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Stage-specific changes in the Krebs cycle network regulate human erythroid differentiation

Manuela Romano

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie-Santé

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Unité de recherche Institut de Génétique Moléculaire de Montpellier (IGMM) – UMR 5535

Stage-specific changes in the Krebs cycle network regulate human erythroid differentiation

Présentée par Manuela ROMANO

Le 20 Décembre 2018

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UNIVERSITÉ
DE MONTPELLIER

Dedico questa tesi alle tre cose buone che me so riuscite int à vita...

...la mia famiglia con gli amici veri che ne fanno parte, per aver sempre creduto in me...

...Benoît, per il tuo amore, il tuo insostituibile sostegno e semplicemente la tua presenza...

...a tutta la strada che ho fatto per arrivare fino a qui!

E come diceva Massimo Troisi: "Ricomincio da tre"

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Régulation des stades d'érythropoïèse humaine par des modifications dans le cycle de Krebs

Résumé

Le processus conduisant à la prolifération et différenciation des cellules souches hématopoïétiques (CSH) en cellules de toutes les lignées sanguines s'appelle l'hématopoïèse. Bien que l'engagement des CSH soit régi par les cytokines, les facteurs de transcription, les modificateurs épigénétiques et la niche des CSH, notre groupe a constaté que leur engagement vers la lignée érythroïde dépendait aussi du métabolisme de la glutamine. La glutaminolyse contribue à la biosynthèse des nucléotides de novo ainsi qu'à la production de l'alpha-kétoglutarate (α KG), intermédiaire métabolique du cycle TCA (Oburoglu et al. 2014). Il est cependant important de noter que la différenciation érythroïde est un processus unique, où chaque cellule fille est structurellement et fonctionnellement différente de sa cellule mère. Chaque division définit un stade de différenciation précis avec un dernier cycle de division produisant un réticulocyte énucléé. Ainsi, nous avons émis l'hypothèse que les réseaux métaboliques mobilisés dans les progéniteurs érythroïdes changent en fonction du stade de différenciation et que ces réseaux régulent la transition des progéniteurs d'un stade à l'autre.

Au cours de ma thèse, j'ai caractérisé les états métaboliques associés aux différents stades de différenciation des progéniteurs érythroïdes. Nous avons ainsi montré qu'aux stades précoces de différenciation érythroïde, avant la différenciation terminale, les progéniteurs hématopoïétiques présentent une activité métabolique accrue avec un niveau de phosphorylation oxydative (OXPHOS) plus élevé. Ces données sont en corrélation avec l'augmentation de la génération de l' α KG à ces stades de différenciation. De plus, nous avons constaté une augmentation de l'OXPHOS de ces progéniteurs en présence d' α KG exogène. Cependant, la différenciation terminale des précurseurs érythroïdes, caractérisée par la perte de la masse mitochondriale et de leur potentiel membranaire, est associée à une diminution du niveau d'OXPHOS. Ainsi,

l'administration exogène d' α KG, a fortement atténué la différenciation érythroïde terminale et l'énucléation, sans affecter la différenciation des pro-érythroblastes. Inversement, un antagoniste de l' α KG (diméthylloxalylglycine, DMOG) n'a pas altéré la différenciation terminale ou l'énucléation, malgré l'abrogation de l'OXPPOS dans les érythroblastes.

Ces données suggèrent que la production d' α KG et sa contribution à l'OXPPOS perturbent l'énucléation des globules rouges. C'est pourquoi, dans le but de réduire les niveaux intracellulaires d' α KG, nous avons inhibé l'expression de l'isocitrate déshydrogénase I (IDH1), enzyme cytosolique catalysant la conversion de l'isocitrate en α KG. Cependant, comme IDH1 peut catalyser les réactions dans les deux sens, la diminution de son expression pourrait également augmenter les niveaux d' α KG. En effet, nous avons constaté que le knockdown d'IDH1 entraînait une forte atténuation de la différenciation terminale et de l'énucléation des précurseurs érythroïdes. Cet effet est probablement dû à un déséquilibre de la disponibilité des substrats ; ainsi l'administration ectopique de l' α KG ainsi que du citrate renforce l'altération de la différenciation terminale des précurseurs érythroïdes IDH1^{-/-} ainsi que leur énucléation. Cette étude identifie donc un rôle crucial pour le métabolite α KG dans la régulation de la fonction mitochondriale et de l'OXPPOS, processus qui sont une condition sine qua non pour la différenciation des précurseurs érythroïdes au stade proérythroblaste. Nous montrons en outre que la suppression d'OXPPOS et la catalyse d'intermédiaires du TCA, substrats d'IDH1, sont requis pour les phases terminales de la différenciation érythroïde et l'énucléation.

En conclusion, les résultats obtenus au cours de ma thèse mettent en évidence la nature dynamique des réseaux métaboliques qui régulent la progression des précurseurs érythroïdes tout au long des différents stades de la différenciation érythroïde.

Mots-clés : érythropoïèse, intermédiaires du cycle de Krebs, fonction mitochondriale, IDH1, alpha-ketoglutarate, énucléation

Stage-specific changes in the Krebs cycle network regulate human erythroid differentiation

Abstract

Hematopoiesis is the process whereby hematopoietic stem cells (HSCs) proliferate and differentiate to all blood cell lineages. While HSC commitment is known to be regulated by cytokines, transcription factors, epigenetic modifiers and the HSC niche, our group found that specification of HSCs to the red cell lineage is dependent on glutamine metabolism. Glutaminolysis contributes to de novo nucleotide biosynthesis and to the generation of the alpha-ketoglutarate (α KG) TCA cycle metabolite (Oburoglu et al. 2014). Importantly though, erythroid differentiation is a unique process as each daughter cell is structurally and functionally different from its parent cell. Each division defines a stage of differentiation with the final division cycle resulting in the production of an enucleated reticulocyte which further matures to a biconcave erythrocyte. Thus, we hypothesized that progenitor metabolic networks change as a function of the erythroid differentiation stage and moreover, that they regulate the transition of progenitors from one stage of differentiation to the next.

During my PhD, I assessed the metabolic alterations that occur as a function of the erythroid differentiation stage. We showed that at early stages of human red cell development, prior to terminal differentiation, hematopoietic progenitors exhibited an increased metabolic activity with a significantly higher level of oxidative phosphorylation (OXPHOS). This correlated with the increased generation of α KG and indeed, we found that ectopic α KG directly augmented OXPHOS in these progenitors. However, the terminal differentiation of erythroid precursors, characterized by the loss of mitochondrial mass and membrane potential, was associated with a decreased level of OXPHOS. Notably, ectopic α KG, which did not alter pro-erythroblast erythroid differentiation, severely attenuated terminal differentiation and enucleation. Conversely, an α KG antagonist (dimethyloxalyl glycine, DMOG) did not negatively impact on terminal differentiation or enucleation despite abrogating OXPHOS in erythroblasts.

These data suggested that the production of α KG and its subsequent contribution to oxidative phosphorylation perturb red cell enucleation. We therefore downregulated isocitrate dehydrogenase I (IDH1), the cytosolic enzyme that catalyzes the conversion of isocitrate to α KG, by an shRNA approach in an attempt to decrease α KG levels. However, because IDH1 can catalyze both the forward and reverse reactions, its downregulation could also increase α KG levels. Indeed, we found that IDH1 knockdown resulted in a severe attenuation of terminal erythroid differentiation and enucleation. This effect was likely due to an imbalance in substrate availability—both ectopic α KG as well as citrate further decreased polychromatic to orthochromatic erythroblast differentiation and the subsequent enucleation of IDH1-knockdown erythroid precursors. Thus, the present study identifies a crucial role for the α KG metabolite in regulating mitochondrial function and oxidative phosphorylation, processes that are a sine qua non for erythroid precursors at the pro-erythroblast stage. We further show that terminal erythroid differentiation and enucleation requires OXPHOS suppression and the IDH1-mediated enzymatic catalysis of its TCA substrates.

To conclude, the results generated during my PhD highlight the dynamic nature of the metabolic networks that regulate the progression of erythroid precursors through the distinct stages of erythroid differentiation.

Keywords: erythropoiesis, Krebs cycle intermediates, mitochondrial function, IDH1, alpha-ketoglutarate, enucleation

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Abbreviations

#

2-HG – 2-hydroxyglutarate
2,3-BPG – 2,3-bisphosphoglycerate
6PGDH – 6-phosphogluconate dehydrogenase
6PGL – 6-phosphogluconolactonase

A

α -KG – α -ketoglutarate
ACC – Acetyl-CoA carboxylase
ACL – ATP-citrate lyase
ACO – Aconitase
ACP – Acyl carrier protein
ACS – Acyl-coA synthetase
ACSBG – Bubbegum acyl-coA synthetase
ACSL – Long-chain acyl-coA synthetase
AGM – Aorta-gonad-mesonephros
AHA – Acute hemolytic anemia
ALA – δ -aminolevulinic acid
ALAS – δ -aminolevulinic acid synthase
AML – Acute myeloid leukemia
AMP – Adenosine monophosphate
Ang-1 – Angiopoietin-1
AQP1 – Aquaporin-1
ASCT - Alanine Serine Cysteine transporter
ATP – Adenosine triphosphate

B

β HB – β -hydroxybutyrate
bHLH – Basic helix-loop-helix
BFU-E – Burst forming unit-Erythroid
BMP – Bone morphogenetic protein
BrdU – 5'-bromo-2'-deoxyuridine

C

CACT – Carnitine/acyl-carnitine translocase
CAR – CXCL12-abundant reticular cell
CAR – Contractile actomyosin ring
CB – Cord blood
CCNA2 – Cyclin A2
CCND3 – Cyclin D3
CFA – Colony forming assay
CFSE – Carboxyfluorescein succinimidyl ester
CFU-E – Colony forming unit-Erythroid
CFU-S – Splenic colony forming unit
CLL – Chronic lymphocytic leukemia
CLP – Common lymphoid progenitor
CMP – Common myeloid progenitor
CoA – Coenzyme A
CoQ – Coenzyme Q
CPT1/2 – Carnitine palmitoyltransferase 1/2
CS – Citrate synthase
CXCL12 – C-X-C motif chemokine ligand 12
Cyt c – Cytochrome C

D

DA – Dorsal aorta
DBA – Diamond Blackfan anemia
DH – β -hydroxyacyl-ACP dehydratase
DHA – Dehydroascorbic acid
DMOG – Dimethyloxallylglycine
DMT1 – Divalent metal transporter 1
DNA – Deoxyribonucleic acid

E

eADA – Adenosine deaminase
EHT – Endothelial-to-hematopoietic transition

EKLF – Erythroid Kruppel-like factor
Emp – Erythroblast macrophage protein
EMP – Erythro-myeloid progenitors
ENO1 – Enolase
EPO – Erythropoietin
EPOR – Erythropoietin receptor
ER – enoyl-ACP reductase
Erk – Extracellular signal regulated kinase
EryP – Primitive erythroid progenitor
EryP-CFC – Primitive erythroid progenitor-colony forming capacity
ESC – Embryonic stem cell
ETC – Electron transport chain

F

FA – Fatty acid
FABP – Fatty acid-binding protein
FACS – Fluorescence activated cell sorting
FADH – Flavin adenine dinucleotide
FAO – Fatty acid oxidation
FAS – Fatty acid synthase
FAT – Fatty acid translocase
FATP – Fatty acid transport protein
FBS – Fetal bovine serum
FC – Ferrochelatase
FFA – Free fatty acid
FH – Fumarate hydratase
FLVCR – Feline leukemia virus C receptor
FOG-1 – Friend of GATA-1
FOXO – Forkhead transcription factor family
FSC – Forward side scatter

G

G6PDH – Glucose-6-phosphate dehydrogenase
GAPD – Glyceraldehyde-3-phosphate

dehydrogenase
GATA-1 – GATA-binding factor 1
GATA-2 – GATA-binding factor 2
GC – Glucocorticoids
Gdf15 – Growth and differentiation factor 15
GDH – Glutamate dehydrogenase
GFP – Green fluorescent protein
GLS – Glutaminase
Glut – Glucose transporter
GlyA – Glycophorin A
GMP – Granulocyte/macrophage progenitor
GOT – glutamate-oxaloacetate transaminase
GPI – Glucose-6-phosphate isomerase
GPL – Glycerophospholipid
GPT – glutamate-pyruvate transaminase
GSH – Reduced glutathione
GSL – Glycosphingolipids
GWAS – Genome-Wide Association Studies

H

Hb – Hemoglobin
HbA – Adult hemoglobin
HbF – Fetal hemoglobin
HDAC – Histone deacetylase
Hh – Hedgehog
HIF – Hypoxia-inducible factor
HK – Hexokinase
HMIT – H⁺/myo-inositol transporter
HSC – Hematopoietic stem cell
HSCT – Hematopoietic stem cell transplantation
HSPC – Hematopoietic stem progenitor cell

I

ICAM – Intercellular adhesion molecule
IDH – Isocitrate dehydrogenase

IGF-1 – Insulin like growth factor 1
IgSF – Immunoglobulin superfamily
IL – Interleukin
IMM – Inner mitochondrial membrane
iPS – Induced pluripotent stem cell

J

Jak2 – Janus kinase 2
JHDM – JmjC domain-containing histone demethylase
Jnk – Jun kinase

K

K - Lysine
KO – Knockout
KGDH – α -ketoglutarate dehydrogenase
KR – β -ketoacyl-ACP reductase
KS – β -ketoacyl-ACP synthase

L

LAT – Leucine-type amino acid transporter
LC3 – Microtubule-associated protein light chain 3
LCFA – Long-chain fatty acid
LDH – Lactate dehydrogenase
LepR – Leptin receptor
LKB1 – Liver kinase B1
LSK – Lin⁻Sca-1⁺c-Kit⁺
LSKT – Lin⁻Sca-1⁺c-Kit⁺Thy-1.1^{lo}
LT-HSC – Long-term HSC
Lu – Lutheran

M

MAPK – Mitogen-activated protein kinase
MAT – Malonyl/acetyl-CoA-ACP transacylase

MCFA – Medium-chain fatty acid
MCV – Mean Corpuscular Volume
MDH – Malate dehydrogenase
MDS – Myelodysplastic syndrome
ME – Malic enzyme
MEP – Megakaryocyte/Erythrocyte progenitor
MFN – Mitofusin
Mfn1 – Mitoferrin 1
MGG – May-Grünwald Giemsa
MPP – Multipotent progenitor
mTOR – Mammalian target of rapamycin
MTP – Mitochondrial trifunctional protein
MUFA – Monounsaturated fatty acid

N

NADH – Nicotinamide adenine dinucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
NEAA – Neutral essential amino acid
NES – Nestin
NFAT – Nuclear factor of activated T -cells
NG2 – Neural/glial antigen 2
NO – Nitric oxide
NOS – NO synthase
NRAMP1 – Natural resistance-associated macrophages protein 1

O

OMM – Outer mitochondrial membrane
OXPHOS – Oxidative phosphorylation

P

PB – Peripheral blood
PC – Phosphatidylcoline
PDH – Pyruvate dehydrogenase

PDK – Pyruvate dehydrogenase kinase
PDK1 – Phosphoinositide dependent kinase-1
PE – Phosphatidylethanolamine
PFK – Phosphofruktokinase
PGK – Phosphoglycerate kinase
PGM – Phosphoglycerate mutase
PHD – Prolyl hydroxylase domain
PHGDH – 3-phosphoglycerate dehydrogenase
PI – Phosphatidylinositol
PI3K – Phosphoinositide 3-kinase
PK – Pyruvate kinase
PKB – Protein kinase B
PLC- γ – phospholipase C gamma
PPAR – Peroxisome proliferator-activated receptor
PPi – Inorganic pyrophosphate
PPP – Pentose phosphate pathway
PROTO – Protoporphyrin IX
PS – Phosphatidylserine
PSAT1 – Phosphoserine aminotransferase 1
PTEN – Phosphatase and tensin homolog
PTPMT1 – PTEN-like mitochondrial phosphatase
PUFA – Polyunsaturated fatty acid

R

R – Arginine
Rb – Retinoblastoma protein
RBC – Red blood cell
RhAG – Rh-associated glycoprotein
RNA – Ribonucleic acid
ROS – Reactive oxygen species
RP – Ribosomal protein
RPE – ribulose-5-phosphate epimerase
RPI – ribose-5-phosphate isomerase

S

SCD – Sickle cell disease
SCF – Stem cell factor
SCFA – Short-chain fatty acid
SCL/TAL1 – stem cell leukemia/T-cell acute lymphoblastic leukemia 1
SCS – Succinyl-coA synthetase
SDF-1 α – Stromal derived factor-1 α
SDH – Succinate dehydrogenase
SEP – Stress erythroid progenitors
SGLT – Na⁺/glucose linked transporter
SH2 – Src homology 2
shRNA – Short hairpin RNA
SLAM – Signaling lymphocyte activation molecule
SLC – Solute carrier
SM – Sphingomyelin
SNARE – Soluble NSF attachment protein receptor
SSO – Sulfo-*N*-succinidyl-oleate
STAT – Signal transducer and activator of transcription
ST-HSC – Short-term HSC

T

TAG – Triacylglycerol
TAL – Transaldolase
TCA – Tricarboxylic acid cycle
TE – Thioesterase
TET – Ten-elven translocation
TfR1 – Transferrin receptor
TGF- β – Transforming growth factor- β
TKL – Transketolase
TPI – Triose phosphate isomerase
TPO – Thrombopoietin

U

UROGEN – Uroporphyrinogen III

V

VCAM – Vascular cell adhesion molecule

VE – Vascular endothelial

VHL – Von Hippel-Lindau

VLA – Very late antigen

VLCFA – Very-long-chain fatty acid

W

WT – Wild type

Y

YS – Yolk sac

Introduction

1 Hematopoietic Stem Cells (HSCs) and Hematopoiesis

1.1 The concept of HSC

Our current understanding of a hematopoietic stem cell (HSC) is the result of at least one century of studies aiming to firstly identify a stem cell and to characterize the function of a HSC. In the second half of the 19th century, the German biologist Ernst Hackel provided a double definition of a stem cell, as “*the unicellular ancestor of all multicellular organisms*” and “*the fertilized egg that gives rise to all cells of the organism*” (Hackel, 1868). While Paul Ehrlich developed a staining procedure allowing the characterization of the different white blood cells (Ehrlich, 1879), Maximow raised the question of whether the hematopoietic system was based on a unique common precursor, able to generate all these cell types (Maximow, 1909). Almost 50 years later, James Till and Ernest McCulloch, amongst others, strongly contributed to the current definition of a HSC as a cell with two fundamental properties: the capacity of self-renewal and to generate all mature blood cells (Till and McCulloch, 1961; Becker *et al.*, 1963; Till *et al.*, 1964). Any stem cell-enriched population should fulfill these two criteria of self-renewal and differentiation (Weissman and Shizuru, 2008), which have allowed their characterization in several additional tissues, such as the skin, the intestine and the central nervous system, amongst others (reviewed in Ramalho-Santos and Willenbring, 2007; Pouzolles *et al.*, 2016).

1.2 HSC origins: from embryo to adult

During development, the process of blood cell production, also known as hematopoiesis, occurs in three waves, one primitive and two definitive, which are distinguished by the origins and the differentiation potential of the hematopoietic progenitors involved (Galloway and Zon, 2003; Medvinsky *et al.*, 2011; reviewed in Ivanovs *et al.*, 2017). The primitive wave, occurring during early embryonic development, mainly produces red blood cells (RBCs), which support the high tissue oxygenation requirements of a rapidly growing embryo (Palis and Yoder, 2001; Orkin and

Zon, 2008; Palis, 2016a) (see section 2.1.1). These embryonic hematopoietic progenitors are not pluripotent and are not capable of self-renewal, likely accounting for the transient nature of primitive hematopoiesis. On the other hand, later in the embryonic development, definitive hematopoiesis leads to the generation of pluripotent HSCs, able to differentiate into all blood cell lineages throughout the life of the organism (Jagannathan-Bogdan and Zon, 2013) (see section 1.4). In this section, I will discuss our current knowledge on the origins of hematopoietic stem and progenitor cells, as well as their differentiation dynamics during development.

1.2.1 Extra- and intra-embryonic hematopoietic progenitors

In the early murine embryo (before E10.0), it has been shown that the primary site of hematopoiesis is the yolk sac (Lux *et al.*, 2008) (**Figure 1**). In fact, in this extra-embryonic structure, macrophages, megakaryocytes and erythroid cells originate together with endothelial cells from the so called blood islands (Sabin, 1920; Maximow, 1924; Moore and Metcalf, 1970) (see section 2.1.1), before the onset of the blood circulation (Jagannathan-Bogdan and Zon, 2013). Interestingly, expression of the Runx1 transcription factor, which is normally associated with hematopoietic programs, has been detected in the yolk sac at E7.5 (Zeigler *et al.*, 2006). In fact, at this developmental stage, a first wave has been shown to originate from the yolk sac, giving rise to the first primitive erythroid and macrophage progenitors (Palis *et al.*, 1999; Palis, 2016a). Nevertheless, in accordance with the transient nature of the primitive wave, extra-embryonic hematopoiesis in the yolk sac only temporarily provides cells with hematopoietic potential (Yamane *et al.*, 2013; Yamane, 2018). In contrast, hematopoietic cells with long-term reconstitution ability have been found in the intra-embryonic mesoderm aortic region of chimeric chicken-quail (Dieterlen-Lievre, 1984) and murine embryos (Dieterlen-Lievre, 1984; Medvinsky and Dzierzak, 1996). Indeed, aorta-gonad mesonephros (AGM)-derived cells exhibit more efficient reconstitution capacity than yolk sac-derived cells following transplantation into lethally irradiated mice. Therefore, extra-embryonic yolk sac-derived cells are fundamental in early development, transiently providing large numbers of erythroid precursors, while AGM cells recapitulate features of definitive HSCs (Medvinsky *et al.*, 1993). Indeed, adult

multipotent HSCs are likely generated from this intra-embryonic region (Matsuoka *et al.*, 2001) (**Figure 1**). Interestingly, GATA2 transcription factor has been recently shown to play an important role in this process, being required for the generation not only of intra-aortic hematopoietic progenitors, but also of HSCs during development (de Pater *et al.*, 2013; Gao *et al.*, 2013; Eich *et al.*, 2018).

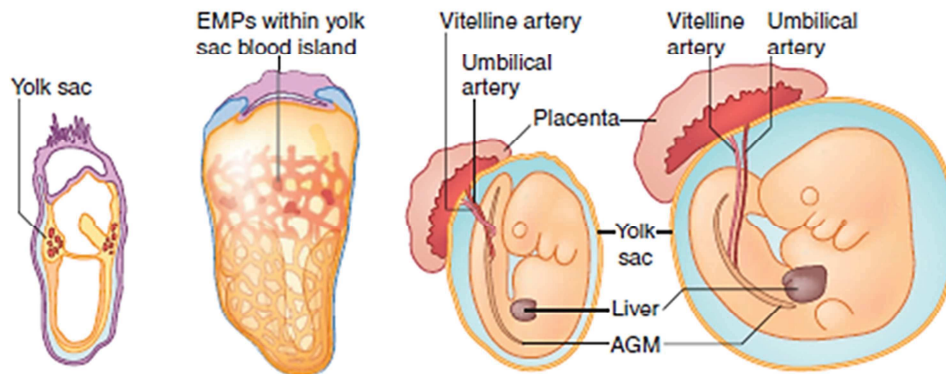


Figure 1. Extra- and intra-embryonic structures involved in hematopoiesis during murine development.

The extra-embryonic yolk sac represents the first site of embryonic hematopoiesis, and the first erythromyeloid progenitors (EMPs) are generated into the blood islands. The two right panels show the localization in the mouse conceptus of the main extra-embryonic hematopoietic sites, such as the umbilical and the vitelline arteries, as well as the placenta. AGM-derived definitive hematopoietic progenitors colonize the fetal liver, which represents the first organ supporting definitive hematopoiesis. Adapted from (Yoder, 2014)

Mammalian vitelline and umbilical arteries link the dorsal aorta (DA) to the yolk sac and the placenta, respectively (Costa *et al.*, 2012). Hematopoietic cells have been shown to reside in both these arteries, contributing to stem cell potential during development (De Bruijn *et al.*, 2000). It is well established that the murine umbilical artery is generated by vasculogenesis in the allantois, and allantois fusion with the chorionic plate generates the placenta (Costa *et al.*, 2012), recently identified as another extra-embryonic hematopoietic organ (Bárcena *et al.*, 2009; Robin *et al.*, 2009). In fact, murine placenta-derived multilineage precursors have been characterized *in vitro* (Alvarez-Silva *et al.*, 2003) and their hematopoietic reconstitution capacity has been then confirmed by transplantation into irradiated recipient mice (Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005).

Further details are here provided regarding the so called process of endothelial-to-hematopoietic transition (EHT) (Slukvin, 2016; Guibentif *et al.*, 2017), allowing HSC formation from the hemogenic endothelium in the dorsal aorta (DA) of the AGM region (Medvinsky and Dzierzak, 1996; Bertrand *et al.*, 2010; Boisset *et al.*, 2010).

1.2.2 Cellular origins of hematopoietic progenitors

The intra-embryonic origins of HSCs have been the object of extensive research over the last decades, leading to several controversial models. First, as blood islands in the yolk sac are able to generate both blood and endothelial cells, the existence of a common mesodermal precursor has been hypothesized. A transient and bi-potent progenitor, called the hemangioblast (Sabin, 1920; Maximow, 1924), has in fact been identified in the posterior primitive streak of the yolk sac mesoderm in mouse (Huber *et al.*, 2004), as well as in zebrafish (Vogeli *et al.*, 2006) and drosophila (Mandal *et al.*, 2004) embryos. Second, as hematopoietic cells are found in close contact with intra-embryonic vessels, and more specifically the dorsal aorta, it was postulated the existence of a “hemogenic endothelium” (Jordan, 1917), constituted of specialized endothelial structures able to generate hematopoietic cells (Dantschakoff, 1909; Jordan, 1917). Notably, approximately 100 years later, single-cell tracking experiments revealed the potential of adherent vascular endothelial (VE) cells to give rise to blood cells (Eilken *et al.*, 2009). Similarly, in transgenic zebrafish embryos, adherent endothelial cells in the aorta vascular bed can convert into round-shaped budding cells expressing hematopoietic markers (Bertrand *et al.*, 2010; Kissa and Herbomel, 2010; Lam *et al.*, 2010). In addition, hemangioblast-like cells with an adherent phenotype can differentiate into round, non-adherent cells *ex vivo*; this process is associated with the loss of endothelial and a gain of hematopoietic markers. Taken together, these studies reveal the potential of the hemangioblast to give rise to hematopoietic progenitors through an intermediate population, represented by the hemogenic endothelium (Eilken *et al.*, 2009; Lancrin *et al.*, 2009). Indeed, the hemogenic endothelium in the AGM has been identified as the site where definitive hematopoietic stem and progenitor cells originate (Chotinantakul and Leeanansaksiri, 2012; K.-D. Choi *et al.*, 2012; Ditadi *et al.*, 2015).

1.2.3 Colonization of fetal liver and bone marrow

The last step in the ontogenesis of definitive hematopoiesis is the migration of hematopoietic progenitors to the definitive hematopoietic organs. These progenitors first colonize an intermediate niche, represented in mammals by the fetal liver and to lesser extent the placenta (Zanjani *et al.*, 1993; Gekas *et al.*, 2005; Mikkola and Orkin, 2006) (**Figure 1**), to then migrate to their final niche in the bone marrow at the end of prenatal life (Zanjani *et al.*, 1993; Mikkola and Orkin, 2006). Therefore, the first organ in which definitive adult-type hematopoiesis occurs is the fetal liver, colonized around E10-E11 (Johnson and Moore, 1975; Houssaint, 1981; Zovein *et al.*, 2010; Zhou *et al.*, 2013) by hematopoietic precursors derived from intra- and extra-embryonic structures (Kieusseian *et al.*, 2012). In fact, after the previously mentioned first wave of yolk-sac-derived primitive progenitors, a second wave of bi- and multi-potent precursors (Böiers *et al.*, 2013; Lin *et al.*, 2014; Palis, 2016a), including erythro-myeloid progenitors (EMPs), originates from the yolk sac at E8.25, and colonize the fetal liver around E10 (Chen *et al.*, 2011; McGrath *et al.*, 2015). This wave is included in the definitive hematopoiesis, together with a third wave of definitive HSCs seeding the fetal liver around E10.5 and being mainly generated from the AGM region (reviewed in Yoder, 2014). Nevertheless, as the absolute number of fetal liver HSCs is not likely to be attained by the sole colonization from the AGM, it has been proposed that these cells also derive from the placenta, umbilical, vitelline and cranial arteries, as well as the yolk sac (Morrison *et al.*, 1995; Kumaravelu *et al.*, 2002; Samokhvalov, 2014; reviewed in Yoder, 2014).

The first HSCs with long-term reconstitution capacity (LT-HSCs) start to be detected in the fetal liver at E11.5, and together with liver intensive growth and progressive vascularization, the HSC pool is significantly expanded (Tavassoli, 1991; Kumaravelu *et al.*, 2002). In fact, in contrast to the quiescent adult counterpart, the amount of fetal liver HSCs increases of 300 to 500 times, between E11.5 and E15.5 (Wang *et al.*, 2018). Interestingly, it has been shown that Nestin⁺NG2⁺ pericytes associated with portal vessels play a fundamental role as fetal liver niche, supporting this intensive proliferation process (reviewed in Cabezas-Wallscheid and Trumpp, 2016; Khan *et al.*, 2016). Contrarily, lack of phosphoinositide dependent kinase-1 (PDK1) significantly

impairs HSC expansion in the fetal liver, because of Akt/FOXO-dependent induction of apoptosis, as well as alterations in cell cycle (Wang *et al.*, 2018). Finally, at the time of birth, corresponding to murine E16.5 to E17.5, HSCs initiate the colonization of the bone marrow (Zanjani *et al.*, 1993; Christensen *et al.*, 2004; Ciriza *et al.*, 2013; Coşkun *et al.*, 2014), leading to the generation of a small stem cell pool, which supports steady-state hematopoiesis throughout adulthood (Godin and Cumano, 2005).

1.3 HSC characterization

The first characterization of murine hematopoietic stem cells, with both self-renewal and differentiation capacity, was derived from transplantation studies in irradiated mice. These studies showed that, following lethal total-body irradiation, intravenously injected donor bone marrow-derived cells were able to generate splenic colony forming units (CFU-S) (Till and McCulloch, 1961). The cells composing these discrete colonies were not only able to proliferate, but also to differentiate into erythrocytic, granulocytic and megakaryocytic lineages (Till and McCulloch, 1961). In addition, according to their proliferation profile, CFU-S were found to be proportional to the number of injected bone marrow cells, therefore suggesting a clonal origin (Till and McCulloch, 1961). This hypothesis was confirmed by injecting irradiated donor bone marrow-derived cells; the presence of specific irradiation-induced aberrations was found in all cells of a given colony, confirming CFU-S origin from a single cell (Becker *et al.*, 1963). The potential of HSCs to undergo both self-renewal and differentiation has resulted in their utilization for the treatment of several blood-related diseases (Daley *et al.*, 2003; Copelan, 2006; Appelbaum, 2007). Thus, it is important to be able to precisely isolate the HSC subsets and a panel of markers expressed on the hematopoietic stem cell surface is now available. However, ongoing research is aimed at developing efficient *ex vivo* systems for the amplification of these extremely rare cell populations.

1.3.1 Long-term HSC (LT-HSC) and short-term HSC (ST-HSC)

Historically, HSCs have been separated into subpopulations of long-term hematopoietic stem cells (LT-HSCs) and short-term hematopoietic stem cells (ST-HSCs), according to their self-renewal potential and bone marrow reconstitution capacity, after

transplantation into lethally irradiated mice (Purton and Scadden, 2007). LT-HSCs represent the most immature progenitors, mainly composed of quiescent cells (Morrison *et al.*, 1997) carrying long-term repopulation capacity, and therefore the ability of a lifetime-lasting production of mature blood cells (Christensen and Weissman, 2001). On the other hand, the ST-HSC subset has been shown to harbor transient reconstitution capacity (8-12 weeks) (Christensen and Weissman, 2001) and to differentiate into multipotent progenitors (MPPs), which lack of self-renewal potential and exhibit higher percentage of cycling cells (Morrison *et al.*, 1997).

1.3.2 Mouse versus human hematopoietic stem and progenitor cells

The above mentioned progenitor populations have been firstly characterized in the mouse bone marrow (Uchida and Weissman, 1992; Morrison *et al.*, 1997), as a lineage in which LT-HSCs give rise to ST-HSCs, subsequently generating MPPs (Morrison *et al.*, 1997). Several studies have led to the definition of murine HSCs as cells lacking lineage (Lin⁻), while expressing Sca-1 and c-kit surface markers (Lin^{-/lo}Sca-1⁺c-kit⁺Thy-1.1^{lo} (LSKT population) (Christensen and Weissman, 2001). In addition, it was found that LT- and ST-HSC subsets were discriminated by the level of expression of the receptor tyrosine kinase Flk-2 (CD135, also known as FLT-3). Consequently, three main populations have been phenotypically characterized in the mouse adult bone marrow, Flk-2⁻Thy-1.1^{lo} LSK (LT-HSCs), Flk-2⁺Thy-1.1^{lo} LSK (ST-HSCs), and Flk-2⁺Thy-1.1⁻ LSK (MPPs) (Christensen and Weissman, 2001). To complete this phenotypic characterization of murine HSCs, other surface markers have been more recently proposed. Indeed, it has been not only shown that 10% of the LSK population (~0.005 % of the bone marrow cells) results CD34⁻ (Osawa *et al.*, 1996; Tian and Zhang, 2016), but also that differential expression of several members of the signaling lymphocyte activation molecule (SLAM) family distinguishes between the murine HSC subsets (**Figure 2**) (Kiel *et al.*, 2005, 2008; Morita *et al.*, 2010; Oguro *et al.*, 2013).

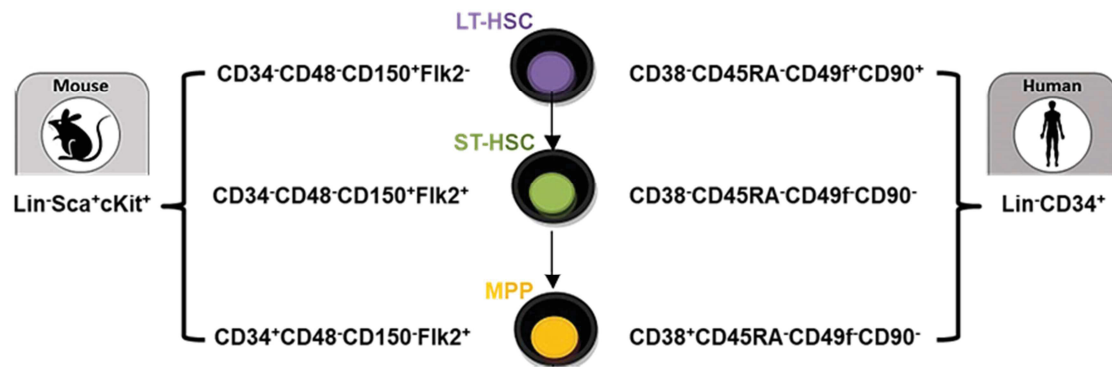


Figure 2. Phenotypic characterization of murine and human hematopoietic stem and progenitor cells. Surface marker expression distinguishing the different subsets of long-term hematopoietic stem cells (LT-HSC), short-term hematopoietic stem cells (ST-HSC) and multipotent progenitors (MPP) in mouse (left) and human (right) bone marrow. *Adapted from* (Doulatov et al., 2012)

SLAM receptors and the majority of the above presented molecules are not included in the phenotypic characterization of human hematopoietic stem and progenitor cells. The latter have been firstly selected as a function of the CD34 surface marker expression, which was shown to identify 1-5% of the bone marrow hematopoietic cells (Civin *et al.*, 1984). Nevertheless, the CD34⁺ population has resulted significantly heterogeneous; therefore, the characterization of these precursors has been improved by the introduction of additional markers, including CD45RA, CD90 (Thy1), and CD38. Consequently, human HSCs with long-term multilineage reconstitution potential are actually described as Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺ cells (Baum *et al.*, 1992; Tian and Zhang, 2016). Conversely, their subsequent differentiation has been associated with reduced CD34 expression, while CD45RA and CD38 upregulation (McKenzie *et al.*, 2007; Doulatov *et al.*, 2012). More recently, another HSC-specific marker has been identified in the CD49f integrin molecule. Long-term multilineage reconstitution occurs upon engraftment of single CD49f⁺ cells, which lose CD49f expression with concomitant progression to the MPP stage (Notta *et al.*, 2011) (**Figure 2**).

1.4 Hematopoiesis

Hematopoiesis is the process through which pluripotent HSCs differentiate into all mature blood cells: starting from the rare subset of LT-HSCs, differentiation occurs through lineage-restricted progenitor stages, showing decreasing self-renewal potential

and increased proliferation (Doulatov *et al.*, 2012). In fact, the hematopoiesis process is significantly dynamic, as able to provide more than 300 billion of differentiated cells per day (Notta *et al.*, 2016). Therefore, this process is tightly regulated in order to allow the maintenance of a quiescent reservoir of HSCs, as well as their differentiation (Rossi *et al.*, 2012). Further details are here below provided regarding the models describing the hematopoiesis hierarchy, as well as the current open debate concerning their validation.

1.4.1 Classical model

Population-based studies, through fluorescence activated cell sorting (FACS) and *in vitro* methylcellulose colony forming assay (CFA), led to the definition of the first hematopoiesis model, also called the canonical hematopoietic tree (Kondo *et al.*, 1997; Akashi *et al.*, 2000; Reya *et al.*, 2001) (**Figure 3**). According to this model, hematopoiesis is a clonal process in which the reconstitution of a complete hematopoietic system is achieved by a single pluripotent HSC, heading the tree (Reya *et al.*, 2001; Patel and Radia, 2017). In fact, through the stepwise differentiation of HSCs into progressively lineage-restricted multi-, oligo- and then uni-potent progenitors, all the mature blood cell populations are produced (Orkin and Zon, 2008; Perié and Duffy, 2016). More precisely, HSCs are able to self-renew and to differentiate into multipotent progenitors (MPPs), the latter generating the two first committed precursors, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) (Kondo *et al.*, 1997; Akashi *et al.*, 2000; Patel and Radia, 2017). Oligopotent CMPs differentiate into bivalent precursors (MEPs and GMPs), which produce platelets/erythrocytes and granulocytes/macrophages (Metcalf and Burgess, 1982; Perié and Duffy, 2016); while mature lymphoid cells, including T and B lymphocytes, originate from CLPs (Orkin and Zon, 2008; Perié and Duffy, 2016).

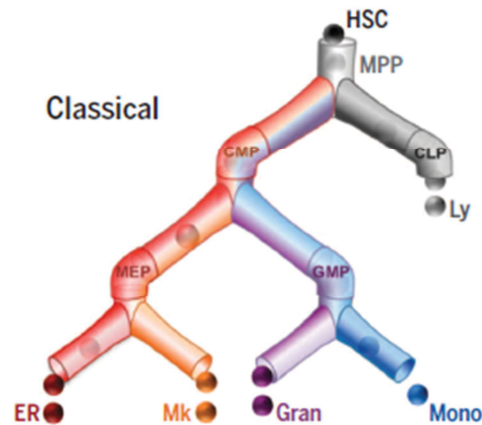


Figure 3. Classical «hematopoiesis tree». In the classical hematopoiesis model HSCs are capable of differentiating into multipotent progenitors (MPPs), which further generate two committed progenitors, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). CMPs then produce megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs), which give rise to mature erythrocytes (ER), platelets (Mk), granulocytes (Gran) and monocyte/macrophages (Mono). CLP differentiation generates different subsets of mature lymphoid cells (Ly). *Adapted from* (Notta et al., 2016)

The above described classical hematopoietic tree provided a reference model of the hematopoiesis process over many years. However, the population-based studies that led to the establishment of this model have recently been questioned, because of their technical limitation in terms of amount of parameters assessed. For instance, flow cytometry allows the detection of a restrained quantity of cell surface markers, compared to the large number of cell features evaluated through the novel single cell-based studies (Pei *et al.*, 2017; Raj *et al.*, 2018; Spanjaard *et al.*, 2018; Wagner *et al.*, 2018). In addition, while methylcellulose colony forming assays allowed the isolation of so-called CMPs and CLPs (Galy *et al.*, 1995; Manz *et al.*, 2002), it was difficult to obtain consistent results for more differentiated bi- or uni-potent precursors (Notta *et al.*, 2016). Therefore, the classical model includes only a limited number of phenotypically identifiable progenitor stages, and it also precludes eventual trans- or dedifferentiation potential (Perié and Duffy, 2016). Contrarily, a current pairwise model of hematopoiesis shows close vicinity between the differentiating programs of several clusters, which would eventually allow their interchange (reviewed in Brown *et al.*, 2018). It is likely that the disparities between different studies are due to an actual functional heterogeneity within subsets that were isolated as phenotypically homogenous, on the basis of specific

surface markers (Zhang *et al.*, 2018). Indeed, single cell-based analyses have allowed the characterization of previously undefined subpopulations (Notta *et al.*, 2016), leading to the redefined hematopoiesis model presented here below.

1.4.2 Single cell-based model

Single cell-based histone modification mapping and *in vitro* colony assays (Paul *et al.*, 2015; Notta *et al.*, 2016) have recently shown that the previously described “homogeneous” population of CMPs is in fact significantly heterogeneous, as composed of several subsets of unilineage myeloid, erythroid and megakaryocytic progenitors (Notta *et al.*, 2016). Similarly, the bivalent MEPs actually include a majority of unipotent erythroid precursors, and to lesser extent megakaryocyte-restricted subsets (Psaila *et al.*, 2016; Miyawaki *et al.*, 2017). Conversely, cells carrying concomitant megakaryocytic/erythroid potential or only megakaryocyte-restricted have been shown to directly originate from the HSC compartment, instead of being generated from HSC-derived oligopotent precursors (Yamamoto *et al.*, 2013; Sanada *et al.*, 2016; reviewed in Woolthuis and Park, 2016). Similarly, several transcriptional analyses of single murine hematopoietic cells have found that even the myeloid differentiation would directly occur through unipotent progenitors, without requiring an intermediate CMP stage (Pronk *et al.*, 2007; Pina *et al.*, 2012; Sanjuan-Pla *et al.*, 2013). Therefore, all these recent findings have clearly challenged the classical hematopoietic tree and led to the characterization of a “two-tier” hierarchy model, in which the first branch-point is represented by the megakaryocyte/erythrocyte lineage (Notta *et al.*, 2016; Ye *et al.*, 2017; Rodriguez-Fraticelli *et al.*, 2018) (**Figure 4**). In addition, while in the fetal liver multipotent precursors, harboring myeloid/erythroid/megakaryocytic and megakaryocytic/erythroid potential, populate the progenitor compartment, the latter includes unipotent precursors in the adult bone marrow (Notta *et al.*, 2016) (**Figure 4**).

