Lgr5; Cdc42 **Abstract:** Hepatocytes cell-based transplantation is a promising strategy for treating liver diseases. However, there are still several limitations for their use in clinical applications among them the high throughput generation of functional hepatocytes, their life span in culture, the age of the donor age and the source of hepatic stem cells (SCs). At present, the challenge lies to develop approaches aiming the identification of the new molecular markers signaling pathways involved in the differentiation of SCs toward functional hepatocytes. In fact, Wnt pathways governs multiple biological processes controlling the differentiation fate of SCs into hepatocytes, some of them result in the activation of small GTPase and the Lgr5 pathway regulators. Indeed, Lgr5 (a target gene of Wnt, the Leucine-rich-repeat-containing G protein-coupled Receptor 5) was shown to be crucial for maintaining long-term expansion of hepatic SC in vitro. In addition, Lgr5 primarily functions as an effector of the Cdc42 GTPases (a RhoGTPase protein, the cell division cycle 42). Higher activity of Cdc42 was reported to be correlated to hematopoietic SCs aging. However, this correlation has never been studied before in adipose tissue Mesenchymal Stem Cells (ADSCs) which were proposed recently as a promising tool for liver regeneration. In this study, we were interested (i) to propose a novel method of reprogramming mouse mature hepatocytes into murine endodermic progenitors (mEndoPCs) that express Lgr5, generate liver-specific organoids and can differentiate into hepatocytes and cholangiocytes in vitro and give arise to bile duct structures and into functional hepatocytes in vivo, and (ii) to study the activity of Cdc42 in human aged-derived hADSCs and the impact of its selective inhibition by ML141 on their hepatic differentiation potential in vitro. In our study we succeeded to generate mEndoPCs and to improve the functionality of the aged-hADSCs derived-hepatocytes. We showed that both Lgr5 and Cdc42 are regulated distinctly by Wnt signaling pathways. In addition, our results revealed that LIFR/STAT3 and LGR5/WNT pathways are important to maintain the unlimited expansion of mEndoPCs in vitro when STAT3 pathway is activated. MAPK/PKA, WNT/ β-catenin pathways and the exosome's production were shown to be deregulated with hADSCs aging. We showed also that a mesenchymal to epithelial transition was crucial to transdifferentiate hADSCs into functional hepatocytes. On the other side, ML141 is proposed as a new pharmacological tool to rejuvenate aged-hADSCs toward functionally younger-like cells thus by promoting cell proliferation, doubling and cell adherence. Finally, the transfer of these methodologies to human could serve the regenerative medicine of the liver as a good tool for hepatocyte-based drug toxicity screening systems and for the liver engineering using a « bio printing »approach.

Titre en français : Le Potentiel Hépatique des Cellules Souches Mésenchymateuses Reversées en Age et des Progéniteurs Endodermiques : Contribution des voies de signalisation de Cdc42 et LGR5 **Mots clés :** Foie ; Hépatocyte ; Différenciation hépatique ; Cellules Souches ; Wnt ; Lgr5 ; Cdc42 **Résumé :** La thérapie cellulaire utilisant une greffe d'hépatocytes est une stratégie prometteuse pour traiter les maladies du foie. Cependant, plusieurs limitations freinent leur transfert pour des applications cliniques, comprenant la production à haut débit d'hépatocytes fonctionnels, leur survie en culture, l'âge du donneur et la source des cellules souches hépatiques (CS). Les avancées scientifiques réalisées à ce jour ont permis d'identifier de nouveaux marqueurs moléculaires et les voies de signalisation impliquées dans la différenciation des CS en hépatocytes fonctionnels. En effet, la voie de signalisation Wnt a montré être importante pour réguler de nombreux processus biologiques des CS permettant de contrôler leur différenciation hépatique dont l'activation des GTPases et les gènes ciblant la voie de signalisation de Lgr5. Récemment, des études ont montré que le marqueur Lgr5 (récepteur 5 couplé à la protéine G contenant une répétition d'acides aminés riche en leucine) est décisif pour maintenir une expansion à long terme des CS hépatiques *in vitro*. En outre, Lgr5 fonctionne principalement comme un effecteur de la Cdc42 (cycle de division cellulaire 42) qui est un membre de la famille des Rho-GTPase. Une forte expression de la Cdc42 a montré être corrélée avec le vieillissement des CSs hématopoïétiques. Néanmoins, cette corrélation n'a jamais été étudiée à ce jour dans les cellules souches mésenchymateuses dérivées du tissu adipeux humain (hADSCs) qui ont été proposées récemment comme une source prometteuse pour la régénération du foie. Au cours de nos travaux de thèse, nous nous sommes intéressés (i) à proposer une nouvelle technologie de reprogrammation d'hépatocytes matures murins en progéniteurs endodermiques (mEndoPCs) exprimant Lgr5 capables de générer des organoïdes spécifiques du foie et de se différencier en hépatocytes et cholangiocytes *in vitro* et en des structures biliaires et hépatiques *in vivo* (ii) à étudier l'activité de Cdc42 dans les hADSCs âgées et l'impact de son inhibition sélective par le ML141 sur leur potentiel de différenciation hépatique *in vitro*. Nous montrons qu'il a été possible de générer des mEndoPCs et à améliorer la différenciation hépatique des hADSCs âgés. Nous montrons également que Lgr5 et Cdc42 sont régulés de façon distincte par la voie de signalisation Wnt. De plus, nos résultats ont révélé que les voies LIFR/STAT3 et Lgr5/WNT sont essentielles pour l'auto renouvellement des mEndoPCs permettant leur expansion illimitée *in vitro* en présence d'activateur de STAT3. Les voies MAPK/PKA, WNT/ β-caténine et la production d'exosomes ont montré une dérégulation liée à l'âge des hADSCs. Nous montrons qu'une transition mésenchymato-épithéliale était nécessaire pour différencier les hADSCs en hépatocytes fonctionnels. D'autre part, ML141 est proposé comme un nouvel outil pharmacologique permettant de reverser l'âge des hADSC âgés et d'amplifier le taux de prolifération, d'adhésion et de fonctionnalité hépatique à un niveau équivalent aux hADSCs jeunes. Enfin, le transfert de ces méthodologies à l'homme pourrait servir pour la médecine régénératrice du foie, comme outil pour évaluer la toxicité hépatique des médicaments et pour l'ingénierie et la reconstitution d'un foie entier par des approches de « bio printing ».

Université Paris-Saclay
Espace Technologique / Immeuble Discovery
Route de l'Orme aux Merisiers RD 128 / 91190 Saint-Aubin, France

Synthèse de la thèse en français

Le potentiel de régénération du foie a été sanctifié il y a des millénaires dans la mythologie grecque de Titan lorsque Prométhée, qui a volé le feu de Zeus et le Dieu l'a donné aux mortels. Prométhée a été accroché à un rocher où un aigle mangeait son foie immortel jour après jour et son foie régénérait continuellement. Cela évoque l'hypothèse de la capacité du foie à s'auto-réparer et à se régénérer. Le foie peut subir une défaillance biologique permanente après des blessures graves, une infection chronique, le cancer et le vieillissement. L'insuffisance hépatique entraîne 1-2 millions de décès par an et est classée comme la 5e cause de décès dans le monde. La transplantation hépatique est actuellement la thérapie efficace pour les maladies hépatiques en phase terminale. Cependant, la pénurie de donneurs crée une limitation sérieuse liée à ce traitement. Ainsi, la liste d'attente du patient pour trouver des donneurs a augmenté à plus de 30% tant en Europe qu'aux États-Unis.

La disponibilité des hépatocytes primaires est un « gold standard » pour la thérapie à base de cellules hépatiques. Les hépatocytes primaires humains ne sont pas faciles à isoler à partir des biopsies de foie et ont également une durée de vie limitée en culture avec une perte progressive de la fonction cellulaire au cours du temps. Par conséquent, l'un des plus grands freins associés à la thérapie hépatique des hépatocytes primaires est la disponibilité d'un nombre suffisant d'hépatocytes fonctionnels de haute qualité. De plus, l'isolation et la culture des hépatocytes peuvent provoquer des altérations majeures de l'expression des gènes et ceci du à la dédifférenciation cellulaire via une transition épithéliale à mésenchymateuse (EMT) ou à l'apoptose cellulaire après seulement quelques jours en culture. De plus, *les* modèles de culture *in vitro* des cellules hépatiques humaines sont cruciaux pour le développement de médicaments. Pour éviter la variabilité dans les hépatocytes humains, une alternative serait de prolonger leur délai de vie *in vitro* en induisant des hépatocytes sains pour (1) Produire des hépatocytes humains à haut débit permettant de meilleurs systèmes de sensibilité aux médicaments, (2) la modélisation des maladies et du développement du foie (3) une source de cellules pour la thérapie cellulaire ,(4) le développement de dispositifs hépatiques bio-artificiels et (5) pour une approche de bio-impression du foie .