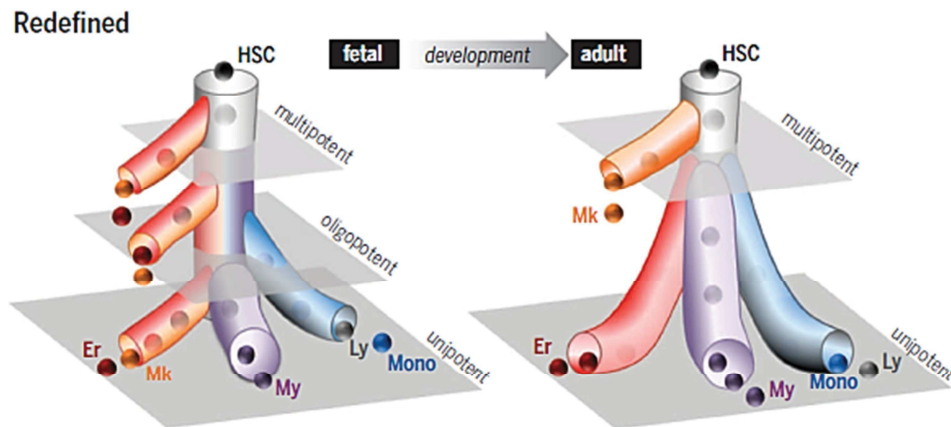


Figure 4. Redefined model of fetal and adult hematopoiesis. The “two-tier” hierarchy model shows the presence of several multipotent progenitors during hematopoiesis in the fetal liver. Contrarily, in the adult the megakaryocyte lineage (Mk) directly branches from the HSC compartment, while the other lineages are generated by dedicated unipotent intermediate precursors. *Adapted from* (Notta et al., 2016)

This redefined model suggests that certain lineage specifications occur before the “classically” defined MPP branching (Pietras *et al.*, 2015), following a differentiation continuum process (Macaulay *et al.*, 2016; Velten *et al.*, 2017; Buenrostro *et al.*, 2018; Dahlin *et al.*, 2018; Karamitros *et al.*, 2018; Laurenti and Göttgens, 2018) (**Figure 5**), which better recapitulates the features of steady-state hematopoiesis (Haas *et al.*, 2018). This hypothesis is also supported by the heterogeneity described in the reconstitution and self-renewal capacity of murine HSCs, upon single cell transplantation into recipient mice (Müller-Sieburg *et al.*, 2002, 2004; Dykstra *et al.*, 2007; Morita *et al.*, 2010; Yamamoto *et al.*, 2013). For instance, lineage “biases” have been observed, with each transplanted HSC able to potentially reconstitute all the mature blood cell types, but preferentially generating one or more specific lineages (Sanjuan-Pla *et al.*, 2013; Yamamoto *et al.*, 2013; Carrelha *et al.*, 2018). Although these data suggest that the HSC subset is functionally heterogeneous (Copley *et al.*, 2012; Crisan and Dzierzak, 2016; Lauridsen *et al.*, 2018), it has to be considered that the characterization of the HSC reconstitution potential through transplantation experiments does not recapitulate a steady-state condition (Haas *et al.*, 2018; Laurenti and Göttgens, 2018).

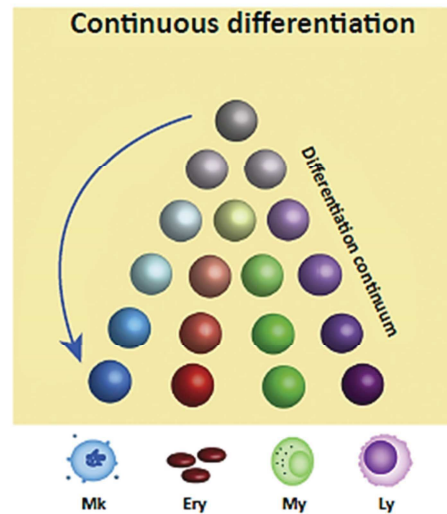


Figure 5. Differentiation continuum of steady-state hematopoiesis. The stepwise differentiation process of HSCs through several intermediate lineage-restricted progenitors has been recently replaced by a «differentiation continuum» representation of steady-state hematopoiesis, in which unipotent progenitors, directly originating from the HSC compartment, generate the different mature blood cell populations. *Adapted from* (Zhang et al., 2018)

To conclude, further investigation is needed as several aspects of the hematopoiesis process still need to be clarified. Indeed, it remains to be defined the number, as well as the requirement of cell divisions for an HSC to become a committed progenitor, as recent work infers a division-independent cell fate “choice” (Brown, Ceredig and Tsapogas, 2018; Grinenko *et al.*, 2018). In addition, it is important to note that also single cell studies show some technical limitations, as some of them involve an *ex vivo* manipulation that could eventually alter the cell differentiation potential. Moreover, these methods provide only a snapshot description of the hematopoiesis process, which is significantly dynamic (Perié and Duffy, 2016) and also exhibits different features between steady-state and stress conditions (Busch *et al.*, 2015; Schoedel *et al.*, 2016; Chapple *et al.*, 2018). Therefore, the possibility of a definitive revision of the classical hematopoietic tree is still on open debate (Pei *et al.*, 2017; Höfer and Rodewald, 2018).

1.5 Regulation of HSC maintenance and commitment

The regulation of HSC quiescence, self-renewal and differentiation involves different mechanisms and factors, acting in the microenvironment of the bone marrow niche. In this section I will provide a general overview of the characterization and modulation of HSC quiescence, as well as of the composition of the bone marrow niche. Some of the most important mediators, such as cytokines (SCF, TPO, SDF-1, Flt3/Flk2 ligand, IL-3), developmental regulators (BMP-4, Tie2/Angiopoietin-1, Notch/Jagged 1, Wnt/ β -catenin, Hedgehog) and transcription factors (SCL, RUNX1, LMO2, GATA2) involved in the regulation of HSC self-renewal and commitment are introduced below (see section 1.5.3), and some of them will be further described in the context of the erythroid differentiation process (see section 2.3). Finally, the metabolism-dependent mechanisms modulating HSC maintenance and differentiation, especially towards the erythroid lineage, will be presented after a general introduction in the following chapters of the erythropoiesis (Chapter 2) and of the main cell metabolic pathways (Chapter 3).

1.5.1 HSC quiescence

Different methods have been implied to assess the number of quiescent HSCs in the bone marrow, providing some controversial results. Indeed, concomitant staining with a DNA-binding dye (Hoechst 33342) and an anti-Ki-67 antibody (proliferating cell marker) found 70% of mouse HSCs in a G0 state (Wilson *et al.*, 2008). On the other hand, only 30% of HSCs have been described as “dormant” (Wilson *et al.*, 2008), according to their capacity to retain BrdU (5'-bromo-2'-deoxyuridine) DNA analog (Fleming *et al.*, 1993; Goodell *et al.*, 1996; Cheshier *et al.*, 1999; Zhang *et al.*, 2003). Similar results have been obtained by measuring the incorporation into nucleosomes of a histone H2B fusion protein with GFP (H2B-GFP) in cycling cells (Kanda *et al.*, 1998). On the other hand, upon single-cell transplantation into non-irradiated mice, steady-state HSC division capacity has been assessed through CFSE (carboxyfluorescein succinimidyl ester) staining (Takizawa *et al.*, 2011), finding an average of 39 days/division.

The maintenance of HSC quiescence is tightly controlled by several transcription factors (SATB1, PU.1, C/EBPa) and cell cycle regulators (E2F1, CDK1, amongst others), as well as different extracellular stimuli (reviewed in Nakamura-Ishizu *et al.*, 2014), such as TGF- β (Jacobsen *et al.*, 1991). HSC quiescence has been shown to be fundamental for the

maintenance of an undifferentiated state (Weissman *et al.*, 2001; Visvader and Clevers, 2016), as demonstrated by the induction of cell proliferation and subsequent HSC exhaustion upon deletion of quiescence-related genes (Trumpp *et al.*, 2010; Pietras *et al.*, 2011; Ito *et al.*, 2012; Nakamura-Ishizu *et al.*, 2014). In addition, HSC quiescence has been found to influence their reconstitution capacity (Suda, Suda and Ogawa, 1983; Morrison and Weissman, 1994; Ema *et al.*, 2005), as defects in HSC repopulation potential have been described upon genetic alterations, leading to loss of quiescence and intensive proliferation (Rossi *et al.*, 2012). Interestingly, the variability observed in the reconstitution capacity (Yamamoto *et al.*, 2013; Ema *et al.*, 2014), according to the HSC classification into long- and short-term subsets, has been correlated to differences in the quiescence properties of these populations (reviewed in Nakamura-Ishizu *et al.*, 2014).

1.5.2 Bone marrow niche

During development, as well as throughout adulthood, HSCs reside in a specific microenvironment called the “niche”, allowing maintenance of the HSC pool and lineage commitment (Nagasawa *et al.*, 2011). The niche is composed of several factors and diverse cell types that provide the environmental signals modulating the equilibrium between survival, self-renewal and differentiation of the HSCs (Chotinantakul and Leraanansaksiri, 2012). The bone marrow niche has been proposed to be divided into the vascular niche and the endosteal niche, with the latter composed of osteoblasts (Chotinantakul and Leraanansaksiri, 2012). Indeed, several studies have highlighted an important crosstalk between osteogenesis or osteoblast function and hematopoiesis, which allows the osteo-progenitor population to support the maintenance of the LT-HSC subset in its quiescent/slow-cycling state (Calvi *et al.*, 2003; Stier *et al.*, 2005; Taichman, 2005; Raaijmakers *et al.*, 2010). Nevertheless, only few HSCs have been actually localized to the bone marrow endosteum; therefore, it has been speculated that the vascular niche is also involved in the regulation of HSC maintenance (Kiel *et al.*, 2007). In accordance, the majority of HSCs in the bone marrow has been localized close to sinusoids, which are characterized by a fenestrated endothelium allowing more efficient exchange of cells and molecules with the bloodstream. Interestingly, in time-lapse microscopy studies of transplantation experiments, committed progenitors were found

to be distant from the endosteal bone surface, unlike the LT-HSC subset (Lo Celso *et al.*, 2009; Xie *et al.*, 2009). Therefore, the vascular niche may represent a transition site (Purton and Scadden, 2008), where differentiated cells find an easy access to the bloodstream.

Among the cell types constituting the niche, bone marrow stromal cells, macrophages, osteoclasts and T cells (Winkler *et al.*, 2010; Chow *et al.*, 2011; Fujisaki *et al.*, 2011) populate the bone marrow extracellular matrix, in which are also dispersed several insoluble and soluble molecular factors. The latter include cytokines and growth factors, nerve transmitters, hormones, as well as nutrients and specific metabolites (Huang and Zhu, 2012). For instance, it has been shown that bone marrow endothelial cells and adipocytes are involved in the secretion of SCF (Ding *et al.*, 2012; Zhou *et al.*, 2017; Xu *et al.*, 2018). On the other hand, the three main mesenchymal cell populations characterized in the bone marrow niche play an important role in the niche and HSC maintenance, as well as in HSC homing (reviewed in Crane *et al.*, 2017; Wei and Frenette, 2018). Interestingly, these populations initially defined as CXCL12 abundant reticular cells (CAR) (Sugiyama *et al.*, 2006; Ding and Morrison, 2013), mesenchymal stem cells expressing the intermediate filament protein nestin (NES⁺ MSCs) (Méndez-Ferrer *et al.*, 2010; Bydlowski *et al.*, 2013; Asada *et al.*, 2017) and Leptin receptor expressing cells (LepR⁺) have been recently shown to share related functions, as well as an overlapping phenotype (reviewed in Boulais and Frenette, 2015; Galán-Díez and Kousteni, 2018; Seike *et al.*, 2018; reviewed in Sugiyama *et al.*, 2018) (**Figure 6**).

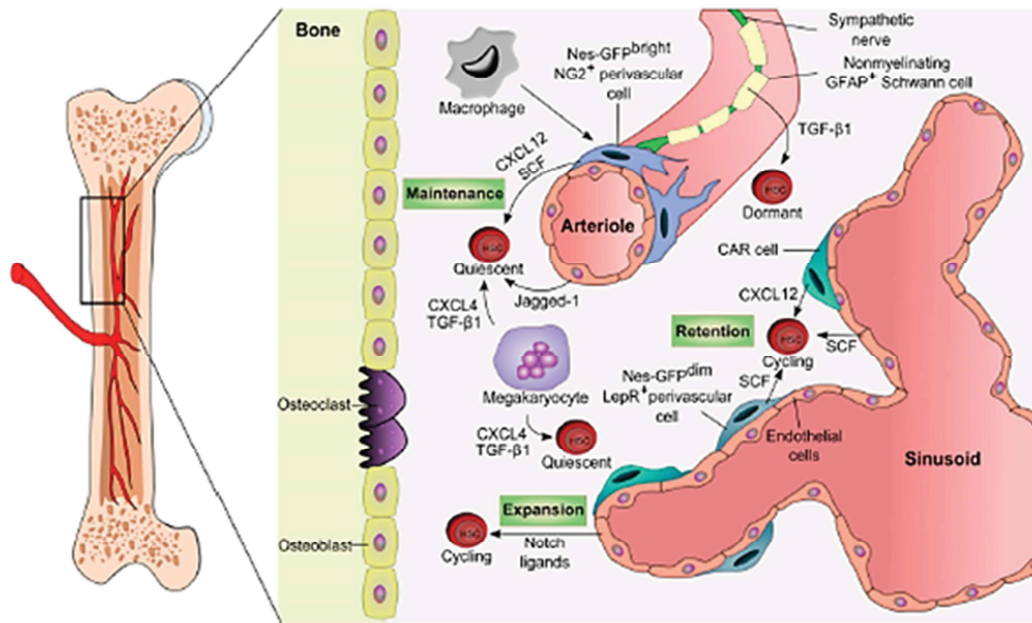


Figure 6. Endosteal and vascular adult bone marrow niche. Schematic representation of the different cell types (osteoclasts and osteoblasts, stromal and mesenchymal cells, macrophages, megakaryocytes, amongst others), soluble factors and consequent signaling pathways involved in HSC maintenance, homing and expansion in the bone marrow niche. CAR cells, specifically producing SDF-1 α (CXCL12), have been found close to sinusoid endothelial cells, but also in contact with HSCs in the endosteum. NES⁺ MSCs are separated into two sub-populations: the arteriole-associated subset (Nes-GFP^{bright}) in the endosteum and the sinusoid-associated NES⁺ cells (Nes-GFP^{dim}), which also express the Leptin receptor (LepR⁺). *Adapted from* (Boulais and Frenette, 2015)

1.5.3 Cytokines, developmental regulators and transcription factors

SCF, or stem cell factor, exists in transmembrane and soluble forms, generated by alternative splicing (Anderson *et al.*, 1991; Huang *et al.*, 1992; reviewed in Broudy, 1997) and binding the tyrosine kinase receptor c-kit (Flanagan and Leder, 1990; Huang *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990). SCF/c-kit signaling pathway plays an important role not only during development, but also in adult bone marrow (Azzoni *et al.*, 2018). Indeed, mutations in SCF or c-kit induce severe macrocytic anemia, leading to embryonic or perinatal death (Tan *et al.*, 1990; reviewed in Broudy, 1997); while, constitutive SCF secretion by bone marrow endothelial cells and fibroblasts regulates steady-state hematopoiesis (Heinrich *et al.*, 1993; Linenberger *et al.*, 1995). In fact, SCF promotes cell cycle entry of HSCs (Leary *et al.*, 1992) and when associated with other

cytokines, it induces their expansion and differentiation (Holyoake *et al.*, 1996; Kent *et al.*, 2008; Ding *et al.*, 2012).

TPO (thrombopoietin) is the ligand of the c-Mpl receptor, expressed on HSCs and to lesser extent on megakaryocytic progenitors, megakaryocytes and platelets (Debili *et al.*, 1995). Upon TPO/c-Mpl binding, the subsequent receptor homodimerization leads to the activation of Janus kinase 2 (JAK2) (Drachman and Kaushansky, 1997). The downstream signaling cascade involves STATs, PI3K, mitogen-activated protein kinases (MAPKs), and extracellular signal regulated kinases (Erk-1 and Erk-2). The induction of this cascade promotes a TPO/c-Mpl-mediated modulation of steady-state HSC maintenance and cell-cycle progression (Qian *et al.*, 2007; Yoshihara *et al.*, 2007), as well as the regulation of megakaryopoiesis (Broudy and Kaushansky, 1995).

BMPs (bone morphogenetic proteins) and angiopoietin-1 (**Ang-1**) play an important role in the maintenance of the HSC pool and the LT-HSC potential, respectively (Zhang *et al.*, 2003; Gomei *et al.*, 2010). BMPs are members of the TGF- β growth factor family (Reddi and Reddi, 2009) and are mainly produced by osteoclasts in the HSC niche (Garimella *et al.*, 2008). During embryonic development, BMP-4 is involved in the generation of hematopoietic cells from mesodermal precursors, while in adulthood it regulates HSC number and function in the bone marrow (Durand *et al.*, 2007; Goldman *et al.*, 2009). Ang-1 binds to the tyrosine kinase receptor Tie2, which is expressed on the surface of endothelial cells and fibroblasts (Makinde and Agrawal, 2008), as well as hematopoietic stem and progenitor cells (HSPCs) (Arai *et al.*, 2004; Rizo *et al.*, 2006). Tie2/Ang-1 interaction leads to increased HSC adhesion to the niche, sustaining their quiescence (Arai *et al.*, 2004). Interestingly, Tie2⁺ HSCs have been shown to upregulate several autophagy-related genes, in a PPAR δ /FAO-dependent manner, highlighting a fundamental role of mitophagy (mitochondrial autophagy) in the self-renewal of this rare cell subset (Ito *et al.*, 2016).

Notch signaling in mammals is mediated through four receptors (Notch 1-4) and five ligands (Jagged1-2 and Delta-like 1, 3, and 4) (Ranganathan *et al.*, 2011; Lampreia *et al.*, 2017), involved in the regulation of cell proliferation, lineage commitment and

differentiation (Artavanis-Tsakonas *et al.*, 1999; Lin and Hankenson, 2011). Notch activation is required for long-term expansion of murine HSPCs, which is sustained by endothelial cells through the expression of the ligand Jagged1 (Butler *et al.*, 2010; Poulos *et al.*, 2013). Similarly, activation of the Notch pathway is involved in the maintenance of human HSCs ($\text{Lin}^- \text{CD34}^- \text{CD38}^- \text{CD93}^{\text{hi}}$) (Anjos-Afonso *et al.*, 2013). Finally, Notch-Notch ligand binding has been shown to induce the expansion of AGM-derived HSCs (Hadland *et al.*, 2015), while impairments in the receptor-ligand interaction are associated with murine HSPC proliferation and exit from the bone marrow (W. Wang *et al.*, 2015).

The stem cell leukemia/T-cell acute lymphoblastic leukemia 1 (**SCL/TAL1**) and a member of the family of runt domain-containing transcription factors (**RUNX1**) are involved in the regulation of hematopoiesis during development (Speck and Gilliland, 2002; Lécuyer and Hoang, 2004). SCL is expressed in blood islands at E7.5 (Kallianpur *et al.*, 1994; Elefanty *et al.*, 1999) and it has been shown that yolk sac hematopoiesis is abolished in $\text{Scl}^{-/-}$ embryos, leading to consequent death around E9.5 (Robb *et al.*, 1995; Shivdasani *et al.*, 1995). Despite the precise dynamics are still under debate, SCL is known to be involved in the generation of hemangioblast-derived hematopoietic progenitors during development (D'Souza *et al.*, 2005; Porcher *et al.*, 2017; Vagapova *et al.*, 2018). Similarly, $\text{Runx1}^{-/-}$ embryos show significant impairment in hematopoietic cell production (Lacaud *et al.*, 2002; North *et al.*, 2002); indeed, it has been found that different expression levels of the RUNX1B isoform regulate the initiation, as well as the progression, of the endothelial-to-hematopoietic transition (EHT) process (Lie-A-Ling *et al.*, 2018). To conclude, the formation of a SCL-dependent transcriptional complex, which is able to regulate RUNX1, has been demonstrated in zebrafish as well as in murine embryos (Nottingham *et al.*, 2007; Patterson *et al.*, 2007; Landry *et al.*, 2008).

As mentioned, the whole hematopoiesis process is tightly regulated, also according to the lineage specification “choice” of the HSCs; in the following chapter I will more precisely focus on the dynamics and the regulation mechanisms of the erythropoiesis.

2 Erythropoiesis

Erythropoiesis is the process whereby HSCs differentiate into mature erythrocytes, also known as red blood cells (RBCs) (reviewed in Nandakumar *et al.*, 2016). This differentiation process is highly dynamic, with 2.5×10^6 RBCs produced each second and representing the most abundant blood cell population (reviewed in Palis, 2014). Developmental studies have identified two types of RBCs: embryonic and fetal/adult, originating from waves of primitive and definitive erythropoiesis, respectively (Dzierzak and Philipsen, 2013; reviewed in Yumine *et al.*, 2017). The ontogeny and morphological features of these RBCs, as well as a characterization of the erythroid progenitors and their differentiation process will be presented in this chapter.

2.1 Erythropoiesis: from embryo to adult

2.1.1 Primitive erythropoiesis

Primitive erythropoiesis occurs in the extraembryonic yolk sac, where early erythroid progenitors (EryP) originate from mesodermal cells (Lawson *et al.*, 1991; Kinder *et al.*, 1999). The latter are derived from epiblast cells, which enter through the primitive streak (Lawson *et al.*, 1991; Kinder *et al.*, 1999), migrate into the yolk sac and once in contact with endodermal cells, form blood islands (Dzierzak and Philipsen, 2013) composed of hematopoietic cells enclosed by endothelium (Ema and Rossant, 2003). The concomitant development of erythroid and endothelial cells suggests their origin from a common mesodermal precursor, the hemangioblast, carrying bi-lineage potential (Sabin, 1920; Murray, 1932; reviewed in Baron *et al.*, 2012; Stefanska *et al.*, 2017). In fact, a subset of mesodermal cells in the extraembryonic region has been found to co-express several endothelial markers, such as PECAM-1, CD34, endoglin (CD105), VE-cadherin and Tie2, as well as the erythroid transcription factor GATA-1 (Ema *et al.*, 2006).

Diverse studies on murine development have identified primitive erythroid progenitors with *in vitro* colony forming capacity (EryP-CFC) at the late primitive streak

stage (Wong *et al.*, 1986; Palis *et al.*, 1999). More precisely, EryP are detected in murine embryos from E7.5 to E8.5 of gestation (Isern *et al.*, 2011) and have been shown to express embryonic globin genes (Wong *et al.*, 1986; Kingsley *et al.*, 2006) (see section 2.2.4.1.1). EryP give rise to the first wave of erythroid precursors; in fact, upon initiation of cardiac contractions, a concomitant loss of progenitor activity (McGrath *et al.*, 2003; Isern *et al.*, 2011) and the release of immature primitive erythroblasts into the bloodstream are observed (Ji *et al.*, 2003; McGrath *et al.*, 2003) (**Figure 7**).

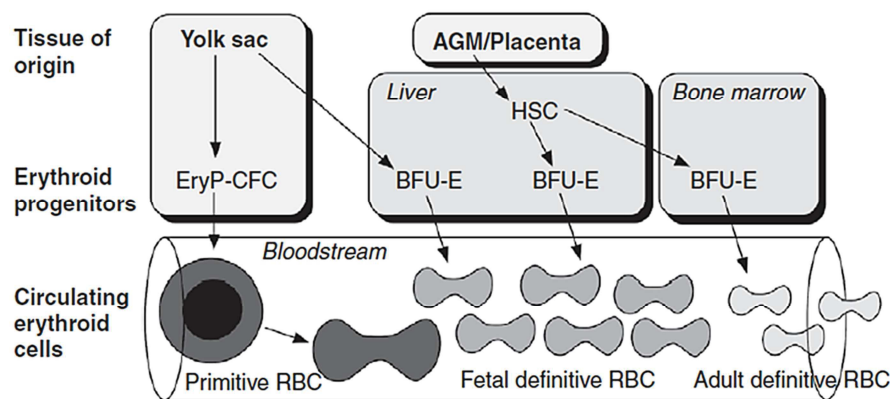


Figure 7. Waves of primitive and definitive erythropoiesis. During early stages of development, yolk sac-derived primitive erythroid progenitors (EryP-CFC) give rise to large primitive circulating erythrocytes. The latter are then progressively replaced by fetal definitive RBCs, which derive from yolk sac erythroid progenitors and AGM/placenta HSCs seeding the fetal liver. Finally, HSCs migrate to their definitive niche into the bone marrow, where adult definitive RBCs are produced. *Adapted from* (McGrath and Palis, 2008)

Morphologic analyses indicate that primitive erythroblasts continue to mature in the bloodstream, undergoing cell division (De la Chapelle *et al.*, 1969; Bethlenfalvay and Block, 1970) accumulating hemoglobin, while decreasing cell size and RNA content, and exhibiting nuclear pyknosis (Sangiorgi *et al.*, 1990; Morioka *et al.*, 1998). Primitive murine erythrocytes have been found to be approximately 6-fold larger than their adult counterpart, and some evidence has reported that they remain nucleated (Kingsley *et al.*, 2004). However, following studies suggest that primitive erythroid cell enucleation occurs between E12.5-E16.5 of gestation (Fraser *et al.*, 2007; Palis *et al.*, 2010), a time point at which primitive pyrenocytes (“extruded nuclei”) have been detected in the mouse embryo bloodstream (McGrath *et al.*, 2008).

Despite, blood islands have been histologically identified in the human conceptus as well (day 16-19 of gestation) (Kelemen and Calvo, 1979; Tavian *et al.*, 1999), the inaccessibility of human embryonic tissues prevents the exhaustive characterization of human EryP (Tavian and Peault, 2005; Baron *et al.*, 2013). Nevertheless, evidence of a significant analogy between mouse and human hematopoiesis has been provided by studies on human embryonic stem cells (hESCs) and induced-pluripotent stem cells (iPS). For instance, the hematopoietic commitment of hESCs has been shown to firstly generate EryP, similarly to the yolk sac-derived primitive erythropoiesis (Zambidis *et al.*, 2005). In addition, hemangioblast-like cells, with both hematopoietic and endothelial colony formation capacity, have been identified at early stages (72 to 96 hours) of hESC-derived embryoid body differentiation (Kennedy *et al.*, 2007). Similarly, differentiation of human pluripotent stem cells, both ESC and iPS, has highlighted that the $KDR^+CD235a^+$ (CD235a or Glycophorin A, GlyA) subset is able to generate primitive erythroid colony-forming cells and hemangioblasts (reviewed in Yoder, 2014). Finally, human EryP have been also found to undergo enucleation, more precisely in the placental villi (Van Handel *et al.*, 2010), where pyrenocytes are engulfed by resident macrophages (Van Handel *et al.*, 2010).

Further investigation is needed to completely characterize the dynamics of primitive erythropoiesis. First, the current definition of primitive erythrocytes has been questioned (Palis *et al.*, 2010; Müllner, 2011), as these cells enucleate like their definitive counterpart and probably circulate into the bloodstream after birth (Kingsley *et al.*, 2004; Fraser *et al.*, 2007; McGrath *et al.*, 2008). Finally, it has been shown that a partial overlap occurs in both mouse and human yolk sac between primitive and definitive hematopoiesis (reviewed in Baron, 2013), as discussed below.

2.1.2 Definitive erythropoiesis

It is generally accepted that the progression from primitive to definitive erythropoiesis during gestation has evolved to fulfill the growing oxygen demands of the fetus. Nevertheless, the developmental origin of definitive hematopoietic progenitors in the mammalian embryo was debated for a long time, as these progenitors could be generated *in vitro* from hemangioblast cultures (Lacaud *et al.*, 2002) while also emerging from hemogenic endothelial cells (Li *et al.*, 2005). Indeed, more recent studies have

found that definitive erythropoiesis has two developmental origins. A first wave of definitive erythroid progenitors, the erythro-myeloid progenitors (EMPs), emerges in the yolk sac at E8.25 (Palis *et al.*, 1999; Bertrand *et al.*, 2005), and colonizes the fetal liver around E10 (Palis *et al.*, 1999; Palis, 2014). Despite not carrying long-term reconstitution potential and not originating from HSCs (McGrath *et al.*, 2011), these progenitors are considered as definitive, as exhibiting the same β -globin gene expression pattern observed in fetal liver erythroid precursors at E10.5 (McGrath *et al.*, 2011). At this developmental stage, in fact, a second wave of definitive erythropoiesis originates from murine hematopoietic stem cells colonizing the fetal liver. These HSCs mainly emerge from the AGM region, but also the placenta, the major arterial vessels (umbilical, vitelline and cranial), as well as the yolk sac (Kumaravelu *et al.*, 2002; Ottersbach *et al.*, 2009; reviewed in Yoder, 2014) (see section 1.2.3), and undergo a first expansion into the fetal liver (Ema and Nakauchi, 2000) (**Figure 7**). Fetal liver erythroid progenitors associate with macrophages to form erythroblastic islands (Sasaki and Sonoda, 2000) (see section 2.3.1); these erythropoiesis niches are also found in the bone marrow cavity, which constitutes the definitive location of HSCs throughout adulthood (see section 1.2.3).

2.2 From erythroid progenitors to mature erythrocytes - How do we get there?

The mature erythrocyte represents the ultimately differentiated and enucleated cell, resulting from a well-defined hierarchy of hematopoietic progenitors, increasingly restricted to the erythroid lineage. The progression through this hierarchy of differentiation stages is generally divided into three phases: early erythropoiesis, terminal erythroid differentiation, and reticulocyte formation and maturation, which will be described in the following dedicated sub-sections.

2.2.1 Early erythropoiesis: BFU-E and CFU-E

Early erythropoiesis begins with the differentiation of hematopoietic stem progenitor cells into the first erythroid-committed progenitors: the erythroid burst-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E), which have been initially

characterized by their behavior in *ex vivo* cell colony assays (Stephenson *et al.*, 1971; Iscove *et al.*, 1974; Gregory and Eaves, 1978). BFU-Es are large colonies, containing several thousand hemoglobinized cells, which are identifiable in methylcellulose cultures after 5-8 and 10-14 days of *ex vivo* stimulation for mouse and human progenitors, respectively (Dzierzak and Philipsen, 2013). BFU-E formation and proliferation are regulated by several cytokines, including stem cell factor (SCF), thrombopoietin (TPO), interleukin 3 (IL3), interleukin 11 (IL11), and FLT3-ligand.

The more mature CFU-E progenitors consist of smaller colonies (16-125 cells) than BFU-E, isolated after 2-3 days (mouse) or 5-8 days (human) of methylcellulose culture (Dzierzak and Philipsen, 2013). Unlike BFU-Es, CFU-Es strictly depend on the erythroid-specific erythropoietin (EPO) cytokine for their survival and colony-forming capacity *in vitro* (Koury and Bondurant, 1990). More recent studies have precisely characterized the immunophenotypic identity of both BFU-Es and CFU-Es (Dulmovits *et al.*, 2017). In mouse fetal liver, BFU-Es and CFU-Es have been identified as c-Kit⁺CD45⁺Ter119⁻CD71^{low} and c-Kit⁺CD45⁻Ter119⁻CD71^{hi}, respectively (Flygare *et al.*, 2011), while murine bone marrow CFU-Es are defined as Lin⁻c-Kit⁺Sca1⁻IL7R α ⁻IL3R α ⁻CD41⁻CD71⁺ cells (Terszowski *et al.*, 2005). Human BFU-E and CFU-E progenitors have been classified as IL3R⁻GlyA⁻CD34⁺CD36⁻ and IL3R⁻GlyA⁻CD34⁻CD36⁺, respectively. The precise identity of these phenotypically-defined populations has been confirmed by their potential of generating pure colonies in methylcellulose culture, as well as a distinct gene expression signature (Li *et al.*, 2014). These advanced techniques have promoted the isolation of all erythroid-committed progenitors, starting from the most immature precursors, from both *in vitro* culture systems and primary human bone marrow cells. Notably though, recent work has found significant differences between erythroid progenitors generated from peripheral blood (PB) and cord blood (CB)-derived human CD34⁺ HSCs, as a function of their differentiation potential and proliferation kinetics. Interestingly, this study led to the identification of a transitory PB-HSC-derived population (IL3R⁻GPA⁻CD34⁺CD36⁺) able to generate both BFU-E and CFU-E *in vitro* (Yan *et al.*, 2018).

2.2.2 Terminal erythroid differentiation: from the pro- to orthochromatic erythroblast

Terminal erythroid differentiation begins with the proerythroblast stage, the first erythroid progenitor morphologically identifiable in the bone marrow. This precursor undergoes sequential mitoses, generating basophilic, polychromatic, and orthochromatic erythroblasts (Liu *et al.*, 2013), which then enucleate to become reticulocytes. Terminal erythroid differentiation is associated with several morphological changes, such as progressive cell size reduction (from 12-20 μ m to 8-10 μ m), cytoplasmic hemoglobin accumulation, as well as chromatin condensation, followed by nuclear pyknosis and enucleation (An and Mohandas, 2011). All these changes, associated with a conversion from a basophilic to acidophilic cytoplasm, allow the precise identification of each erythroblast stage by May-Grünwald Giemsa staining (MGG) (Rozenberg, 2011). In addition, an immunophenotypic characterization of the terminal erythroid progenitors has been recently provided, both in mice and humans (Chen *et al.*, 2009; Hu *et al.*, 2013). Indeed, by combining the decrease in cell size with the expression level of the murine erythroid-specific marker Ter119 and the adhesion molecule CD44, it was possible to discriminate between mouse pro-, basophilic, polychromatic, and orthochromatic erythroblasts, as well as reticulocytes and mature RBCs (Chen *et al.*, 2009) (**Figure 8**).

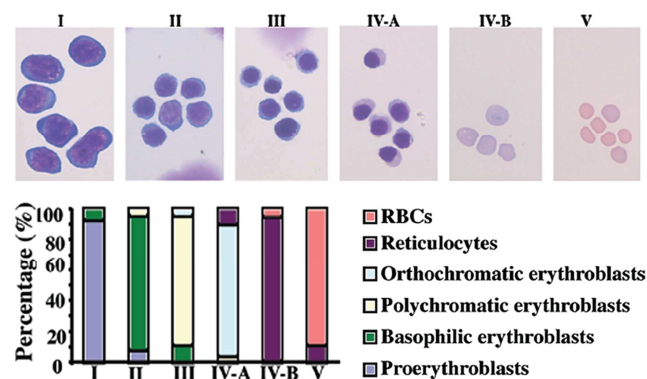


Figure 8. Isolation of murine terminal erythroid progenitors. May-Grünwald Giemsa staining showing homogenous populations of murine terminal erythroid progenitors (proerythroblasts, basophilic, polychromatic and orthochromatic erythroblasts, as well as reticulocytes and RBCs), isolated as a function of cell size, associated with Ter119 and CD44 surface marker expression. *Adapted from* (Chen *et al.*, 2009)

Similarly, human terminal erythroid progenitors have been characterized by associating cell size with the expression level of the human erythroid-specific marker Glycophorin A (GlyA), the adhesion molecule $\alpha 4$ -integrin (CD49d) and the transmembrane protein Band 3 (Hu *et al.*, 2013). This strategy, applied to adult human bone marrow-derived cells, has allowed separating pure populations of proerythroblasts, early and late basophilic, polychromatic and orthochromatic erythroblasts, as well as reticulocytes (**Figure 9**). Notably, each population showed the expected proliferation profile of one cell division per stage, following a 1:2:4:8:16 ratio (Hu *et al.*, 2013).

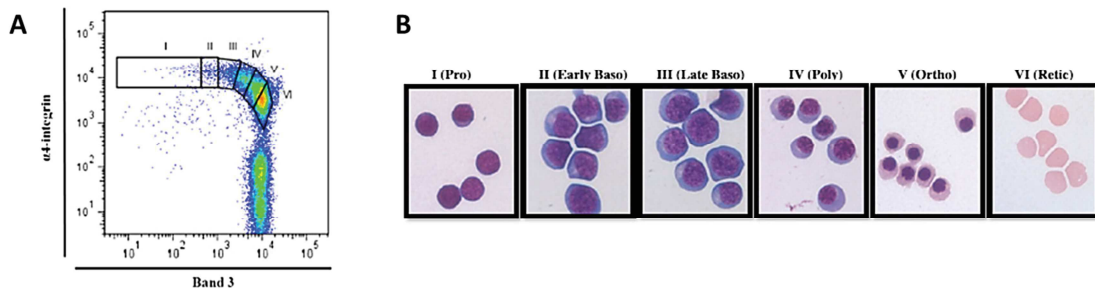


Figure 9. Immunophenotypic characterization and isolation of terminal erythroid progenitors from human bone marrow. Panel A describes the isolation strategy of human bone marrow-derived GlyA⁺ terminal erythroid progenitors, as a function of $\alpha 4$ -integrin and Band 3 expression levels. The corresponding homogenous populations of proerythroblasts, early and late basophilic, polychromatic and orthochromatic erythroblasts, as well as reticulocytes are shown by May-Grünwald Giemsa staining in panel B. *Adapted from* (Hu *et al.*, 2013)

The above mentioned isolation strategy has been also employed for transcriptomic analyses of both murine and human terminal differentiating erythroid progenitors. These analyses have led to the identification of the most expressed genes at each differentiation stage, and have highlighted species-specific differences in the gene expression profiles of human and murine erythroblasts (An *et al.*, 2014). Interestingly, transcriptomic and proteomic studies have also shown that several key genes remain active throughout early and terminal erythropoiesis, suggesting that some features of the erythroid differentiation program are likely defined at the very early stages (Kingsley *et al.*, 2013; Gautier *et al.*, 2016). Nevertheless, further investigation is needed to

precisely characterize the extensive and tightly regulated mechanisms that orchestrate the erythroid differentiation process, both in physiological and pathological conditions.

2.2.3 Reticulocyte formation and maturation

Mature RBCs show different morphological features as a function of the species; indeed, enucleation only occurs in mammals. Therefore, mammalian erythrocytes circulate in the bloodstream as enucleated cells, while bird, amphibian and fish erythrocytes maintain a condensed pyknotic nucleus. Despite the reason of this developmental “choice” has not yet been elucidated, it has been shown that nucleated erythroblasts are more susceptible to hemolysis in the circulation, especially into the small capillaries (Gaehtgens, Schmidt and Will, 1981; Gaehtgens, Will and Schmidt, 1981).

Once at the orthochromatic stage, erythroblasts enucleate and consequently differentiate into immature reticulocytes. More precisely, orthochromatic erythroblasts definitively exit the cell cycle (Hsieh *et al.*, 2000; Pop *et al.*, 2010) and after completing chromatin condensation, they polarize their nucleus to one side of the cell (Keerthivasan *et al.*, 2011). Subsequently, the nucleus is expelled, giving rise to a reticulocyte and a “pyrenocyte”, “*the extruded nucleus with a thin rim of cytoplasm surrounded by a plasma membrane*” (McGrath *et al.*, 2008). Because of their composition and their consequent inability to produce energy, pyrenocytes are highly unstable and are rapidly engulfed by macrophages in the erythroblastic islands (Lee *et al.*, 2004; Yoshida *et al.*, 2005) (see section 2.3.1).

Reticulocytes are morphologically distinguished from mature RBCs through the staining of their cytoplasmic ribosomal RNA and reticulon, by methylene blue. Four reticulocyte stages (R1 to R4) have been characterized, by combining two features of their maturation process: the progressive RNA loss and transferrin receptor (TfR1 or CD71) clearance from the plasma membrane (Malleret *et al.*, 2013; Ovchinnikova *et al.*, 2017). In the adult human bone marrow, the newly formed R1 reticulocytes are motile, multi-lobular, and retain some intracellular organelles as well as endocytic activity (Mel *et al.*, 1977; Malleret *et al.*, 2013). More mature reticulocytes lose their motility, exhibit

increased membrane deformation capacity, and exit the bone marrow to enter into the bloodstream (Mel *et al.*, 1977; Chasis *et al.*, 1989; Waugh *et al.*, 1997).

The last steps of reticulocyte maturation include remodeling of the plasma membrane, volume and surface area reduction, as well as degradation/elimination of the remaining cytoplasmic organelles, such as mitochondria and ribosomes. All these steps occur in the circulation and lead to the generation of biconcave discoid erythrocytes (Keerthivasan *et al.*, 2011; Griffiths, Kupzig *et al.*, 2012). Indeed, enucleation and subsequent reticulocyte maturation into RBCs are the result of many different processes. First, cytoskeletal and membrane remodeling lead to ~20% loss of the plasma membrane (Waugh *et al.*, 1997, 2001; Da Costa *et al.*, 2001), probably by involving the endosome-exosome pathway and consequently promoting nucleus extrusion (Blanc *et al.*, 2005). Interestingly, CD71 shedding occurs through exocytosis (Pan *et al.*, 1985; Johnstone *et al.*, 1987), following a mechanism which is also regulated by Ca^{2+} flux (Savina *et al.*, 2003). Second, protein sorting systems have a crucial function. Indeed, cytoskeletal proteins (spectrins, ankyrin and protein 4.1R), as well as cytoskeletal-associated membrane proteins (GlyA, RhAG and Band3, amongst others) are preferentially distributed to reticulocytes (Wickrema *et al.*, 1994; Lee *et al.*, 2004; Salomao *et al.*, 2010). Similarly, the majority of the proteasome particles segregate to the reticulocyte, while all histones, except the H4, to the pyrenocyte (Gautier *et al.*, 2016). Finally, mechanisms of organelle degradation, by autophagy, via vesicle trafficking and exocytosis, participate to the final generation of a mature red blood cell (Kang *et al.*, 2012; Zhang *et al.*, 2015). The intriguing and still open debate on the relative contributions of these different pathways to the proper progression of the last erythroid differentiation step will be discussed in the following sections.

2.2.3.1 Open debate on mechanisms of enucleation: importance of vesicle trafficking

Initial studies aiming to characterize the mechanisms of enucleation raised the hypotheses that this process occurs following mechanisms commonly described during apoptosis or asymmetric cytokinesis.

The first hypothesis is based on the identification of a critical role for caspase activation in erythroid differentiation (Zermati *et al.*, 2001; Tran *et al.*, 2017), as well as the evidence of nuclear pyknosis and membrane shedding dynamics during enucleation (Simpson and Kling, 1967). Nevertheless, an apoptosis-independent effect has been described upon death-receptor-mediated activation of caspases, which are involved in GATA-1 cleavage and have been associated to differentiation arrest at the basophilic erythroblast stage (De Maria *et al.*, 1999). In addition, caspase downregulation has been shown to affect enucleation, but mainly as an indirect effect of the differentiation blockage between pro- and basophilic erythroblast stage, induced by the lack of caspase (Carlile *et al.*, 2004). Finally, during human terminal erythroid differentiation, caspase-3 induction participates to nuclear remodeling by acting on several substrates, such as lamin B (Zermati *et al.*, 2001). Similarly, caspase-3 activation mediates the formation of transient nuclear openings, allowing histone release into the cytoplasm of murine terminal erythroid progenitors, and consequent chromatin/nuclear condensation (Zhao *et al.*, 2016). Taken together, these studies demonstrate that despite caspase expression and activity play a role in the regulation of terminal erythroid differentiation, enucleation does not rely on an apoptosis-dependent mechanism.

The “asymmetric cytokinesis” hypothesis has been based on the proposition that enucleation is a form of cell division, in which the cytokinetic machinery separates the cell nucleus from the cytoplasm. In fact, the formation of a cleavage furrow has been detected during erythroblast enucleation (Koury *et al.*, 1989), as well as the presence of a contractile actomyosin ring (CAR) (Konstantinidis *et al.*, 2012), as observed in cell mitosis (Barr and Gruneberg, 2007). Indeed, actin was found to accumulate in the region between the extruding nucleus and the cytoplasm (Griffiths *et al.*, 2012) and inhibitors of actin polymerization decreased enucleation (Koury *et al.*, 1989). However, it has been shown that cytokinesis inhibitors only indirectly affect enucleation, by actually preventing erythroblast cell cycle progression (Keerthivasan *et al.*, 2010).