Plusieurs études montrent l'existence de cellules souches résidentes dans le foie adulte. Des progéniteurs hépatiques humains ont été observés au cours de maladies hépatiques aiguës et chroniques, mais leur nombre était insuffisant et ils étaient incapables d'effectuer une régénération complète du foie. Les stratégies basées sur les cellules souches sont par conséquent explorées comme une approche alternative attrayante pour la réparation du foie. Ainsi, divers types de cellules souches ont été utilisés dans la période récente pour produire un grand nombre d'hépatocytes

fonctionnels *in vitro*. Plusieurs études ont décrit la différenciation des cellules souches embryonnaires (ESC) vers des hépatocytes-like (HLCs). Malgré le potentiel de différenciation des CES, leur application clinique reste limitée compte tenu du risque de formation de tératomes et des limitations éthiques. Plus tard, les cellules souches pluripotentes induites (CSPi) ont été introduites comme une approche innovante dans le domaine de la thérapie cellulaire du foie. Cependant, les HLCs dérivées des CSPi ont montré un statut de différenciation incomplet par rapport aux hépatocytes primaires puisqu'elles maintiennent une expression des transgènes exogènes qui peuvent interférer avec les protocoles de différenciation. En outre, la génération d'hépatocytes induits (iHep) à partir de cellules souches pluripotentes présente une sécurité élevée mais des soucis éthiques limitant leurs utilisations cliniques pour la médecine régénérative à base de cellules. Ainsi, des études cliniques fondamentales tentent d'ouvrir de nouveaux défis pour la génération d'hépatocytes à partir de cellules souches adultes telles que les cellules souches mésenchymateuses (MSC).

Plus récemment, la reprogrammation des hépatocytes primaires chez l'homme et les rongeurs a produit des cellules souches hépatiques exprimant un gène cible de la voie Wnt appelé Lgr5 (récepteur 5 couplé à la protéine G contenant la leucine -rich-repeat) . Cette technologie a ont émergé comme une nouvelle plate-forme pour la médecine régénératrice du foie à base de cellules souches; en fait, les cellules souches ou progéniteurs *Lgr5*$^{+}$ ont activement contribué à la régénération du foie en induisant *de novo* hépatocytes ceci via la signalisation Wnt.

En effet, les voies de signalisation Wnt canoniques et non-canoniques régit une myriade de processus biologiques sous-jacents cruciaux pour le développement et le maintien de l'homéostasie tissulaire adulte et la régulation de l'auto-renouvellement et la différentiation des cellules souches.

La régulation des voies de Wnts peut déclencher de multiples cascades de signalisation, dont certaines entraînent l'activation de petites GTPase Rho, Rac et Cdc42. Des souris transgéniques où la protéine Rho-GTPase Cdc42 a été génétiquement modifiée et réprimée dans leurs hépatocytes, présentent de graves anomalies du foie. Cela indique que Cdc42 joue un rôle dans le foie et pourrait être nécessaire pour la fonction hépatique. Plusieurs rapports ont discuté du rôle de la Cdc42 dans la prolifération des MSCs humaines, leur migration, leur différenciation et notamment son implication dans vieillissement cellulaire.

De ce fait, notre stratégie était basée sur une nouvelle technologie de reprogrammation des hépatocytes primaires murines vers des cellules souches murines des souris exprimant la LGR5^{+} et capable de donner des hépatocytes *in vitro*. D'une autre part, nous étions intéressés à étudier le rôle de la Cdc42 dans le vieillissement des MSCs humains

dérivées du tissu adipeux (ADSCs). La corrélation entre le rôle de la Cdc42, l'âge des ADSCs et leur potentiel de différentiation hépatique a été évalué en inhibant la Cdc42 avec un inhibiteur spécifique appelé ML141.

Mon projet de thèse inclut deux grandes parties, la première partie décrit un nouvel outil de génération de cellules souches hépatiques bipotentes via un processus de reprogrammation hépatocytaire. Ces progéniteurs sont destinés à se redifférencier en hépatocytes et cholangiocytes après une expansion *à* long terme *in vitro*. Quant à la seconde partie elle présente le rôle de la Cdc42 dans l'âge la sénescence et le potentiel hépatocytaire *in vitro* pour une première fois dans les ADSCs. Ces deux parties seront introduites par la suite brièvement.

Partie I

1. Génération de cellules souches Lgr5 + à partir d'hépatocytes murins primaires

Le foie montre deux mécanismes de régénération dépendamment du type de lésion. L'hépatectomie partielle (PH) implique un rôle important des hépatocytes échappant à la lésion qui s'engagent dans la régénération du foie.

Cependant, les lesions toxiques du foie impliquent des cellules souches du foie adultes, appelées cellules ovales chez les rongeurs. Ces cellules souches sont situées au niveau du canal biliaire (chez l'animal) et le canal de Hering (chez l'homme). Elles sont capables de donner naissance à la fois aux hépatocytes et aux cholangiocytes. Plusieurs voies ont été montrées pour réguler la régénération hépatique et la prolifération hépatocytaires telles que la voie canonique Wnt / β-caténine, le complexe membranaire E-cadhérine / β-caténine , la voie IL6 / STAT- et la TGF β . Malheureusement, dans la plupart des temps toutes ces voies sont loin d'être efficaces après PH et ne parviennent pas à régénérer le foie en raison des accumulations d'espèces réactives de l'oxygène (ROS), des lésions de l'ADN dans les hépatocytes et de l'activation insuffisante des cellules souches hépatiques. De l'autre côté, les cellules souches du foie ovales ne sont activées qu'après une toxicité du foie. Elles prolifèrent et redonnent naissance à des hépatocytes et des cholangiocytes. Bien que les cellules ovales soient activées, leur implication occasionnelle dans la régénération du foie est insuffisante lorsque l'atteinte hépatique est massive.

Ainsi, la recherche sur la régénération hépatique s'est intéressée à mimer les mécanismes de la régénération hépatique *in vitro*. La mise en place de protocoles pour maintenir une prolifération à long terme des hépatocytes humains primaires et d'autre part l'isolation, l'expansion et l'activation des cellules souches bipotentes humaines ont été les principaux objectifs de la recherche de la médecine régénérative du foie pour plusieurs années.

Cependant, ces deux sources cellulaires présentent de fortes limitations et sont loin d'être maintenues ex-vivo. En effet, l'isolation des hépatocytes primaires est soumise à une procédure très compliquée et ces hépatocytes pénètrent rapidement dans un cycle de sénescence. D'un autre côté, l'isolation des cellules souches hépatiques nécessite une caractérisation moléculaire des marqueurs de surface. Malheureusement, ces progéniteurs ne sont pas une population distincte du foie puisqu'ils expriment des marqueurs communs avec les hépatoblastes (AFP et CK19) [3], les hépatocytes (HNF4-β et Alb), les cellules souches stromales hépatiques (c-kit, Sca-1, Thy1 (CD90) et CD34) et des marqueurs épithéliaux hépatiques (EpCAM). Le traçage du linéage cellulaire *in vivo* des cellules souches hépatiques bipotentes a été réalisé en utilisant des systèmes promoteurs inductibles-Cre chez des souris transgéniques tels que les promoteurs de Foxl1, Sox9 et. Ces progéniteurs ont été capables de repeupler le foie blessé et de procréer des hépatocytes et des cholangiocytes.

Malheureusement, ces cellules souches bipotentes activées ne contribuent qu'à 2,5% de la régénération des lésions hépatiques. De ce fait, le développement de stratégies pour isoler et proliférer ces progéniteurs *in vitro* semble être essentiels pour la médecine à base de cellules souches hépatiques.

La plasticité des hépatocytes a été beaucoup discutée dans le concept de la régénération hépatique. Dans ce champs, le traçage des hépatocytes humains chez les souris FAH-/- montre qu'après induction de lésion chronique de foie, les hépatocytes présentent une capacité élevée à se dédifférencier vers des cellules souches exprimant CK19 +/ EPCAM+/OPN+/SOX9+.