Despite the asymmetric cytokinesis hypothesis is not completely disproved, the current enucleation model suggests the concomitant contribution of vesicle trafficking and autophagy into the processes of nucleus extrusion and organelle degradation. First evidence came from the detection of cytoplasmic vacuoles surrounding the condensed nucleus, just before the enucleation. The latter is directly linked to the formation of

these vacuoles, as inhibition of the endocytic vesicle trafficking and the endosome/lysosomal secretory pathways significantly reduced enucleation (Keerthivasan *et al.*, 2010). Similar effect was observed in orthochromatic erythroblasts under conditions where the clathrin heavy chain is downregulated (Keerthivasan *et al.*, 2010). Indeed, a tightly controlled vesicle trafficking directly contributes to the enucleation process, as a proper membrane quantity and composition is required for the pyrenocyte/reticulocyte segregation (Keerthivasan *et al.*, 2011). As mentioned above, autophagy also plays a critical role. Autophagy is a physiological process, also induced upon fasting (Gubbiotti *et al.*, 2018), allowing the catabolism and recycling of damaged intracellular organelles or aberrant proteins, through the formation of specialized vacuoles, the autophagosomes (Zhang *et al.*, 2015; Grosso *et al.*, 2017) (see section 2.2.3.2). The conjugated form of the LC3 protein (LC3-II) is considered the main autophagy marker, as associated with autophagosome formation (Kissová *et al.*, 2007; Zhang *et al.*, 2007). Interestingly, transmission electron microscopy analyses showed that the LC3-II marker is present in reticulocyte cytoplasmic vacuoles, containing degraded intracellular organelles. In addition, the surface of these vacuoles was shown to harbor integral membrane proteins, such as GlyA, aquaporin-1 (AQP1), Glut1 and Band 3 (Griffiths *et al.*, 2012). This suggests that the intracellular organelles within immature reticulocytes are mainly degraded by autophagy, and that endocytic plasma membrane-derived vesicles participate to the formation of the autophagosomes. Finally, these GlyA-positive vacuoles are then able to fuse with the plasma membrane, discarding their content by exocytosis and complete reticulocyte maturation (Griffiths *et al.*, 2012) (**Figure 10**). Further details about the dynamics of reticulocyte maturation into erythrocytes will be provided in the next section.

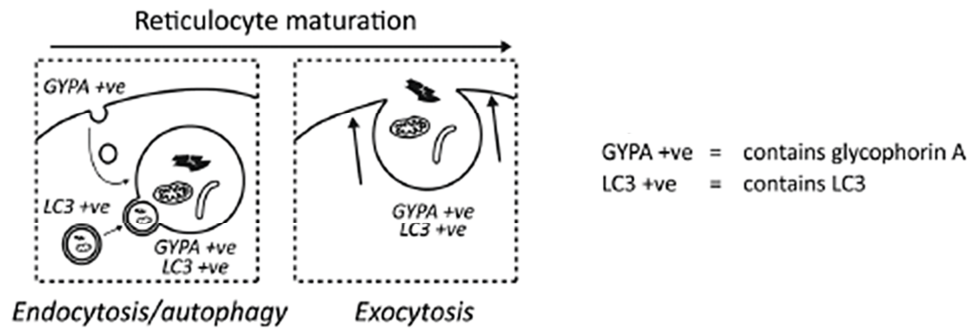


Figure 10. Vesicle trafficking in reticulocyte maturation. During reticulocyte maturation, degradation of intracellular organelles mainly occurs through autophagy. Subsequent fusion of autophagic LC3⁺ vacuoles with GlyA⁺ endosomes leads to the formation of specialized vesicles, which fuse with the plasma membrane and discard their content through exocytosis. *Adapted from* (Griffiths et al., 2012)

2.2.3.2 Mitochondrial clearance: fundamental step in terminal erythroid differentiation

Several cellular mechanisms operate during the 48h that separate enucleation and consequent reticulocyte generation to the complete reticulocyte maturation into circulating RBCs. As previously mentioned, one of these mechanisms involves the autophagy process, which is composed of different steps. First, the degradation target is surrounded by a specialized membrane, the phagophore (Axe *et al.*, 2008; Lamb *et al.*, 2013), and subsequently included into the autophagosome vacuole (Militello and Colombo, 2011; Lamb *et al.*, 2013). The latter fuses with multivesicular body/late endosome/lysosome, to form the autophagolysosome, in which the cargo is degraded by proteolytic enzymes (Eskelinen and Saftig, 2009; Fader and Colombo, 2009) (**Figure 11**). The conversion of the soluble form of the microtubule-associated protein 1 light chain 3B (LC3B-I) to the lipidated LC3B-II form is a fundamental step in the autophagosome formation; LC3B-II/LC3B-I ratio being therefore a direct measure of autophagy (Klionsky, 2012).

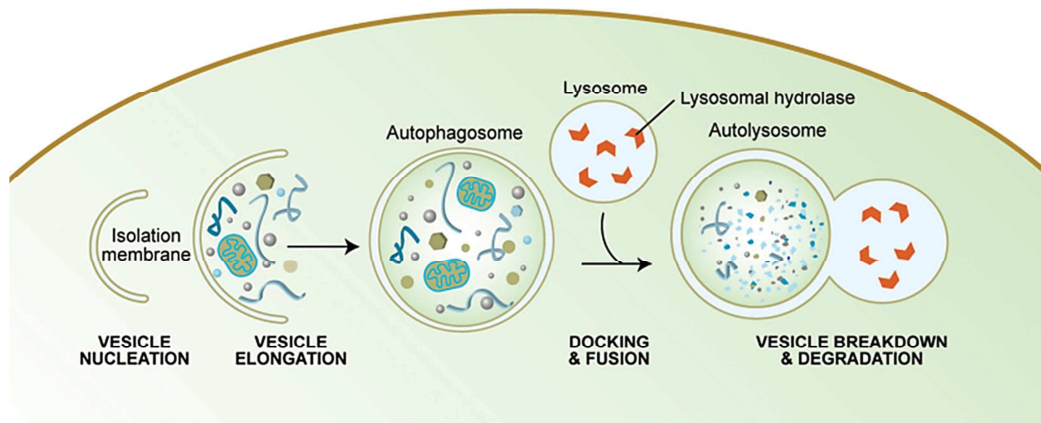


Figure 11. Autophagy process. Intracellular organelles to be degraded are surrounded by the isolation membrane, which progressively generates the autophagosome vacuole. The latter fuses with lysosome to form the autolysosome, where the degradation target is digested by the lysosomal hydrolase enzymatic activity. *Adapted from (Meléndez et al., 2005)*

Interestingly, autophagy has been described as a major mechanism of mitochondrial clearance (mitophagy) in maturing reticulocytes and recently linked to the regulation of terminal erythroid differentiation and enucleation (Zhang *et al.*, 2015; Grosso *et al.*, 2017). Indeed, the autophagy-related genes Ulk1 and Nix (Bnip3l) are significantly upregulated in late erythroblasts (Schweers *et al.*, 2007; Kundu *et al.*, 2008) and their activity is required for terminal differentiation (Mortensen *et al.*, 2010; Honda *et al.*, 2014). More precisely, Ulk1 is involved in mitochondrial and ribosome clearance (Kundu *et al.*, 2008), while Nix act as receptor for the recruitment of LC3 and consequent targeting of mitochondria to mitophagy (Aerbajinai *et al.*, 2003; Novak *et al.*, 2010). Indeed, Nix^{-/-} mice exhibit defects in mitochondrial clearance, which are associated with altered reticulocyte maturation into RBCs and, therefore, anemia (Sandoval *et al.*, 2008). Similarly, KO of several molecules involved in the autophagy pathway, such as Atg7 and Ulk1, has been shown to impair mitochondrial clearance, leading to alterations in the number of mature erythrocytes (Kundu *et al.*, 2008; Zhang and Ney, 2009; Zhang *et al.*, 2009). For instance, impaired autophagy is involved in the pathogenesis of several diseases associated with defective erythropoiesis, such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Watson *et al.*, 2011; Jiang *et al.*, 2018).

Finally, it has been recently found that GATA-1 induces a member of the Forkhead transcription factor family (FOXO), FOXO3, to positively regulate the transcription of

several autophagy-related genes (Kang *et al.*, 2012). In fact, in FOXO3 mutant erythroblasts, significantly reduced expression of Nix and Ulk1 genes is accompanied by a decreased LC3B-II/LC3B-I ratio, suggesting inhibition of autophagy; indeed, mitochondrial retention is observed in FOXO3 mutant reticulocytes (Liang *et al.*, 2015). These mechanisms are associated with a blockage in terminal differentiation, as demonstrated by the accumulation of orthochromatic erythroblasts among FOXO3 mutant bone marrow cells (Marinkovic *et al.*, 2007).

These findings identify in the mitochondrial clearance a fundamental step for reticulocyte maturation into RBCs (reviewed in Moras *et al.*, 2017; Ovchynnikova *et al.*, 2018), but also an important relationship between the mechanisms of organelle degradation and the transition from orthochromatic erythroblasts to reticulocytes. Indeed, additional aspects of this intriguing relationship will be object of speculation in the experimental part of this thesis.

2.2.4 Erythrocytes

Centuries of studies have aimed to characterize the functional and morphological features of circulating erythrocytes (Van Leeuwenhoek, 1675): enucleated cells exhibiting a discoid biconcave shape. Normal human RBCs are 8 μm in diameter, with a 90 fL volume and a 140 μm^2 surface area (Migliaccio *et al.*, 2009). During their 120-day life span, RBCs travel 150km in the peripheral circulation, continually passing through large arterial axis and narrow capillaries. As erythrocytes do not contain nucleus and cytoplasmic organelles, their main structural component is represented by the plasma membrane. The latter has been extensively studied, in order to characterize the membrane protein and lipid composition, as well as the mechanical features underlying its extensive deformation capacity (reviewed in Huisjes *et al.*, 2018).

2.2.4.1. Structure

The RBC membrane shows a peculiar organization in which the bilayer structure, composed of cholesterol, phospholipids and transmembrane proteins, is anchored to the cytoskeleton network to ensure specific mechanical features. Phospholipids show an asymmetric partition between the two leaflets of the membrane, with sphingomyelin

(SM) and phosphatidylcholine (PC) mainly distributed in the outer monolayer, while phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner one (Verkleij *et al.*, 1973). Different types of transmembrane proteins are found in the RBC plasma membrane, including adhesion molecules, cytokine receptors and transport proteins, as well as blood group antigens, amongst others.

Noteworthy among the RBC membrane transport proteins are the anion exchanger Band 3, the water carrier aquaporin 1, the gas transporter RhAG and Glut1, which mediates both glucose and L-dehydroascorbic acid uptake (Montel-Hagen *et al.*, 2008). Band 3 is the most abundant protein ($\sim 1 \times 10^6$ copies/cell) of the erythrocyte membrane (Burton and Bruce, 2011), where its localization is helped by the chaperone-like activity of the Glycophorin A (van den Akker *et al.*, 2010), which is also present at 1×10^6 copies per cell. Glut1 is found in the human RBC membrane (200-700,000 copies per cell), not only as part of the ankyrin and 4.1R complexes, but also bound to stomatin (Salzer and Prohaska, 2001; Rungaldier *et al.*, 2013). Moreover, some of the mentioned transport proteins are also members of membrane macromolecular complexes linked to the cytoskeleton matrix. For instance, in the ankyrin complex, Band 3 tetramers interact with ankyrin, which is then linked to the cytoskeleton network. Similarly, in the 4.1R complex, the interaction with the cytoskeleton is mediated by Glycophorin C, XK, Rh, and Duffy binding to the 4.1R protein (Bennett, 1983; Nicolas *et al.*, 2003; Salomao *et al.*, 2008) (**Figure 12**).

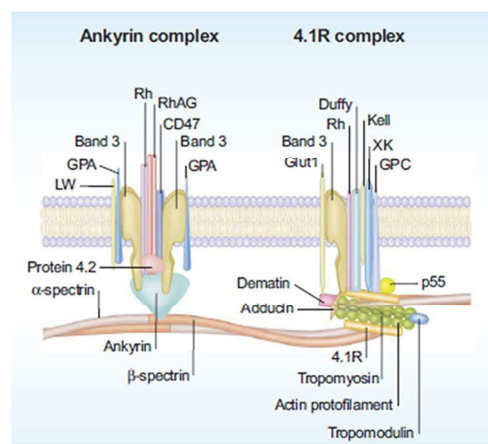


Figure 12. Ankyrin and 4.1R complexes in human erythrocyte plasma membrane. Membrane macromolecular complexes linked to the spectrin cytoskeletal network in mature circulating RBCs, playing an important role in membrane stability and deformability. Ankyrin and 4.1R protein bind the cytoskeletal components, while interacting with other proteins in the respective complex, including Band 3 and RhAG

for the ankyrin complex, whereas Glycophorin C, XK, Rh, and Duffy for the 4.1R complex. *Adapted from* (Mohandas and Gallagher, 2008)

The RBC membrane cytoskeletal network is mainly composed of α - and β -spectrin antiparallel heterodimers, which assemble in spectrin tetramers, together with other proteins, such as actin, adducin, dematin, tropomyosin and tropomodulin (Karinch *et al.*, 1990; Mohandas and An, 2006). The cytoskeleton interactions with the transmembrane proteins of the macromolecular complexes, mainly through ankyrin and 4.1R, allow the formation of a solid but flexible structure fundamental for maintaining erythrocyte shape and elasticity. This mechanical stability of the erythrocyte membrane avoids any risk of fragmentation or vesiculation, upon the stresses that RBCs encounter in the circulation. Indeed, it has been shown that a transient increase in intracellular calcium (Ca^{2+}) concentration occurs after deformation and that a subsequent Ca^{2+} efflux is required for RBC reshaping (Dyrda *et al.*, 2010; Bogdanova *et al.*, 2013). In addition, specific membrane lipid domains seem to be involved in this process. Upon reshaping, cholesterol-enriched domains are re-localized to cell edges and areas of the plasma membrane characterized by an increased curvature. Moreover, RBC shape and volume restoration involves the formation of sphingomyelin-enriched domains, which is consequent to Ca^{2+} efflux (Leonard *et al.*, 2017).

At the end of their 120-day life span, senescent erythrocytes show a progressive decrease in membrane integrity, including the loss of cholesterol- and sphingomyelin-enriched domains due to membrane vesiculation (Leonard *et al.*, 2017). The consequent reduced biconcavity and deformability lead to the clearance of these senescent cells from the peripheral circulation (Mohandas and Gallagher, 2008; Leonard *et al.*, 2017). Indeed, damaged RBCs are characterized by Band 3 clustering or PS exposure in the outer leaflet of the plasma membrane, which allow their recognition and retention into the spleen, where they are phagocytized by macrophages (Mohandas and Gallagher, 2008; reviewed in Klei *et al.*, 2017).

2.2.4.1.1 Hemoglobin: isoforms and globin gene expression

Hemoglobin (Hb) is the most represented intracellular protein in mature circulating erythrocytes and plays a fundamental role in oxygen transport. Adult hemoglobin is a tetramer composed of two α - and two β -globin chains, each associated with a prosthetic heme group containing an iron atom (see section 2.2.4.1.2). Globin chains are encoded by the α - and β -globin gene clusters, which are respectively localized on the chromosome 16 and 11 (Palis and Segel, 1998), and are composed of several genes as well as pseudogenes. A transcriptional switch mediates differential expression of the genes in each cluster, leading to the production of diverse Hb isoforms during development and adulthood. In fact, in yolk sac-derived primitive erythrocytes the Gower1 ($\zeta_2\varepsilon_2$) is the main Hb form, and small amounts of the Gower2 ($\alpha_2\varepsilon_2$) are also detected (Peschle *et al.*, 1985). A first switch occurs with the onset of definitive erythropoiesis in the fetal liver, with fetal hemoglobin HbF ($\alpha_2\gamma_2$) becoming predominant throughout fetus development (Sankaran *et al.*, 2010). Subsequently, γ -globin is progressively replaced by β -globin chain synthesis (γ to β , fetal-to-adult globin switch) and by 6 months after birth, adult hemoglobin HbA ($\alpha_2\beta_2$) is mainly observed in circulating RBCs (Palis and Segel, 1998) (**Figure 13**). Small amounts of HbF are still found in adult erythrocytes, less than 2% (Rochette *et al.*, 1994), but they often increase in case of β -globin chain synthesis defects, such as in sickle cell disease (SCD) and β -thalassemia (Platt *et al.*, 1991).

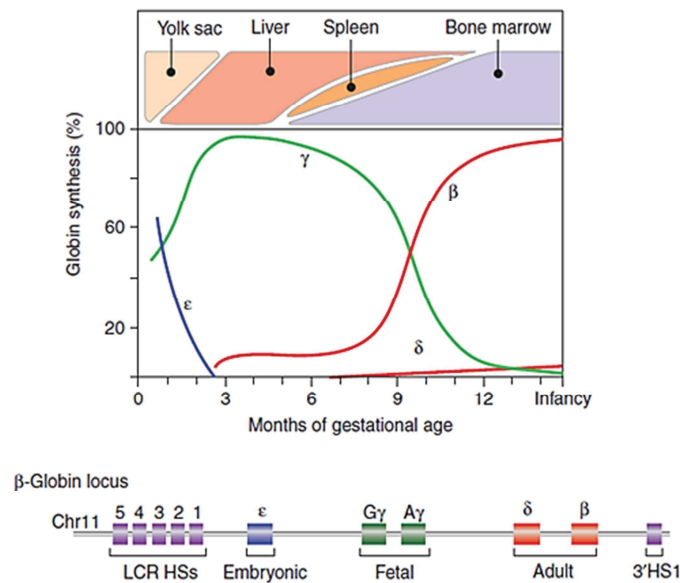


Figure 13. Globin gene expression switch and β -globin gene cluster. Embryonic to fetal (ϵ to γ) and fetal to adult (γ to β) globin switch occurring during human development are shown in the upper panel. Bottom panel represents the different genes and pseudogenes composing the human β -globin gene cluster on chromosome 11 (Chr11). LCR, locus control region (enhancer of the β -globin locus); HS, DNase I hypersensitivity site; 3'HS1, downstream hypersensitivity site. *Adapted from* (Sankaran and Orkin, 2013)

Several erythroid transcription factors, such as GATA-1, Klf1, and SCL/TAL1 play a role in the globin gene expression regulation (Cantor and Orkin, 2002). However, BCL11A has been identified as the specific modulator of the fetal-to-adult (γ to β) globin switch (Sankaran *et al.*, 2008; Xu *et al.*, 2010; Basak *et al.*, 2015; Dias *et al.*, 2016). More precisely, BCL11A acts as a repressor, by recognizing a specific sequence motif at the level of the γ -globin gene promoter (Liu *et al.*, 2018); in fact mice lacking BCL11A show persistent expression of γ -globin in definitive erythrocytes (Sankaran *et al.*, 2009; McGrath *et al.*, 2011). Interestingly, BCL11A expression is directly upregulated by the erythroid specific transcription factor Klf1 (Borg *et al.*, 2010; Zhou *et al.*, 2010) (see section 2.3.4). Finally, DNA methylation is also involved in γ -globin gene silencing in adult erythroid progenitors (van der Ploeg and Flavell, 1980); moreover, specific DNA and histone modifications, as well as chromatin remodeling and transcription factors participate to the regulation of fetal and adult globin gene expression (Hong *et al.*, 2005; Xu *et al.*, 2010; Stadhouders *et al.*, 2014; Iarovaia *et al.*, 2018).

2.2.4.1.2 Heme: biosynthesis and iron metabolism

Heme biosynthesis begins with the condensation of succinyl-CoA and glycine, catalyzed by the mitochondrial δ -aminolevulinic acid synthase (ALAS) enzyme (Fanica-Gaignier and Clement-Metral, 1973), to generate δ -aminolevulinic acid (ALA). The latter represents the first “building block” for the consequent cytosolic biosynthesis of a cyclic tetrapyrrole intermediate, the uroporphyrinogen III (UROGEN) (Battersby, 2000). Subsequently, decarboxylation (Cornah and Smith, 2009) and aromatization reactions, occurring between the cytoplasm and the mitochondria, give rise to the protoporphyrin IX (PROTO), which becomes substrate of the last enzymatic step in heme biosynthesis. Indeed, the insertion of ferrous iron (Fe^{2+}) into protoporphyrin IX, catalyzed by the mitochondrial ferrochelatase (FC) enzyme, definitely generates heme (Layer *et al.*, 2010; Yien *et al.*, 2018).

Iron delivery into the cells is ensured by the binding of the transferrin receptor, CD71 or TfR1, with the plasma glycoprotein transferrin, which vehicles Fe^{3+} into the blood circulation. The transferrin-TfR1 interaction induces internalization of this complex and subsequent iron release to the intracellular compartments (Hentze *et al.*, 2010). During erythropoiesis, CD71 is increasingly expressed on cell surface, mainly starting from the pro-erythroblast stage (Hu *et al.*, 2013), allowing iron import for the progressive Hb accumulation required for a proper differentiation. Indeed, alterations in TfR1 function lead to microcytic anemia in adult mice, while severe anemia causes mouse embryo death, in absence of TfR1 during development (Iolascon *et al.*, 2009).

In erythroblasts, it has been shown that after transferrin-TfR1 internalization, iron would be straightly released into the mitochondria, through a “kiss and run” mechanism of direct interaction between endosomes and mitochondria (Sheftel *et al.*, 2007). Subsequently, the inner mitochondrial membrane protein mitoferrin 1 (Mfrn1), which is member of the solute carrier family 25 (SLC25A37), mediates iron transport into the mitochondria (Shaw *et al.*, 2006). Interestingly, erythroblasts are also able to export heme, through the FLVCR (feline leukemia virus C receptor) transporter (Keel *et al.*, 2008; Byon *et al.*, 2013).

Iron availability is tightly regulated through a loop of absorption/recycling, release into the circulation and iron storage. Indeed, iron is mainly stored into the liver and its circulating levels are regulated by hepatocyte-dependent secretion of the hepcidin hormone (reviewed in Reichert *et al.*, 2017). 1-2 mg of dietary iron per days are absorbed, as reduced form (Fe^{2+}), through the divalent metal transporter 1 (DMT1/SLC11A2) expressed on the surface of duodenal enterocytes (Gunshin *et al.*, 1997). On the other hand, macrophages recycle ~25 mg of iron/day, by phagocytizing senescent erythrocytes and digesting heme through hemoxygenase enzymatic activity. Subsequently, the NRAMP1 (natural resistance-associated macrophages protein 1) divalent metal transporter mediates iron export from phagocytic vesicles (Soe-Lin *et al.*, 2009). Finally, cytosolic iron (Fe^{2+}) is released into the circulation through the exporter, ferroportin (also SLC40A1) (Donovan *et al.*, 2000; McKie *et al.*, 2000). The latter is not only localized on the surface of macrophages, but also on the basolateral membrane domain of duodenal enterocytes. After being exported by ferroportin, Fe^{2+} is oxidized by the membrane protein hephaestin, allowing Fe^{3+} to interact with transferrin. Surface levels of ferroportin are regulated by the hepcidin hormone, which by binding to ferroportin induces its internalization, ubiquitination, and consequent degradation into the lysosomes (Nemeth *et al.*, 2004). Thus, hepcidin is expressed upon high iron circulating levels, to limit iron export into the bloodstream by promoting ferroportin endocytosis. In contrast, low levels of plasmatic iron inhibit hepcidin production, subsequently increasing iron export (Hentze *et al.*, 2010). Finally, hepcidin expression is also suppressed by another hormone, the erythroferrone, which is directly secreted by erythroblasts upon anemia (Kautz *et al.*, 2014, 2015).

2.2.4.2 Function

The main function of circulating RBCs consists in maintaining the proper oxygenation level of all peripheral tissues, through a tightly regulated mechanism of oxygen delivery, in exchange of CO_2 . The gas carrier capacity of erythrocytes is directly linked to the cytoplasmic Hb amount and functionality; this functionality differs between Hb isoforms. For instance, HbF ($\alpha_2\gamma_2$) has a higher oxygen affinity than adult HbA ($\alpha_2\beta_2$), which allows the increased oxygen uptake from maternal circulation, required for the correct fetal tissue oxygenation (Murphy, 2005).

The mechanism of gas exchange involves different proteins: Hb, the carbonic anhydrase enzyme, as well as the anion transporter Band 3. In fact, when red blood cells reach the peripheral capillaries, carbonic anhydrase in the RBC cytoplasm catalyzes the CO_2 hydration into H_2CO_3 . Subsequently, H_2CO_3 dissociates into H^+ and HCO_3^- , and Band 3 operates the so called “chloride shift”, by exchanging cellular HCO_3^- with plasmatic Cl^- . This anion exchange converts the weak acid H_2CO_3 into the strong acid HCl, lowering the RBC intracellular pH. Acidification is an important step of the so called “Bohr effect”, in which the low pH triggers O_2 dissociation from the oxygenated Hb (oxyhemoglobin HbO_2), while intracellular protons bind and stabilize the deoxyhemoglobin (HbH^+) (**Figure 14**). Therefore, O_2 is delivered to metabolically active tissues, and the RBC intracellular pH is restored in order to avoid further O_2 release from HbO_2 (Wagner, 1977; Wieth *et al.*, 1982; Hamasaki and Okubo, 1996). Interestingly, the whole described process is normally occurring in a range of 0.3 to 0.7 second.

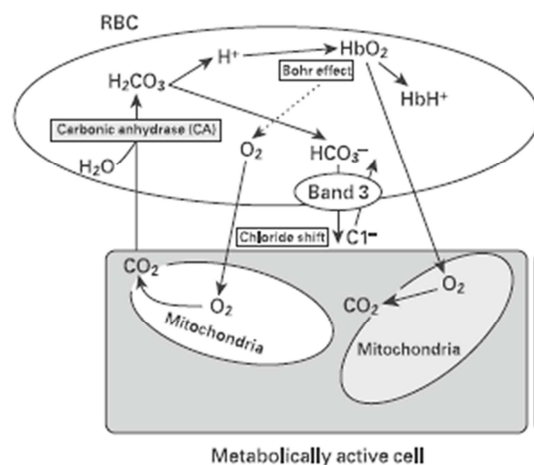


Figure 14. RBC gas exchange mechanism. O_2 delivery to metabolically active tissues starts with carbonic anhydrase-dependent reaction in the RBC cytoplasm: CO_2 hydration into H_2CO_3 , which then dissociates into H^+ and HCO_3^- ; Band 3 operates the so called “chloride shift”, by exchanging cellular HCO_3^- with plasmatic Cl^- . The consequent acidification of RBC intracellular pH participates to the “Bohr effect”: low pH induces O_2 dissociation from oxyhemoglobin (HbO_2) and intracellular protons bind to deoxyhemoglobin (HbH^+). *Adapted from* (Hamasaki and Yamamoto, 2000)

Another important function of circulating erythrocytes is the regulation of the vascular tension, through several mechanisms controlling nitric oxide (NO) levels. Firstly, RBCs are able to uptake NO produced by vascular endothelial cells, therefore reducing

its levels to prevent the NO-dependent relaxation of vascular smooth muscles and the consequent vasodilatation (Furchgott and Zawadzki, 1980; Cortese-Krott and Kelm, 2014). In addition to this “NO sink” function, erythrocytes synthesize, store and transport several products of NO catabolism (Helms and Kim-Shapiro, 2013). Finally, RBCs are able to induce NO-dependent vasodilatation, upon hypoxia and shear stress (Cosby *et al.*, 2003; Crawford *et al.*, 2006; Webb *et al.*, 2008). Under mechanical deformation, RBCs release ATP, which activates the endothelial NO synthase (eNOS) and the consequent increase in NO levels induces vasodilatation as well as increased blood flow (Ellsworth *et al.*, 1995; Wan *et al.*, 2011).

2.2.4.3 Metabolism in erythrocytes

Oxidative metabolism in mature RBCs is prevented by the lack of mitochondria; therefore, glucose remains the major source for erythrocyte energy production, through the Embden-Meyerhof pathway (see section 3.1.2) (**Figure 15**). Anaerobic glycolysis allows adenosine triphosphate (ATP) biosynthesis, as well as nicotinamide adenine dinucleotide (NAD⁺) reduction to NADH (Beutler, 1990). ATP energy is required for maintaining membrane integrity and the cationic osmotic gradient across the plasma membrane. On the other hand, reduced nicotinamide cofactors play an important role in preserving cell redox state and preventing oxidative damage. In fact, glycolysis-derived NADH is employed in the methemoglobin reductase pathway: methemoglobin reductase enzymatic reaction maintains heme iron in the reduced state (Fe²⁺) and prevents its oxidation (Fe³⁺), which would hamper Hb binding to O₂ (Brown, 1996). In addition, glucose metabolism through the pentose phosphate pathway (PPP, also referred as hexose monophosphate shunt; see section 3.1.3) is mainly involved in the biosynthesis of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (reviewed in Stincone *et al.*, 2015). NADPH then participates to the conversion of oxidized to reduced glutathione (GSH), which mediates the most important RBC antioxidant mechanism: upon glutathione oxidation, Hb is maintained in its reduced and active form (**Figure 15**). Protection from lipid and protein oxidative damage is also provided by the enzymatic activity of catalases and peroxidases, which convert hydrogen peroxide (H₂O₂) into water (H₂O) (Tavazzi *et al.*, 2008).

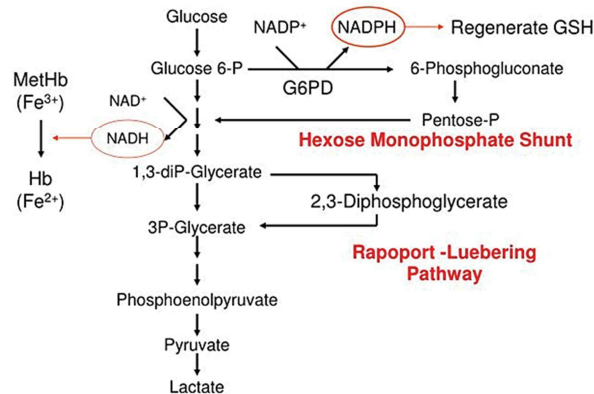


Figure 15. Metabolic pathways in circulating erythrocytes. RBCs rely on glycolysis for energy production in the form of ATP. Glycolysis-derived NADH cofactor participates to the methemoglobin reductase pathway, which controls iron oxidation state to allow O₂ binding to Hb. Glucose utilization through the hexose monophosphate (PPP) shunt allows NADPH production, to sustain the biosynthesis of reduced glutathione (GSH) and thereby RBC antioxidant mechanisms. Glucose metabolism through the Rapoport-Luebering pathway generates 2,3-bisphosphoglycerate which regulates Hb oxygen releasing function. Adapted from (www.slideserve.com)

Finally, another side pathway of glycolysis is the Rapoport-Leubering cycle, in which the glycolytic reactions do not contribute to the biosynthesis of ATP, but to the generation of 2,3-bisphosphoglycerate (2,3-BPG) (**Figure 15**). Through this metabolic cycle, excess of glucose not required for energy production is diverted towards the biosynthesis of 2,3-BPG, for the regulation of RBC oxygen releasing function through Hb. In fact, 2,3-BPG is able to bind Hb and, by reducing its oxygen affinity, leads to an increased oxygen release to peripheral tissues. Indeed, this mechanism is observed upon hypoxic conditions, including high altitude and anemia, as well as in fetal tissues, to enhance oxygen supply (Mulquiney *et al.*, 1999; reviewed in Huisjes *et al.*, 2018).

2.3 Regulation of erythropoiesis

2.3.1 Erythroblastic islands

The erythropoiesis niches, referred as erythroblastic islands (Bessis, 1958; Mohandas and Prenant, 1978) have been firstly described through transmission electron microscopy analyses in 1958 (Bessis, 1958). More recent studies have identified these niches throughout the fetal liver and the adult bone marrow, as well as in some sites of

stress erythropoiesis (see section 2.4.1), such as the red pulp of mouse spleen (Sonoda and Sasaki, 2012; Jacobsen *et al.*, 2014). Erythroblastic islands are composed of erythroblasts at different stages of differentiation physically surrounding a central nursing macrophage (Giger and Kalfa, 2015; Hom *et al.*, 2015; Heideveld and van den Akker, 2017). Cell-cell contacts allow macrophages to induce erythroblast proliferation and prevent their apoptosis (Rhodes *et al.*, 2008). In fact, erythroblasts establish several direct interactions, by integrin $\alpha 4\beta 1$ binding to VCAM-1 on the central macrophage (Chow *et al.*, 2011) and to ICAM4 on other erythroblasts present in the island (Lee *et al.*, 2006; Palis, 2016b; Seu *et al.*, 2017). In addition, both erythroblasts and macrophages express the erythroblast macrophage protein (Emp) and blocking their interaction through this protein leads to erythroblast apoptosis and impaired erythropoiesis *in vitro* (Hanspal *et al.*, 1998). Moreover, macrophages are able to sustain BFU-E and CFU-E growth, by secreting insulin-like growth factor and erythroid burst-promoting activity (Sawada *et al.*, 1989; Chasis and Mohandas, 2008), but they also release several erythropoiesis inhibitors, such as TGF- β , TNF- α and INF- γ (Zamai *et al.*, 2000; Zermati *et al.*, 2000).

It has been shown that orthochromatic erythroblasts are more often associated with erythroblastic islands close to sinusoids (Mohandas and Prenant, 1978; Yokoyama *et al.*, 2003). This finding suggests that a migration process towards these vessels, either of the whole island or of maturing erythroblasts, occurs in concomitance with erythroblast progression through terminal erythroid differentiation (Yokoyama *et al.*, 2003). Indeed, macrophages in the erythroblastic islands not only support erythroblast proliferation and provide iron for Hb biosynthesis (Pollycove, 1991; Leimberg *et al.*, 2008), but also help erythroblast enucleation. In fact, pyrenocytes are engulfed and then digested by macrophages, which recognize the PS exposure and several adhesion molecules, such as EMP and $\beta 1$ integrin, on their surface (Yoshida *et al.*, 2005).

2.3.2 Extracellular matrix-dependent regulation

Further mechanisms of terminal erythroid differentiation regulation involve specific interactions between adhesion molecules on erythroblasts and extracellular matrix proteins, such as fibronectin and laminin. Indeed, erythroblast membrane contains two

fibronectin-binding integrins, $\alpha 4\beta 1$ and $\alpha 5\beta 1$, with $\alpha 4\beta 1$ being the only expressed at late stages of differentiation (Roseblatt *et al.*, 1991; Hanspal, 1997). It has been shown that murine erythroleukemia cells differentiate more efficiently into reticulocytes, when cultured on a fibronectin matrix (Patel and Lodish, 1987). In fact, interactions with fibronectin are required for terminal differentiation, but then decrease at the very late stages, as demonstrated by final reticulocyte detachment from the fibronectin matrix (Vuillet-Gaugler *et al.*, 1990).

The laminin-binding glycoproteins of the Lutheran (Lu) blood group include two isoforms, 85 kDa and 78 kDa, members of the immunoglobulin superfamily (IgSF) (Parsons *et al.*, 1995; Rahuel *et al.*, 1996). These glycoproteins are expressed by late terminal differentiating erythroblasts (El Nemer *et al.*, 1998; Udani *et al.*, 1998) and participate to high-affinity interactions with laminin10/11, which contain the $\alpha 5$ polypeptide chain (Parsons *et al.*, 2001). Intriguingly, these laminins are localized at the level of the bone marrow sinusoids, more specifically in the sub-endothelial basement membrane (Gu *et al.*, 1999). Therefore, it has been proposed that Lu-laminin10/11 interactions would help reticulocyte location to sinusoids, prior to their release into the bloodstream (Chasis and Mohandas, 2008).

2.3.3 Cytokines

The hierarchical progression through early and late erythroid-committed progenitors is also orchestrated by a balanced equilibrium of cytokines and growth factors, with some stages relying more than others on specific mediators. For instance, EPO alone is sufficient for CFU-E colony formation, while several other mediators, such as SCF, insulin like growth factor 1 (IGF-1), glucocorticoids (GCs), IL3, and IL6 are able to support BFU-E growth, even in absence of EPO (Hattangadi *et al.*, 2011). However, multiple studies have identified EPO and SCF as the only strictly required factors for the proper development of mammalian erythroid progenitors. Indeed, severe anemia is observed in mice carrying mutations in either EPO or SCF receptor, while the absence of IL3R does not perturb erythropoiesis (Nishinakamura *et al.*, 1995; Wu *et al.*, 1995; Broudy, 1997). Therefore, further details about these factors and their related intracellular signaling pathways are here provided.

Erythropoietin (EPO)

Erythropoietin, a ~30 kDa glycoprotein, is mainly secreted by a subset of cortical interstitial cells localized close to the proximal tubules in the kidney (Obara *et al.*, 2008; Frede *et al.*, 2011). However, in the adult, EPO is also produced by a population composed of hepatocytes and interstitial cells in the liver (Koury *et al.*, 1991; Obara *et al.*, 2008), which rather represents the main EPO-producing organ during development (Sherwood, 1984; Jelkmann, 1992).

A single gene localized on chromosome 7 (mouse chromosome 5) encodes the human EPO, which is rapidly secreted without intracellular accumulation (Law *et al.*, 1986). Normal EPO serum concentrations fall into the range of 5-30mU/mL (Vandekerckhove *et al.*, 2009), but quickly increase in conditions where oxygen tension decreases (hypoxia, anemia), through a hypoxia inducible factor (HIF)-mediated EPO gene expression regulation (see section 2.3.4). The resulting rapid induction of erythropoiesis occurs through EPO-dependent promotion of CFU-E survival, by mainly preventing their apoptosis, and transition to terminal differentiation (Koury and Bondurant, 1990; Molineux *et al.*, 2009; Hattangadi *et al.*, 2011). In agreement with the direct EPO effect on CFU-E, several *in vivo* studies have shown that EPO and EPO receptor KO mice lack post-CFU-E erythroid progenitors, without alterations in earlier differentiation stages (Wu *et al.*, 1995; Lin *et al.*, 1996).

The EPO specific receptor (EPOR), which is member of the type I cytokine receptor family, is a 55 kDa transmembrane protein, encoded by a single gene localized on human chromosome 19 (mouse chromosome 9) (D'Andrea *et al.*, 1989; Jones *et al.*, 1990). Several studies in mice have shown that EPOR mRNA and protein expression increase during the transition from BFU-E to CFU-E stage, and then start to decrease in basophilic erythroblasts, to become undetectable on mature reticulocytes and RBCs (Hattangadi *et al.*, 2011). Upon EPO direct binding to an EPOR homodimer, a conformational change of this receptor occurs, leading to the activation of the EPOR-associated Janus kinase 2 (Jak2) tyrosine kinase. Jak2 phosphorylates specific EPOR tyrosine residues, which represent anchoring sites for enzymes containing Src homology 2 (SH2) domains,

including STAT5, PI3K/Akt, MAPK and PLC- γ (phospholypase C gamma) (Remy *et al.*, 1999; Watowich, 1999; Wojchowski *et al.*, 1999), involved in the downstream EPO-dependent signaling cascades.

Upon Jak2-dependent phosphorylation, activated STAT5 consequently homodimerize and translocate to the nucleus, to regulate the expression of genes involved in cell survival, proliferation and differentiation (Vandekerckhove *et al.*, 2009). It has been found that constitutive activation of STAT5 is able to support erythroid progenitor self-renewal and differentiation, even in absence of EPOR or Jak2 signal (Grebien *et al.*, 2008). EPOR activation induces PI3K signaling and consequent activation of the serine/threonine protein kinase B (PKB)/Akt, which not only lead to cell cycle progression, but also promote erythroid progenitor proliferation and survival by acting on several downstream targets (Sivertsen *et al.*, 2006). Indeed, PI3K requirement in erythropoiesis has been demonstrated by the decreased number of BFU-E and CFU-E progenitors observed in mice lacking the regulatory PI3K subunit, p85. Finally, the activation of different MAP kinases, such as extracellular regulated kinases 1/2 (Erk1/2), Jun kinase (Jnk) and p38, downstream of EPO signaling is involved in cell cycle progression and cell survival. In addition, it has been shown that Ras is not only required for erythropoiesis, more specifically for the differentiation step into late basophilic erythroblasts, but also for hematopoietic progenitor proliferation (Khalaf *et al.*, 2005).

Stem cell factor (SCF)

As previously discussed, SCF is produced by several cell types within the bone marrow niche and signals via its receptor c-Kit. In the context of erythropoiesis, c-Kit is expressed at high levels on BFU-E and during the transition to CFU-E, while a decrease is subsequently observed with loss of c-Kit expression in polychromatic and orthochromatic erythroblasts (Broudy, 1997). A recent proteomic analysis has confirmed that c-Kit levels start to decrease at the basophilic erythroblast stage, while a progressive increase in the expression of globins, Band 3, and GlyA is observed (Gautier *et al.*, 2016). SCF binding to c-Kit induces a dimerization of the receptor and autophosphorylation on specific tyrosine residues. This represents the first step for the initiation of several downstream cascades, involving PI3K p85 subunit, Ras GTPase

activating protein, as well as Src kinases, which play a role in cell proliferation and survival. In fact, SCF promotes self-renewal of early erythroid progenitors CFU-E, as well as proerythroblasts. Indeed, similar effect with increased numbers of early progenitors is found upon c-Kit activating mutations; contrarily, sustained c-Kit signal results then deleterious by preventing erythroblast terminal differentiation (Haas *et al.*, 2015).

2.3.4 Transcription factors

The lineage restricted expression of erythroid-specific genes, as well as the dynamic regulation of their expression throughout the differentiation process are mediated by several transcription factors (TFs), including GATA-1, SCL/TAL1, Klf1, LMO2, LDB1, Gfi-1b and HIF (Hattangadi *et al.*, 2011; Stadhouders *et al.*, 2015).

GATA-1 is the first discovered member of a zinc finger family of TFs able to bind the DNA consensus sequence 5' A/T GATA A/G 3', named GATA motif (Orkin, 1992). This family is composed of six members, GATA-1 to -6. More specifically, GATA-2 regulates the proliferation of early erythroid progenitors, while GATA-1 the terminal differentiation. In agreement with mouse studies showing the importance of a GATA-2/GATA-1 switch for a proper erythropoiesis (Ikonomi *et al.*, 2000), a recent transcriptomic analysis has confirmed that this switch occurs between the human BFU-E and CFU-E stage. Indeed, while GATA-1 and GATA-2 are coexpressed in BFU-E, only GATA-1 remains highly expressed starting from the CFU-E stage and throughout the erythroid differentiation process (Li *et al.*, 2014).

GATA-1 plays a central role in regulating the expression of erythroid-specific genes, such as EPOR (Zon *et al.*, 1991), but also in cell survival. Indeed, GATA-1 KO mice develop lethal anemia, as erythroid progenitors blocked at the proerythroblast stage die of apoptosis (Pevny *et al.*, 1991). GATA-1 activity is regulated by several post-translational modifications. For instance, acetylation by P300 or CREB-binding protein enhances GATA-1 transcriptional activity, as well as inducing degradation via the ubiquitin/proteasome pathway, if GATA-1 is concomitantly phosphorylated by MAPK (Hernandez-Hernandez *et al.*, 2006). On the other hand, phosphorylation by Akt increases GATA-1 activity (Zhao *et al.*, 2006).

GATA-1 is involved in the formation of transcriptional complexes, which not only include different factors, but also differentially regulate the expression of specific erythroid genes. First, FOG-1 (Friend of GATA-1) is considered an essential factor for GATA-1-dependent modulation of erythropoiesis, as mice carrying mutations that prevent GATA-1/FOG-1 interactions develop severe and lethal anemia (Tsang *et al.*, 1998). Indeed, the GATA-1/FOG-1 complex activates transcription, whereas the GATA-1/FOG-1/MeCP1 complex mainly shows a repressive effect (Snow and Orkin, 2009). Several other proteins, including LMO2, SCL/TAL1, Gfi-1b, and PU-1, take part to transcriptional complexes by directly interacting with GATA-1 (Vandekerckhove *et al.*, 2009). Finally, GATA-1-binding sites are surrounded by DNA consensus sequences for SCL/TAL1, highlighting a cooperation mechanism between these two factors (Kassouf *et al.*, 2010).