D'une manière intéressante, ces cellules souches bipotentes dérivées des hépatocytes ont été montré à exprimer *LGR5*, un gène cible Wnt fortement exprimé dans les cellules souches en division dans les tissus épithéliaux comme l'intestin grêle, le côlon, l'estomac et les follicules des cheveux. LGR5 exige la présence de son ligand Rspondin1 (Rspo1) pour médier la signalisation Wnt –Frizzled/B caténin. Plus intéressant, dans une culture de système 3D favorable à la voie de conduite Wnt, des cellules souches Lgr5+ individuelles isolées formaient des organoïdes retenant l'expression génique du foie sur plusieurs passages. Ces derniers étaient capables de s'auto-renouveler et se différencier en hépatocytes fonctionnels lors d'une transplantation dans un model FAH - / - de souris.

Se basant sur toutes ces preuves, Huch et al, en 2015 a isolé des cellules souches EpCAM + à partir de biopsies de foie humain. Dans la structure 3D et en présence de Rspo1 et de certaines petites molécules inhibant les voies Notch, FGF4 et BMP, les cellules EpCAM + ont pu former des organoïdes exprimant LGR5 +. Les hépatocytes dérivés des cellules EpCAM+ LGR5 + se différenciaient vers des hépatocytes fonctionnels et régénéraient le foie de souris endommagé chimiquement par le CCL4. Plus récemment, Katsuda et al. , ont trouvé une combinaison de petites molécules capables de convertir les hépatocytes primaires en progéniteurs hépatiques bipotents et exprimant

LGR5+ ayant un potentiel de différenciation hépatocytaire et cholangiocytaire élevé. Cette stratégie a dépassé la complexité de l'isolation des cellules EpCAM + et la formation d'organoïdes dans les structures 3D. Cependant, Rspo1 et d'autres activateurs Wnt / β-caténine canoniques tels que Wnt3a étaient essentiels pour élargir de manière stable les cellules souches LGR5 + avant leur différenciation.

2. Objectifs de la Partie I

Notre objectif général était d'évaluer une nouvelle technologie de génération de cellules souches bipotentes du foie *in vitro* via une reprogrammation des hépatocytes murins primaire et de la suggérer comme un outil prometteur pour la thérapie hépatique à base de cellules souches.

Il était évident dans une première étape d'évaluer l'identité moléculaire spécifique des EndoPC par rapport aux cellules souches pluripotentes, aux cellules souches ovales Lgr5+ et aux hépatocytes hépatiques primaires.

Donnant l'expression Lgr5 authentique dans EndoPCs , nous avions l'intention de trouver les meilleures conditions d'activation Wnt pour dériver des organoïdes spécifiques et maintenir leur auto-renouvellement lors du retrait du facteur inhibiteur de la leucémie (LIF). Dans une prochaine étape, nous visions à évaluer le potentiel hépatique des EndoPC chez un modèle de souris à lésion hépatique et de confirmer leur sécurité de greffe. En outre, nous avions l'intention d'étudier la communication entre les axes de signalisation Lgr5 / Wnt et IL6 / LIFR / STAT3 au sein des EndoPC en présence de RSPO-1 en culture.

En addition, nous voulions étudier le potentiel hépatogénique des EndoPCS dans des matrices bi et tri-dimensionnelles.

3. Structure de la Partie I

La première partie implique quatre chapitres

Chapitre 2. L'Organogenèse du foie, fonctions et maladies

L'anatomie du foie est présentée dans ce chapitre. En outre, la transition épithéliale mésenchymateuse associée à la spécification du linéage hépatocytaire à partir de l'endoderme définitif est présentée. En dernière section de ce chapitre, les fonctions du foie et les maladies connexes ont été mises en évidence brièvement.

Chapitre 3. Les Mécanismes de régénération hépatique et la **contribution des progéniteurs de foie bipotent (LPs)**

Deux mécanismes de régénération du foie sont décrits. Les voies de signalisation cellulaire et moléculaires impliquées dans la régénération des hépatocytes sont largement détaillées. En outre, les limitations bloquant la capacité régénératrice du foie sont discutées. Les mots clés sont définis tels que Cdc42, Lgr5, le vieillissement cellulaire, les hépatocytes, Wnt / β-caténine et IL6 / STAT3. En plus, les cellules souches du foie sont introduites comme une source potentielle pour la science de la régénération du foie à base de cellules souches. Lgr5 sera énoncé comme un nouveau marqueur de cellules souches hépatiques bipotent crucial pour activer la voie Wnt et pour maintenir l'auto-renouvellement à long terme des progéniteurs LGR5+.