SCL/TAL-1 is a member of the basic helix-loop-helix (bHLH) family of transcription factors, which is present in hematopoietic stem cells, as well as erythroid and megakaryocytic progenitors (Hall *et al.*, 2003) (see section 1.5.3). During erythropoiesis, SCL is upregulated in human polychromatic and orthochromatic erythroblasts, while earlier in mice at the basophilic erythroblast stage (Pishesha *et al.*, 2014; An *et al.*, 2015). SCL is part of a pentameric complex, together with the ubiquitously expressed E-proteins, as well as LMO2, Ldb1 and GATA-1 or GATA-2. Association with GATA-2 in CD34⁺ hematopoietic progenitor cells positively regulates c-Kit transcription (Lécuyer *et al.*, 2002), while showing repressive activity in early erythroid progenitors. Contrarily, the switch to GATA-1 in terminally differentiating cells is associated with increased expression of fundamental erythroid genes, such as human GlyA and murine α -globin chain (Fujiwara *et al.*, 2009; Tripic *et al.*, 2009; Pimkin *et al.*, 2014). Finally, it has been shown that not only SCL, but also LMO2 KO cause severe anemia in mice (Shivdasani *et al.*, 1995; Crispino and Weiss, 2014).

Klf1 (formerly called EKLF, Erythroid Kruppel-like factor) is a master regulator of erythropoiesis, member of the zinc finger family of TFs, which recognizes the DNA consensus motif CACCC (Miller and Bieker, 1993). Mutations in CACCC sequence are associated with β -thalassemia, as this sequence is present in the human β -globin gene

promoter. In fact, Klf1 plays a fundamental role in the fetal to adult globin switch, but it also modulates important genes for hemoglobin metabolism and membrane stability (Vandekerckhove *et al.*, 2009). More recently, Klf1 has been shown to be required for erythroblast enucleation (Parkins *et al.*, 1995; Magor *et al.*, 2015), by regulating the expression of cell cycle and nuclear membrane proteins, as well as deacetylases and caspases (Gnanapragasam *et al.*, 2016; reviewed in Gnanapragasam and Bieker, 2017). Finally, Klf1 and GATA-1 binding sites are found in close vicinity, but Klf1 consensus sequences overlap SCL/TAL-1 ones, suggesting mutual exclusivity of these two factors in GATA-1 transcriptional complexes (Kerenyi and Orkin, 2010).

HIF (Hypoxia inducible factor) is responsible for the oxygen tension-mediated regulation of EPO gene expression. HIF is a heterodimer composed of HIF α and HIF β subunit (Jiang *et al.*, 1996), and HIF α protein levels are regulated through hydroxylation by the enzyme HIF-prolyl hydroxylase (Schofield and Ratcliffe, 2004; reviewed in Haase, 2017). This modification induces HIF α ubiquitination by the Von Hippel-Lindau (VHL) factor, which targets for proteasome-mediated degradation (Ohh *et al.*, 2000; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). In fact, the HIF-prolyl hydroxylase enzymatic activity is positively regulated by high oxygen tension, as well as the two cofactors iron and α -ketoglutarate. Thus, in normoxia HIF α hydroxylation leads to its degradation, while the lack of oxygen under hypoxic conditions allows the stabilization of the non-hydroxylated HIF α subunit (Schofield and Ratcliffe, 2004), therefore inducing EPO gene expression. More precisely, HIF heterodimer is able to bind cis-regulatory elements in several hypoxia-responsive genes, which also include the majority of the glycolytic enzymes (Semenza *et al.*, 1994, 1996). The latter are in fact induced under hypoxia, which represents a main regulator of glycolysis in erythrocytes, as well as other cell types.

2.3.5 Cell cycle regulation

During terminal erythroid differentiation, each cell division is simultaneously coupled with a stage of differentiation, which identifies a unique feature of the erythropoiesis process (Chen *et al.*, 2009; Hu *et al.*, 2013). Nevertheless, these cell division dynamics are significantly different as compared to early erythropoiesis, in which cell division

leads to the expansion of a certain population (reviewed in von Lindern, 2006). Several studies have also highlighted different length of the cycle phases, as well as the involvement of diverse cell cycle regulators between early and terminal erythropoiesis (Dolznig *et al.*, 1995; Ciemerych *et al.*, 2002; Malumbres *et al.*, 2004). Interestingly, the retinoblastoma protein (Rb) is involved in the transition from early to late progenitors and its absence leads to differentiation blockage, which is also associated with impaired induction of mitochondrial biogenesis (Sankaran *et al.*, 2007). On the other hand, Genome-Wide Association Studies (GWAS) have shown that cyclin D3 (CCND3) and A2 (CCNA2) play an important role in terminal differentiation. Firstly, upon cyclin D3 KO or *in vitro* downregulation, the number of terminal divisions is decreased, leading to an anemic phenotype in CCND3 deficient mice, with reduced RBC counts while increased MCV (Sankaran *et al.*, 2012). Secondly, cyclin A2 is involved in the progression through the cytokinesis phase and its downregulation affects erythroblast cell size (Ludwig *et al.*, 2016). Finally, several erythroid-restricted transcription factors have been found to play a role in cell cycle regulation. More precisely, GATA-1 is involved in the mechanisms modulating G1-phase arrest (Rylski *et al.*, 2003), while Klf1 is required for efficient cell cycle exit in orthochromatic erythroblasts. In fact, in absence of Klf1, its targets p18 and p27 show low expression levels, which lead to blockage at the orthochromatic erythroblast stage and consequent impaired enucleation (Gnanapragasam *et al.*, 2016).

2.3.6 Epigenetic regulation

Specific changes in the gene expression profile occur upon hematopoietic progenitor cell commitment towards the erythroid lineage, as well as throughout the erythropoiesis process. Thus, spectrin and membrane cytoskeleton, as well as hemoglobin-related genes are progressively upregulated during erythroid differentiation, while the expression of genes involved in protein synthesis and ribosome biogenesis decreases (Li *et al.*, 2014). Interestingly, the most complex transcription profile is observed in proerythroblasts, while a general decrease in transcription occurs upon transition from basophilic to orthochromatic stage (Waggoner and Liebhaber, 2003; Siatecka and Bieker, 2011). Finally, few remaining transcripts are found in reticulocytes, both in primitive and definitive erythropoiesis (Kingsley *et al.*, 2013).

Dynamic changes in several histone methylation marks, including H3K27me3 (repressive mark) as well as H3K4me2 and H3K4me3 (activating marks), have been observed upon erythroid commitment of hematopoietic progenitor cells. Indeed, “poised” genes localized in the so called “bivalent chromatin domains”, simultaneously marked by H3K27me3 and H3K4me3 (Cui *et al.*, 2009), are activated upon erythroid differentiation by losing the H3K27me3 repressive mark (Hattangadi *et al.*, 2011). In addition, upon induction of erythropoiesis, non-erythroid-specific genes are repressed by losing the H3K4me2 mark; while, H3K4me3 addition on erythroid-specific genes induces their expression (Hattangadi *et al.*, 2011). Indeed, during murine erythroid differentiation several histone modifications considered as active transcription marks (H3K4me2, H3K4me3 and H3K9Ac) or repressive marks (H3K27me3) are found at the promoter of highly induced or repressed genes, respectively (Wong *et al.*, 2011). Interestingly, H3K79 methylation, observed within the globin locus and operated by Dot1 methyltransferase (Guenther *et al.*, 2007), plays an important role in erythropoiesis, as decreased number and size of BFU-E colonies lead to impaired primitive and definitive erythropoiesis in absence of Dot1 (Feng *et al.*, 2010).

It has been also observed that global levels of histone acetylation marks, such as H3K9Ac, H4K5Ac, H4K8Ac and H4K12Ac are reduced during terminal erythroid differentiation (Popova *et al.*, 2009). Accordingly, treatment of human erythroid-committed progenitors with histone deacetylase (HDAC) inhibitors hampered terminal differentiation (Fujieda *et al.*, 2005). More precisely, downregulation of HDAC2 levels or activity specifically affects mouse fetal liver erythroblast differentiation, by impairing chromatin condensation and enucleation (Ji *et al.*, 2010; Hattangadi *et al.*, 2011). Defects in chromatin condensation and terminal erythroid differentiation have also been observed upon histone methyltransferase Setd8 deletion, which is associated with lethal anemia (Malik *et al.*, 2017).

Hypermethylation of the transcription factor binding sites in the promoter region is generally associated with gene silencing; therefore, it was expected that hypermethylation is involved in nuclear condensation upon terminal differentiation. However, the analysis of a global cytosine methylation profile found a progressive loss of methylation during human erythropoiesis (Y. Yu *et al.*, 2013). In addition, several DNA demethylases have been shown to play an important role in erythroid differentiation. In

the chicken, the expression of the β A globin locus is modulated through demethylation of the promoter by the cTET1 (Ten Eleven Translocation isoform 1) methylcytosine dioxygenase (Okuzaki *et al.*, 2017). Impaired erythropoiesis is observed upon knockdown of Zebrafish TET2, which demethylates regulatory sites in the gene promoter of several transcription factors, including SCL/TAL1, MYB and GATA-1 (Ge *et al.*, 2014). Finally, it has been recently shown that knockdown of human TET3 inhibits terminal erythroid differentiation, while TET2 downregulation mainly affects early progenitors (Yan *et al.*, 2017). Indeed, TET2 deficiency leads to impaired differentiation of human CFU-E progenitors, associated with the expansion of a population showing CFU-E phenotype but altered function (Qu *et al.*, 2018).

The role of TET methylcytosine dioxygenases and other demethylases in hematopoietic malignancies, as well as their metabolism-mediated regulation will be further discussed in this thesis (see section 2.4.3 and 3.2.3.4).

2.4 Deregulated erythropoiesis

The homeostatic physiological renewal of circulating erythrocytes, referred as steady-state erythropoiesis, is characterized by low EPO plasma levels, which are finely regulated to constantly induce CFU-E bone marrow progenitor differentiation into mature RBCs. On the contrary, the extreme erythrocyte deficiencies observed in case of anemia, prolonged hypoxia and recovery from bone marrow irradiation induce the so called “stress erythropoiesis” response (Liao *et al.*, 2018; Mairbäurl, 2018). The latter, as well as different types of disordered erythropoiesis will be further described in this section.

2.4.1 Stress erythropoiesis

Under conditions of acute blood loss or significant reduction in tissue oxygenation, a massive red cell production occurs in response to enhanced EPO biosynthesis and secretion (Paulson *et al.*, 2011). Indeed, stress erythropoiesis is observed upon anemia and hemolysis, but also at high altitude, where the air oxygen content is lower

(Mairbäurl, 2018). In general, prolonged hypoxia triggers HIF α -mediated expression of EPO, leading to the expansion of the erythroid progenitor pool in the bone marrow (Gupta *et al.*, 2017). In fact, upon stress erythropoiesis a large number of CFU-Es is produced from their immediate precursor, BFU-Es, which show significantly higher proliferation rate than CFU-Es (Paulson *et al.*, 2011).

Stress erythropoiesis has been mainly studied in mice, demonstrating that some erythroid progenitors migrate to extramedullary sites, such as the fetal liver, as well as the adult liver and spleen, to complete their differentiation (Paulson *et al.*, 2011; Liao *et al.*, 2018). Indeed, stress erythroid progenitors (SEPs) rapidly derive from ST-HSCs, which become erythroid-primed upon migration to the extramedullary sites (Harandi *et al.*, 2010). Subsequently, SEPs undergo extensive proliferation and differentiate into BFU-Es, as stimulated by the significant increase in EPO production (Perry *et al.*, 2007; Xiang *et al.*, 2015). These processes of migration and proliferation require several non-canonical erythropoiesis signals, including bone morphogenetic protein 4 (Bmp4), Hedgehog (Hh) and growth and differentiation factor 15 (Gdf15) (Perry *et al.*, 2007, 2009; Millot *et al.*, 2010). On the other hand, the subsequent terminal differentiation is similarly regulated as steady-state erythropoiesis, showing GATA-1 activation, as well as progressive increase in Hb biosynthesis (Whitelaw *et al.*, 1990; Pevny *et al.*, 1991; Liao *et al.*, 2018).

In human, stress erythropoiesis mainly occurs in the bone marrow and shares some features with fetal erythropoiesis, as demonstrated by the increased number of HbF positive progenitor and mature cells (Paulson *et al.*, 2011). Because of the rapid release of a significant number of newly synthesized erythrocytes, aiming to rescue the RBC deficiency, stress erythropoiesis is associated with increased circulating reticulocyte counts (Aoki and Tavassoli, 1981; Rhodes *et al.*, 2016).

2.4.2 Anemia

Conditions where the amount of circulating erythrocytes, the level of hemoglobin or RBC function are reduced lead to a state of anemia (reviewed in Sankaran and Weiss, 2015). In fact, three general causes of anemia are identified: significant loss of circulating RBCs upon bleeding, reduced or altered RBC production and increased hemolysis (Hillman and Ault, 2001). A decrease in erythroid differentiation or reticulocyte release

into the bloodstream could derive from lack of regulating hormones, as well as key nutrients required for a proper differentiation, including iron, vitamin B12 and folic acid (Broadway-Duren and Klaassen, 2013). In addition, altered RBC production is also the result of different types of bone marrow failure, such as Diamond Blackfan anemia (DBA) (reviewed in Sakamoto and Narla, 2018) (see section 2.4.2.2) and myelodysplastic syndrome (MDS) (see section 2.4.3).

Anemia is also consequence of hemolysis, a non-physiological and premature RBC clearance before the end of their 120-day life span (Dhaliwal *et al.*, 2004; Chen and Zieve, 2012). Anemia resulting from increased hemolysis is observed in diverse genetic diseases, such as sickle cell disease (SCD) and thalassemia, as well as metabolism-related disorders, including glucose-6-phosphate dehydrogenase (G6PDH) and pyruvate kinase (PK) deficiencies (Broadway-Duren and Klaassen, 2013).

2.4.2.1 Glucose-6-phosphate dehydrogenase deficiency

The most common inherited form of acute hemolytic anemia (AHA) is the G6PDH deficiency, with more than 400 million people affected worldwide. Up to 160 mutations have been characterized in the G6PDH gene, localized on the X chromosome and encoding for the rate-limiting enzyme of the pentose phosphate pathway (PPP) (Cappellini and Fiorelli, 2008). Defects in the G6PDH activity strongly impair RBC metabolism, which relies on the PPP for the biosynthesis of NADPH, required for the production of reduced glutathione, the most important reducing agent in mature erythrocytes (see section 2.2.4.3). Indeed, G6PDH deficiency leads to decreased amount of NADPH, and consequently of reduced glutathione, rendering RBCs more susceptible to oxidant-induced injury (Grace and Glader, 2018). Thus, free radical-mediated oxidative damage leads to RBC premature clearance by the splenic reticuloendothelial system, therefore generating anemia (Rifkind, 1965).

Most of the G6PDH-deficient individuals are asymptomatic, but oxidative stress-inducing agents, such as drugs, infections, and ingestion of fava beans can lead to clinical manifestations, including dark urine, fatigue, tachycardia, shortness of breath, and jaundice. Life expectancy of G6PDH-deficient patients is not significantly affected if acute hemolysis events are prevented, by avoiding oxidant-induced injury. Events of acute

hemolysis rarely develop into severe anemia, which can be eventually treated by RBC transfusions (Tavazzi *et al.*, 2008).

2.4.2.2 Diamond-Blackfan anemia (DBA)

Diamond-Blackfan anemia (DBA) is a rare form of congenital hypoplastic anemia, normochromic and usually macrocytic, due to a differentiation blockage in bone marrow erythroid progenitors and consequent reticulocytopenia. This differentiation blockage, likely occurring between the BFU-E and CFU-E stage, leads to erythroblastopenia with paucity of erythroid progenitors in the bone marrow, while not affecting the other blood cell populations (reviewed in Da Costa *et al.*, 2018). Clinical manifestations, including pallor and lethargy, mainly appear during the first year of life (90% of the cases); moreover, about 50% of affected individuals show congenital malformations (reviewed in Ball, 2011). In addition, increased activity of the erythrocyte adenosine deaminase (eADA) enzyme, which is involved in the purine salvage pathway, has been detected in more than 75% of the cases (Fargo *et al.*, 2013; Narla *et al.*, 2016). Patient phenotype ranges from mild (mild or no anemia with slight abnormalities in the erythroid lineage) to severe forms, with the latter including fetal anemia and hydrops fetalis (Ball, 2011; Wlodarski *et al.*, 2018). Furthermore, DBA has been associated with an increased risk of developing acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), as well as solid tumors, including colon carcinoma and osteosarcoma (Lipton *et al.*, 2001; Vlachos *et al.*, 2012; Lipton and Alter, 2017; reviewed in Sakamoto and Narla, 2018).

DBA is a polygenic disease, linked to mutations or deletions in different genes encoding for ribosomal proteins (RPs), with the RPS19 of the small ribosome subunit being the first identified (Gustavsson *et al.*, 1997; Draptchinskaia *et al.*, 1999) and showing the higher incidence. “Non-RP” genes also found altered in DBA patients include GATA-1, the pre-rRNA-processing protein TSR2 and EPO (reviewed in Da Costa *et al.*, 2018). The majority of the mutations observed are sporadic with 55% to 60% of the patients carrying *de novo* pathogenic variants, while 40% to 45% showing an inherited form of DBA, mainly transmitted in autosomal dominant manner (reviewed in Ball, 2011). Indeed, all the RP mutations already characterized are heterozygous, while in case of homozygosity a lethal phenotype has been observed in different DBA models (Matsson *et al.*, 2006). After diagnosis, one year old affected children are treated with

corticosteroids, which have been found to improve RBC counts in about 80% of the cases (reviewed in Ball, 2011). On the other hand, no corticosteroid-responsive patients undergo chronic transfusions, while hematopoietic stem cell transplantation (HSCT) still remains the only curative therapy (reviewed in Ball, 2011).

2.4.3 Myelodysplastic syndrome (MDS)

Myelodysplastic syndrome (MDS) comprises a heterogeneous group of clonal HSC malignancies characterized by ineffective clonal hematopoiesis and dysplasia of blood progenitor cells (Vardiman, 2006). Indeed, several dysplastic figures, such as binucleate erythroid precursors and ring sideroblasts, are detected in bone marrow aspirates, as a result of the altered progenitor cell differentiation and maturation (reviewed in Steensma, 2015). These alterations lead to cytopenia, mostly represented by macrocytic or normocytic anemia, and main symptoms described in MDS patients include fatigue, dyspnea, poor exercise tolerance, bleeding, as well as susceptibility to infection (Germing *et al.*, 2008; reviewed in Steensma, 2015).

MDS represents the most common acquired bone marrow failure, showing a prevalence of 7:100 000, with incidence increasing with age (Williamson *et al.*, 1994; Sekeres, 2010; Neukirchen *et al.*, 2011). The majority of MDS cases (85% to 90%) are idiopathic, as consequence of spontaneous mutations, which show increased frequency in concomitance with advancing age (Genovese *et al.*, 2014; McKerrell *et al.*, 2015). Chromosome abnormalities and gene alterations have been found even in the most primitive stem cell compartment, defining MDS as a “stem cell disease” (Elias *et al.*, 2014). These aging-related founder mutations, which are normally followed by other lesions, confer selective advantage to a subset of stem or progenitor cells, mainly leading to the expansion of myeloid precursors (Jaiswal *et al.*, 2014; Xie *et al.*, 2014; reviewed in Dussiau and Fontenay, 2018).

In addition to cytogenetic aberrations (del(5q), del(7q), trisomy 8, amongst others), a panel of MDS driver mutations (discussed in section 2.4.3.1) has been identified in genes encoding for signaling molecules and factors involved in mRNA splicing, DNA methylation, as well as chromatin remodeling (Papaemmanuil *et al.*, 2013; reviewed in Shallis *et al.*, 2018). Moreover, it has been shown that changes in the bone marrow

stroma and secreted cytokines contribute to the ineffective clonal hematopoiesis (Raza *et al.*, 1995; Raaijmakers *et al.*, 2010). For instance, bone marrow macrophages of MDS patients produce increased levels of TNF- α (Flores-Figueroa *et al.*, 2002), consequently inhibiting the macrophage-mediated regulation of erythroblast proliferation (Chasis and Mohandas, 2008). Indeed, defects in erythroblast enucleation are also observed in MDS patients (Garderet *et al.*, 2010; Park *et al.*, 2016).

To conclude, the mechanisms involved in MDS development highlight the importance of a tight hematopoiesis regulation, allowing balanced equilibrium between the different lineage commitments. In MDS, perturbation of this equilibrium leads to myeloid progenitor expansion and impaired erythropoiesis, through a process that is still under investigation. It has been also shown that MDS elevates the risk of acute myeloid leukemia (AML) development (Germing *et al.*, 2001, 2008), especially in presence of mutations leading to granulo-monocytic progenitor amplification (Elias *et al.*, 2014; reviewed in Dussiau and Fontenay, 2018). Further insights regarding the genetic heterogeneity of MDS, as well as the treatment and outcome of MDS patients are provided in the following section.

2.4.3.1 Genetic background in MDS

The main founder mutations in MDS patients affect molecules involved in DNA methylation (TET2 and DNMT3A), chromatin remodeling (ASXL1 and EZH2), as well as RNA splicing (SF3B1, SRSF2, U2AF1, or ZRSR2) (Delhommeau *et al.*, 2009; Gelsi-Boyer *et al.*, 2009; Ernst *et al.*, 2010; Yoshida *et al.*, 2011). On the other hand, upon MDS transformation into AML, major alterations are observed in genes related to cell differentiation and proliferation, such as RUNX1, GATA-2, BCOR, N/KRAS and CBL (Damm *et al.*, 2013; Mazumdar *et al.*, 2015). Finally, mutations in DNMT3A, TET2, IDH1/2, and TP53 genes have been identified in MDS-derived AML and *de novo* AML (reviewed in Dussiau and Fontenay, 2018) (**Figure 16**).

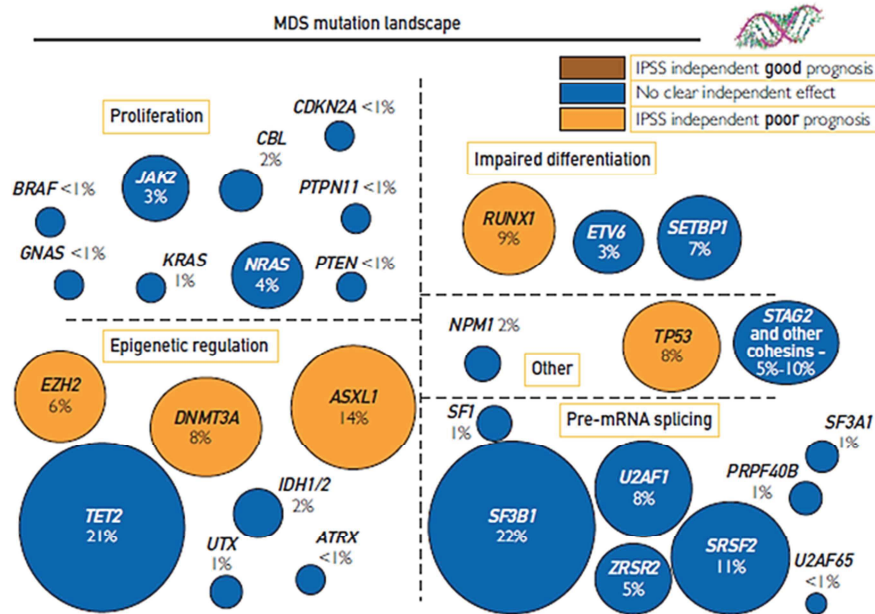


Figure 16. Principal founder mutations in myelodysplastic syndrome (MDS). MDS founder mutations have been described in different types of genes, involved in DNA methylation (TET2 and DNMT3A), chromatin remodeling (ASXL1 and EZH2) and RNA splicing (SF3B1, SRSF2, U2AF1, or ZRSR2), but also in molecules participating to the regulation of cell differentiation and proliferation (RUNX1, GATA-2, BCOR, N/KRAS and CBL), as well as metabolism-related genes (IDH1/2). *Adapted from* (Steensma, 2015)

The different MDS subtypes show significant clinical heterogeneity. This heterogeneity derives from the specific founder mutation, but also from the combination and occurrence order of additional genetic alterations (reviewed in Dussiau and Fontenay, 2018), which induce the clonal expansion of a selected progenitor population (Will *et al.*, 2012). For instance, mutations in SF3B1 are correlated to the expansion of the LT-HSC compartment (Obeng *et al.*, 2016) and generally lead to sideroblastic anemia (Mortera-Blanco *et al.*, 2017). This genetic and phenotypic heterogeneity is responsible for the significant variability observed in MDS outcome, which influences patient treatment and prognosis (Germing *et al.*, 2008). For instance, lenalidomide is a FDA accepted drug for del(5q)-derived lesions, leading to amelioration of anemia symptoms and increased survival (Komrokji and List, 2016). This drug is less effective in non-del(5q) MDS cases, where a relapse is often observed and associated with the onset of additional mutations (Chesnais *et al.*, 2016). On the other hand, DNA methyltransferase 1 inhibitors, azacitidine and decitabine (aza-substituted analogues of cytosine nucleoside), are an approved treatment for patients carrying mutations in TET2

or DNMT3A (Itzykson *et al.*, 2011; Bejar *et al.*, 2014). These alterations are normally associated with a hypermethylation and consequent gene silencing phenotype, which are reversed upon treatment with azacitidine and decitabine (McDevitt, 2012; reviewed in Steensma, 2015). Because of MDS occurrence during elderly, HSCT does not represent a main treatment, while current research aims to developing more efficient targeted therapies, adapted to the genetic and clinical heterogeneity of the MDS patients (reviewed in Dussiau and Fontenay, 2018).

2.4.3.2 IDH mutations: MDS and progression to acute myeloid leukemia

Recent work has shown prevalence of mutant isocitrate dehydrogenase (IDH) in MDS patients at the leukemic transformation stage, suggesting a role for IDH defects into the development and progression of hematological malignancies (N. Wang *et al.*, 2017). Indeed, alterations in IDH are often associated with *de novo* AML, as well as with MDS transformation into AML (secondary AML or sAML). IDH catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG), with concomitant production of reduced nicotinamide adenine dinucleotides. Indeed, IDH exists in three different isoforms, with IDH1 localized into the cytosol and peroxisome, while IDH2 and IDH3 into the mitochondria (reviewed in Al-Khallaf, 2017) (see section 3.2.3). It has been shown that IDH mutations mainly occur in the exon 4, at the level of specific arginine residues (Tefferi *et al.*, 2010) and therefore affecting the enzyme catalytic site (Ye *et al.*, 2018). In fact, these gain-of-function mutations confer a neomorphic catalytic activity, allowing reduction of α -KG to the 2-hydroxyglutarate (2-HG) oncometabolite (Dang *et al.*, 2009; Ward *et al.*, 2010; Krell *et al.*, 2013). Increased 2-HG levels have been linked to alterations in hematopoietic differentiation and consequent malignant transformation, as a result of modifications in the DNA methylation profile (Figueroa *et al.*, 2010) (see section 3.2.3.4). Indeed, worse prognosis has been associated with higher serum levels of 2-HG in sAML patients, carrying mutations in IDH2. The latter are often associated with defects in other genes, such as DNMT3A and SRSF2 (Lin *et al.*, 2018), supporting previous evidence that IDH2 mutation alone is not sufficient for inducing malignant transformation to AML (Ogawara *et al.*, 2015).

3 Cell energy sources: main metabolic pathways

One of the main interests in our laboratory consists in the metabolism-dependent modulation of human HSC specification towards the erythroid lineage. The role of metabolism and metabolic regulators in the maintenance and commitment of the HSCs has started to be investigated very recently, focusing on the main metabolic sources not only providing cell energy, but also constituting the cell biomass. Indeed, proteins, sugars, lipids and DNA/RNA derive from several metabolic pathways, which mainly involve glucose, glutamine and other amino acids, as well as fatty acids (**Figure 17**). These nutrients are either acquired from the extracellular environment or intracellularly synthesized, in order to support the metabolic requirements of different cell types, in physiological, stress and pathological conditions. In this chapter, I will first present the principal cell energy sources and related metabolic pathways. I will then introduce the current knowledge regarding the modulation of these metabolic pathways in the HSC maintenance *versus* commitment, and more specifically in the context of the human erythroid differentiation (see section 3.4).

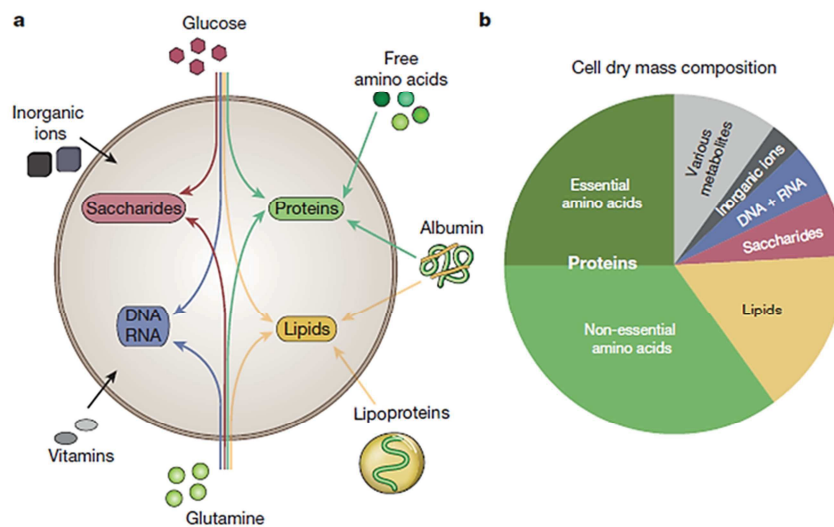


Figure 17. Cell energy sources. Panel **a** Main energy sources and molecules contributing to the cell mass composition (represented in panel **b**). Adapted from (Palm and Thompson, 2017)

3.1 Glucose

Glucose is the most abundant sugar found in nature, representing a fundamental energy source for all the mammalian cells (Thorens and Mueckler, 2009). In fact, this 6-carbon monosaccharide (or hexose) is substrate for catabolic and anabolic biochemical reactions, as well as for the biosynthesis of the energy storage carbohydrate polymer, glycogen (Adeva-Andany *et al.*, 2016). In addition, glycosylation constitutes a major post-translational modification, which has been shown to regulate protein function and stability (reviewed in Arey, 2012). According to its important role, all mammalian cells are capable of absorbing glucose from the extracellular compartment, despite the fact that this polar and large molecule cannot diffuse through the plasma membrane (Navale and Paranjape, 2016). Indeed, all mammalian cells express surface transporters, allowing glucose uptake, but differing in terms of transport kinetics and regulation mechanisms (Thorens and Mueckler, 2009). Further insights into the process of glucose uptake and its intracellular metabolism will be provided in the following sections.

3.1.1 Glucose transport

Glucose uptake requires the expression on cell surface of specific glucose transporters, which include many structurally related proteins, divided into two main families: facilitated diffusion glucose transporters (GLUTs) and sodium-glucose linked transporters (SGLTs) (Navale and Paranjape, 2016).

The GLUT transporter family, also referred as SLC2 (for **S**olute **C**arrier 2), includes 13 members in human (Uldry and Thorens, 2004), which share several related features with the recently characterized structure of the first member discovered, GLUT1 (Kasahara and Hinkle, 1977; Baldwin *et al.*, 1981; Mueckler *et al.*, 1985; Deng *et al.*, 2014). GLUT transporters are composed of approximately 500 amino acids and are characterized by 12 membrane-spanning regions, with both C- and N-terminal domains located in the intracellular compartment (Mueckler and Thorens, 2013; Navale and Paranjape, 2016). According to the degree of homology identified by alignment of their protein sequences, GLUT transporters have been divided into three classes (I, II and III) (Joost *et al.*, 2002) (see Table 1). The class I is composed of 4 members, GLUT1 to GLUT4, with GLUT1 being

one of the most represented molecules on the erythrocyte cell surface (200-700.000 copies/cell), corresponding to 10% of the total plasma membrane protein mass (Helgerson and Carruthers, 1987; Mueckler, 1994; Rungaldier *et al.*, 2013). The class II includes GLUT5, GLUT7, GLUT9 and GLUT11; while GLUT6, GLUT8, GLUT10, GLUT12 and GLUT13 (also known as HMIT or H⁺-driven myoinositol transporter) constitute the class III. To conclude, GLUT family members adopt a facilitated diffusion transport mechanism, which allows the energy-independent uptake of glucose, down its concentration gradient (Mueckler and Thorens, 2013; Navale and Paranjape, 2016). Not only glucose but also other metabolites, including further hexose sugars, dehydroascorbic acid (DHA) (Montel-Hagen *et al.*, 2008), glucosamine and urate, are substrates for GLUT transporters (Mueckler and Thorens, 2013).

Family	Isoform	Gene name	Tissue distribution	Substrate specificity
GLUT	GLUT1	SLC2A1	Ubiquitous (brain, red blood cells, colon, placenta)	Glucose/galactose
	GLUT2	SLC2A2	Intestine, liver, kidney, beta cells	Glucose/fructose/galactose
	GLUT3 (GLUT14)	SLC2A3	Brain, testis, kidney, placenta	Glucose/galactose
	GLUT4	SLC2A4	Skeletal and cardiac muscle cells, adipose cells	Glucose
	GLUT5	SLC2A5	Intestine, kidney, muscle, brain, testis	Fructose
	GLUT6	SLC2A6	Brain, spleen	Glucose
	GLUT7	SLC2A7	Small intestine, colon, testis, prostate, liver	Fructose, glucose
	GLUT8	SLC2A8	Testis, brain, fat, liver, spleen	Glucose/fructose
	GLUT9	SLC2A9	Kidney, liver, placenta, colon	Fructose/glucose
	GLUT10	SLC2A10	Heart, lung	Glucose
	GLUT11	SLC2A11	Muscle, heart, placenta, kidney, pancreas, fat	Glucose
	GLUT12	SLC2A12	Heart, prostate	Glucose/fructose
SGLT	SGLT1	SLC5A1	Intestine, trachea, kidney, heart, brain, testis, prostate	Glucose/galactose
	SGLT2	SLC5A2	Kidney, brain, liver, thyroid, muscle, heart	Glucose
	SGLT3	SLC5A4	Intestine, testis, uterus, lung, brain, thyroid	Glucose

Table 1. GLUT and SGLT families of glucose transporters Adapted from (Keating and Martel, 2018)

The second main family of glucose transporters is represented by the sodium-glucose linked transporters (SGLTs), also referred as SLC5 (see Table 1). SGLT1 has been the first member of this family to be identified and its structure is characterized by 14 transmembrane regions, with both C- and N-terminal domains towards the extracellular compartment (Navale and Paranjape, 2016). Amongst the 12 members of the human SGLT family, only SGLT1 and SGLT2 are actually involved in glucose uptake (Wright *et al.*, 2011; Yamazaki *et al.*, 2018). Both these molecules operate an active glucose transport, against its concentration gradient, following a symport mechanism of glucose uptake together with Na⁺ ions. The energy required for this mechanism is provided by the electrochemical potential of the Na⁺ concentration gradient across the plasma

membrane, which is generated by the ATP-dependent activity of the Na^+/K^+ ATPase (Navale and Paranjape, 2016).

3.1.2 Glycolysis

Glucose catabolism through the enzymatic reactions of the glycolytic pathway, also called glycolysis or Embden-Meyerhof pathway, mainly contributes to cell energy production, through the biosynthesis of ATP. Glycolysis is one of the mostly conserved metabolic pathways and almost all the cells of the human body have been found to express glycolytic enzymes, which catalyze their reactions in the cytoplasmic compartment (Akram, 2013). More precisely, a single molecule of glucose, with two NAD^+ and two ATP molecules are converted into two molecules of pyruvic acid (or pyruvate), two NADH and four ATPs (Kim and Dang, 2005). According to the enzymatic reactions involved, the glycolytic pathway has been divided into the three phases described here below: energy investment (or priming phase), splitting and energy-generation phase (**Figure 18**).

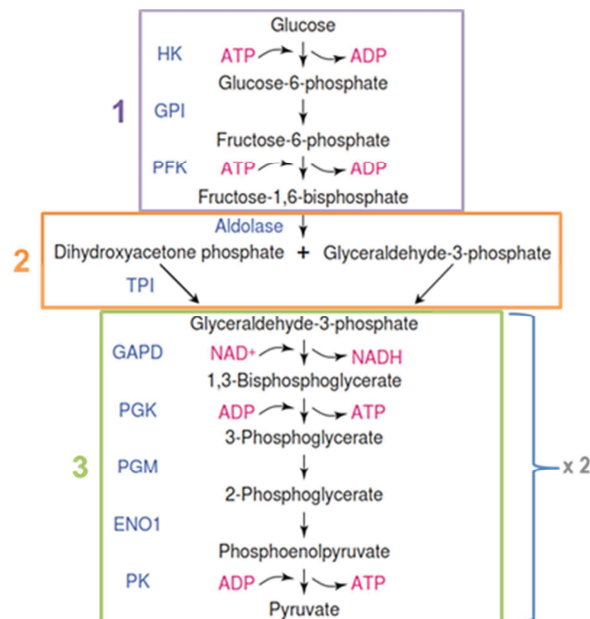


Figure 18. Three phases of the glycolytic pathway. Energy investment phase (1): glucose is phosphorylated to glucose-6-phosphate, then isomerized to fructose-6-phosphate, which is further phosphorylated to fructose-1,6-bisphosphate. HK, hexokinase; GPI, glucose-6-phosphate isomerase; PFK, phosphofructokinase. Splitting phase (2): fructose-1,6-bisphosphate is cleaved into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate; the latter then isomerized to glyceraldehyde-3-phosphate. TPI, triose phosphate isomerase. Energy-generation phase (3): glyceraldehyde-3-phosphate is converted

into 1,3-bisphosphoglycerate. A substrate-level phosphorylation produces ATP from ADP by converting 1,3-bisphosphoglycerate into 3-phosphoglycerate; the latter is further converted into 2-phosphoglycerate and consequently phosphoenolpyruvate, which undergoes another substrate-level phosphorylation leading to the biosynthesis of ATP and pyruvate. GAPD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO1, enolase; PK, pyruvate kinase. Adapted from (Kim and Dang, 2005)

The glycolytic pathway has been first described in species developing in absence of oxygen and, therefore, considered as anaerobic glucose catabolism (Canback *et al.*, 2002). Indeed, glycolysis also occurs in presence of oxygen (aerobic glycolysis), and the glycolysis-derived pyruvate is metabolized in different catabolic pathways, as a function of oxygen levels. In anaerobic conditions, pyruvate is converted to lactate by the lactate dehydrogenase (LDH), in presence of the NADH cofactor (Goldblatt and Cameron, 1953), which eventually originates from the GAPD glycolytic reaction. This mechanism allows lactate production with concomitant NAD⁺ regeneration, therefore providing a substrate loop able to continuously sustain anaerobic glycolysis. Conversely, in aerobic conditions, glucose fuels the tricarboxylic acid cycle (TCA cycle) through the oxidative decarboxylation of pyruvate. This reaction, catalyzed by the pyruvate dehydrogenase (PDH) and requiring NAD⁺ reduction to NADH, generates acetyl-CoA, which enters the TCA cycle (see section 3.2.3.1) (Akram, 2013) (**Figure 19**). Thus, anaerobic glycolysis remains the main energy producing pathway in mitochondria-devoid cells that lack aerobic oxidative metabolism, such as erythrocytes, as well as cornea and lens cells (Akram, 2013). On the other hand, aerobic glycolysis has been shown to support the growth of rapidly proliferating cells, as reported in cancer cells through the so called “Warburg effect” (Vander Heiden *et al.*, 2009).

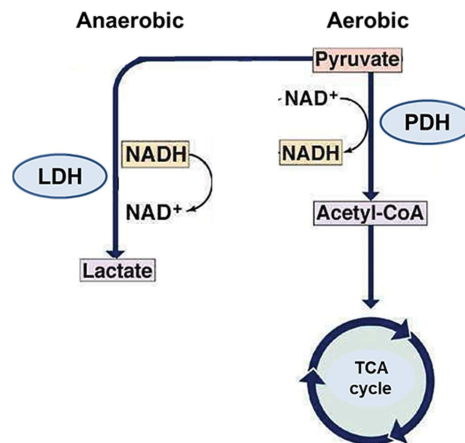


Figure 19. Outcome of glycolysis-derived pyruvate in anaerobic and aerobic conditions. In anaerobic conditions, pyruvate is reduced to lactate while NADH is oxidized to NAD⁺ by the lactate dehydrogenase (LDH). Conversely, in aerobic conditions, the oxidative decarboxylation of pyruvate is catalyzed by the pyruvate dehydrogenase (PDH), with concomitant NAD⁺ reduction to NADH. This reaction generates acetyl-CoA, which fuels the TCA cycle. *Adapted from* (McGraw-Hill)

It is important to note that after being produced through the hexokinase-dependent phosphorylation, glucose-6-phosphate is not only catabolized through glycolysis for the production of ATP, but eventually shunted towards the pentose phosphate pathway (PPP) (Berg et al., 2002). Further details about this fundamental and highly conserved metabolic pathway are here below provided.

3.1.3 Pentose Phosphate Pathway (PPP)

Glucose catabolism through the pentose phosphate pathway plays a dual role by supporting anabolic metabolism, especially for nucleotide and amino acid production, as well as maintaining the cell redox homeostasis (reviewed in Stincone *et al.*, 2015). In fact, the PPP converts glucose into 5 and 4 carbon-sugars (ribose-5-phosphate and erythrose-4-phosphate), which are important precursors for the biosynthesis of the RNA/DNA backbone and aromatic amino acids, respectively (Zimmer, 1992; Zhao *et al.*, 1995; Wang *et al.*, 2006; Cadière *et al.*, 2011; Clasquin *et al.*, 2011). In addition, this pathway represents a fundamental source of NADPH, which sustains the production of several reducing agents, such as glutathione, involved in the cellular antioxidant response (Riganti *et al.*, 2012). The PPP enzymatic reactions occur in the cytoplasmic

compartment, and are divided into the here presented oxidative and non-oxidative branch (reviewed in Stincone *et al.*, 2015) (**Figure 20**).

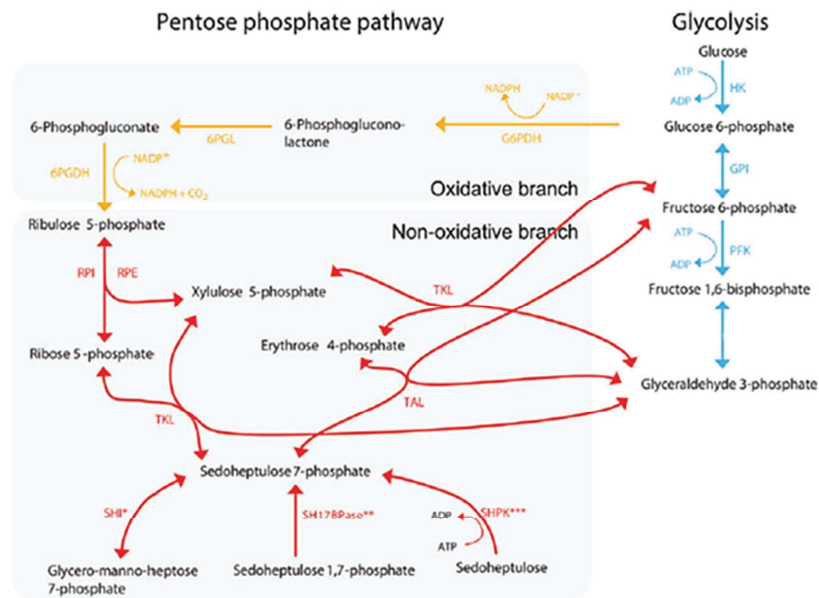


Figure 20. Pentose phosphate pathway (PPP). The oxidative branch converts glucose-6-phosphate into ribulose-5-phosphate through the consecutive reactions catalyzed by the glucose-6-phosphate dehydrogenase (G6PDH), the 6-phosphogluconolactonase (6PGL) and the 6-phosphogluconate dehydrogenase (6PGDH), with concomitant generation of two NADPH molecules. In the non-oxidative branch ribulose-5-phosphate is converted into either ribose-5-phosphate or xylulose-5-phosphate, by the ribose-5-phosphate isomerase (RPI) and the ribulose-5-phosphate epimerase (RPE), respectively. Subsequent multi-substrate interconversion reactions, mediated by the transketolase (TKL) and the transaldolase (TAL), generate glycolytic intermediates glyceraldehyde-3-phosphate and fructose-6-phosphate. HK, hexokinase; GPI, glucose-6-phosphate isomerase; PFK, phosphofructokinase. *Adapted from* (Stincone *et al.*, 2015)

3.2 Glutamine

Glutamine represents the most abundant circulating amino acid (extracellular concentration 0.2 to 0.8 mM) (Bode, 2001; Newsholme *et al.*, 2003), constituting more than 20% of the free amino acid pool in plasma. Despite the fact that the endothelium of the small intestine absorbs more than 30% of dietary glutamine, this non-essential amino acid is also synthesized by several organs (Krebs, 1980), including skeletal muscle, lungs, and adipose tissue, amongst others (Felig *et al.*, 1973; Stumvoll *et al.*, 1999;

Hensley *et al.*, 2013). Proliferating cells, including lymphocytes, small intestine enterocytes and especially cancer cells, employ glutamine not only for energy production, but also as source of carbon and nitrogen for their cell growth-induced increase in biomass (Windmueller and Spaeth, 1974). In fact, glutamine catabolism is implicated in numerous biological processes, including supplementation of TCA intermediates, synthesis of glutathione and control of redox balance, protein and nucleotide biosynthesis, as well as gene expression regulation.

3.2.1 Glutamine transport

Glutamine transport into mammalian cells is operated by several membrane-anchored amino acid transporters, including members of the SLC1, SLC6, SLC7, and SLC38 families (reviewed in Pochini *et al.*, 2014), which are characterized by different transport mechanisms and kinetics, as well as tissue-specific expression (Bhutia *et al.*, 2015) (see Table 2).

HUGO Name	Alternative name	Mode of transport	Coupling ion
SLC1A5 ^a	ASCT2	Exchange	Na ⁺
SLC6A14 ^b	ATB ^{Na+}	Symport	Na ⁺ , Cl ⁻
SLC6A19 ^c	B ⁰ AT1	Symport	Na ⁺
SLC7A5 ^d	LAT1	Exchange	None
SLC7A6 ^e	y ⁺ LAT2	Exchange	Na ⁺
SLC7A7 ^e	y ⁺ LAT1	Exchange	Na ⁺
SLC7A8 ^d	LAT2	Exchange	None
SLC38A1 ^c	SNAT1/ATA1	Symport	Na ⁺
SLC38A2 ^c	SNAT2/ATA2	Symport	Na ⁺
SLC38A3 ^f	SNAT3/SN1	Symport	Na ⁺ , H ⁺
SLC38A4 ^c	SNAT4/ATA3	Symport	Na ⁺
SLC38A5 ^f	SNAT5/SN2	Symport	Na ⁺ , H ⁺

^a Na⁺-amino acid/Na⁺-amino acid exchange.
^b Na⁺/Cl⁻/amino acid symport.
^c Na⁺/amino acid symport.
^d Neutral amino acid/neutral amino acid exchange.
^e Na⁺-neutral amino acid/cationic amino acid exchange.
^f Na⁺-amino acid/H⁺ exchange.

Table 2. Glutamine transporters in mammalian cells Adapted from (Bhutia *et al.*, 2014)

One of the most extensively studied glutamine transporters, playing an important role in human erythropoiesis (Oburoglu *et al.*, 2014), is SLC1A5, also referred as ASCT2 (alanine/serine/cysteine transporter 2), according to initial studies showing ASCT2 ability to transport several neutral amino acids (Utsunomiya-Tate *et al.*, 1996). Indeed, it has been then found that glutamine constitutes the main substrate of this transporter, while

cysteine would more likely play a role in modulating its function (Scalise *et al.*, 2015). More precisely, ASCT2-mediated uptake implies a Na⁺-dependent antiporter mechanism (Utsunomiya-Tate *et al.*, 1996; Pingitore *et al.*, 2013; reviewed in Scalise *et al.*, 2018), with concomitant efflux of neutral amino acids, including asparagine, threonine and serine (reviewed in Scalise *et al.*, 2018). A recent study has solved the ASCT2 3D structure, represented by a homotrimer in which each monomer is further composed of a scaffold domain, allowing the assembly, and a transport domain for substrate binding (Garaeva *et al.*, 2018).

The two members of the SLC7 family, SLC7A5 and SLC7A6, eventually referred as LAT1 and LAT2 (Leucine-type amino acid transporter 1 and 2), also play an important role in glutamine transport (Mastroberardino *et al.*, 1998; Bode, 2001). These transporters exist as heterodimers, associated with CD98 (SLC3A2) chaperon protein, which is not involved in the transport function *per se* (Costa *et al.*, 2013; Rosell *et al.*, 2014; Napolitano *et al.*, 2015). LAT1/CD98 heterodimer acts as Na⁺-independent antiporter, mediating simultaneous glutamine secretion and leucine uptake, but also allowing the uptake of essential amino acids (valine, isoleucine, histidine and tyrosine, amongst others) (Yanagida *et al.*, 2001). Intriguingly, it has been shown that glutamine and leucine, as well as the ASCT2 and LAT1 transporters, would cooperate in the activation of the mTOR protein kinase (Nicklin *et al.*, 2009; Laplante and Sabatini, 2012), an important cell sensor for nutrients, and especially amino acids. Interestingly, SLC38A9 is involved in glutamine and arginine transport across the lysosomal membrane, which is also associated with the induction of the mTOR signaling pathway (Rebsamen *et al.*, 2015; S. Wang *et al.*, 2015). Noteworthy, LAT1 has also been found to localize to the lysosomes (Milkereit *et al.*, 2015), and it is well established that glutamine catabolism (glutaminolysis) induces mTORC1 translocation to the lysosomes, which constitutes a fundamental step for its Rag-dependent activation (Durán *et al.*, 2012).

3.2.2 Glutamine metabolism

Once transported into the intracellular compartment, glutamine has been shown to fuel several metabolic pathways required for cell survival, growth and proliferation

(Dang, 2010). In fact, glutamine catabolism (glutaminolysis) not only contributes to energy production, but also provides nitrogen, sulfur and carbon units to several anabolic pathways, including nucleotide, amino acid and lipid biosynthesis (Reitzer *et al.*, 1979; Medina, 2001; Lu *et al.*, 2010; Wise and Thompson, 2010). For instance, glutamine-derived nitrogen units enter the purine/pyrimidine biosynthesis pathway (reviewed in DeBerardinis and Cheng, 2010) and glutamine also participates to the production of hexosamine and non-essential amino acids (Wellen *et al.*, 2010; Hensley *et al.*, 2013). Furthermore, cancer cells cultured under hypoxia or cells carrying mitochondrial defects utilize the reductive carboxylation reactions (see section 3.2.3.3) to convert glutamine-derived α -KG into citrate (Mullen *et al.*, 2012, 2014). This mechanism has been shown to support lipid (Metallo *et al.*, 2011) and nucleotide biosynthesis (Birsoy *et al.*, 2015; Sullivan *et al.*, 2015), as well as participating to the control of the redox state of mitochondria (Jiang *et al.*, 2016). Indeed, glutaminolysis contributes to several mechanisms involved in the maintenance of cell redox homeostasis, such as NADPH and glutathione production. In fact, it has been recently shown that glutamine-derived glutamate is fundamental for *de novo* glutathione biosynthesis, required for the control of ROS production upon T cell differentiation (Lian *et al.*, 2018). Importantly, glutaminolysis can directly replenish the tricarboxylic acid (TCA) cycle, through the reactions catalyzed by the glutaminase (GLS), the glutamate dehydrogenase (GDH), as well as several transaminases (GPT, GOT and PSAT1), which lead to the production of α -ketoglutarate (α -KG) (see section 3.2.3) (Reitzer *et al.*, 1979) in the so called process of anaplerosis (**Figure 21**).

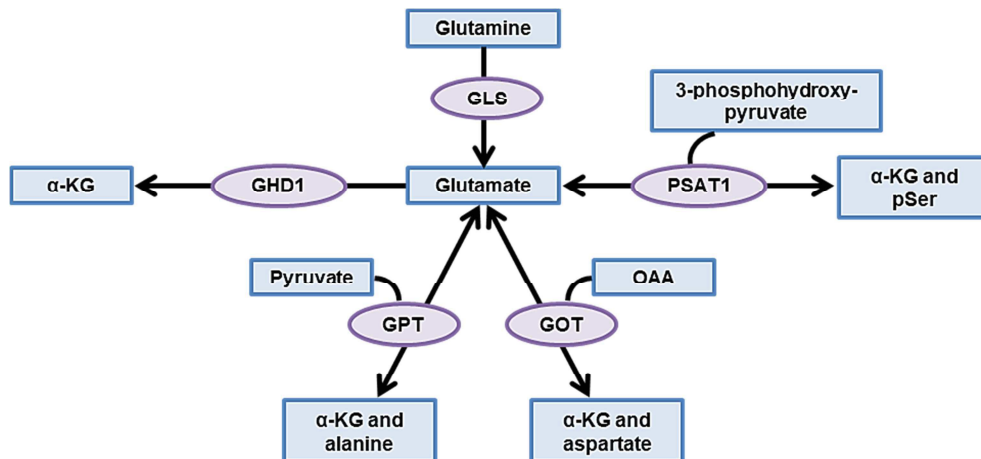


Figure 21. Glutamine anaplerosis and α -ketoglutarate biosynthesis. After glutamine entry into the mitochondria, glutamine deamination into glutamate is catalyzed by the glutaminase (GLS). Glutamate is then converted into α -KG through several reactions. Glutamate dehydrogenase (GDH1) mediates oxidative deamination of glutamate into α -KG. Alternatively, α -KG is produced by transaminase reactions, catalyzed by the glutamate-pyruvate transaminase (GPT or alanine aminotransferase), the glutamate-oxaloacetate transaminase (GOT or aspartate aminotransferase) or the phosphoserine aminotransferase 1 (PSAT1), which involve different additional substrates/products. OAA, oxaloacetate; pSer, phosphoserine. Adapted from (Altman *et al.*, 2016)

Interestingly, a recent study has shown downregulation of GDH expression, while transaminases are upregulated in highly proliferating mammary epithelial and human breast cancer cells. More specifically, cell growth was supported by the increased metabolic flux through transaminase-dependent instead of the GDH-mediated reaction, by allowing the recycling of glutamine-derived nitrogen into non-essential amino acids (Wang *et al.*, 2011). This study as well as others have shown that different mechanisms are involved in the “choice” between alternative reactions dedicated to the biosynthesis of α -KG, which will be further described in the next section.

3.2.3 Alpha-ketoglutarate: a central metabolite

The TCA cycle intermediate α -KG derives from multiple metabolic reactions, showing different kinetics and generating diverse byproducts. For instance, the GDH irreversible reaction not only produces α -ketoglutarate and ammonium, but also NADH or NADPH (Botman *et al.*, 2014), which contribute to the TCA cycle (see section 3.2.3.1), the OXPHOS (see section 3.2.3.2), as well as to antioxidant mechanisms and lipid

biosynthesis. Contrarily, the transaminase-dependent reactions (α -keto acid converted into the corresponding amino acid), mediated by the GPT, the GOT and the PSAT1 are reversible (Altman *et al.*, 2016) (**Figure 21**).

The oxidative decarboxylation of isocitrate, which is catalyzed by the isocitrate dehydrogenase (IDH) enzyme, is another important source of α -KG. For instance, the IDH family includes three isoforms (IDH1, IDH2, and IDH3), with IDH1 localized into the cytoplasm and peroxisome, while IDH2 and IDH3 found into the mitochondria. In addition to exhibiting different structure and kinetics, these enzymes have also been shown to require diverse cofactors. More precisely, IDH1 and IDH2 have been characterized as NADP^+ -dependent homodimeric complexes, while IDH3 is a NAD^+ -dependent heterotetramer, composed of two IDH3A, one IDH3B and one IDH3G subunit (Chandel, 2015). Finally, IDH1 and IDH2 are able to mediate both direct and reverse enzymatic reactions; while, IDH3 irreversibly catalyzes isocitrate oxidization to α -KG in the context of the TCA cycle (reviewed in Al-Khallaf, 2017) (**Figure 22**).

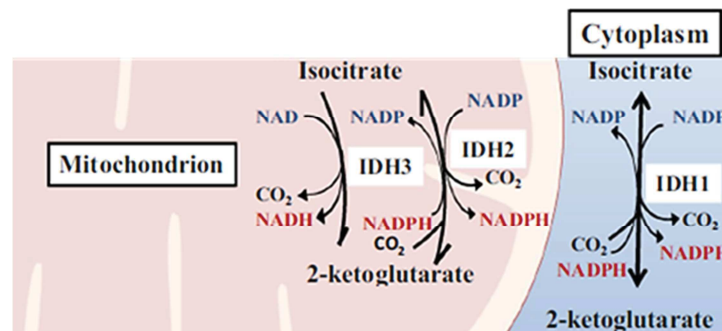


Figure 22. IDH isoforms and enzymatic reactions. The isocitrate dehydrogenase (IDH) exists in three isoforms, which show different subcellular localization, kinetics and cofactors. IDH1 is localized into the cytoplasmic compartment, while IDH2 and IDH3 into the mitochondria. IDH1 and IDH2 are NADP^+ -dependent enzymes, able to catalyze both direct and reverse reactions; whereas IDH3 is NAD^+ -dependent and mediates the isocitrate conversion into α -KG as part of the TCA cycle reactions (not shown). *Adapted from* (Al-Khallaf, 2017)

All the above described pathways allow α -KG biosynthesis from either glutamine or glucose, with α -KG intracellular concentrations in proliferating cells ranging from 0.5 to 3mM (Chowdhury *et al.*, 2011; Thirstrup *et al.*, 2011). In addition, α -KG levels, both in the cytoplasm and the mitochondria, are regulated by its exchange between these two

compartments, through the malate/aspartate shuttle (Hautecler *et al.*, 1994; Locasale and Cantley, 2011). Finally, all the mechanisms modulating α -ketoglutarate biosynthesis and availability are likely to be influenced by the cell metabolic requirements, which also regulate the outcome of α -KG. The latter, in fact, is either further catabolized through the TCA cycle or utilized as substrate of the anabolic biosynthesis of fatty acids (see section 3.3.4), via reductive carboxylation.

3.2.3.1. Alpha-KG as TCA cycle intermediate

As mentioned before, α -ketoglutarate is an important intermediate of the tricarboxylic acid cycle. This pathway, playing a key role in the aerobic energy-producing metabolism (Bodner, 1986), is also called Krebs cycle as discovered by Hans Adolf Krebs in 1937 (Krebs *et al.*, 1938). For instance, the catabolic reactions of the TCA cycle provide several electron donors, which enter the electron transport chain (ETC) (reviewed in Chaban *et al.*, 2014), therefore contributing to the OXPHOS-dependent production of ATP. Nevertheless, the TCA cycle is an amphibolic pathway, as it also participates to anabolic cellular processes, by providing precursors for many biosynthetic metabolisms (Baldwin and Krebs, 1981). The biochemical reactions of the TCA cycle occur in the mitochondrial matrix, starting from the first metabolite, the acetyl-CoA, which is generated from different sources (Meléndez-Hevia *et al.*, 1996; reviewed in Anderson *et al.*, 2018). In fact, glucose replenishes the TCA cycle through pyruvate decarboxylation into acetyl-CoA (see section 3.1.2), which also represents the end product of the fatty acid catabolism through the β -oxidation pathway (see section 3.3.3). In addition, several TCA intermediates, including oxaloacetate and α -KG, can be derived from the majority of the amino acids, while isoleucine, leucine and tryptophan are directly converted into acetyl-CoA (Berg *et al.*, 2002).

The first reaction of the TCA cycle, catalyzed by the citrate synthase (CS), is represented by acetyl-CoA condensation with oxaloacetate in order to produce citrate. The latter is then isomerized to isocitrate, through subsequent dehydration and rehydration (generating *cis*-aconitate as intermediate metabolite), mediated by the aconitase (ACO) (Costello and Franklin, 1981). Next step is the oxidative decarboxylation of isocitrate to α -ketoglutarate, catalyzed by the isocitrate dehydrogenase (IDH), with

concomitant production of NADH. Then, α -ketoglutarate is converted into succinyl-CoA through another oxidative decarboxylation, mediated by the multi-enzymatic complex of the α -ketoglutarate dehydrogenase (KGDH) (Guffon *et al.*, 1993). Subsequently, the succinyl-CoA synthetase (SCS) catalyzes a substrate-level phosphorylation, generating succinate, as well as GTP from GDP. Concomitant with FADH_2 production is the succinate dehydrogenase (SDH)-mediated conversion of succinate into fumaric acid (Brière *et al.*, 2005). Subsequently, the stereospecific hydration of the fumarate double carbon-carbon bond is catalyzed by the fumarate hydratase (FH, also known as fumarase) to release L-malate (Akiba *et al.*, 1984). The latter is finally dehydrogenated, while NAD^+ is reduced to NADH, by the malate dehydrogenase (MDH) enzymatic activity to produce oxaloacetate. Therefore, oxaloacetate is regenerated and available for starting another round of the TCA cycle, through the condensation with acetyl-CoA leading to citrate biosynthesis (Srere, 1975) (**Figure 23**).

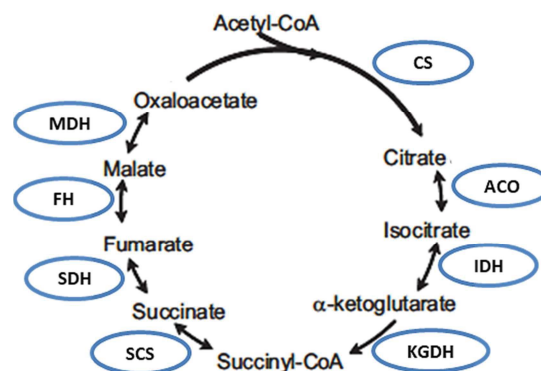


Figure 23. TCA cycle. First reaction of the TCA cycle is the condensation of acetyl-CoA and oxaloacetate molecules, generating citrate. The latter is isomerized to isocitrate, which undergoes two subsequent oxidative decarboxylation reactions, giving rise to α -ketoglutarate and then succinyl-CoA. Succinyl-CoA is further converted into succinate, fumarate and malate to finally reconstitute oxaloacetate. CS, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; KGDH, α -ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; FH, fumarate hydratase; MDH, malate dehydrogenase. Adapted from (Guay *et al.*, 2007)

Interestingly, several of the above presented enzymes play a dual role, in addition to their function in the mitochondrial TCA cycle. Indeed, the heterotetrameric SDH complex (SDHA, SDHB, SDHC, and SDHD subunits) also constitutes the complex II of the electron transport chain (Chandel, 2015) (see section 3.2.3.2). Furthermore, the FH has been also

found to localize into the cytoplasm, where it catalyzes biochemical reactions of the urea cycle and it is also involved in the nucleotide and amino acid metabolism (Ratner *et al.*, 1953; Yogev *et al.*, 2011).

3.2.3.2. Oxidative phosphorylation (OXPHOS)

The electron transport chain (ETC) reactions coupled to the oxidative phosphorylation (OXPHOS) play a main role in the aerobic cell energy production (Chance and Williams, 1955; Boveris *et al.*, 2000). Several reduced cofactors, such as TCA cycle-, glycolysis- or other metabolism-derived NADH and FADH₂, act as electron donors for the ETC, which involves 5 oxidoreductase enzymatic complexes (complex I to V), localized into the inner mitochondrial membrane (IMM) (reviewed in Chaban *et al.*, 2014). As a result, an electron transfer cascade leads to O₂ reduction into H₂O (Chance and Williams, 1956) and generates a proton gradient across the IMM, which is utilized by the ATP synthase enzyme for the biosynthesis of ATP (OXPHOS) (reviewed in Chaban *et al.*, 2014). The above mentioned electron donors enter the ETC at the level of the complex I and II, following the cascade of reactions here below described.

As first step, the complex I (CI, also known as NADH-ubiquinone oxidoreductase) mediates NADH oxidation, with concomitant transfer of 2 electrons to the first mobile electron carrier of the chain, the ubiquinone (coenzyme Q or CoQ) (Janssen *et al.*, 2006; reviewed in Mimaki *et al.*, 2012). The complex II (CII or succinate-ubiquinone oxidoreductase) mediates a similar electron transfer to CoQ, following FADH₂ oxidation (reviewed in Cecchini, 2003). This step is also associated with succinate oxidation through the TCA cycle, which is catalyzed by the complex II itself (succinate dehydrogenase; see section 3.2.3.1). The two obtained molecules of ubiquinol (reduced ubiquinone) transfer electrons to the complex III (CIII, also known as ubiquinol-cytochrome c oxidoreductase or cytochrome bc₁), and consequently to the mobile electron shuttle, the cytochrome c (Cyt c) (reviewed in Chaban *et al.*, 2014). The latter interacts with the complex IV (CIV or cytochrome c oxidase), which utilizes the cytochrome c-derived electrons to catalyze O₂ reduction into H₂O (reviewed in Chaban *et al.*, 2014). In addition, coupled to the electron transfer is the concomitant pumping of protons (H⁺) through the complexes I, III and IV into the mitochondrial intermembrane

space (Yano, 2002; Janssen *et al.*, 2006; Zickermann *et al.*, 2009). This process generates an electrochemical potential (ΔP) across the inner mitochondrial membrane (Ghezzi and Zeviani, 2018), which allows the complex V (CV, ATP synthase) conformational change, responsible for the CV-dependent biosynthesis of ATP (Schultz and Chan, 2001) (**Figure 24**).

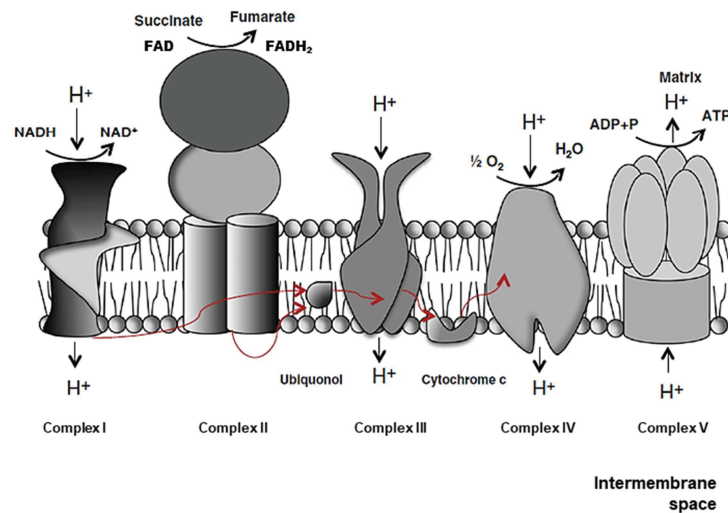


Figure 24. Electron transport chain (ETC) and oxidative phosphorylation (OXPHOS). Complex I and complex II respectively oxidize NADH and FADH₂, generating two molecules of ubiquinol (reduced ubiquinone), which transfer electrons to the complex III, and consequently to the mobile electron shuttle, the cytochrome c. Upon cytochrome c interaction with complex IV, the latter catalyzes O₂ reduction into H₂O. These reactions are coupled to pumping of protons (H⁺) through the complexes I, III and IV into the mitochondrial intermembrane space. The consequent electrochemical potential across the inner mitochondrial membrane is utilized for the complex V conformational change, which is coupled to the biosynthesis of ATP. *Adapted from* (Grimm, 2012)

3.2.3.3 Reductive carboxylation

Under specific metabolic conditions, the alternative pathway of reductive carboxylation provides precursors for fatty acid and lipid biosynthesis, through the reductive metabolism of TCA cycle intermediates. It has been shown that upon alterations in TCA cycle or mitochondrial function (Mullen *et al.*, 2012; Chen *et al.*, 2018; Gaude *et al.*, 2018), as well as prolonged hypoxic conditions (Metallo *et al.*, 2011; Wise *et al.*, 2011; Gameiro *et al.*, 2013), an enhanced anaerobic glycolytic flux is used to support energy production (reviewed in Halbrook *et al.*, 2018). However, this

mechanism leads to reduced pyruvate entry into the TCA cycle, with consequent decreased levels of acetyl-CoA, a fundamental precursor for fatty acid and lipid biosynthesis (Maus and Peters, 2017). These biosynthetic pathways are therefore supported by acetyl-CoA production, starting from the TCA cycle intermediate citrate (Maus and Peters, 2017). The consequent withdrawal of citrate is eventually rescued by α -ketoglutarate shunting through the reversal TCA cycle reactions of the reductive carboxylation (Filipp *et al.*, 2012) (**Figure 25**). In fact, it has been shown that isocitrate dehydrogenases (IDH1 and IDH2) are able to catalyze the reverse reaction of α -ketoglutarate reduction to citrate, with concomitant oxidation of the NADPH cofactor (Ward *et al.*, 2010). More precisely, in a glioblastoma cell line cultured under hypoxia, it has been demonstrated that IDH2 catalyzes the reductive carboxylation of glutamine-derived α -KG into isocitrate (Wise *et al.*, 2011; Mullen *et al.*, 2012). In addition, another study has identified a TCA cycle-independent pathway of α -KG reductive carboxylation, which occurs in the cytoplasm and involves the IDH1 enzymatic activity (Metallo *et al.*, 2011). This pathway also leads to the production of citrate, and subsequently acetyl-CoA, in order to support fatty acid and lipid biosynthesis. Therefore, under these conditions, glucose is used for energy production, while glutamine-derived citrate becomes an important source of cytosolic acetyl-CoA (Metallo *et al.*, 2011; Mullen *et al.*, 2012), mainly through the ATP-citrate lyase (ACL) enzymatic reaction. The latter also generates oxaloacetate, which is converted into malate by the cytosolic malate dehydrogenase (MDH1), with concomitant oxidation of NADH to NAD⁺. This mechanism has been recently shown to support the NAD⁺ regeneration, required to sustain the mentioned glycolysis-dependent energy production upon mitochondrial defects (Gaude *et al.*, 2018; reviewed in Halbrook *et al.*, 2018) (**Figure 25**).

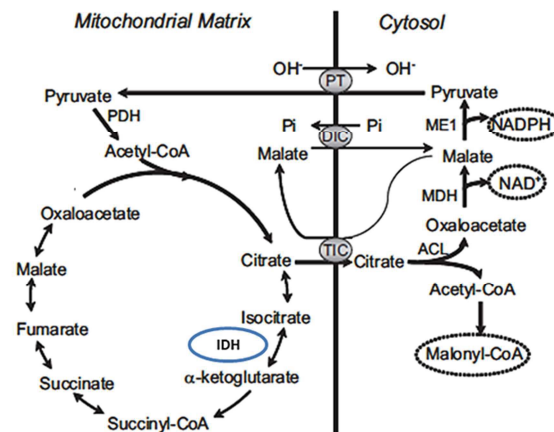


Figure 25. Reductive carboxylation. IDH reverse reaction of reductive carboxylation converts α -ketoglutarate into isocitrate, which is then isomerized to citrate and transported into the cytoplasmic compartment. Eventually citrate is directly produced into the cytoplasm by the cytosolic IDH isoform (not shown), IDH1. The ATP-citrate lyase (ACL) generates acetyl-CoA, which is the main substrate for malonyl-CoA and consequently lipid biosynthesis, and oxaloacetate, which is further converted into malate and pyruvate through the activity of the malate dehydrogenase (MDH) and the malic enzyme (ME1), respectively. *Adapted from* (Guay *et al.*, 2007)

3.2.3.4 Alpha-KG as demethylase and dioxygenase co-factor

The metabolic state of a cell is influenced by numerous environmental stimuli, among which nutrient availability (Vander Heiden *et al.*, 2009). Conversely, it is only recently that numerous studies have highlighted a relationship between metabolic and epigenetic state of a cell, by showing the importance of several endogenous metabolites and cofactors in regulating the activity of DNA and chromatin-modifying enzymes (reviewed in Ryall *et al.*, 2015; Li *et al.*, 2018). These enzymes mediate different types of modifications, such as methylation and hydroxymethylation of DNA cytosine residues, as well as acetylation, methylation, phosphorylation, ubiquitination and SUMOylation of histone lysine (K) and/or arginine (R) residues, which determine the chromatin conformation (reviewed in Li *et al.*, 2018). Therefore, by indirectly influencing genome accessibility to the transcriptional machinery, several metabolites and cofactors participate to the mechanisms of gene expression regulation. Interestingly, it has been also found that the enzymes dedicated to the biosynthesis of these metabolites eventually localize to the nucleus, facilitating the metabolism/epigenetic crosstalk (reviewed in Li *et al.*, 2018).

First evidence regarding this crosstalk has been provided through the finding that glucose-derived acetyl-CoA is incorporated in half of the H4K16 acetyl marks, upon Myc activation in fibroblasts (Morrish *et al.*, 2010). Similarly, glycolysis-dependent production of acetyl-CoA has been found to sustain the high histone acetylation levels, required for embryonic stem cell (ESC) pluripotency (Moussaieff *et al.*, 2015). On the other hand, the sirtuin family of deacetylases is constituted by several enzymes, which catalyze the removal of DNA acetylation marks, in a NAD⁺-dependent manner (Cantó *et al.*, 2015). Furthermore, the activity of class I and IIa histone deacetylases (HDACs) is regulated by the fatty acid derivatives butyrate and β -hydroxybutyrate (β HB) (Shimazu *et al.*, 2013).

Further studies have additionally demonstrated the requirement for specific metabolites, in order to support DNA and chromatin modifications. For instance, glutamine-derived α -ketoglutarate is required for the histone/DNA demethylation of embryonic stem cells (ESCs), maintaining their pluripotency (Carey *et al.*, 2015). More generally, α -ketoglutarate has been identified as fundamental cofactor of JmjC domain-containing histone demethylases (JHDM) and Ten-eleven translocation (TET) family of DNA dioxygenases (Klose and Zhang, 2007; Guengerich, 2015). In fact, these enzymes catalyze a reaction requiring not only α -ketoglutarate, but also O₂ and Fe²⁺ as cofactors, while producing succinate and formaldehyde (Shi and Tsukada, 2013). Indeed, the JHDM and TET demethylation activity is metabolically regulated, as a function of their cofactor availability and through a feedback inhibition mechanism, by the TCA cycle intermediates α -KG and succinate, respectively. Despite some evidence highlighting only a weak succinate-dependent inhibition of JHDMs *in vitro* (Rose *et al.*, 2008), it has been shown that modifications in the histone methylation state are induced in mammalian cells, upon significant increase of intracellular succinate levels (Cervera *et al.*, 2009). Indeed, inactivating mutations of the succinate dehydrogenase enzyme have been associated with a DNA hypermethylation phenotype in several types of cancer (Killian *et al.*, 2013). Additional studies have shown that defects not only in succinate dehydrogenase (SDH), but also in fumarate hydratase (FH) as well as isocitrate dehydrogenase (IDH) lead to metabolite-dependent inhibition of several chromatin-modifying enzymes, which is responsible of cancer development (Frezza *et al.*, 2011; Oermann *et al.*, 2012; Sciacovelli *et al.*, 2016). In fact, SDH and FH inactivating mutations promote the accumulation of succinate and fumarate, respectively (Pollard *et al.*, 2005;

Xiao *et al.*, 2012), which act as inhibitors of the α -KG-dependent enzymes (Xiao *et al.*, 2012; Meier, 2013). Similar mechanism has been observed upon mutations in IDH1 and IDH2, which are associated with the pathogenesis of several tumors, including gliomas and acute myeloid leukemia (AML) (Mardis *et al.*, 2009; Yan *et al.*, 2009; reviewed in Molenaar *et al.*, 2018), showing a DNA hypermethylation profile as well (Noushmehr *et al.*, 2010; Turcan *et al.*, 2012). In fact, IDH mutations mainly affect crucial arginine residues of the enzyme active site (Parsons *et al.*, 2008; Dang *et al.*, 2009; Mardis *et al.*, 2009; Yan *et al.*, 2009) and have been shown to confer a neomorphic enzymatic activity, leading to the biosynthesis of an oncometabolite, the 2-hydroxyglutarate D enantiomer (D-2-HG) (Dang *et al.*, 2009; Ward *et al.*, 2010). The latter is an α -KG structural analogue, showing antagonist effect on α -KG-dependent demethylases, by interacting with the α -KG pocket in their active site (Xu *et al.*, 2011; Koivunen *et al.*, 2012) (**Figure 26**). The consequent modifications in histone and DNA methylation lead to altered gene expression. This mechanism is responsible for the effects on cell differentiation and the predisposition to malignant transformation observed upon IDH mutation (Figuerola *et al.*, 2010; Ye *et al.*, 2018).

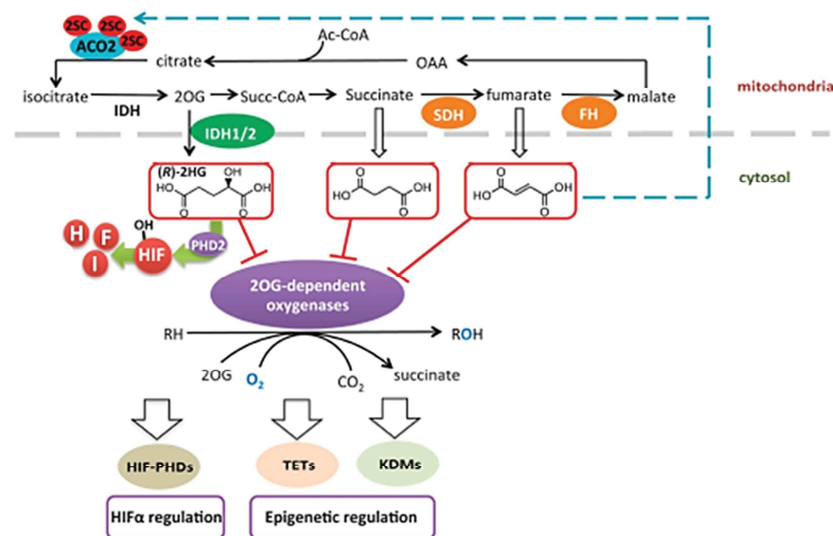


Figure 26. α -ketoglutarate cofactor activity. α -ketoglutarate (2OG or α -KG) is produced through the oxidative decarboxylation of isocitrate, catalyzed by the isocitrate dehydrogenase (IDH). α -ketoglutarate acts as fundamental cofactor for lysine demethylases (KDM), Ten-eleven translocation (TET) DNA dioxygenases, as well as prolyl hydroxylase domain (PHD)-containing proteins (which regulate HIF function). Upon IDH1 and IDH2, as well as SDH and FH mutations, the R-2HG oncometabolite (or D-2-HG), succinate and fumarate accumulates, respectively. Because of a significant homology in their structure,

these metabolites act as inhibitors for the α -ketoglutarate-dependent enzymes. *Adapted from* (Adam et al., 2014)

Finally, α -KG has been found to modulate the activity of another family of enzymes, the prolyl hydroxylase domain (PHD)-containing proteins. The latter, in fact, function in an O_2 -, α -KG- and Fe^{2+} -dependent manner to catalyze the hydroxylation of HIF-1 (Schofield and Ratcliffe, 2004; Kaelin and Ratcliffe, 2008) and this modification induces the proteasome-mediated degradation of the HIF transcription factor (Jaakkola *et al.*, 2001). It has been shown that not only α -KG, but also succinate and fumarate, are able to influence HIF levels and function, by regulating the activity of the PHD enzymes. In fact, HIF-1 stabilization is observed as a consequence of the PHD2 inhibition, mediated by succinate and fumarate accumulation upon SDH and FH mutations, respectively (Isaacs *et al.*, 2005; Selak *et al.*, 2005; King *et al.*, 2006). Similar effect has been also shown in presence of IDH1 mutations, where the D-2-HG-dependent inhibition of PHD2 activity is associated with increased levels of HIF-1 (Zhao *et al.*, 2009; Chowdhury *et al.*, 2011). However, more recent studies have highlighted that in cancer cells producing D-2-HG, as well as in T lymphocytes which uptake D-2-HG, the PHD2 enzyme is actually activated, therefore inducing HIF-1 degradation (Losman and Kaelin, 2013; Böttcher *et al.*, 2018).

3.3 Fatty acids

3.3.1 Fatty acid definition and structure

Fatty acids represent an important source of metabolic energy and are also involved in diverse cell functions, such as the regulation of membrane structure and intracellular signaling pathways. These nutrients are either absorbed through the diet or produced by *de novo* biosynthesis, as well as by the catabolism of triacylglycerol (TAG)/phospholipids (Tang *et al.*, 2018). The fatty acid molecular structure is composed of a carbon chain with variable length, with a methyl group at one end (also named omega, ω) and a carboxyl group at the other (referred as C1) (Lehninger *et al.*, 2008). According to the length of the chain, different classes of fatty acids have been defined: short-chain (SCFAs),

medium-chain (MCFAs), long-chain (LCFAs) and very-long-chain fatty acids (VLCFAs) (Rustan and Drevon, 2005). In addition, the absence or the presence of double carbon-carbon bonds identifies saturated and unsaturated species, respectively. Unsaturated fatty acids are classified as *cis* or *trans* isomers, according to the geometric configuration of the hydrogen atoms on the sides of the double bond, oriented either in the same or in the opposite direction, respectively (Rustan and Drevon, 2005) (**Figure 27**). The length of the carbon chain, as well as the degree of unsaturation and the double bond configuration contribute to the thermodynamic properties of the fatty acid molecules (Lehninger *et al.*, 2008). Monounsaturated fatty acids (MUFAs) contain a single carbon-carbon double bond, mainly in a *cis* configuration, and the most common MUFAs show a 16-22 carbon chain length. The principal polyunsaturated fatty acids (PUFAs) ω -3 and ω -6 are essential nutrients, which need to be absorbed through the diet, as no biosynthetic pathway has been characterized for these fatty acids in human cells (Wallis *et al.*, 2002).

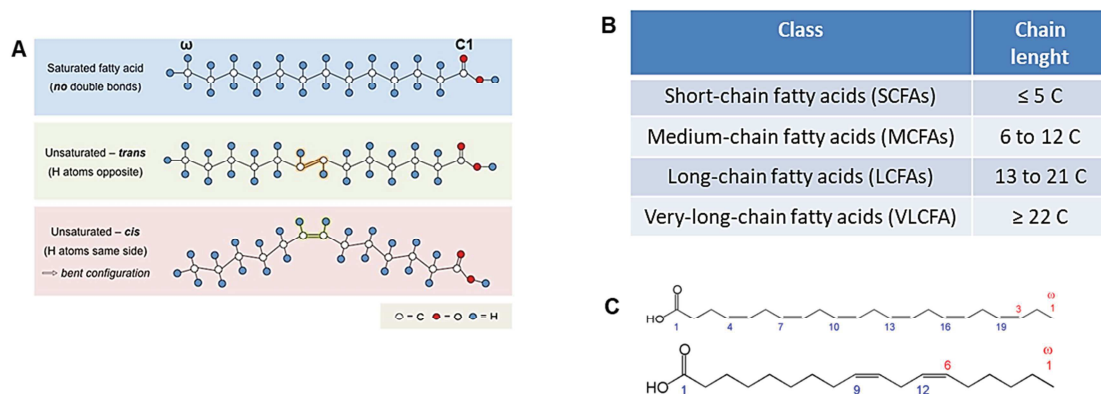


Figure 27. Fatty acid structure. Panel **A** The carboxyl group of the fatty acid chain is referred as C1, while the terminal methyl group as ω . The structure of saturated and unsaturated, both *cis* and *trans*, fatty species are shown. Panel **B** Table resuming the fatty acid classification according to the length of their carbon chain. Panel **C** Structure of the most common PUFAs, classified as ω -3 (upper) or ω -6 (bottom), according to the position of the first double bond between the third and the fourth or the sixth and seventh carbon of the chain, in respect of the ω carbon (end methyl group). *Adapted from* (ib.bioninja.com; vector.childrenshospital.org)

The most important steps of fatty acid metabolism, including their uptake, intracellular transport, as well as their involvement in both biosynthetic and catabolic pathways will be discussed in the following sections.

3.3.2 Fatty acid transport

3.3.2.1 Fatty acid uptake and activation

Dietary-derived long-chain fatty acids or *de novo* synthesized fatty acids are transported through the lymphatic vessels and the blood circulation as components of different classes of lipoproteins (chylomicrons, VLDL, IDL, LDL and HDL). In addition, free fatty acids, mainly released from triacylglycerols (TAGs) stored in the adipocytes, are carried to peripheral energy requiring tissues through the bloodstream, bound to the serum protein albumin (Rustan and Drevon, 2005). However, SCFAs and MCFAs enter the blood circulation as free fatty acids as well (Schönfeld and Wojtczak, 2016).

The mechanism of fatty acid uptake into the cells has been initially described as a simple passive diffusion across the plasma membrane, allowed by the amphipathic nature of the fatty acid molecules (Hamilton, 2007). Despite some short- and medium-chain fatty acids actually enter into the cell via diffusion (Charney *et al.*, 1998; Kamp and Hamilton, 2006), different types of fatty acid transporters have been identified for the long-chain forms. Indeed, several membrane-associated proteins are involved in fatty acid uptake, by either binding long-chain fatty acids or directly facilitating their passage through the plasma membrane (reviewed in Schwenk *et al.*, 2010) (**Figure 28**).

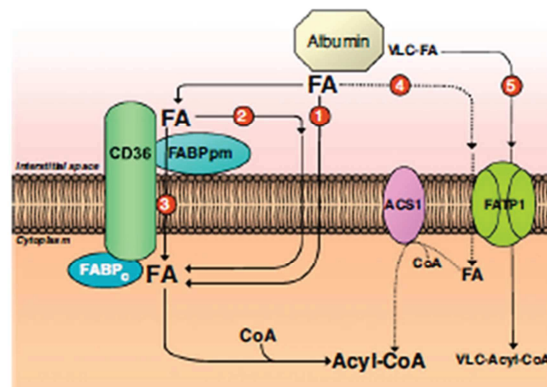


Figure 28. Fatty acid uptake and activation. Fatty acids are transported into the bloodstream bound to the plasma protein albumin. Passive fatty acid diffusion through the plasma membrane (1). Plasma membrane-associated fatty acid binding proteins (FABPpm) and CD36 act as fatty acid acceptors, facilitating the transport through passive diffusion (2) or a dedicated transporter (such as CD36 itself) (3); cytoplasmic fatty acid binding proteins (FABPc) participate to the intracellular fatty acid transport. Acyl-CoA synthetase (ACS1) (4) and fatty acid transport protein (FATP1) (5) respectively participate to long-

chain and very long-chain fatty acid transport/activation. Activation occurs through the esterification of the fatty acid chain to a coenzyme A molecule. *Adapted from* (Schwenk et al., 2010)

The fatty acid translocase (FAT) CD36 is a scavenger receptor (Martin *et al.*, 2007), which has been first identified as LCFA transporter by its ability to bind to the oleate ester sulfo-*N*-succinimidyl-oleate (SSO) (Abumrad *et al.*, 1993; Harmon and Abumrad, 1993; Coort *et al.*, 2002). Interestingly, CD36 has been found to function either as fatty acid acceptor or as direct fatty acid transporter (reviewed in Schwenk *et al.*, 2010) (**Figure 28**). Indeed, CD36 is involved in LCFA transport in adipocytes, heart, as well as skeletal muscle (Kerkhoff *et al.*, 2001; Pohl *et al.*, 2005). In addition, several studies have shown an important role for CD36 in the regulation of the mitochondrial fatty acid oxidation in skeletal muscle tissues (Campbell *et al.*, 2004; Bezaire *et al.*, 2006; Holloway *et al.*, 2006; Georgiou *et al.*, 2015). Interestingly, increased fatty acid uptake in cancer cells is also mediated by CD36, as demonstrated by the induction of its surface expression in chronic lymphocytic leukemia (CLL) cells (Rozovski *et al.*, 2018). Several aspects of CD36 function still remain to be elucidated, but it is generally accepted that this transporter plays a fundamental role in the regulation of long-chain fatty acid uptake and metabolism in diverse tissues (reviewed in Glatz and Luiken, 2015).