Chapitre 4. Les Avancées dans Reprogramming des hépatocytes matures vers les progéniteurs de foie bipotent (LPs) via l'activation de la voie WNT-LGR5

La traçabilité des lignées de cellules souches bipotentes a été décrite en détail. Ce chapitre explique aussi que la plasticité des hépatocytes peut donner lieu à une population de cellules souches hépatiques LGR5 + bipotentes dans la zone du canal biliaire. En conséquence, de nouvelles stratégies sont décrites et montrent le mécanisme de génération des progéniteurs LGR5+ à partir d'hépatocytes primaires. Trois stratégies sont rapportées: (i) les hépatocytes dédifférenciés vers les descendances bipotentes via l'expression des gènes de pluripotence ; (ii) Isolation direct des cellules EpCAM à partir de biopsies du foie suivi d'une induction de LGR5 + via l'activation de la voie Wnt et (iii) Conversion chimique d'hépatocytes en cellules souches hépatiques bipotentes .

Chapitre 5. Introduction à la publication scientifique 1.

Le premier article scientifique intitulé **: "Direct reprogramming of LGR5+ Liver Progenitors cells responding to both gp130/JAK/STAT3 and Wnt/β-catenin the signaling pathways"** est introduit puis inséré.

Partie II

1. *Impact de l'inhibition de la Cdc42 sur l'âge des cellules souches mésenchymateuses âgées.*

De nombreuses approches ont mis en évidence un lien entre les cellules souches mésenchymateuses (MSCs) et leur haut potentiel hépatique. Les MSCs humaines (hMSC) résident dans divers tissus et organes tels que la moelle osseuse, le tissu adipeux, le muscle, le foie, le cerveau, les tissus parodontaux et le pancréas. Elles ont

été considérées récemment un nouvel espoir pour la thérapie cellulaire autologue. Les hMSC dérivées des tissus adipeux humains (ADSC) ont montré un potentiel de prolifération plus élevé et un effet immunomodulateur plus puissant comparées aux hMSCs dérivées de la moelle osseuse (BMSC). Les ADSCs ont été décrits comme une source idéale en thérapeutique médicale pour leur innocuité et leur efficacité *in vivo*. Récemment, les ADSCs ont été décrites comme une source prometteuse de MSCs pour être utilisées dans la médecine régénérative du foie. Les hépatocytes dérivant des ADSCs humains sont considérés comme un outil potentiel pour étudier la toxicité des médicaments. En outre, à ce jour, plusieurs études cliniques chez l'homme sont menées pour explorer les effets des ADSCs chez les patients atteints d'une maladie du foie (NCT02705742 / NCT00913289 / NCT01062750). De plus, dans des études récentes, les exosomes dérivés d'ADSCs sont considérés comme un nouvel outil dans les thérapies basées sur les exosomes tels que les troubles neurologiques et de manière intéressante pour les maladies hépatiques soulignant les mécanismes moléculaires forts partagés entre les ADSC et le foie. «Pourquoi les vieilles cellules sont-elles plus vulnérables à la pathologie et aux maladies que les jeunes cellules?», Une question posée par Hayflik L. en 1998, juste après la découverte de l'immortalité cellulaire. A cette époque, on considérait que les changements moléculaires liés à l'âge cellulaire se produisent de manière intracellulaire sans aucune explication supplémentaire. Aujourd'hui, la science de l'âge cellulaire implique la connaissance des mécanismes moléculaires et cellulaires des maladies, appelés biomarqueurs liés à l'âge. Ainsi, les recherches sur le l'âge cellulaire ont montré une progression extraordinaire au cours des 15 dernières années et ont défini de nouveaux marqueurs moléculaires. De nos jours, le l'âge cellulaire est défini comme le déclin fonctionnel qui affecte les organismes vivants et la fonctionnalité cellulaire associée à un ou plusieurs des mécanismes suivants: instabilité génomique, altérations épigénétiques, dérégulation des nutriments, dysfonction mitochondriale, sénescence cellulaire et épuisement des cellules souches. En outre, chaque marqueur distinctif est considéré lorsque son inhibition montre un retard dans le processus de vieillissement et une augmentation de la durée de vie de la cellule et une diminution d'autres biomarqueurs liés à l'âge régulés positivement.