Several studies have found that the 6 members of the fatty acid transport protein (FATP) family are involved in the uptake of very-long-chain fatty acids (Schaffer and Lodish, 1994; Abumrad *et al.*, 1999; Frohnert and Bernlohr, 2000). It has been also shown that FATPs carry an acyl-CoA synthetase (ACS) activity (Jia *et al.*, 2007) and, similarly to the long-chain acyl-CoA synthetases (ACSLs), they are involved in the so called process of “fatty acid activation”. This process consists in fatty acid thioesterification to coenzyme A, which is required before fatty acid transfer to the intracellular organelles in which their metabolism occurs (Ellis *et al.*, 2010; Grevenkoed *et al.*, 2014; Adeva-Andany *et al.*, 2018). Indeed, several acyl-CoA synthetase (ACS) enzymes mediate a thioester linkage between the substrate fatty acyl moiety and the coenzyme A sulfur atom, in presence of ATP and magnesium (Mg^{2+}), while producing inorganic pyrophosphate (PPi) and adenosine monophosphate (AMP) (**Figure 28**). More precisely, the ACS family includes about 25 members, which are divided into 3 main

groups, according to the chain length of their preferential substrates (Ellis *et al.*, 2010; Grevengoed *et al.*, 2014): long-chain acyl-CoA synthetase (ACSL), very-long-chain acyl-CoA synthetase (FATP), and bubblegum acyl-CoA synthetase (ACSBG) (Steinberg *et al.*, 2000). In mammals, long-chain ASCs (ACSLs) exist in five isoforms (ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6) and catalyze the activation of the most abundant fatty acids (13-21 carbons) (Grevengoed *et al.*, 2014; Neess *et al.*, 2015).

Our current knowledge is that FATPs, but also ACSLs, actually contribute to fatty acid uptake following two mechanisms (**Figure 28**). While plasma membrane associated FATPs and ACSLs directly mediate fatty acid transport, the intracellular isoforms influence fatty acid uptake through their ACS activity. Indeed, fatty acid conversion into acyl-CoA esters allows their entering into the cellular metabolic pathways, consequently reducing the amount of intracellular free fatty acids (FFAs), as well as their efflux (Mashek and Coleman, 2006). Interestingly, ACSL subcellular localization and their interaction with specific transporter systems have been shown to regulate fatty acid “commitment” towards anabolic or catabolic metabolisms (Digel *et al.*, 2009; Ellis *et al.*, 2010). In fact, ACSL1, ACSL4 and ACSL5, localized in the mitochondria, are involved in fatty acid synthesis and β -oxidation; while ACSL1 and ACSL4 in the peroxisome support alkyl lipid biosynthesis, as well as β -oxidation (reviewed in Tang *et al.*, 2018).

3.3.2.2 Intracellular fatty acid transport

Once into the cell and activated, fatty acids need to be transported into several intracellular compartments, mainly mitochondria, but also peroxisomes, as well as ER. In human, no membrane transporter is required for fatty acids with chain lengths up to 12 carbons to enter into the mitochondria (Knottnerus *et al.*, 2018). On the other hand, the majority of the dietary- or adipose tissue-derived FFAs, which are composed of 14 or more carbons, are transferred across the mitochondrial membrane by the L-carnitine shuttle system (Nakamura, Yudell and Loor, 2014). The latter is composed by the carnitine palmitoyltransferases 1 and 2 (CPT1 and CPT2) and the carnitine/acyl-carnitine translocase (CACT or CAT) (Adeva-Andany *et al.*, 2018) (**Figure 29**). This mechanism of fatty acid transport across the mitochondrial membrane plays an important role in the regulation of the subsequent fatty acid oxidation (FAO), as CPT1 is inhibited by malonyl-CoA, which is the first intermediate of the fatty acid biosynthesis pathway (see section

3.3.4) (Foster, 2012; Knottnerus *et al.*, 2018). Three isoforms of human CPT1 have been identified, CPT1A, CPT1B and CPT1C, respectively expressed in the liver, muscles and brain, with CPT1C mainly localized to the endoplasmic reticulum (Rinaldi *et al.*, 2015).

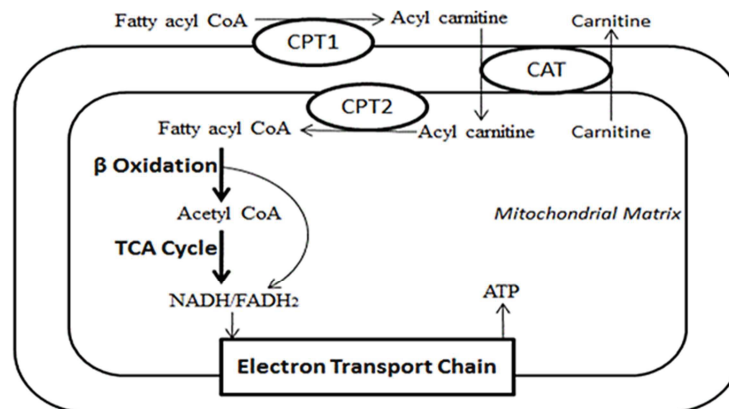


Figure 29. Fatty acid transport across the mitochondrial membrane. CPT1 in the outer mitochondrial membrane (OMM) catalyzes acyl group transfer from coenzyme A to L-carnitine, to generate acyl-carnitine esters. Carnitine/acyl-carnitine translocase (CAT) exchanges acyl-carnitine with free L-carnitine, allowing acyl-carnitine transport into the mitochondria, with concomitant reconstitution of the cytosolic pool of free L-carnitine. CPT2 in the inner mitochondrial membrane (IMM) mediates the acyl group transfer from acyl-carnitine to coenzyme A, reconstituting fatty acyl-CoA esters into the mitochondria. Adapted from (Fillmore *et al.*, 2011)

3.3.3 Fatty acid catabolism: fatty acid oxidation (FAO)

Fatty acid oxidation (FAO) contributes to the cell energy production, through the mitochondrial enzymatic cascade of the β -oxidation pathway (Knottnerus *et al.*, 2018). During this process saturated fatty acyl-CoA esters are converted into several molecules of acetyl-CoA, with concomitant production of NADH and FADH₂, which enter the TCA cycle and the OXPHOS to support the biosynthesis of ATP (Knottnerus *et al.*, 2018; Tang *et al.*, 2018). Here below described are the four biochemical reactions of the β -oxidation pathway, which progressively shorten the acyl-CoA ester chain, by being repeated in successive rounds: each round releases acetyl-CoA and a two carbon-shorter acyl-CoA ester (Adeva-Andany *et al.*, 2018; Knottnerus *et al.*, 2018) (**Figure 30**). In human, it has been shown that a mitochondrial trifunctional protein (MTP), carrying enoyl-CoA hydratase, 3-L-hydroxyacyl-CoA dehydrogenase and 3-ketoacylCoA thiolase enzymatic

activity, is located in the inner membrane and mediates the three last reactions of the β -oxidation pathway (Adeva-Andany *et al.*, 2018).

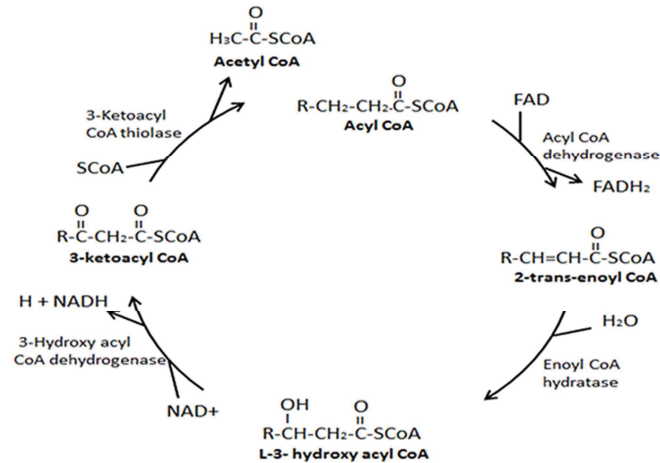


Figure 30. β -oxidation pathway of fatty acid oxidation (FAO). In the first step of the pathway, the acyl-CoA dehydrogenase introduces a double bond in the saturated acyl chain, to produce a 2-trans-enoyl-CoA derivative (α/β -trans-enoyl-CoA thioester). The enoyl-CoA hydratase mediates the hydration of the α/β -trans-enoyl-CoA thioester into the L-3-hydroxyacyl-CoA derivative. The latter is then oxidized into the correspondent 3-ketoacyl-CoA by the 3-hydroxyacyl-CoA dehydrogenase; subsequent thiolitic cleavage, by the thiol group of a second molecule of coenzyme A, is catalyzed by the 3-ketoacyl-CoA thiolase. Adapted from (Fillmore *et al.*, 2011)

3.3.4 *De novo* fatty acid biosynthesis

As mentioned in the previous section, acetyl-CoA is the main product of fatty acid oxidation, but this molecule also represents the “building block” in fatty acid biosynthesis, through the production of malonyl-CoA (reviewed in Röhrig and Schulze, 2016). In fact, *de novo* fatty acid biosynthesis is the process through which several 3-carbon molecules of malonyl-CoA are condensed to generate acyl chains, with the saturated 16-carbon palmitate (16:0) being the first synthesized precursor (Lehninger *et al.*, 2008). Eventually, palmitate is elongated or converted into unsaturated forms, by the activity of several elongase and desaturase enzymes (Jakobsson *et al.*, 2006; Igal, 2010). In human, this process mainly occurs in the liver and the adipose tissue, with liver-derived FAs involved in energy supply to other tissues, while FAs accumulating in the adipose tissue representing a form of energy storage (reviewed in Nguyen *et al.*, 2007).

First step in the cytosolic pathway of *de novo* fatty acid biosynthesis is the irreversible reaction of malonyl-CoA production from acetyl-CoA and bicarbonate (HCO_3^-), catalyzed by the acetyl-CoA carboxylase (ACC) in an ATP- and biotin-dependent manner (Kim, 1997; Brownsey *et al.*, 2006). Subsequently, the acyl groups involved in the above mentioned condensation reactions need to be “charged” onto the multifunctional system catalyzing these reactions, the fatty acid synthase (FAS I in vertebrates) (Smith *et al.*, 2003; Maier *et al.*, 2008). The latter, in fact, includes several active sites with different catalytic functions, linked through the shuttle activity of the acyl carrier protein (ACP) (Maier *et al.*, 2006, 2008). While the saturated intermediate acyl groups remain covalently attached to the ACP, the fatty acyl chain is progressively extended by two carbons, through successive rounds of the four enzymatic reactions here below described (Lehninger *et al.*, 2008) (**Figure 31**).

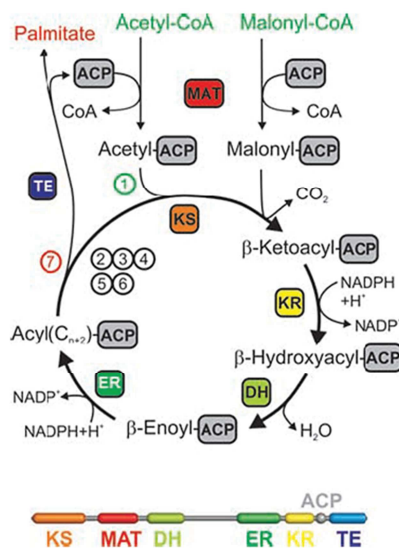


Figure 31. *De novo* fatty acid biosynthesis. The acetyl group of acetyl-CoA is transferred to the KS domain, while the malonyl group from malonyl-CoA to the ACP. After condensation of the acetyl and malonyl groups to produce acetoacetyl-ACP (β -ketoacyl-ACP), the latter is reduced with concomitant NADPH oxidation. Subsequent dehydration introduces a double carbon-carbon bond, which is then reduced (saturated), generating the first acyl-ACP intermediate. The latter is further elongated until palmitate (16:0) is released from the FAS I system. The catalytic domains of the mammalian FAS complex are showed in a linear fashion together with the ACP (bottom). KS, β -ketoacyl-ACP synthase; MAT, malonyl/acetyl-CoA-ACP transacylase; DH, β -hydroxyacyl-ACP dehydratase; ER, enoyl-ACP reductase; KR, β -ketoacyl-ACP reductase; TE, thioesterase. *Adapted from* (Maier *et al.*, 2006)

3.4 Regulation of HSC maintenance: a metabolic “choice”?

Several lines of evidence have revealed that long-term HSCs are not only characterized by cell cycle quiescence, but also by a metabolically “inactive” state. More precisely, anaerobic glycolysis is now thought to be both sufficient and required for the maintenance of HSCs in the hypoxic microenvironment of the endosteal bone marrow niche. This metabolic state avoids the production of reactive oxygen species (ROS), which could induce DNA damage and subsequent alterations in such a rare and indispensable cell population (Simsek *et al.*, 2010; Takubo *et al.*, 2013; Ito and Suda, 2014; Lara-Astiaso *et al.*, 2014; Qian *et al.*, 2016). In fact, it has been shown that increased ROS levels affect HSC long-term reconstitution capacity, by inducing their proliferation and finally leading to stem cell exhaustion (Ito *et al.*, 2004, 2006). Therefore, several mechanisms allowing the maintenance of a low oxidative phosphorylation (OXPHOS) rate participate to the “metabolic control” of LT-HSC self-renewal potential (see section 3.4.1). In addition, it has been demonstrated that the “metabolically inactive” state of LT-HSCs concerns protein synthesis as well, and long-term reconstitution potential results affected by alterations in proteostasis (Signer *et al.*, 2014).

Not only the structure, but also the metabolic composition of the bone marrow niche plays an important role in the regulation of HSC maintenance. For instance, amongst the niche soluble factors, the amino acid valine has been shown to be required for the maintenance of the LT-HSC pool (Taya *et al.*, 2016). Moreover, the vasculature architecture of the bone marrow participates to the control of ROS production (Itkin *et al.*, 2016). In addition, the lower oxygen levels in the endosteal niche, as compared to the vascular niche, foster a G0 cell-cycle phase of the HSCs in the former (Parmar *et al.*, 2007). Interestingly, dormant cells in the bone marrow have been shown to depend on retinoic acid signaling, which prevents HSC stress-induced entry into cell cycle, and eventual exhaustion (Cabezas-Wallscheid *et al.*, 2017). On the other hand, it is well established that HSC migration through the vascular niche is associated with loss of HSC potential, cell cycle entry and increased hematopoiesis (Parmar *et al.*, 2007). Under these conditions, as well as upon induction of differentiation, there is an increase in biosynthetic pathways and mitochondrial biomass (Mantel *et al.*, 2010; Maryanovich *et*

al., 2015), with a concomitant switch from glycolytic to oxidative metabolism (Suda *et al.*, 2011; Takubo *et al.*, 2013; Ito and Suda, 2014; Walter *et al.*, 2015).

3.4.1 Metabolic switch: glycolysis, OXPHOS and FAO

Recent studies have led to our appreciation that different cell types exhibit distinct metabolic requirements, but also that the metabolic state of a given cell type is modulated by cell cycle entry (Oburoglu *et al.*, 2016). As mentioned above, HSCs are characterized by a high glycolytic rate with limited mitochondrial metabolism and biogenesis; in fact, low mitochondrial activity has been reported in LT-HSCs (Sukumar *et al.*, 2015; Vannini *et al.*, 2016). In agreement with the importance of glycolysis in HSCs, it has been shown that pyruvate dehydrogenase kinase (PDK) activity increases stem cell potential, by preventing the utilization of glycolytic intermediates in mitochondrial oxidative metabolism (Takubo *et al.*, 2013). Similarly, differentiation arrest and a subsequent increase in the size of the HSC pool are observed upon depletion of a PTEN-like mitochondrial phosphatase (PTPMT1), promoting glycolysis and decreasing oxidative phosphorylation (W.-M. Yu *et al.*, 2013). Furthermore, mitochondrial clearance through autophagy (mitophagy) is involved in the maintenance of a “low-OXPHOS” state, by removing not only damaged (Nguyen *et al.*, 2016) but also active mitochondria (Ho *et al.*, 2017). On the contrary, alterations in mitochondrial biogenesis, which is regulated by mTORC1 (Chen *et al.*, 2008), as well as Akt1 and PTEN (Zhang *et al.*, 2006; Kharas *et al.*, 2010), impair HSC self-renewal potential by increasing OXPHOS and ROS production (Chen *et al.*, 2008; Qian *et al.*, 2016). Indeed, proliferation and subsequent stem cell exhaustion are associated with increased oxidative phosphorylation (Wilson *et al.*, 2008; Foudi *et al.*, 2009; Pietras *et al.*, 2011). Recent evidence has also shown that fatty acid oxidation (FAO) plays a crucial role in the balance between the maintenance and commitment of dividing HSCs. While the mechanisms have not yet been completely described, it has been demonstrated that FAO induces asymmetric cell division, giving rise to one committed and one self-renewing daughter cell. Conversely, when FAO is impaired (for example by inhibiting carnitine palmitoyltransferase 1 (CPT1)), there is a symmetric division of HSCs and the generation of two committed progenitors (Ito *et al.*, 2012; Yusuf and Scadden, 2012). Further evidence has been provided by the finding that

depletion of the tumor suppressor liver kinase B1 (LKB1) (Ito and Suda, 2014), an inducer of PPAR γ coactivator 1 (PGC-1) and downstream FAO-related genes, impairs HSC maintenance (Kleiner *et al.*, 2009; Gan *et al.*, 2010; Gurumurthy *et al.*, 2010; Nakada *et al.*, 2010). Thus, the equilibrium between FAO and glycolysis versus oxidative phosphorylation represents a fundamental mechanism, regulating the balance between HSC maintenance and commitment, respectively (reviewed in Oburoglu *et al.*, 2016) (Figure 32).

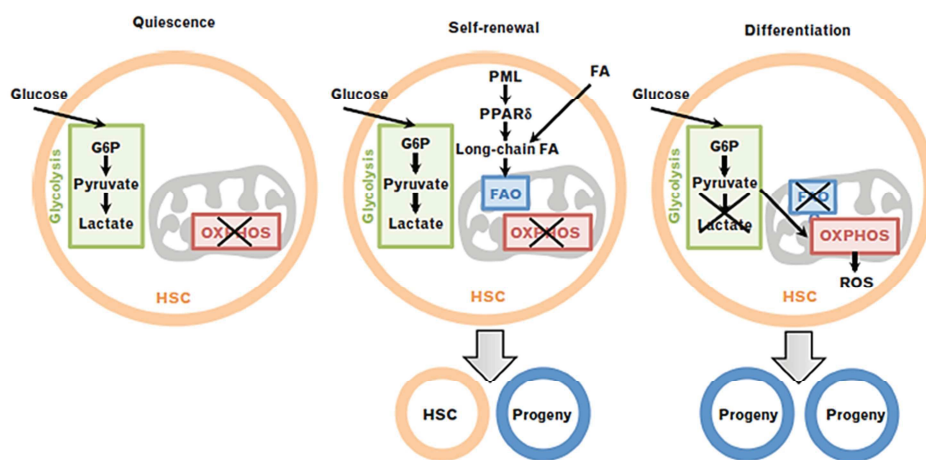


Figure 32. Metabolic regulation of HSC maintenance and commitment. Quiescent HSCs show a mainly glycolytic metabolism. Glycolysis and FAO, which concomitantly inhibit oxidative phosphorylation (OXPHOS) are associated with HSC self-renewal. A switch towards an oxidative metabolism is observed upon HSC differentiation. Adapted from (Oburoglu *et al.*, 2016)

3.4.2 Mitochondrial dynamics in HSC maintenance vs commitment

Despite the precise dynamics of cell division are still under debate (Ito *et al.*, 2016; Turcotte *et al.*, 2017; Grinenko *et al.*, 2018), the importance of FAO has been confirmed in recent single-cell studies, focusing on a homogenous LT-HSC population, the Tie2⁺ subset (CD34⁻CD150⁺CD48^{low/-}CD135⁻LSK). These cells show significantly increased expression of not only HSC- but also metabolism-related genes, involved in FAO and including carnitine palmitoyl-transferase 1 (CPT1A) as well as peroxisome proliferator-activated receptor delta (PPAR δ), as compared to Tie2⁻ HSCs (Ito *et al.*, 2018). In addition, initial cell divisions of Tie2⁺ HSCs have been found to be symmetric, therefore generating two daughter cells carrying long-term reconstitution potential (Ito *et al.*,

2016; Turcotte *et al.*, 2017). While defective organelles, such as damaged mitochondria, usually segregate to differentiated daughter cells upon asymmetric cell divisions (Katajisto *et al.*, 2015), they result equally distributed between the two daughter cells in case of a symmetric division (Ito and Ito, 2016; Ito *et al.*, 2016). Interestingly, mitophagy has been found to play a fundamental role in self-renewing HSC expansion, highlighting a relationship between self-clearance mechanisms and symmetric cell division (Ito *et al.*, 2018). In fact, enhanced Tie2⁺ HSC self-renewal potential has been observed upon activation of PPAR-FAO pathway, which subsequently induces mitophagy, through Parkin protein recruitment into the mitochondria (Ito *et al.*, 2016). On the contrary, silencing of Parkin, consequently leading to mitophagy inhibition, results in reduced Tie2⁺ HSC expansion potential (Ito *et al.*, 2018). Finally, mitochondrial dynamics, as well as intracellular Ca²⁺ levels, have also been identified as other important factors, involved in the modulation of HSC maintenance and commitment (Tan and Suda, 2017). In fact, the mitochondrial protein mitofusin-2 (MFN2) plays a role in HSC fate specification, not only by regulating mitochondrial fusion, but also by acting on NFAT transcriptional activity, through intracellular Ca²⁺ levels (Luchsinger *et al.*, 2016). The latter, when increased have been shown to induce HSC cell division, while being downregulated by exogenous adenosine in the modulation of HSC fate (Umemoto *et al.*, 2018).

Further insights into the metabolism-dependent modulation of HSC fate specification towards the erythroid lineage are provided in the next section.

3.4.3 Metabolic requirements in erythroid lineage commitment

Amongst the recent research highlighting the importance of metabolic regulation in the HSC commitment towards a precise lineage fate, our laboratory is interested in the specificities of erythropoiesis. Initial metabolic studies of erythropoiesis focused mainly on iron metabolism, required for the biosynthesis of hemoglobin (Hb), the principal erythrocytic protein (Hb) (see sections 2.2.4.1.1 and 2.2.4.1.2). However, mammalian erythroid cells have other metabolic specificities including the lack of a nucleus and mitochondria, resulting in a reliance on glycolysis-dependent energy production (see section 2.2.4.3). In fact, the RBC plasma membrane contains more than 300.000 copies

of the glucose transporter Glut1 (Mueckler, 1994). Notably though, several studies have shown that Glut1 expression in ascorbic acid-defective species (higher primates, fruit bats and guinea pigs) also allows the transport of the oxidized form of vitamin C (DHA) (Himmelreich *et al.*, 1998; May *et al.*, 2001; Montel-Hagen *et al.*, 2008, 2009; Ogawa, 2008). During erythroid commitment, Glut1 surface expression is only observed in terminal erythroid progenitors, suggesting a role for other cell nutrients/metabolites as main energy sources during the differentiation process. Interestingly, research from our laboratory has described expression of the ASCT2 glutamine transporter on human hematopoietic and erythroid progenitor cells, with levels maintained throughout the erythroid differentiation (Oburoglu *et al.*, 2014). In addition, an impaired ASCT2-dependent glutamine uptake or glutamine catabolism diverts the differentiation of erythroid-primed progenitors towards the myelomonocytic lineage (Oburoglu *et al.*, 2014). It was then established that glutamine-dependent nucleotide biosynthesis is required for the erythroid commitment of human HSCs, as confirmed by an enhanced erythropoiesis upon glucose utilization in the pentose phosphate pathway (PPP) and subsequent production of nucleotide precursors (Oburoglu *et al.*, 2014). Recent work has also shown that the succinyl-CoA required for heme biosynthesis is mainly produced from glutamine-derived α -ketoglutarate, and not as intermediate of the TCA cycle. This mechanism involves a direct interaction between the α -ketoglutarate dehydrogenase (KGDH) complex and the first enzyme of the heme biosynthesis pathway, the δ -aminolevulinic acid synthase (or 5-aminolevulinic acid synthase 2, ALAS2) (Burch *et al.*, 2018). On the other hand, higher expression levels of hemoglobin genes have been observed upon supplementation of erythroleukemia K562 cells with lactic acid (lactate), which also increased the erythroid differentiation of K562 cells. Indeed, this effect is linked to lactic acid-dependent ROS production, which has been found to foster erythroid differentiation in lactic acid-treated mice as well (Luo *et al.*, 2017). Hemoglobin biosynthesis has also been shown to be regulated by neutral essential amino acid (NEAA) availability, especially L-leucine, via the mTOR signaling pathway (Chung *et al.*, 2015). Finally, recent work has identified an important role for lipid metabolism in the terminal steps of erythroid differentiation, with reduced enucleation levels upon impairment in phosphocoline catabolism. This alteration leads to changes in the metabolic balance with reduced OXPHOS, while increased glycolysis, which is shunted towards the

biosynthesis of serine and glycine (reviewed in Gibson and Rees, 2018; Huang *et al.*, 2018).

Taken together, these results support a critical role for nutrients and their metabolism in the commitment of hematopoietic stem and progenitor cells to specific lineage fates. Therefore, further investigation is needed to better characterize the mechanisms of “metabolic control” orchestrating the hematopoietic stem cell “choice” to each differentiation fate.

Objectives

Human HSC commitment towards the erythroid lineage is orchestrated by diverse factors, including cytokines, transcription factors, epigenetic regulators, as well as the stem cell niche. Several recent studies have highlighted an important relationship between metabolic regulation and cell differentiation potential, suggesting that metabolic heterogeneity represents another mechanism governing the fate specification of HSCs (Oburoglu *et al.*, 2014; Haas *et al.*, 2018; Kratchmarov *et al.*, 2018). However, an intriguing question—whether there are specific metabolic features that characterize early versus late erythroid progenitor stages—remains open.

Recent findings in our laboratory have identified a requirement for glutamine catabolism in the erythroid commitment of human HSCs, with differentiation skewing towards the myeloid lineage upon impaired glutamine uptake or utilization (Oburoglu *et al.*, 2014). Indeed, the group has demonstrated that glutamine catabolism, together with glucose shunting towards the PPP, contribute to nucleotide biosynthesis, which is a *sine qua non* condition for erythropoiesis. Intriguingly, Oburoglu *et al.* have also shown that, upon human erythroid differentiation, glutamine anaplerosis fuels the TCA cycle via the biosynthesis of a crucial intermediate, the α -ketoglutarate (α -KG).

These interesting findings have prompted us to investigate the role of the α -KG metabolite, which is not only involved in the TCA cycle and the consequent oxidative phosphorylation (OXPHOS)-dependent metabolism, but is also an important cofactor for several DNA and chromatin-modifying enzymes (Klose and Zhang, 2007; Shi and Tsukada, 2013; Guengerich, 2015). During my PhD, I first assessed the contribution of oxidative metabolism in the early *versus* late phases of human erythropoiesis. I became particularly interested in the metabolic networks regulating the progression through terminal erythroid differentiation and the enucleation process, which are both linked to important mechanisms of mitochondrial clearance and chromatin condensation (Ji *et al.*, 2010; Hattangadi *et al.*, 2011; Zhang *et al.*, 2015; Grosso *et al.*, 2017). I therefore studied the erythroid commitment of human CD34⁺ progenitor cells in order to address the following questions:

- Does α -KG play a role in the early phase of erythroid differentiation?
- How are mitochondrial function and oxidative metabolism (OXPHOS) regulated throughout human erythropoiesis?
- Is there a relationship between mitochondrial function, OXPHOS and the enucleation process during terminal erythroid differentiation?
- Do isocitrate dehydrogenase (IDH) activity and TCA cycle intermediate levels regulate terminal erythroid differentiation?

Results

ARTICLE:

Human erythroid differentiation is regulated by stage-specific changes in the Krebs cycle network

Abstract

The commitment of a hematopoietic stem cell to an erythroid lineage fate is governed by the extracellular niche. One important component of this niche is its nutrient composition and indeed, amino acid availability is critical for erythroid lineage specification. However, following commitment, the differentiation of an immature proerythroblast to an enucleated red blood cell is accompanied by distinct changes in cell size and morphology. We hypothesized that this process is dependent upon metabolic alterations at each stage of differentiation. Differentiation of human CD34⁺ progenitors to the proerythroblast stage was associated with an increased glutamine catabolism. The glutamine-derived generation of the alpha-ketoglutarate (α KG) Krebs cycle intermediate directly increased oxidative phosphorylation (OXPHOS) in these erythroblasts. Notably, ectopic α KG did not alter pro-erythroblast differentiation but significantly attenuated terminal differentiation and enucleation, stages that were associated with physiological decreases in mitochondrial function and OXPHOS. Conversely, an α KG antagonist (dimethylxalylglycine, DMOG) abrogated OXPHOS in erythroblasts but did not negatively impact on terminal differentiation. Surprisingly, downregulation of the cytoplasmic isocitrate dehydrogenase I (IDH1) enzyme, converting isocitrate to α KG, also inhibited terminal orthochromatic erythroblast generation and enucleation. This effect was likely due to an imbalance in substrate availability as IDH1 catalyzes both the forward and reverse reactions; both ectopic α KG as well as citrate further decreased the attenuated enucleation of IDH1-knockdown erythroid precursors. Thus, α KG-mediated OXPHOS is crucial for early erythroid differentiation but the dampening of this metabolic network is a *sine qua non* for red cell maturation.

Introduction

Hematopoietic stem cells (HSCs) must be tightly regulated throughout the lifetime of an individual, allowing the sustained production of all mature blood lineages under physiological as well as pathological conditions. This regulation is finely balanced by an interplay between external and internal stimuli. During the past decades, extensive research has shown the importance of cytokines, transcription factors, and cell-cell contacts in the maintenance and differentiation of HSCs. However, it is only recently that we have envisaged the possibility that nutrient metabolism contributes to HSC quiescence and fate commitment and indeed, this is clearly the case; stem cell quiescence, required for efficient repopulating ability, is promoted by a specific metabolic state, namely anaerobic glycolysis (Takubo et al., 2013; Yu et al., 2013). Consistent with this finding, genetic manipulations that enhance glycolysis, at the expense of oxidative phosphorylation, increase HSC activity (Takubo et al., 2013; Yu et al., 2013) while alterations that augment mitochondrial biogenesis promote HSC differentiation (Chen et al., 2008; Mantel et al., 2010; Maryanovich et al., 2015; Yu et al., 2013). Furthermore, HSC commitment is itself regulated by metabolism; fatty acid oxidation (FAO) promotes the asymmetric division of HSCs, resulting in the generation of one committed daughter cell and one daughter cell with self-renewal potential (Ito et al., 2012; Yusuf and Scadden, 2012). Conversely, inhibition of FAO together with increased oxidative phosphorylation lead to a symmetric division with the generation of two committed progenitors.

The data presented above indicate that the balance between HSC maintenance and the generation of all blood lineages is conditioned by the cell's metabolism. However, they do not provide information on the metabolic parameters that regulate differentiation to a specific lineage fate and moreover, the metabolic parameters governing the successful generation of a mature lineage cell. Erythropoiesis, the process by which a hematopoietic erythroid progenitor

proliferates and differentiates into an enucleated reticulocyte, is a metabolically daunting process when evaluated at the level of cell numbers. In healthy adults, committed erythroid progenitors support the production of 2.4 million erythrocytes per second via a synchronized regulation of iron, glucose, fatty acid and amino acid metabolism. Iron is indispensable for heme biosynthesis in erythroblasts (Oburoglu et al., 2016) and is interconnected with amino acid metabolism via amino acid-induced mTOR signaling (Chung et al., 2015). Furthermore, we previously showed that the utilization of both glutamine and glucose in *de novo* nucleotide biosynthesis is a *sine qua non* for erythroid differentiation (Oburoglu et al., 2014).

It is important though to note that erythropoiesis is also distinctive in that each mitosis results in the production of daughter cells that differ, morphologically and functionally, from their parent cells. This ordered maturation is tightly regulated with each stage of erythroid differentiation defined by a decreased cell size, enhanced chromatin condensation, progressive hemoglobinization, changes in membrane organization, and transcriptome specificities (An et al., 2014; Chen et al., 2009; Hu et al., 2013a; Li et al., 2014). We therefore hypothesized that progenitor metabolic networks evolve as a function of the erythroid differentiation stage and moreover, that they regulate the transition of progenitors from one stage of differentiation to the next.

Here, we demonstrate that at early stages of human red cell development, prior to terminal differentiation, hematopoietic progenitors exhibited an increased metabolic activity with a significantly higher level of oxidative phosphorylation (OXPHOS). This correlated with the increased generation of α KG and indeed, we found that α KG, the TCA cycle intermediate generated by the anaplerotic utilization of glutamine, directly augmented OXPHOS in these progenitors. However, the terminal differentiation of erythrocyte precursors, with the loss of

mitochondrial mass and membrane potential, resulted in a decreased level of OXPHOS. Under these latter conditions, but not prior to the pro-erythroblast stage, α KG severely attenuated terminal differentiation and red cell precursor enucleation. Conversely, an α KG antagonist (dimethylxalylglycine, DMOG) which abrogated OXPHOS in erythroblasts did not negatively affect terminal differentiation or enucleation.

These data strongly suggested that the late generation of α KG through glutamine metabolism and the TCA cycle is detrimental to the production of mature red cells. Surprisingly though, the lentiviral-mediated downregulation of isocitrate dehydrogenase I, the cytosolic enzyme that catalyzes the reversible conversion of isocitrate to α KG, also resulted in a severe attenuation of terminal erythroid differentiation and enucleation. This effect was likely due to an imbalance in substrate availability as both citrate and α KG synergized with IDH1 knockdown to further decrease red cell enucleation. Thus, the present study shows that the α KG metabolite regulates mitochondrial function and oxidative phosphorylation and this process is critical for early erythroid differentiation. However, at late erythroblast stages, decreased cellular respiration and subsequent enucleation are dependent on the catalysis of these Krebs cycle intermediates. The ensemble of these data strongly suggests that metabolic networks leading to an increased cellular respiration during the terminal stages of development will result in a pathological erythroid differentiation.

Methods and Materials

CD34⁺ cell isolation and *ex vivo* differentiation assays. CD34⁺ cells were isolated from umbilical cord blood obtained immediately following delivery of full-term infants after informed consent and approval by the “Committee for the Protection of Persons” (IRB) and from peripheral blood from the New York Blood Center or the French Blood Center (EFS). CD34⁺ cells were isolated using the human CD34 MicroBead kit (MACS Miltenyi Biotec, CA, USA) according to the manufacturers’ recommendations. CD34⁺ cells (5×10^5 cells/ml) were expanded 7 days in StemSpan media (StemCell Technologies Inc) supplemented with 5% fetal bovine serum (FBS), 25 ng/ml rhuSCF (Amgen), 10 ng/ml rhIL-3 and 10 ng/ml rhIL-6 (R&D) at 37°C with a humidified 5% CO₂ atmosphere. After 6-7 days of expansion, cells were differentiated in IMDM media supplemented with BSA (1.5 g/L, Sigma Aldrich), human holo-transferrin (60 mg/L, Sigma-Aldrich), human insulin (4.4 mg/L, Sigma-Aldrich), rhuSCF (10 ng/ml, Amgen), rhIL-3 (1 ng/ml). Erythropoiesis was induced by addition of 3 U/ml recombinant human erythropoietin (rEPO; Eprex, Janssen-Cilag). Cytokines were supplemented every 3 days and cell concentrations were maintained between 0.5 and 1.5×10^6 cells/ml. In some experiments, progenitors were differentiated in the presence of cell permeable dimethyl α -ketoglutarate (DMK) (Sigma-Aldrich) or dimethylxalylglycine (DMOG) (Sigma-Aldrich) at a concentration of 3.5mM and 0.5mM, respectively. Cultures were supplemented with α -KG or DMOG every 3 days.

Cytospin preparation. Cytospins were prepared on glass slides (5×10^4 cells in 200 μ l of PBS), using the Thermo Scientific Shandon 4 Cytospin. Slides were stained with May-Grünwald solution (Sigma-Aldrich) for 5 min, rinsed in 40 mM Tris buffer for 90 sec, and subsequently stained with Giemsa solution (Sigma-Aldrich) for 15 minutes. Cells were imaged using a Leica DM2000 inverted microscope under 100 \times objective magnification.

Flow cytometry. Expression of the CD36, CD71, CD49d, and Glycophorin A surface markers was monitored using the appropriate fluorochrome-conjugated monoclonal antibodies (mAb)

(Beckman Coulter and Becton Dickinson), a mAb against Band 3 was used as previously described (Hu et al., 2013b) and Glut1 expression was monitored by binding to its retroviral envelope ligand fused to eGFP (Metafora biosystems), as reported (Kim et al., 2004; Kinet et al., 2007; Manel et al., 2003; Swainson et al., 2005). Mitochondrial biomass and mitochondrial transmembrane potential levels were assessed by staining with MitoTrackerGreen, MitoTrackerRed, respectively (0.5 μ M; Invitrogen, Molecular Probes). Enucleation was evaluated by staining with the SYTO-16 or SYTO-60 nucleic acid stain (1 μ M and 5 μ M, ThermoFisher) for 15 minutes in PBS 2% FBS at RT. Cell sorting was performed on a FACS ARIA high-speed cell sorter (Becton-Dickison) and analyses were performed on FACSCalibur (BD Biosciences) or FACS-Canto II (BD Biosciences) cytometers. Data analyses were performed using FlowJo software (Tree Star, Ashland, OR).

Virus production and transduction of CD34⁺ progenitor cells. Lentiviral pLKO.1 plasmids harboring shRNAs directed against IDH1 (Clone ID: TRCN0000027284 and ID:TCRN0000312463) were obtained from Sigma-Aldrich. Cells were transduced with the first IDH1 shRNA lentiviral plasmid or the second shIDH1 plasmid where the 714bp sequence encoding for EGFP was inserted in place of the puromycin gene at the unique BamHI and KpnI restriction sites. Virions were generated by transient transfection of 293T cells with these vectors together with the Gag-Pol packaging construct 8.91 and a plasmid encoding the VSV-G envelope, pCMV-VSV-G, as described previously (Loisel-Meyer et al., 2012). Viral supernatant was harvested 48 hours post-transfection and virions were concentrated by ultra-centrifugation through a 20% sucrose cushion for 2h at 4°C at 25,000 rpm. Virions were resuspended in RPMI with 1% BSA and stored at -80°C. Titers were determined by serial dilutions of vector preparations on Jurkat cells and are expressed as Jurkat transducing units (TU/ml).

For transduction of CD34⁺ progenitors, cells were expanded for 3 days in Stem Span medium (Stem cell Technologies Inc) supplement with 5% fetal bovine serum (FBS), 25 ng/ml rhuSCF

(Amgen), 10 ng/ml rhIL-3 and 10 ng/ml rhIL-6 (R&D) at 37°C with a humidified 5% CO₂ atmosphere. 5×10^5 cells were then exposed to viral supernatants containing 4.8×10^5 TU to 9.9×10^5 TU (representing a multiplicity of infection of 1-2). 48h after transduction, erythroid differentiation was initiated by the addition of rEPO (3 U/ml). Transduction efficiency was monitored as a function of GFP expression or following puromycin selection at 48h post transduction.

Quantitative Real Time PCR. Total RNA was isolated at the indicated time points using the RNeasy Mini kit (Qiagen). Quantitative PCR of cDNAs was performed using the Quantitect SYBR green PCR Master mix (Roche) with 10 ng of cDNA and 0.5 μ M primers in a final volume of 10 μ l. Primer sequences are as follows: IDH1: 5'- GTTAGCCCACAGAGCAAAGC-3' (forward)/ 5'- TAATGCAAGCAGCCAAGTC-3' (reverse); 18S: 5'-CGGCTACCACATCCAAGGAA-3' (forward)/ 5'- GGGCCTCGAAAGAGTCCTGT-3' (reverse); and β -actin: 5'-GTCTCCCTCCATCGTG-3' (forward)/ 5'- TTCTCCATGTCGTCCCAG-3' (reverse). Amplification of cDNAs was performed using the LightCycler 480 (Roche). Cycling conditions were comprised of a denaturation step for 5 min at 95°C, followed by 40 cycles of denaturation (95°C for 10 sec), annealing (63°C for 10 sec) and extension (72°C for 10 sec). After amplification, melting curve analysis was performed with denaturation at 95°C for 5 sec and continuous fluorescence measurement from 65°C to 97°C at 0.1°C/s. Each sample was amplified in duplicate. Relative expression was calculated by normalization to 18S or β -actin as indicated.

Mass spectrometry (LC-MS). For profiling of intracellular metabolites, 10^6 cells were incubated in IMDM medium without glutamine and were supplemented with uniformly labeled [U-¹³C₅-¹⁵N₂] for 6 hours. Cells were rapidly washed in ice-cold PBS and metabolites were extracted in a 50% methanol/ 30% acetonitrile solution. For profiling of metabolite exchange rates, cells were cultured in the same conditions and media derived from wells devoid of cells were used as a reference. All cultures were performed in triplicate. Both cell extracts and media were

centrifuged at 16 000g for 10min at 4°C and supernatants were analyzed by HPLC-MS. Samples were separated on a Thermo Scientific Accela liquid chromatography system coupled with a guard column (Hichrom) and a ZIC-pHILIC column (Merck). The separation was performed using a gradient elution in a solution of 20 mM ammonium carbonate, with 0.1% ammonium hydroxide and acetonitrile. Metabolites were detected using a Thermo Scientific Exactive Orbitrap mass spectrometer with electrospray ionization, operating in a polarity switching mode. The raw data were analyzed with Xcalibur 2.2 and LCquan 2.7 Thermo scientific software. The peak areas resulting from the intracellular samples were normalized to the number of cells obtained at the end of the 6-hour incubation period.

Seahorse analysis. OCR and ECAR were measured using the XFe-96 Extracellular Flux Analyzer (Seahorse Bioscience). Cells (2.5×10^5) were placed in XF media (non-buffered DMEM containing 10 mM glucose and 2 mM L-glutamine) and monitored in basal conditions and in response to oligomycin (1 μ M), FCCP (1 μ M), rotenone (100 nM) and antimycin A (1 μ M; Sigma). In some experiments, α KG (to a final concentration of 3.5 mM) or DMOG (to a final concentration of 0.5mM) was injected directly into the well before the sequential addition of oligomycin, FCCP, rotenone and antimycin A during the Mitostress test.

Statistical analyses. Data were analyzed with GraphPad Software (GraphPad Prism), and P values were calculated by one-way ANOVA (Tukey's post hoc test) or t-tests, as indicated.

Results and Discussion

Early erythroid differentiation is associated with an increased generation of the alpha-ketoglutarate intermediate and augmented oxidative phosphorylation

As glutaminolysis is required for erythroid lineage specification (Oburoglu et al., 2014), we evaluated the utilization of glutamine during the early stages of human erythroid differentiation, at initial stages of Glycophorin A upregulation (**Figure 1A**). The conversion of glutamine to glutamate can be evaluated by tracing the utilization of carbons from labelled glutamine ($^{13}\text{C}_5$) into glutamate ($^{13}\text{C}_5$). As shown in Figure 1B, there was a significantly higher generation of m+5 glutamate from glutamine following erythropoietin-induced erythroblast generation of human CD34^+ progenitors, from a mean of 0.08 ± 0.007 to 0.10 ± 0.005 ($p < 0.05$; **Figure 1B**). Furthermore, the utilization of glutamine was augmented upon erythroid differentiation—the percentage of glutamate derived from labelled glutamine was $64 \pm 0.6\%$ as compared to $40 \pm 1\%$ in progenitors expanded in the absence of erythropoietin ($p < 0.0005$). This correlated with a higher ratio of αKG generation from glutamate ($p < 0.05$; **Figure 1B**).