Il y a quelques mois, *Florian et al. , estima* que d'ici 2050, le nombre de personnes de plus de 80 ans triplera au niveau mondial, proposant que des approches spéciales doivent être établies pour accompagner les maladies de l'âge cellulaire.

Dans les maladies du foie et les contextes de l'âge, il a également été discuté du déclin de la population de cellules progénitrices hépatiques pourrait être une des raisons de la défaillance de la régénération hépatique. De plus, des rapports récents ont considéré l'âge du donneur (DA) comme un facteur de risque d'échec de transplantation. Par ailleurs, les BMSCs ont montré l'exemple caractéristique du dysfonctionnement des MSCs dans le

microenvironnement âgé où le potentiel ostéogénique est remplacé vers une différenciation vers des adipocytes causant les phénomènes d'ostéoporose dans les os. Au cours des dernières années, de nombreuses études ont rapporté l'impact négatif du DA du tissu adipeux sur la capacité de régénération des ADSC chez la souris et l'homme. D'autres biomarqueurs liés à l'âge, tels que les gènes suppresseurs de tumeurs p53, p16ink4a et p21, l'activité de la télomérase et le taux de prolifération se sont révélés être régulés négativement dans les ADSCs dérivés de donneurs âgés. Ainsi, l'identification des biomarqueurs valides liés à l'âge sera un must pour soutenir les thérapies basées sur les cellules souches chez les personnes âgées. D'une manière intéressante, l'âge des cellules souches hématopoïétiques a été corrélé avec la forte expression d'une protéine RhoGTPase appelée cell division cycle 42 (Cdc42). Cdc42 régule strictement la migration cellulaire et les mécanismes moléculaires d'adhésion. Comme toute la famille des protéines RhoGTPase, Cdc42 agit comme un complexe de commutation binaire entre la forme active liée au GTP et la forme inactive liée au GDP. Le Knockout de GTPase Activating Protein (GAP) , un régulateur négatif du complexe Cdc42 GTP, est montré à réduire significativement le temps d'expansion, améliorer la réparation des lésions de l'ADN et à supprimer les p53, $p16^{Ink4a}$, p21, et la bêta- galactosidase associée à la sénescence (SA- ß-gal) dans plusieurs tissus âgés chez la souris, tels que le coeur, le cerveau, le poumon, le foie, la rate, le rein et la moelle osseuse . Consécutivement, Cdc42 -GTP a été suggérée pour réguler le vieillissement des cellules souches de la souris. Plus tard, avec *Carrillo-Garcia et al.* En 2012, l'inhibition de l'activité de Cdc42 a montré un phenotype jeune des cellules souches âgées. Sur la base de cette hypothèse, l'activité de Cdc42 a été inhibée pharmacologiquement avec un inhibiteur de l'activité de Cdc42 (CASIN). CASIN a travaillé comme un inhibiteur de l'histone désacétylase (HDAC), d'une autre manière et a abouti à une réparation des dommages de l'ADN dans les cellules souches hématopoïétiques (HSC). De ce fait, lors de la transplantation, les CSH traitées par CASIN se sont révélées potentiellement identiques aux CSH isolées des jeunes donneurs. Pour validé que l'activation de Cdc42 peut être un biomarqueur dans le vieillissement humain, le même groupe, *Florian et al.* (2017), ont rapporté une étude de cohorte sur 196 donneurs humains âgés de HSCs et ont montré une corrélation élevée entre l'expression de Cdc42-GTP et le vieillissement des cellules hématopoïétiques.

2. Objectifs de la Partie II

Nous avons d' abord émis l'hypothèse qu'un certain déséquilibre peut se produire dans l'activité du Cdc42 entre les MSCs issus de sujets jeunes et âgés. Puisque la présence et la fonction du tissu adipeux sont extrêmement

dépendantes de l'âge, il était important d'étudier les mécanismes d'action existants dans les hADSC indifférenciés, corrélant l'activité de Cdc42 en particulier à la signalisation MAPK et Wnt (s).