Glutamine supports the Krebs cycle via the anaplerotic generation of αKG . As such, it was of interest to determine whether the increased generation of the αKG intermediate is associated with an augmented OXPHOS. Indeed, the basal oxygen consumption rate (OCR), an estimation of OXPHOS, as well as the spare respiratory capacity (SRC) of the cell, were significantly increased upon erythroid differentiation (**Figure 1C**). Moreover, we found that αKG directly regulates the metabolic state of progenitors as the injection of a cell-permeable αKG augmented the respiratory capacity of erythroblasts within 30 minutes of administration ($p < 0.01$; **Figure 1D**). Thus, the increased metabolism of an early erythroblast is associated with higher intracellular levels of the αKG metabolite.

Terminal erythroid differentiation is associated with decreased mitochondrial function

As indicated above, each stage of erythroid differentiation is defined by a decreased cell size and the final orthochromatic erythroblast to reticulocyte transition results in the extrusion of the nucleus and mitochondria. Indeed, we found that both mitochondrial biomass and transmembrane polarization, monitored as a function of MitoTrackerGreen and MitoTrackerRed staining, respectively, was significantly decreased between days 0 and 10 of differentiation; measures of biomass decreased from 560 ± 42 to 97 ± 62 while transmembrane potential decreased from 671 ± 20 to 67 ± 36 ($p < 0.01$, **Figure 2A**). This had functional consequences for the cell as both the basal OCR and the SRC of erythroblasts decreased significantly between days 4 and 10 of erythroid differentiation, with the former diminishing from 185 ± 11 to 105 ± 14 pmol O_2 /min (**Figure 2B**). However, the erythroblast populations that are present at any given day of *ex vivo* rEPO-induced erythroid differentiation are mixed and may therefore not accurately reflect the respiratory status of a specific erythroblast subset. We therefore FACS-sorted specific GlycophorinA (GlyA⁺) erythroblast populations on the basis of their CD49d/Glut1 staining profiles. As previously shown, the subsets presented in **Figure 2C** are basophilic, polychromatic and orthochromatic erythroblasts, respectively. Notably, these purified subsets exhibited significant differences in their respiratory capacities with oxygen consumption decreasing drastically between the basophilic and the orthochromatic stages of differentiation.

α -Ketoglutarate negatively regulates terminal erythroid differentiation and enucleation by increasing mitochondrial function

To assess whether the Krebs cycle intermediate α KG differentially alters erythroid differentiation under the high oxidative conditions of early erythroblasts as compared to the low oxidative conditions of late erythroblasts, we supplemented erythroblasts with a cell permeable α KG ester

(dimethyl α -ketoglutarate). Prior to the erythroblast stage, ectopic α KG did not alter the generation of either CD34⁻CD36⁺ committed erythroid colony forming unit (CFU-E) progenitors or GlyA⁺ erythroblasts (**Figure 3A**). However, addition of α KG at day 7 of erythroid differentiation, a time point at which progenitors had already reached the basophilic erythroblast stage, significantly inhibited erythroblast progression to the CD49d⁻Glut1⁺ orthochromatic stage at day 10, from 43 \pm 13% to 34 \pm 10% ($p < 0.5$; **Figure 3B**). This phenomenon was associated with a significantly attenuated level of enucleation, decreasing from a mean of 51 \pm 3% to 23 \pm 7% in the presence of α KG at day 14 ($p < 0.005$, **Figure 3C**).

To determine the precise impact of α KG on distinct subsets, basophilic, polychromatic and orthochromatic erythroblasts, corresponding to populations p2-p4 respectively, were isolated by FACS sorting (**Figure 3D**). Sorted erythroblast subsets were then activated with rEPO for 24h in the absence or presence of α KG and enucleation was monitored. As shown in **Figure 3D**, enucleation was not detected in the most immature P2 population, irrespective of the presence of α KG. Notably though, α KG dramatically inhibited enucleation from sorted P3 erythroblasts. As expected, the most mature polychromatic/ orthochromatic erythroblast sorted subset (P4) exhibited the highest level of enucleation but even in this subset, α KG had a negative effect on enucleation. This negative impact on late stage terminal differentiation was also detected visually by May-Grünwald-Giemsa staining of erythroblasts, revealing a significantly lower percentage of enucleated reticulocytes in the presence of α KG (**Figure 3E**).

Collectively, these data demonstrated that a high level of α KG is detrimental to late stage erythroid differentiation. To assess whether this was associated with increased mitochondrial activity during terminal erythroid stages, we first monitored mitochondrial biomass and transmembrane polarization. While no changes in staining were detected at day 3 of

differentiation, ectopic α KG significantly inhibited the decrease in mitochondrial parameters at day 10 of differentiation, with mean biomass and polarization parameters altered from 124 ± 27 to 149 ± 35 and 85 ± 10 to 110 ± 17 , respectively ($p < 0.05$; **Figure 4A**). These alterations correlated with a significant increase in basal respiration as well as SRC in erythroblasts exposed to ectopic α KG from days 3-10 of differentiation, with the latter increasing from 16 ± 7 to 68 ± 4 pmol O_2 /min ($p < 0.005$). Moreover, a similar impact was revealed upon short term exposure to α KG, from days 7-10 of differentiation ($p < 0.005$, **Figure 4B**). Thus, the negative impact of α KG on orthochromatic erythroblast differentiation and enucleation was associated with an increased respiratory capacity of these cells.

Abrogating α KG-mediated oxidative phosphorylation attenuates early erythroid differentiation but does not impact on late differentiation and enucleation

The data presented above revealed a negative regulatory role of α KG in late stage erythroid differentiation. α KG function can be inhibited by antagonists such as the cell permeable α KG analog, dimethylxalylglycine (DMOG). While this analog has been used to activate hypoxia inducible factors (HIFs), recent work has shown that it directly suppresses OXPHOS in cancer cells (Zhdanov et al., 2015). Upon evaluation of this α KG antagonist on day 3 erythroblasts, we found that it directly inhibits OXPHOS in these cells, within 30 minutes of its addition (**Figure 5A**). Furthermore, DMOG abrogated OXPHOS in EPO-induced CD34⁺ progenitor cells, between days 0 and 3 of differentiation (**Figure 5B**). Interestingly, this antagonist was not toxic at the concentration used (0.5 mM) but did inhibit erythroblast generation, as monitored by the upregulation of Glycophorin A (**Figure 5C**).

A negative impact of an α KG antagonist on early erythroid differentiation correlated with the increased α KG generation and OXPHOS that we detected at these early stages (**Figures 1, 3**).

However, at late stages, OXPHOS was decreased (**Figure 4**) and it was thus important to determine the impact of this α KG antagonist during this terminal differentiation. To this end, the DMOG α KG antagonist was added to erythroblasts at day 7 of EPO-induced differentiation, a time point at which all cells had proceeded to a GlycophorinA⁺ basophilic stage, as monitored by their CD49d⁺Glut1⁺ profile (**Figure 5D**). Notably, while DMOG inhibited OXPHOS to minimal levels (**Figure 5E**), differentiation to the CD49d⁺Glut1⁺ orthochromatic stage was not inhibited and indeed, there was a trend towards an enhanced progression (43 \pm 3% vs 64 \pm 12%), albeit not statistically significant. Furthermore, this α KG antagonist did not negatively impact on enucleation (52 \pm 21% vs 45 \pm 8%, **Figure 5D**). Together, these results reveal an important role for α KG in promoting erythroid commitment and specification. However, the continued generation of α KG is detrimental to terminal differentiation and indeed, this latter process is not perturbed when α KG function is antagonized and cellular respiration is abrogated.

IDH1 activity is required for terminal erythroid differentiation, balancing the inter-conversion between α KG and citrate

The generation of α KG from isocitrate is catalyzed in the mitochondria by isocitrate dehydrogenase 2 (IDH2) and IDH3 enzymes, while IDH1 resides in the cytoplasm and peroxisome (**Figure 6A**). Transcriptomic and proteomic analyses revealed high levels of the NADP⁺-dependent IDH1 and IDH2 enzymes but only low levels of IDH3 (An et al., 2014; Gautier et al., 2016; Yan et al., 2018). Because late stage erythroblasts exhibit decreased mitochondrial function, we assessed the role of the cytoplasmic IDH1 enzyme as a function of erythroid differentiation.

In order to investigate the specific effects of IDH1, we pursued an shRNA-mediated knockdown approach using an shIDH1-GFP lentiviral vector. Transduction of more than 50% of progenitors

was obtained, resulting in decrease in IDH1 transcripts of approximately 65% (**Figure 6B**). CD34⁺ progenitors harboring the shIDH1 vector were maintained in the absence of EPO, monitored as a function of GFP expression (**Figure 6C**, left), but IDH1 knockdown under conditions of erythroid differentiation was associated with a dramatic selective disadvantage. The percentage of cells harboring the shIDH1 vector decreased from 57% to 25% between days 3 and 10 of differentiation in a representative experiment (**Figure 6C**). Despite the selective disadvantage, proerythroblast differentiation was not impacted and shIDH1⁺ progenitors progressed to a GlyA⁺ stage at a rate similar to that detected in cells transduced with a control vector (**Figure 6D**). Notably though, the rate of enucleation was significantly decreased in progenitors with downregulated IDH1, decreasing from 50% to 20% in a representative experiment (**Figure 6E**).

These results were surprising as we had hypothesized that a downregulation of IDH1 would result in lower levels of α KG and would potentially accelerate terminal differentiation and enucleation. However, our data revealed a lower level of enucleation following IDH1 knockdown, evoking the interplay of other parameters in this effect. Indeed, IDH1 catalyzes both the forward and reverse reactions suggesting that IDH1 knockdown could increase α KG at the expense of isocitrate/citrate levels. Furthermore, recent studies have shown that alterations in the ratios of TCA cycle metabolites impact on the balance between ES cell self-renewal and differentiation (Carey et al., 2014; Rose et al., 2011; Xiao et al., 2012). To assess whether this was the case, we increased α KG levels in IDH1 knockdown cells and found a synergistic effect on orthochromatic differentiation and enucleation. Using very low levels of α KG (2 mM), the impact on enucleation was lower than the >50% decrease we initially detected (**Figure 3C**), ranging at approximately 25% (**Figure 7A**). However, in the context of the IDH1 knockdown, ectopic α KG reduced orthochromatic erythroblast generation and enucleation by >75% (**Figure 7A**). Moreover, while increasing citrate levels did not impact on enucleation in control erythroblasts,

they clearly inhibited enucleation following IDH1 knockdown (**Figure 7B**). Notably, these IDH1-mediated changes in the intracellular concentrations of Krebs cycle intermediates also resulted in the generation of aberrant multinucleated erythroblasts (**Figure 7**).

Mutations and lack of function of IDH1/IDH2 are strongly associated with myelodysplastic syndromes (MDS) that are characterized by ineffective erythropoiesis (80% of cases) (reviewed in (Bejar and Steensma, 2014; Fenaux et al., 2014)). Anemia dominates the early course of MDS and these patients exhibit an aberrant erythroid differentiation with the generation of multinucleated erythroblasts (Abdel-Wahab and Levine, 2013) similar to that which we detected here (**Figure 7A**). Our group and others have previously shown that the initial stages of erythroblast differentiation require glutaminolysis with generation of the α KG intermediate (Burch et al., 2018; Oburoglu et al., 2014). Notably, carbons from α KG, but not from succinate, fuel the generation of succinyl coA which is required for heme biosynthesis (Burch et al., 2018). However, we show here that there is a dynamic regulation of these processes during erythroid differentiation—at late stages, IDH1 activity and α KG is detrimental for red blood cell formation. Rather, a regulated diminution of α KG levels is required for the progression of normal erythroblasts to an enucleated reticulocyte stage. It is therefore tempting to speculate that the anemia associated with MDS is due to an aberrant regulation of the Krebs cycle metabolic network at late stages of the erythroid differentiation process.

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Figure Legends

Figure 1. Increased alpha-ketoglutarate generation during early erythroid differentiation is associated with an augmented oxidative phosphorylation.

(A) The phenotype of CD34⁺ progenitors differentiated in the absence (-) or presence (+) of recombinant erythropoietin (EPO). Representative histograms showing expression of GlyA, CD71, and CD36 are presented. (B) A schematic representation of glutamine conversion to α -ketoglutarate (α -KG), showing the metabolism of [¹³C] heavy-labelled glutamine carbons. Ratios of the different isotopomers in the two conditions (\pm SD) are presented and horizontal lines represent mean levels. * p <0.05; **** p <0.001 (C) Oxygen consumption rates (OCR), a measure of OXPHOS, were determined using a Seahorse Xfe96 analyzer by sequential injection of oligomycin, FCCP and antimycin A/rotenone into CD34⁺ progenitors differentiated for 3 days in the absence (-, green) or presence (+, black) of EPO. (D) OCR and extracellular acidification rates (ECAR), a measure of glycolysis, were monitored at day 3 of EPO-induced erythroid differentiation. As indicated, α -KG (3.5 mM, blue line) was directly injected. Mean levels \pm SEM are presented (n=5-6 per time point).

Figure 2. Erythroid differentiation results in decreased mitochondrial activity and oxidative phosphorylation.

(A) Mitochondrial biomass and polarization were evaluated by MitoTrackerGreen and MitoTrackerRed staining, respectively, at days 0, 3, 7 and 10 of erythroid differentiation. Representative histograms at the indicated time points of differentiation are shown and mean MFIs are indicated. Mean levels at each time point are shown (n=3, 1-way ANOVA with Tukey's post-hoc test). ** p <0.01; *** p <0.005, ns-non significant (B) OCR was monitored in EPO-stimulated CD34⁺ progenitors at days 4, 7 and 10 of differentiation. Mean levels \pm SEM in the different conditions are presented (n=6, 2-tailed t-test). * p <0.05; ** p <0.01 (C) The profile of

Glut1 expression as a function of $\alpha 4$ -integrin is shown on GlyA⁺ erythroblasts at day 10 of differentiation. Three cell populations, designated P3, P4 and P5, and identifying basophilic, polychromatic and orthochromatic erythroblasts, were isolated on a FACSaria. OCR was monitored on purified subsets 15h post sorting and mean levels \pm SEM are presented (n=6, 1-way ANOVA with Tukey's post-hoc test). ***p<0.001

Figure 3. Ectopic α -ketoglutarate inhibits terminal erythroid differentiation and enucleation.

(A) CD34⁺ progenitors were stimulated with rEPO in the absence (-) or presence (+) of α KG (3.5 mM). CD34/CD36 profiles, allowing the assessment of CD34⁻CD36⁺ CFU-E, were monitored at day 3 and representative plots are shown (top). Expression of GlyA was monitored at days 3 and 7 of differentiation and representative histograms are shown (grey histograms, isotype controls; black lines, specific staining) and the percentages of GlyA⁺ cells at day 3 are indicated. (B) Erythroid differentiation was induced for 7 days with rEPO and erythroblasts were then cultured in the absence or presence of α KG. CD49d/Glut1 profiles of GlyA⁺ erythroblasts were monitored at days 10 and 14 of differentiation and representative dot plots are shown (top). Quantification of the mean percentages \pm SEM of CD49d⁺Glut1⁺ (polychromatic) and CD49d⁻Glut1⁺ (orthochromatic) erythroblasts are presented (n=5, 2-tailed t-test). *, p<0.05 (C) Erythroblast enucleation was evaluated as a function of Syto16 nuclear staining at days 10 and 14 of differentiation and representative dot plots are shown. The percentages of Syto16⁻ enucleated cells are indicated (top). Percentage enucleation in the absence or presence of α KG was quantified and means levels \pm SEM are presented (n=3, 1-way ANOVA with Tukey's post-hoc test). **, p<0.01; ***p<0.005 (D) CD49d/Glut1 staining of GlyA⁺ erythroblasts at day10 of differentiation was used to sort erythroblast subsets as a function of increasing maturity, labelled from P2 to P4 as indicated, on a FACSaria flow cytometer. Isolated subsets were cultured overnight in the absence or presence of α -KG (3.5 mM) and enucleation was monitored

as a function of Syto16 staining. Representative plots are shown and the percentages of enucleated reticulocytes are indicated. (E) Representative cytopsin images of day 16 erythroblasts, differentiated in the absence or presence of α -KG, are shown.

Figure 4. α -ketoglutarate increases mitochondrial function and oxidative phosphorylation at late stages of erythroid differentiation.

(A) CD34⁺ progenitors were stimulated with EPO and at day 7, erythroid differentiation was continued in the absence (-) or presence (+) of α KG. Mitochondria biomass and membrane potential were monitored by MitoTrackerGreen and MitoTrackerRed staining respectively, at day 10 of differentiation and representative dot plots are shown (top). Mean mitochondrial biomass and polarization levels (\pm SEM) of erythroblasts cultured in the presence of α KG between days 0-3 and 7-10 of differentiation are presented (bottom; n=3, t-test). *,p<0.05 (B) OCR was monitored in EPO-stimulated CD34⁺ progenitors at day 10 of differentiation following addition of α KG between days 3-10 (top) or 7-10 (bottom; n=6, 2-tailed t-test). ***p<0.005

Figure 5. α -KG-mediated oxidative phosphorylation is not required for late differentiation and enucleation.

(A) OCR was monitored in CD34⁺ progenitors following 3 days of EPO-induced differentiation. The α -KG antagonist DMOG was injected as indicated (red line) and mean levels \pm SEM are presented (n=6, 2-tailed t-test). ***p<0.001 (B) OCR was monitored in EPO-induced CD34⁺ progenitors differentiated in the absence (black line) or presence (red line) of the α KG antagonist DMOG (0.5 mM). Mean levels \pm SEM are presented (n=6, 2-tailed t-test). ***p<0.005 (C) Expression of the GlyA, CD36 and CD71 erythroid markers was monitored at day 4 of differentiation in the absence (-) or presence (+) of the α KG antagonist and representative histograms are presented. Black lines show specific staining and grey histograms depict the

isotype control. Data are representative of 3 independent experiments. **(D)** Erythroid differentiation was induced for 7 days in the presence of EPO. Erythroblast differentiation was monitored as a function of GlyA upregulation and induction of a CD49d⁺Glut1⁺ polychromatic erythroblast stage. Representative plots are presented (top). At day 7, erythroblasts were exposed to the DMOG α KG antagonist (+) and erythroblast differentiation and enucleation were evaluated as a function of CD49d/Glut1 and Syto16 staining, respectively. Representative plots are presented (middle). Mean levels \pm SEM of enucleation in the two conditions are presented (n=4, t-test; bottom). **(E)** OCR was monitored at day 10 of erythroid differentiation in the absence (black line) or presence (red line) of the DMOG α KG antagonist. Mean levels \pm SEM are presented (n=6, 2-tailed t-test). **p<0.01; ***p<0.005

Figure 6. IDH1 knockdown does not impact on erythroblast generation but attenuates enucleation.

(A) Schematic representation of the catalytic reactions regulating glutamine catabolism. Glutamine can be converted to glutamate and further to cytosolic α KG (α -KG_c). Isocitrate dehydrogenase 1 (IDH1), a cytosolic enzyme, can further produce isocitrate from α -KG. IDH1 can catalyze both the forward and reverse reactions. **(B)** Progenitors were transduced with an shIDH1 lentiviral vector and IDH1 mRNA levels were monitored by qRT-PCR. Mean levels \pm SEM relative to 18S are presented. **(C)** The evolution of sh-IDH1-expressing progenitors, monitored as a function of GFP expression, was monitored at days 3, 6 and 10 of differentiation in the absence (-) or presence (+) of EPO and the percentage of GFP⁺ cells are presented. **(D)** The expression of GlyA and CD36 was monitored in shControl- and shIDH1-transduced progenitors at days 3 and 6 of EPO-induced differentiation. Representative histograms (grey, isotype control; black, specific staining) are shown and the percentages of positively stained cells are indicated. **(E)** Enucleation

of shControl- and shIDH1-transduced progenitors was assessed on FACS-sorted GFP⁺ cells at day 14 by Syto60 staining and representative histograms are presented.

Figure 7. IDH1 substrate accumulation exacerbates the negative impact of IDH1 downregulation on orthochromatic erythroblast differentiation and enucleation.

(A) Late stage erythroblast differentiation was monitored in shControl and shIDH1-transduced progenitors as a function of exogenous α KG (2 mM). CD49d/Band 3 profiles of erythroblasts was monitored at day 10. Enucleation was assessed at day 16 as a function of Syto16 staining and cytopins were performed. Representative images are presented. (B) Erythroid differentiation was monitored as described above in the absence (-) or presence (+) of exogenous citrate (4 mM).

Figure 1

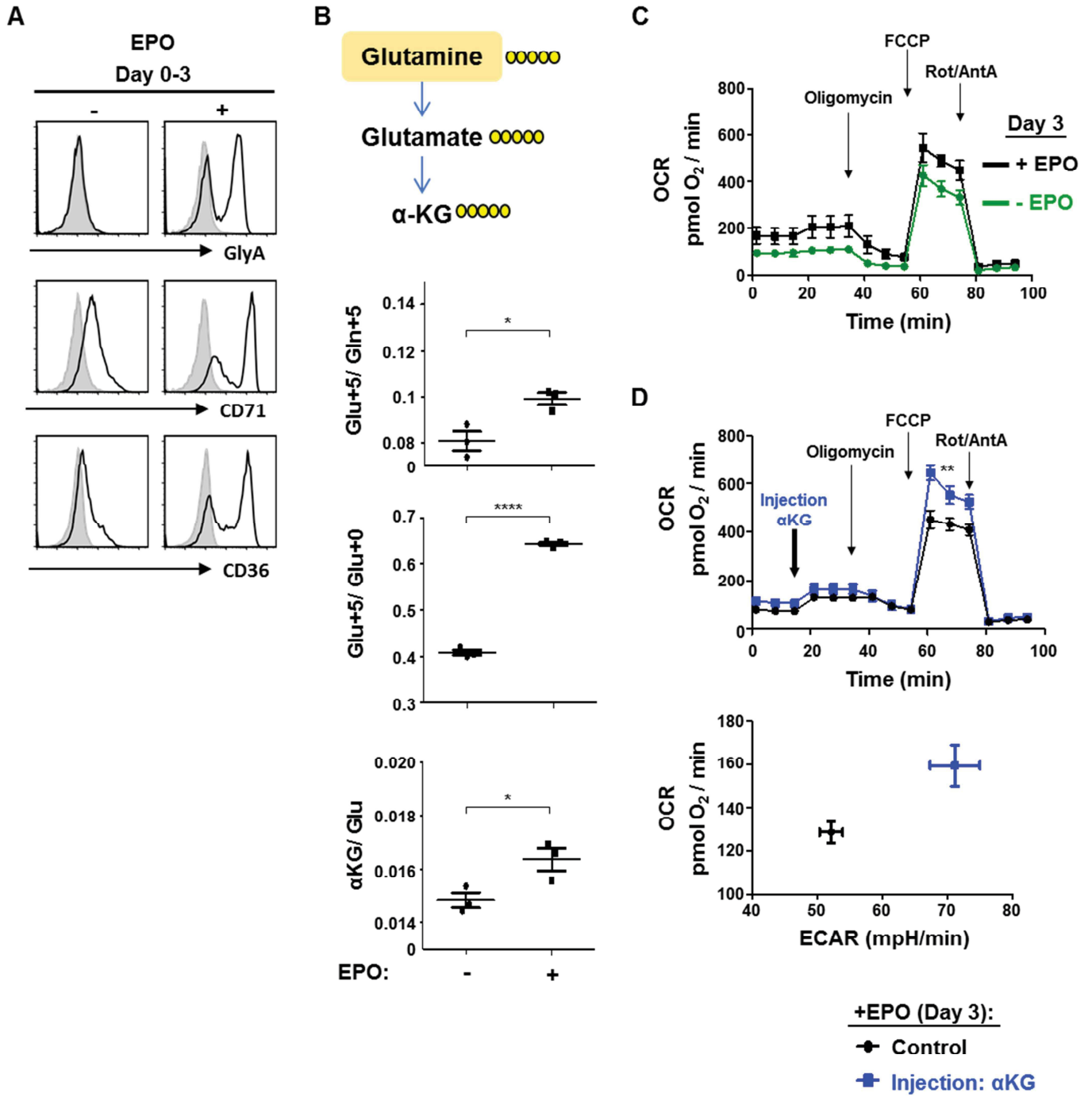


Figure 2

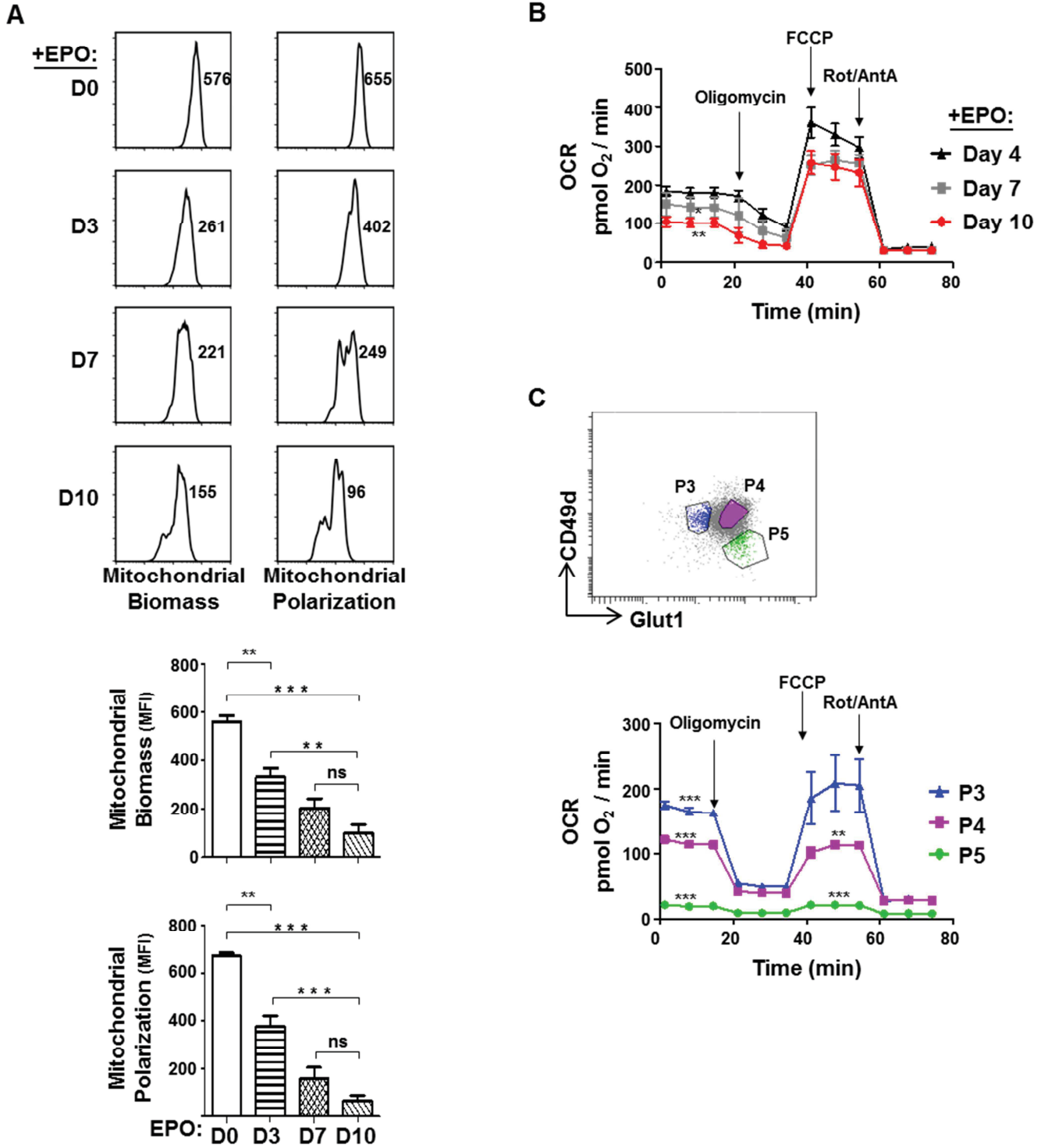


Figure 3

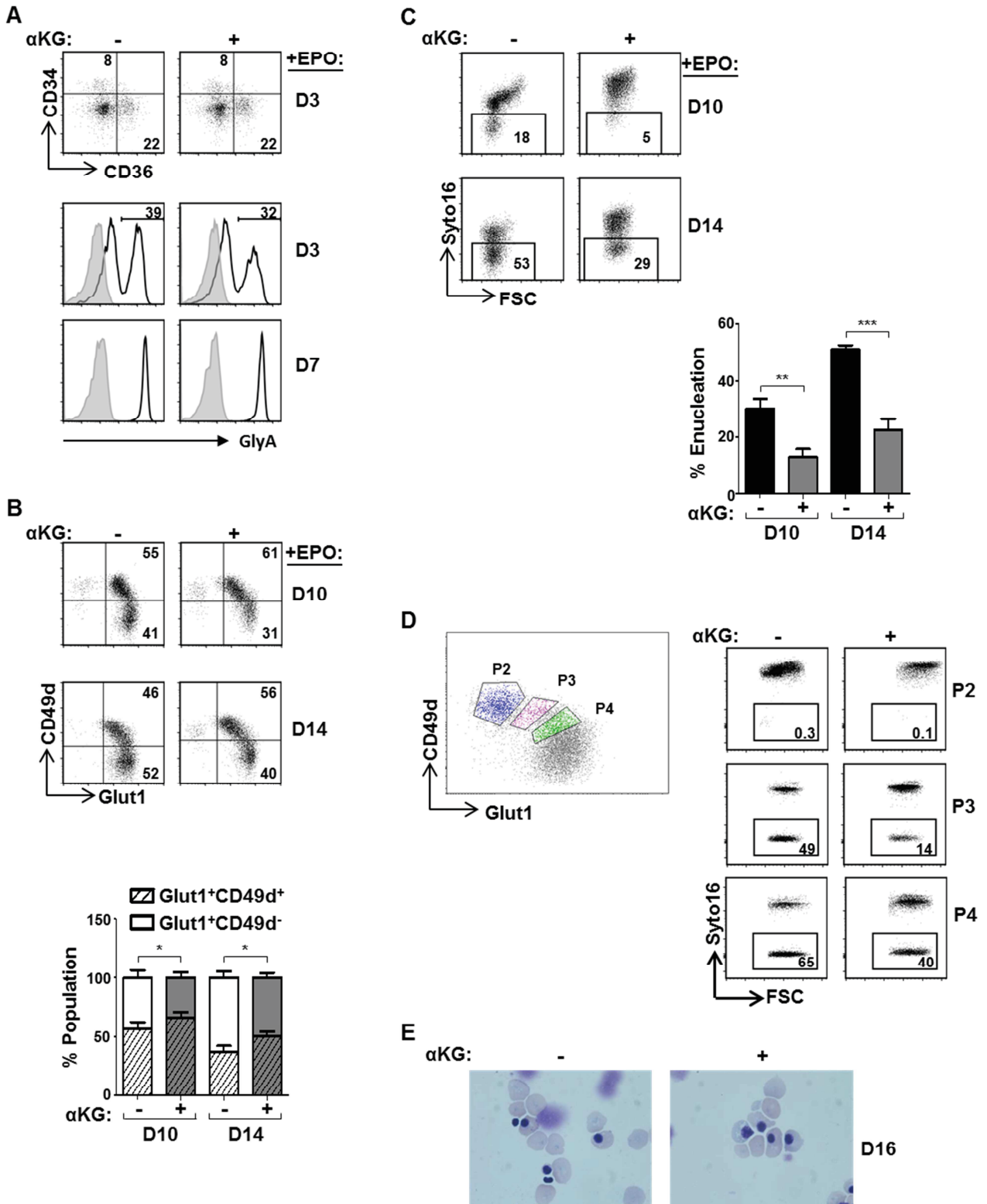


Figure 4

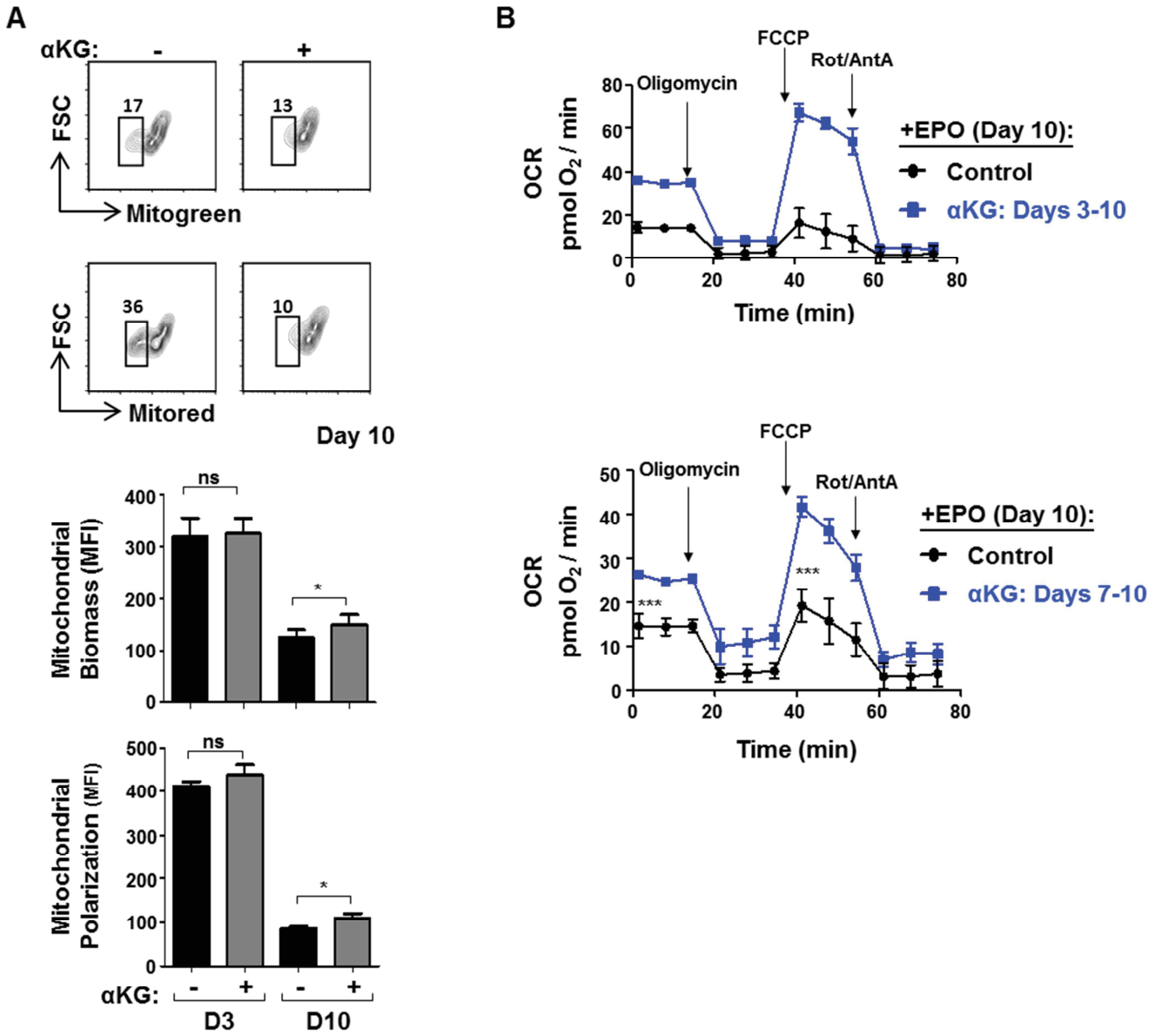


Figure 5

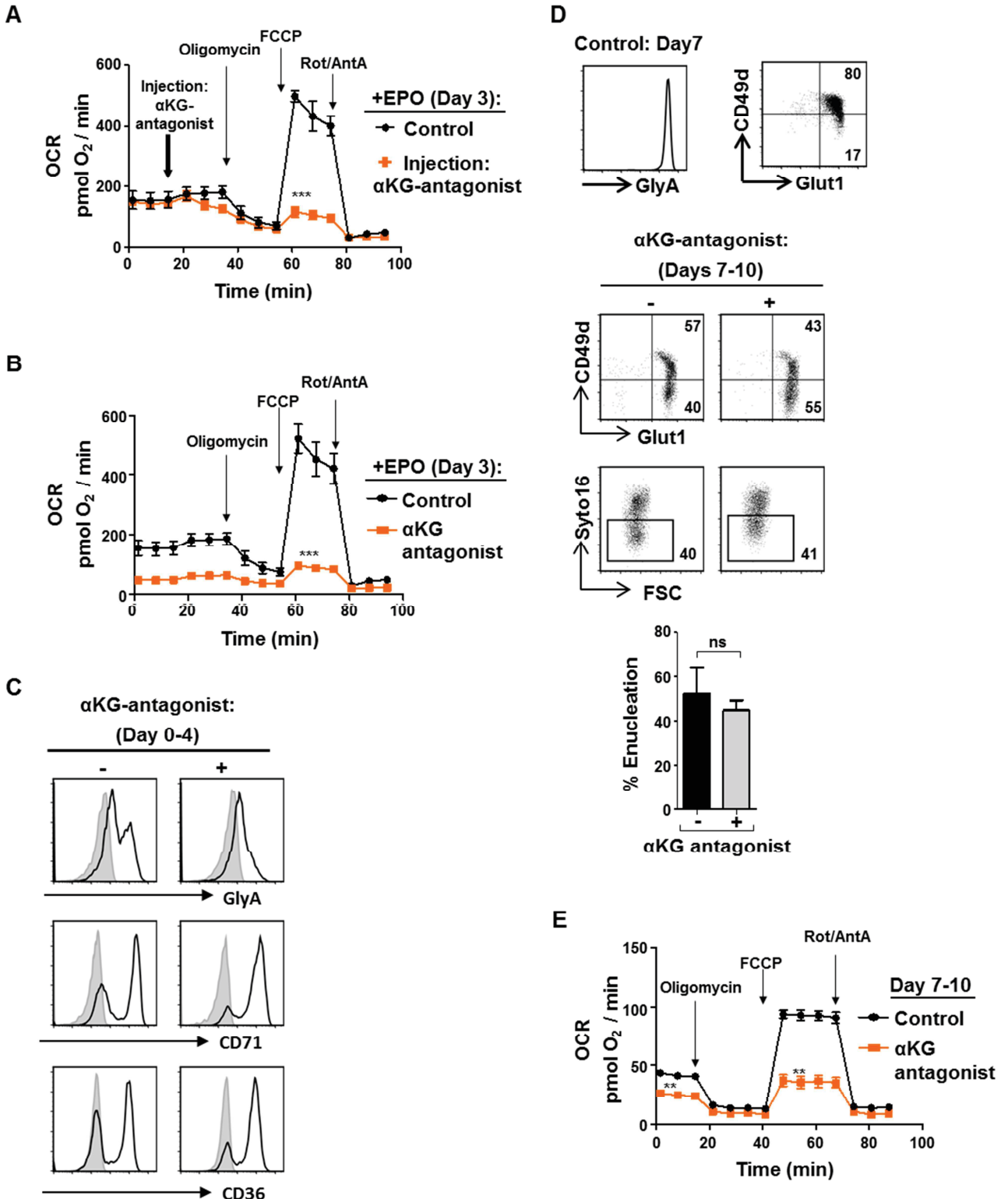


Figure 6

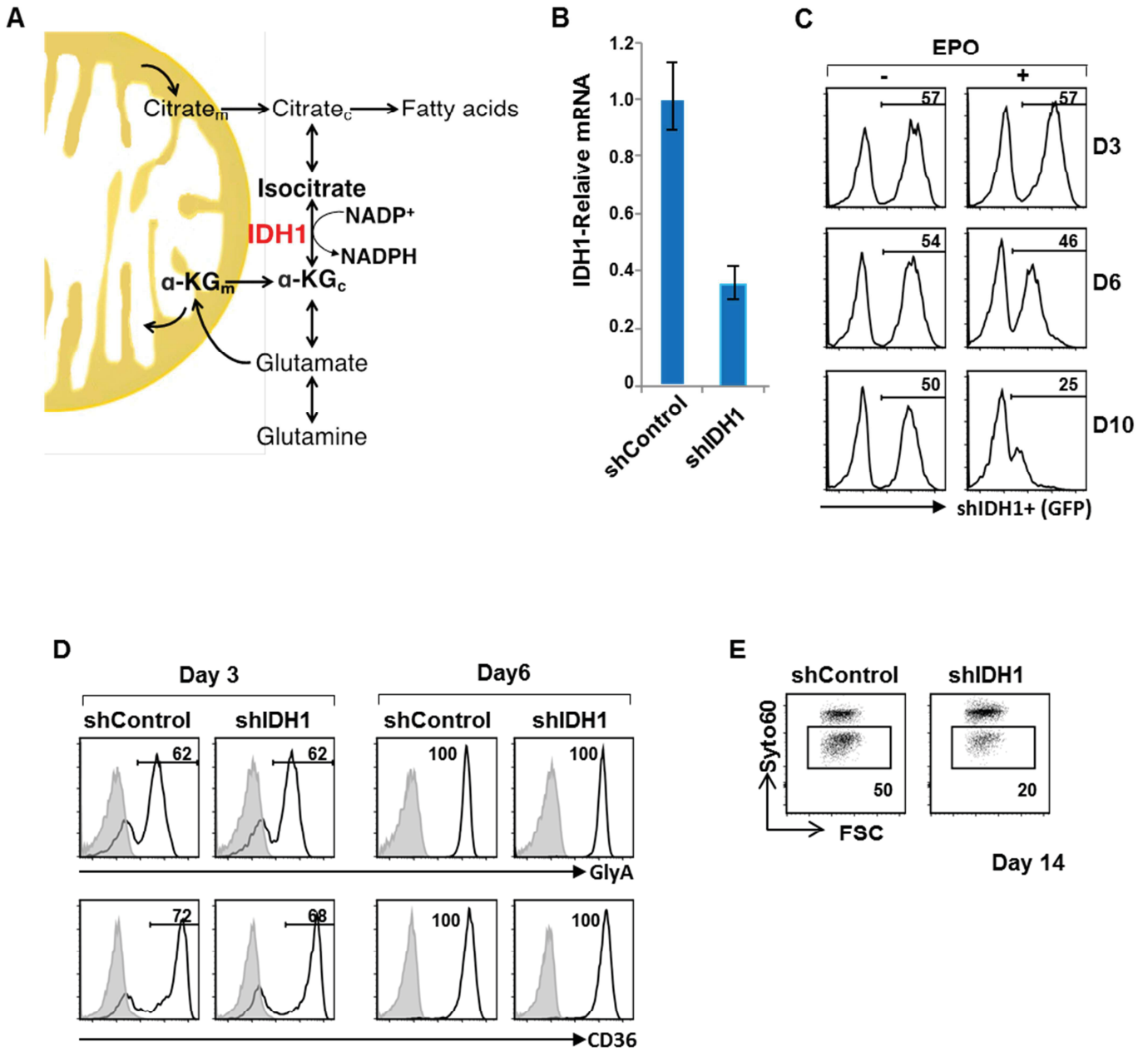
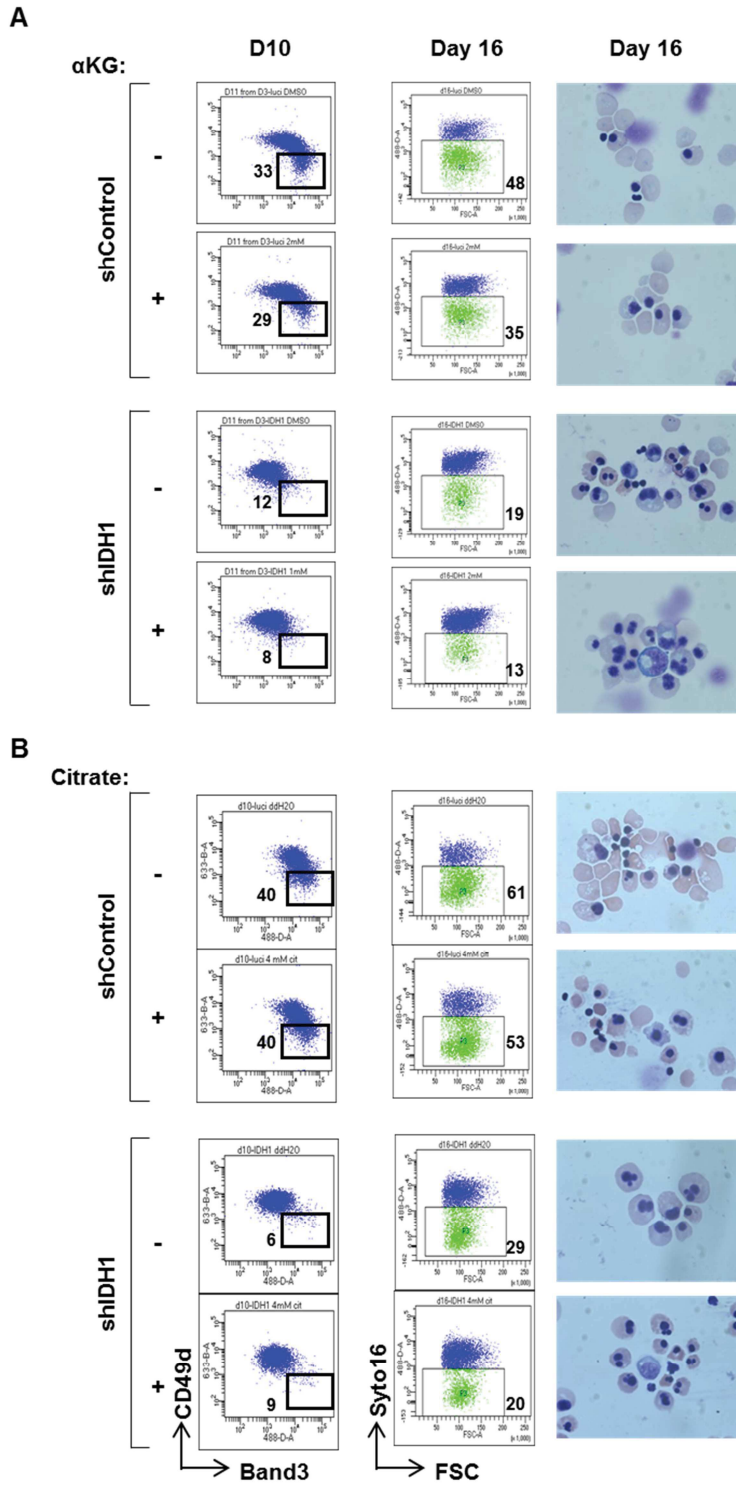


Figure 7



Discussion

Regulation of mitochondrial function and metabolism during erythroid differentiation

It has been recently shown that metabolic regulation mechanisms involving mitochondrial oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) play an important role in the HSC “choice” between maintenance and commitment (reviewed in Oburoglu *et al.*, 2016). On the other hand, the role of mitochondria has been extensively studied in order to characterize the mechanisms regulating the mitochondrial dynamics of biogenesis *versus* clearance in the context of the erythroid differentiation (Ahlqvist *et al.*, 2015; reviewed in Liang and Ghaffari, 2017; Liu *et al.*, 2017). The progression through the erythroid progenitor stages and more precisely through terminal erythroid differentiation is accompanied by significant changes in cell size, morphology and intracellular content (An and Mohandas, 2011). Indeed, after orthochromatic erythroblast enucleation, reticulocyte maturation into RBCs involves several processes allowing complete organelle degradation (Keerthivasan *et al.*, 2011; Griffiths, Kupzig and *et al.*, 2012). Among these processes, mitochondrial clearance has been shown to play a fundamental role, as alterations in the mechanisms of mitochondrial degradation lead to impaired terminal erythroid differentiation and enucleation (Zhang *et al.*, 2015; Grosso *et al.*, 2017; reviewed in Moras *et al.*, 2017). Indeed, significant differentiation defects have been observed under conditions of altered expression or function in several autophagy-related genes, demonstrating that mitochondrial clearance through autophagy is required for terminal erythropoiesis (Kundu *et al.*, 2008; Sandoval *et al.*, 2008; Zhang and Ney, 2009; Zhang *et al.*, 2009). Moreover, altered autophagy has been correlated to the pathological impaired erythropoiesis observed in patients affected by acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (Watson *et al.*, 2011; Jiang *et al.*, 2018). Finally, FOXO3 transcription factor and mTOR signaling have been shown to cooperate in erythroid differentiating progenitors for the regulation of several metabolic networks not only involved in oxidative metabolism, but also in the response to cell oxidative stress (Marinkovic *et al.*, 2007; Kang *et al.*, 2012; Zhang *et al.*, 2014; Liang *et al.*, 2015).