Ensuite, nous avons émis l'hypothèse que les variations dans l'activité de Cdc42 dans les hADSCs peuvent affecter leur différenciation vers des hépatocytes. Dans ce domaine, nous avons inhibé pharmacologiquement l'activité de la Cdc42 et avons évalué si la régulation négative de Cdc42 pourrait influer leur hépatogenèse.

3. Structure de la Partie II

Les données scientifiques d'appui pour la partie II sont élaborées dans trois chapitres différents comme suit:

Chapitre 6. Impact de l'âge du donneur (DA) sur la fonctionnalité des cellules souches mésenchymateuses

Dans cette section, je discute des biomarqueurs liés à l'âge dans les différents sous-types de MSCs. En outre, les mécanismes impliqués dans l'altération du cycle cellulaire et le potentiel de différenciation dans les MSCs âgés sont cités. Ensuite, la contribution des niches âgées à l'impact négatif sur les MSCs est détaillée.

Chapitre 7. Potentiel hépatogène des MSCs de tissu adipeux (ADSCs)

Le potentiel hépatogénique des hMSC est d'abord discuté, suivie d'une description de l'impact négatif du vieillissement sur le devenir hépatique des hADSCs *in vitro*. Dans ce contexte, il était évident de discuter certaines indications de culture cellulaire à prendre en compte durant la transition mésenchymato-épithéliale (MET) *in vitro* régulant la différenciation des ADSCs vers des hépatocytes. À la fin de ce chapitre, Cdc42 est présentée comme un nouveau marqueur pour les cellules souches multipotentes telles que les cellules souches hématopoïétiques.

Chapitre 8. Cdc42: Un nouveau biomarqueur du vieillissement cellulaire. Ce chapitre a été crucial pour faire appui à nos objectifs; par conséquent, j'ai discuté de l'impact des mécanismes d'activation de la Cdc42 sur l'âge des cellules souches. Dans une deuxième étape j'ai discuté des voies de signalisation régulant la Cdc42 dans le vieillissement des cellules souches.

La focalisation sur la signalisation Wnt et l'activation de Cdc42 était importante puisque la voie Wnt est impliquée dans la sénescence et le vieillissement du cycle cellulaire des MSCs. Pour valider le rôle de la Cdc42 dans le vieillissement des ADSCs, j'ai décri dans ce chapitre des stratégies récentes visant l'inhibition de Cdc42 dans les cellules souches âgées par des molécules pharmacologiques.

Chapitre 9. **Introduction à la publication scientifique 2**

Nos données présentées dans le deuxième article scientifique intitulé**: *"ML141 reverses the negative impact of the RhoGTPase Cdc42-dependent donor's age on hepatogenic differentiation of hADSCs"*** ont été introduites suivi du papier scientifique.

Discussion et perspectives

Chapitre 10. Les résultats obtenus dans les deux parties ont été discutés dans ce chapitre. Ainsi, les deux stratégies étaient évaluées dans le contexte des nouvelles innovations et progrès au sein de la médecine régénérative à base de cellules souches du foie, de la pharmacotoxicologie à bases d'hépatocytes et des nouvelles stratégies dans la bio - imprimerie du foie.

CONCLUSION

En résumé, nous proposons une approche simple de reprogrammation des hépatocytes murins qui permette leur conversion vers des cellules souches hépatiques exprimant LGR5. Cette technologie pourra constituer une alternative idéale pour échapper au rejet immunologique lors d'une transplantation d'hépatocytes chez l'homme. En effet, l'expansion de ces progéniteurs Lgr5+ dérivées d'une petite biopsie de foie ouvre les horizons vers une banque de cellules souches hépatiques autologues pour servir les patients présentant des maladies du foie.

Dans une deuxième perspective nos résultats proposent le ML141 comme un nouvel outil pharmacologique ciblant le vieillissement des cellules souches mésenchymateuses humaines.L'inhibition de la Cdc42 par ML141 dans les ADSCs propose une nouvelle approche efficace pour :

(1) Rajeunir les mécanismes paracrines et moléculaires liés à l'âge avant l'utilisation des ADSCs humaines dérivées de donneurs âgés

(2) Diriger la différenciation hépatique des hADSCs qui pourra servir la thérapie cellulaire des maladies du foie à base de MSCs et les stratégies de bio-ingénierie du foie.