According to these studies, showing an important regulation of mitochondrial dynamics and function during erythroid differentiation, one major goal of my PhD was to

further investigate the mechanisms modulating mitochondrial metabolism and activity throughout this differentiation process. We now find that human erythropoiesis exhibits a general decrease in mitochondrial activity and function, which is associated with a reduction in cell oxidative metabolism at late stages (D10) as compared to early erythroid progenitors (D3). More precisely, a progressive decrease in both mitochondrial biomass and transmembrane potential is associated with a reduction in OXPHOS throughout the erythropoiesis process (**Article - Figures 2A-B**). In addition, according to their immunophenotypic characterization (Hu *et al.*, 2013), we isolated homogenous populations of basophilic, polychromatic as well as orthochromatic erythroblasts, and confirmed that the progression through the progenitor stages of terminal erythroid differentiation is associated with decreased cell respiration capacity and OXPHOS (**Article - Figure 2C**).

These results highlight important differences in the mitochondrial activity between early and late phases of the erythroid differentiation process, with a significant decrease in oxidative metabolism concomitant with terminal differentiation. This decrease in mitochondrial function could be required for the mitochondrial clearance process observed at this stage, by addressing “non-functional” mitochondria to degradation via the autophagy pathway. Further evidence is needed to understand whether this represents a specificity of the erythroid differentiation process, according to the unique features of mature RBCs, lacking intracellular organelles and consequently relying on a strictly glycolytic metabolism (see section 2.2.4.3). We have started to address this question, by investigating the regulation of mitochondrial function in erythropoiesis, not only as compared to non-erythroid committed cells, but also by studying early *versus* late erythroid progenitor stages.

α -KG intermediate production and function in early erythropoiesis

Previous work in our laboratory has shown an important role of the anaplerotic metabolism of glutamine, via the biosynthesis of the TCA cycle intermediate α -KG, during the HSC differentiation towards the erythroid lineage. More precisely, inhibition of glutamine-derived α -KG production via the transaminase reaction led to an erythroid differentiation blockage, which was partially rescued by ectopic α -KG (Oburoglu *et al.*,

2014). Supporting the importance of α -KG biosynthesis in erythropoiesis, we now find that the significant production of the α -KG intermediate during early stages of human erythroid differentiation contributes to cell oxidative metabolism. These results have been obtained by comparing the metabolic profile of human CD34⁺ cells cultured either in absence or in presence of EPO. More precisely, while erythropoietin fosters the erythroid commitment of human CD34⁺ progenitors; in absence of EPO, differentiation towards the myeloid lineage occurs by default. Under these two conditions of erythroid vs myeloid commitment (+EPO and -EPO), the incorporation of glutamine-derived carbon into several metabolites, such as glutamate and α -KG, has been assessed through heavy-labeled glutamine tracing experiments. In fact, mass spectrometry analyses (LC-MS) have shown an increased production and utilization of α -KG during early erythroid differentiation (D3), as compared to cells cultured in absence of erythropoietin (-EPO) (**Article - Figure 1B**). According to the α -KG function as TCA cycle intermediate and its consequent contribution to the oxidative phosphorylation (OXPHOS), we asked whether the increased production of α -KG in erythroid-committed cells could be associated with a specific oxidative metabolism status. Therefore, oxidative metabolism has been assessed in erythroid- *versus* myeloid-committed progenitors (+EPO and -EPO) through Seahorse assay, as a function of the cell oxygen consumption rate (OCR). Indeed, higher basal respiration and spare respiratory capacity (SRC) have been observed in early erythroid differentiating progenitors (D3), as compared to -EPO cells (**Article - Figure 1C**).

To further characterize the role of cell oxidative metabolism in erythropoiesis and the eventual contribution of α -KG to the OXPHOS regulation, we investigated the effect of ectopic α -KG in the early phases of the differentiation process. Interestingly, under these conditions, the respiratory capacity of early erythroid progenitors (D3) was significantly increased (**Article - Figure 1D**), despite no major changes have been observed in the differentiation profile between cells cultured in absence and in presence of ectopic α -KG. Indeed, we found comparable amount of BFU-E and CFU-E progenitors, characterized as a function of CD36 and CD34 expression levels (Li *et al.*, 2014), as well as similar upregulation of Glycophorin A (GlyA) upon transition to terminal differentiation (**Article - Figure 3A**).

Taken together, these results suggest that glutamine anaplerosis, as well as OXPHOS and mitochondrial metabolism are differentially regulated between myeloid- and erythroid-committed precursors. Interestingly, glutamine anaplerosis via the biosynthesis of α -KG has been recently shown to directly contribute to succinate production, required for heme biosynthesis during erythropoiesis (Burch *et al.*, 2018). On the other hand, our current results show that α -KG biosynthesis in early erythroid progenitors is linked to the cell respiratory capacity, which is modulated upon changes in α -KG levels. Therefore, we asked whether similar α -KG-dependent effect could be observed in late erythroid progenitors and whether this could affect the progression through terminal differentiation, which we found to be characterized by decreased mitochondrial function and OXPHOS.

Is there an interplay between OXPHOS regulation and terminal erythroid differentiation?

In accordance with the previously observed α -KG-dependent increase in OXPHOS (**Article - Figure 1D**), we have found that ectopic α -KG increases the cell respiration capacity of late erythroid progenitors, and this increase in cell respiration was associated with concomitant augmentation in mitochondrial biomass and transmembrane potential (**Article - Figures 4A-B**). On the contrary, while not altering the differentiation profile of early progenitors (**Article - Figure 3A**), ectopic α -KG impaired terminal erythroid differentiation, as well as erythroblast enucleation (**Article - Figures 3B-C**). These results were further confirmed on FACS-sorted basophilic, polychromatic and orthochromatic erythroblasts (Hu *et al.*, 2013); more precisely, polychromatic and orthochromatic erythroblast enucleation was significantly decreased after 24h in presence of ectopic α -KG (**Article - Figure 3D**).

Taken together, these data suggest that the α -KG-dependent increase in mitochondrial function and OXPHOS impairs terminal erythroid differentiation and enucleation. Therefore, these results further confirm the importance of a specific regulation of the mitochondrial oxidative metabolism, not only to support the progression from early to late erythropoiesis, but more specifically to achieve and complete the terminal erythroid differentiation phase. Indeed, while high levels of TCA

cycle intermediates, such as α -KG, and cell respiration are observed during the early stages, the downregulation of these metabolic pathways seems to be required for the progression through terminal differentiation. These intriguing findings prompted us to further investigate the effect of altering the α -KG-dependent oxidative metabolism or the production/utilization of the TCA cycle intermediates during the early *versus* late phases of the human erythropoiesis process.

It has been shown that the enzymatic activity of the α -KG-dependent prolyl hydroxylase domain (PHD)-containing proteins is inhibited by the α -KG antagonist dimethyloxalylglycine (DMOG) (Jaakkola *et al.*, 2001; Cummins *et al.*, 2008), which has also been recently found to block OXPHOS in cancer cells (Zhdanov *et al.*, 2015). Intriguingly, by assessing the effect of this α -KG antagonist on human erythropoiesis, we observed that DMOG-dependent impairment in OXPHOS affects early erythropoiesis, without altering the progression through terminal erythroid differentiation. Despite DMOG was found to significantly reduce cell respiration capacity of both early and late erythroid precursors (**Article - Figures 5A-B-E**), this α -KG antagonist showed dissimilar effect on the differentiation capacity of these progenitors. Indeed, in presence of DMOG, we observed decreased number of early BFU-E and CFU-E progenitors, as well as lower percentage of GlyA⁺ cells (**Article - Figure 5C**); contrarily, no significant changes have been found in terminal erythropoiesis, as well as in erythroblast enucleation (**Article - Figure 5D**). Taken together, these results confirm that early phases of the erythroid differentiation process rely on α -KG-dependent oxidative metabolism, which is no longer required upon progression through terminal erythroid differentiation. Intriguingly, α -KG and its related metabolism have been also found to play a role in the regulation of the differentiation potential of other cell types, such as pluripotent stem cells and mesenchymal stromal precursors (TeSlaa *et al.*, 2016; Singh *et al.*, 2017). In addition, mechanisms of “metabolic switch”, associated with a specific modulation of the OXPHOS-dependent metabolism, have been described upon induction of differentiation or specific cell functions (Shum *et al.*, 2016; Zheng *et al.*, 2016; Price *et al.*, 2018). Therefore, to further characterize the α -KG role in the context of terminal erythroid differentiation and enucleation, we decided to investigate the effect of altering the intracellular biosynthesis of α -KG.

Isocitrate dehydrogenase function and levels of TCA cycle intermediates regulate terminal erythroid differentiation

Intracellular α -KG biosynthesis occurs through several enzymatic reactions, including the glutamate dehydrogenase (GDH)- or transaminase-dependent conversion of glutamate into α -KG (Botman *et al.*, 2014; Altman *et al.*, 2016). On the other hand, α -KG is also produced in the context of the TCA cycle reactions through isocitrate decarboxylation, which is catalyzed by different isoforms of the isocitrate dehydrogenase enzyme (IDHs). Indeed, three IDH isoforms have been characterized, with IDH1 localized into the cytoplasm and peroxisome, while IDH2 and IDH3 into the mitochondria. While the NAD^+ -dependent IDH3 only mediates isocitrate decarboxylation to α -KG in the context of the TCA cycle, the NADP^+ -dependent IDH1 and IDH2 isoforms are able to reversibly catalyze isocitrate conversion into α -KG (Chandel, 2015; reviewed in Al-Khallaf, 2017). Therefore, these enzymes result also allosterically regulated in a different manner, as a function of their substrate, cofactor and product availability (Xu *et al.*, 2004; Ma *et al.*, 2017). To further characterize the mechanisms involved in the ectopic α -KG-dependent impairment in terminal erythroid differentiation, we investigated the effect of altering the intracellular biosynthesis of this metabolite via the IDH-dependent reactions. To this purpose, we downregulated the expression of the isocitrate dehydrogenase I (IDH1) isoform through shRNA approach.

Upon IDH1 downregulation we did not observe alterations in early erythropoiesis, as well as in the transition to terminal erythroid differentiation, with progressive GlyA^+ upregulation (**Article - Figure 6D**). Surprisingly, IDH1 knockdown, which we expected to be associated with reduced levels of α -KG and to have opposite effect than ectopic α -KG, showed a similar phenotype with decreased enucleation (**Article - Figure 6E**). Consequently, we hypothesized that this effect could be linked to the drastic alteration in the α -KG intracellular biosynthesis and we asked whether ectopic α -KG could rescue the effect of IDH1 downregulation. Intriguingly, the impaired enucleation phenotype was worsened, and a comparable negative impact on enucleation was observed under conditions of ectopic citrate supplementation (**Article - Figures 7A-B**). These results suggest that IDH1 enzymatic activity is required for terminal erythroblast enucleation. In

addition, we have identified a crucial role for the equilibrium between biosynthesis/utilization of α -KG and citrate, as impaired metabolism of these TCA cycle intermediates strongly affects the erythroblast enucleation capacity. Therefore, among the future perspectives of this project, it would be interesting to more precisely characterize the metabolic profile of terminal erythroblasts (levels of α -KG, citrate, as well as other TCA cycle intermediates) and eventual changes in this metabolic profile in presence of ectopic α -KG and citrate, as well as upon IDH1 knockdown.

The above presented results regarding the α -KG-mediated modulation of terminal erythropoiesis raised several intriguing questions. Indeed, we asked whether the observed effects were not only linked to the α -KG-dependent alterations in the oxidative metabolism, but also consequence of the increased intracellular levels of α -KG. The latter, in fact, could either contribute to alternative metabolic pathways, leading to the biosynthesis of other crucial intermediates and metabolites, or play a role as enzymatic cofactor. Thus, several hypotheses have been raised and some of them have been partially investigated.

OXPHOS-independent functions of α -KG: reductive carboxylation

It has been shown that under certain metabolic conditions α -KG utilization is diverted towards a reductive metabolism and α -KG becomes substrate of the reductive carboxylation pathway (see section 3.2.3.3) (reviewed in Halbrook *et al.*, 2018). The latter allows reversal conversion of α -KG into citrate, consequently leading to the production of acetyl-CoA, which is a fundamental precursor for the biosynthesis of fatty acids (Filipp *et al.*, 2012). Changes in the intracellular fatty acid content could eventually alter the fatty acid composition of the plasma membrane phospholipids, whose structure and unsaturation degree have been shown to influence the fluidity properties of the membrane, as well as the formation of specific domains (see Annex section). This could consequently affect the mechanisms of vesicle trafficking and membrane segregation, which are involved in the processes of enucleation and subsequent reticulocyte maturation (Keerthivasan *et al.*, 2010; Griffiths *et al.*, 2012). Indeed, recent work has found that alterations in lipid metabolism, more precisely in the catabolism of phosphatidylcholine (PC)-derived phosphocoline, impair terminal stages of both human

and mouse erythropoiesis (Huang *et al.*, 2018). In addition, unpublished data obtained during my PhD show different enucleation levels between cells cultured in presence of fetal bovine serum (FBS) *versus* human serum and plasma (see Annex section). These sera are characterized by a distinct composition in fatty acids, with human serum being enriched in unsaturated species (Lagarde *et al.*, 1984). In fact, we found that increased enucleation in presence of human serum and plasma was associated with higher incorporation of unsaturated fatty acid species in the cell membrane phospholipids (see Annex section). We therefore hypothesized that increased intracellular levels of α -KG could lead to increased flux through the reductive carboxylation pathway and that the consequent alteration in lipid biosynthesis could be responsible for the impaired enucleation observed in presence of ectopic α -KG. In order to assess ectopic α -KG contribution to the reductive carboxylation pathway, we measured intracellular $\text{NADP}^+/\text{NADPH}$ levels, as NADPH conversion to NADP^+ occurs upon IDH-dependent reduction of α -KG into isocitrate. On the other hand, mass spectrometry analyses have been performed in order to detect eventual changes in the membrane lipid composition, between cells cultured in presence and in absence of ectopic α -KG. Indeed, we did not observe significant changes in both $\text{NADP}^+/\text{NADPH}$ levels and fatty acid composition of the membrane phospholipids (data not shown). We could then conclude that the altered enucleation observed in presence of ectopic α -KG is probably not linked to changes in the fatty acid composition, and consequently the fluidity properties, of the cell plasma membrane.

OXPHOS-independent functions of α -KG: cofactor role

The phenotype observed in presence of ectopic α -KG and upon IDH downregulation, with impaired terminal erythroid differentiation and enucleation, recapitulates features of the ineffective erythropoiesis described in myelodysplastic syndrome (MDS) patients (reviewed in Steensma, 2015). In fact, almost all MDS patients develop anemia as a consequence of a clonal hematopoiesis process and altered bone marrow progenitor differentiation. Indeed, these patients carry aging-related mutations, which confer selective advantage to a specific progenitor population, mainly leading to the expansion of the myeloid lineage (Jaiswal *et al.*, 2014; Xie *et al.*, 2014; reviewed in Dussiau and Fontenay, 2018) (see section 2.4.3). Among the main founder mutations described in

MDS patients, genetic alterations have been described in the TET2 family of α -KG-dependent DNA dioxygenases, as well as in IDH1 and IDH2 (reviewed in Steensma, 2015) (see sections 2.4.3.1 and 2.4.3.2). Therefore, second intriguing question raised by our results concerns the function of α -KG as cofactor for several DNA and chromatin-modifying enzymes, including JmjC domain-containing histone demethylases (JHDM) and the above mentioned TET DNA dioxygenases (Klose and Zhang, 2007; Guengerich, 2015). In this context, the effect of α -KG could either be direct or consequence of α -KG conversion into strictly related metabolites, which are either involved in DNA and chromatin modifications or are able to modulate the function of DNA and chromatin-modifying enzymes as well.

Several line of evidence led us to hypothesize a potential role for α -KG as demethylase cofactor in erythropoiesis. First, alterations in the different isoforms of TET DNA demethylases have been shown to significantly affect both human *ex-vivo* and Zebrafish erythroid differentiation (Ge *et al.*, 2014; Yan *et al.*, 2017; Qu *et al.*, 2018). In addition, the finding of a specific pattern of cytosine methylation during erythropoiesis suggests an important role for the regulation of the DNA methylation state during this differentiation process (Y. Yu *et al.*, 2013). Indeed, histone methyltransferase Setd8 deletion has been associated with impaired chromatin condensation and terminal erythroid differentiation (Malik *et al.*, 2017). It is well established that DNA and chromatin organization influence the activity of transcriptional complexes, regulating the expression of lineage-specific genes during the erythroid differentiation process (Stadhouders *et al.*, 2014, 2015). In addition, upon terminal erythroid differentiation, the modulation of chromatin structure plays a crucial role, as chromatin condensation is required prior to nucleus polarization and extrusion (Keerthivasan *et al.*, 2011). Therefore, we hypothesized that the effect of ectopic α -KG on erythroblast enucleation could be linked to the modulation of the chromatin structure, via the regulation of the α -KG-dependent DNA and chromatin-modifying enzymes.

This hypothesis remains to be investigated among the future perspectives of my PhD project. Nevertheless, the importance of α -KG-dependent changes in DNA methylation has been confirmed in diverse studies, in the context of the T cell Th1 polarization (Chisolm *et al.*, 2017), as well as the regulation of stem cell pluripotency (Tischler *et al.*,

2018; Tran *et al.*, 2018). More generally, recent work has highlighted the involvement of diverse metabolism-dependent mechanisms of epigenetic regulation in the stem cell maintenance and commitment (reviewed in Ryall *et al.*, 2015; Li *et al.*, 2018). For instance, glycolysis-derived acetyl-CoA has been shown to contribute to the mechanisms of histone acetylation controlling embryonic stem cell (ESC) potential (Moussaieff *et al.*, 2015). Interestingly, HDAC2-dependent histone deacetylation is required for chromatin condensation and enucleation in mouse fetal liver erythroblasts (Ji *et al.*, 2010; Hattangadi *et al.*, 2011). Therefore, another effect of ectopic α -KG on the chromatin structure regulation could be linked to its conversion into acetyl-CoA. Alternatively, the increased α -KG contribution to oxidative metabolism could in turn alter glucose metabolism and consequently glucose utilization for the biosynthesis of acetyl-CoA. Indeed, tracing experiments of heavy-labeled α -KG or heavy-labeled glucose in presence of ectopic α -KG could help addressing these questions. More generally, amongst the future perspective of this research project, it would be interesting to understand whether specific metabolism-dependent mechanisms of epigenetic regulation are involved in the modulation of terminal erythroid differentiation. To address this question, we could start by characterizing the DNA and histone methylation and/or acetylation profile in presence of ectopic α -KG, as well as upon IDH downregulation.

As previously mentioned, an indirect effect of ectopic α -KG on the modulation of the DNA and chromatin structure could be linked to its conversion into several related metabolites, which also play a role in different mechanisms of epigenetic regulation. Whether in our study there is an altered equilibrium in the biosynthesis of these α -KG-derived metabolites is not known yet, but a characterization by mass spectrometry of the cell metabolic profile in presence of ectopic α -KG is ongoing. Nevertheless, we considered different options according to diverse recent published studies. First, it has been shown that the activity of several α -KG-dependent DNA and chromatin-modifying enzymes is regulated by the equilibrium between the levels of succinate and α -KG (see section 3.2.3.4). Indeed, succinate has been recently found to modulate the cell epigenetic state not only in embryonic stem cells (Carey *et al.*, 2015), but also in the context of cancer-associated mutations in the succinate dehydrogenase (SDH) enzyme (Rose *et al.*, 2008; Cervera *et al.*, 2009; Killian *et al.*, 2013). According to the increased

oxidative metabolism observed in presence of ectopic of α -KG, we could hypothesize that this intermediate is rapidly catabolized into the TCA cycle, where the α -ketoglutarate dehydrogenase (KGDH) reaction converts α -KG into succinate. Interestingly, the KGDH has been also found to localize to the nucleus, allowing *in situ* production of succinyl-CoA, which supports histone succinylation (Y. Wang *et al.*, 2017).

In addition to the biosynthesis of succinate, we hypothesized an eventual α -KG conversion into the 2-HG oncometabolite, which has also been described as inhibitor of the α -KG-dependent enzymes and therefore able to affect the cell epigenetic state (reviewed in Reitman and Yan, 2010; Xu *et al.*, 2011). As previously mentioned, upon IDH mutations, which are involved in the pathogenesis of MDS, as well as in MDS progression to acute myeloid leukemia (Lin *et al.*, 2018) (see section 2.4.3.2), an increased production of 2-HG has been observed (Gross *et al.*, 2010; Choi *et al.*, 2012). However, it has been recently shown that this oncometabolite is also generated via promiscuous enzymatic reactions occurring in physiological conditions (reviewed in Ye *et al.*, 2018). Indeed, the mitochondrial and cytoplasmic malate dehydrogenases (MDH2/mMDH and MDH1/cMDH, respectively) mediate a NAD⁺-dependent “side reaction”, that reduces α -KG to L-2-HG (Rzem *et al.*, 2007). Similarly, a promiscuous reductase activity of the 3-phosphoglycerate dehydrogenase (PHGDH) allows R-2-HG biosynthesis from α -KG, which not only occurs in several human breast cancer cell lines (Fan *et al.*, 2015), but also in yeast (Becker-Ketterer *et al.*, 2016). Finally, increased levels of L-2-HG have been observed in transformed cell lines cultured under hypoxia (Intlekofer *et al.*, 2015; Oldham *et al.*, 2015), as a result of promiscuous reactions of the LDHA enzyme (Intlekofer *et al.*, 2015) (**Figure 33**).

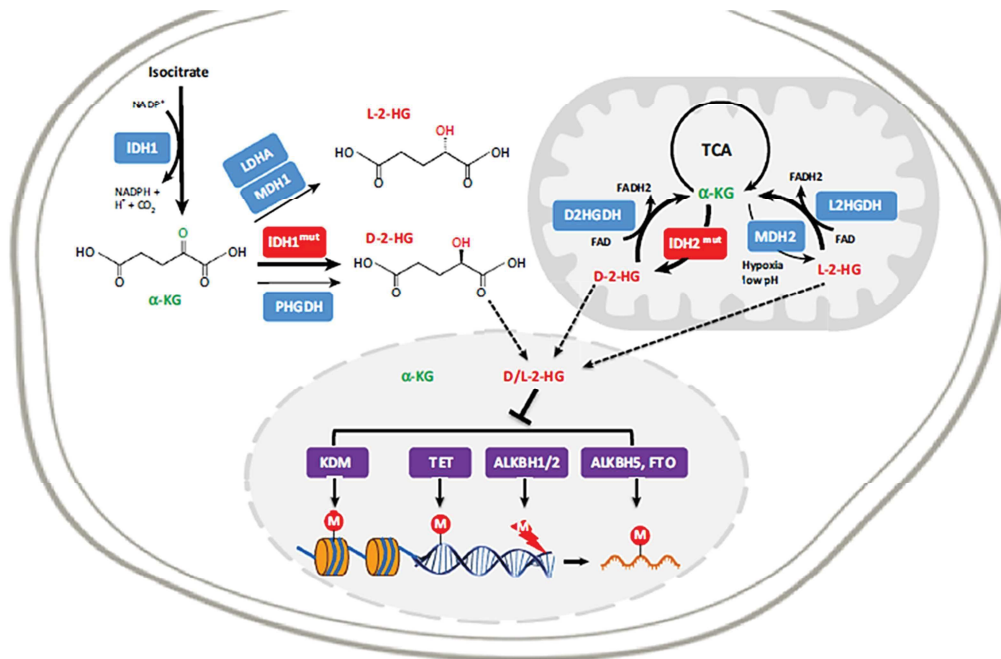


Figure 33. Oncometabolite 2-HG biosynthesis in physiological and pathological conditions. Gain-of-function mutations in isocitrate dehydrogenases (IDH1/2) lead to the biosynthesis of the 2-HG oncometabolite, which is also produced through promiscuous enzymatic reactions involving malate dehydrogenases (MDH1/2), 3-phosphoglycerate dehydrogenase (PHGDH) and lactate dehydrogenase (LDHA). 2-HG intracellular levels are controlled by its conversion to α -ketoglutarate, catalyzed by the D-2-HG and L-2-HG dehydrogenases (D2HGDH, L2HGDH). Adapted from (Ye et al., 2018)

Whether in our study increased intracellular levels of α -KG foster the above described promiscuous enzymatic reactions still needs to be assessed. Therefore, further investigation is needed to understand whether the ectopic α -KG-dependent effect could be linked to its conversion into related metabolites which, by modifying the DNA and chromatin structure, impair fundamental steps required for terminal erythroid differentiation and/or enucleation.

Conclusion

The results generated during my PhD have highlighted an important role for the specific regulation of mitochondrial function and oxidative metabolism in the early *versus* late phases of human erythroid differentiation. Indeed, these results identify previously undescribed mechanisms modulating the TCA cycle-related and oxidative metabolism-dependent networks, over the human erythropoiesis process in a stage-specific manner. These findings provide new insights into the metabolism-dependent regulation of HSC commitment towards a specific lineage fate and the dynamic nature of this regulation as a function of the progression through the lineage specification itself. Understanding how metabolism influences and eventually enhances the mechanisms of HSC maintenance and differentiation could help ameliorating the current techniques of HSC manipulation *in vitro*. Indeed, the enucleation process is still object of extensive research, as human erythroid differentiation *in vitro* does not completely reproduce yet the levels of enucleation and consequent generation of mature RBCs observed *in vivo*. This represents a challenging perspective, as *in vitro* RBC production for transfusions could solve the current limits of compatibility, availability and storage of the donor-derived blood samples.

Annexes

Lipid metabolism in human erythroid differentiation

The role of lipid membrane composition in circulating RBCs has been studied in the context of their unique capacity of shape deformation into the vasculature (Leonard *et al.*, 2018), but also in atherosclerosis (reviewed in da Silva Garrote-Filho *et al.*, 2017). Nevertheless, little is known about the importance of lipids, not only as membrane components, but also in general on their metabolism, during the process of erythroid differentiation. Importantly, fatty acids represent one of the main cell energy sources, and the balance between FAO and *de novo* lipogenesis or vs glycolysis- and OXPHOS-dependent metabolism has been shown to play an important role in stem cell proliferation and differentiation (Ito *et al.*, 2012; Yusuf and Scadden, 2012; Knobloch *et al.*, 2013, 2017). In this section, I will provide an overview of the main membrane lipids and their contribution to the cell membrane properties; I will then present some intriguing results in the context of human erythroid differentiation.

Membrane lipids: classification

Three major classes of lipids have been identified as main components of the cell membranes: glycerophospholipids (GPLs), sphingolipids and sterols (cholesterol in mammals) (van Meer *et al.*, 2008; reviewed in Harayama and Riezman, 2018). Despite these molecules share an amphipathic nature, with both a hydrophobic side and a polar head group, they show some structural differences (Berg *et al.*, 2002).

First, phospholipids are divided into glycerophospholipids (GPLs) and sphingolipids according to the nature of their backbone: a glycerol (3-carbon alcohol) or a sphingosine (amino alcohol carrying an unsaturated hydrocarbon chain) molecule, respectively (Berg *et al.*, 2002) (**Figure 34, panels a-b**). The GPL structure includes two fatty acid chains esterified on the hydroxyl groups at C1 (sn-1) and C2 (sn-2) of the glycerol backbone; while the C3 carries a phosphoric group (Berg *et al.*, 2002; reviewed in Harayama and Riezman, 2018). According to the nature of the alcohol molecules which are in turn bound to the phosphoric group, different classes of GPLs have been identified: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (van Meer *et al.*, 2008). On the other hand, the majority of sphingolipids found in mammalian cells are represented by sphingomyelin (SM) and

glycosphingolipids (GSLs) (van Meer and Lisman, 2002; reviewed in Harayama and Riezman, 2018).

Finally, sterols are characterized by a rigid structure, constituted of four fused hydrocarbon rings (Griffiths *et al.*, 2017); this steroid group is linked to a hydrocarbon chain at one end and a hydroxyl group at the other end to form cholesterol (Berg *et al.*, 2002) (**Figure 34, panel c**).

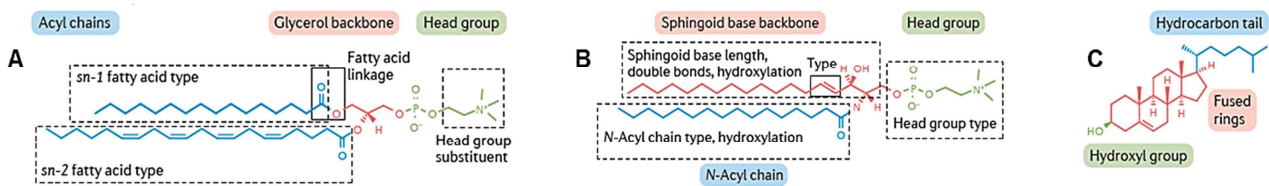


Figure 34. Membrane lipid classes. Panel **A** Glycerophospholipid structure, including a glycerol backbone, linked to 2 fatty acid chains and a phosphoric group, which is in turn bound to an head group substituent. Panel **B** Sphingolipid structure, with the sphingosine backbone carrying a fatty acid chain and a phosphoric head group. Panel **C** Cholesterol structure, composed of four fused hydrocarbon rings bound to a hydrocarbon chain and a hydroxyl group. *Adapted from* (Harayama and Riezman, 2018)

In addition to the above mentioned structural differences, glycerophospholipids and sphingolipids are also composed of fatty acids with different chain lengths, as well as distinct number and position of the double carbon-carbon bonds (reviewed in Harayama and Riezman, 2018). Glycerophospholipids often include saturated or monounsaturated fatty acids in the sn-1 position, while monounsaturated or polyunsaturated forms in the sn-2 (Yamashita *et al.*, 2014). For instance, it has been shown that the majority of the phosphatidylinositol (PI) species are composed of stearic and arachidonic acid chains (18:0 and 20:4), while phosphatidylserine (PS) often carries stearic acid at the sn-1 position (Hicks *et al.*, 2006; Yamashita *et al.*, 2014). On the other hand, sphingolipid acyl chains are usually longer and exhibit lower unsaturation degree, as compared to GPLs (Grösch *et al.*, 2012).

In general, phospholipids (with PC representing above 50% of the species) account for the major class of structural lipids in eukaryotic membranes (van Meer *et al.*, 2008). Further details about their contribution to the cell membrane organization and properties are here below provided.

Membrane lipids: phospholipid-dependent membrane properties

It has been shown that phospholipids exhibit a different shape, as a function of the relative size of the polar head group and the hydrophobic chain; indeed, the molecular geometry is nearly cylindrical for PC, while conical for PE molecules (van Meer *et al.*, 2008). Interestingly, this shape is able to influence the spontaneous curvature of the membrane (Ernst *et al.*, 2016). For instance, a negative spontaneous curvature is observed upon inclusion of PE molecules into the membrane, and this curvature stress has been shown to be involved in the processes of budding, fission and fusion (Marsh, 2007) (**Figure 35, panel a**). In addition, *in vitro* studies have demonstrated an important role for conical lipids in the soluble NSF attachment protein receptor (SNARE)-mediated fusion mechanism (Zick *et al.*, 2014). Therefore, it has been proposed that under conditions where membrane deformation is required, a spontaneous membrane curvature could be induced through the regulation of its lipid composition (reviewed in Harayama and Riezman, 2018).

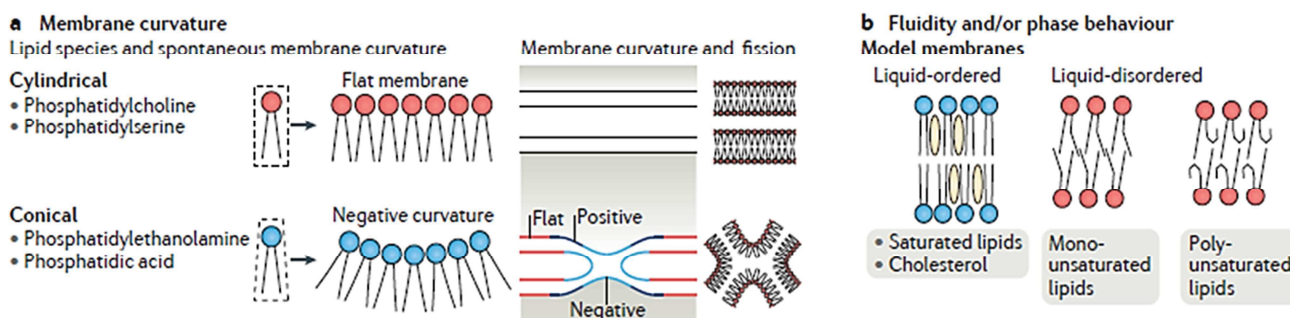


Figure 35. Contribution of phospholipid structure to membrane properties and organization. Panel **a** The phospholipid molecular geometry (cylindrical vs conical) influences the spontaneous membrane curvature, which is linked to the processes of budding, fission and fusion. Panel **b** The degree of unsaturation in the fatty acid chains of membrane phospholipids determines the “membrane packing” into liquid-ordered or liquid-disordered structures. Saturated fatty acid chains show stable interactions, leading to increased membrane compaction and lower fluidity; whereas, unsaturated fatty acid species induce higher fluidity. *Adapted from* (Harayama and Riezman, 2018)

Not only the relative amount of the different phospholipid species, but also the specific features of their fatty acid chains play an important role in defining the plasma membrane structure and properties. It is generally accepted that the stable interactions between long and saturated fatty acid chains lead to increased membrane compaction

and lower fluidity (van Meer *et al.*, 2008; Sezgin *et al.*, 2017). On the contrary, introduction of PUFAs has been found to decrease membrane bending rigidity (Rawicz *et al.*, 2000; Barelli and Antonny, 2016), suggesting that membrane deformation could be favored by regulating the GPL content in PUFAs. Saturated-, unsaturated- and cholesterol-rich lipids have been shown to form different types of domains in artificial membranes, as a function of their level of packaging (van Meer *et al.*, 2008) (**Figure 35, panel b**). In addition, the lipid composition influences the localization and conformation of several transmembrane proteins; indeed, proteins containing lipid-binding domains have been found to associate with specific membrane compartments or subdomains (Saliba *et al.*, 2015; reviewed in Harayama and Riezman, 2018). Taken together, all these studies demonstrate the importance of lipid composition in defining the membrane organization and structure, which are then linked to specific membrane physical properties and functions.

Membrane lipids: is enucleation linked to the phospholipid fatty acid composition?

Upon rEPO-dependent erythroid commitment of CD34⁺ progenitor cells, we have observed some intriguing results under cell culture conditions including either 5% fetal bovine serum (FBS) or a combination of 3% AB human serum and 2% human plasma. Indeed, we identified a delayed terminal differentiation kinetic, assessed as a function of CD49d/Glut1 expression levels, in presence of FBS (**Figure 36**). This differentiation delay was associated with significant differences in the enucleation levels between cells cultured in presence of FBS or human serum and plasma (**Figure 36**).

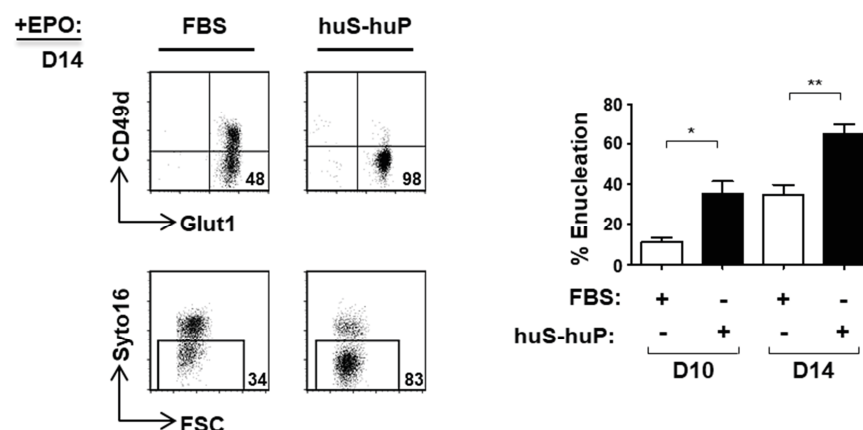


Figure 36. Erythroblast terminal differentiation and enucleation in presence of FBS or human serum and plasma (huS-huP). CD34⁺ progenitors were stimulated with rEPO and cultured in presence of either 5%

FBS or a combination of 3% AB human serum and 2% human plasma (huS-huP). CD49d/Glut1 profiles of GlyA⁺ erythroblasts and enucleation, as a function of Syto16 nuclear staining, were monitored at day 14 of differentiation and representative dot plots are shown. Percentages of Glut1⁺/CD49d⁻ (orthochromatic) erythroblasts and of Syto16⁻ enucleated cells are indicated (left). Percentage enucleation in presence of FBS or huS-huP was quantified at day 10 and day 14 of differentiation (p<0.5 day10, p<0.05 day14) (right).

Interestingly, several studies have shown that changes in the fatty acid or serum content of the culture medium influence the composition of intracellular lipids and membrane phospholipids in diverse cell types (Spector *et al.*, 1979; Lagarde *et al.*, 1984; Stoll and Spector, 1984; Pazouki *et al.*, 1992; García-Pelayo *et al.*, 2003). In fact, sera of different species exhibit distinct fatty acid content (Stoll and Spector, 1984), with human serum being enriched in polyunsaturated fatty acids (PUFAs) as compared to FBS (Lagarde *et al.*, 1984) (**Figure 37**).

		FBS	HS
	Saturated		
Palmitic	16:0	52	42
Stearic	18:0	18	11
Arachidic	20:0	0	0
	Unsaturated		
Oleic	18:1	20	27
Linoleic	18:2n-6	3	11
Gamma-linolenic	18:3n-6	0	ND
Eicosadienoic	20:2n-6	0	0
Arachidonic	20:4n-6	3	3

Figure 37. FBS and human serum (HS): percentage composition in saturated and unsaturated fatty acid species. Adapted from (Lagarde *et al.*, 1984)

As previously discussed in this section, phospholipid structure and their fatty acid composition play an important role in determining the fluidity properties of the plasma membrane. It is important to note that in the context of terminal erythroid differentiation, the enucleation step is also linked to mechanisms of vesicle trafficking and fusion with the plasma membrane, which participate to the process of organelle degradation (see section 2.2.3.1). Therefore, we asked whether the observed differences in the enucleation levels, between cells cultured in presence of FBS or human serum and

plasma, could be linked to changes in the fatty acid composition of the cell membrane phospholipids. Intriguingly, we found an increased degree of unsaturation of the main phospholipid species (PC, PE, PS and PI) under conditions where the culture medium was supplemented with human serum and plasma (**Figure 38**). Therefore, we hypothesize that higher incorporation of unsaturated fatty acid chains could increase membrane fluidity, which could in turn influence the enucleation process.

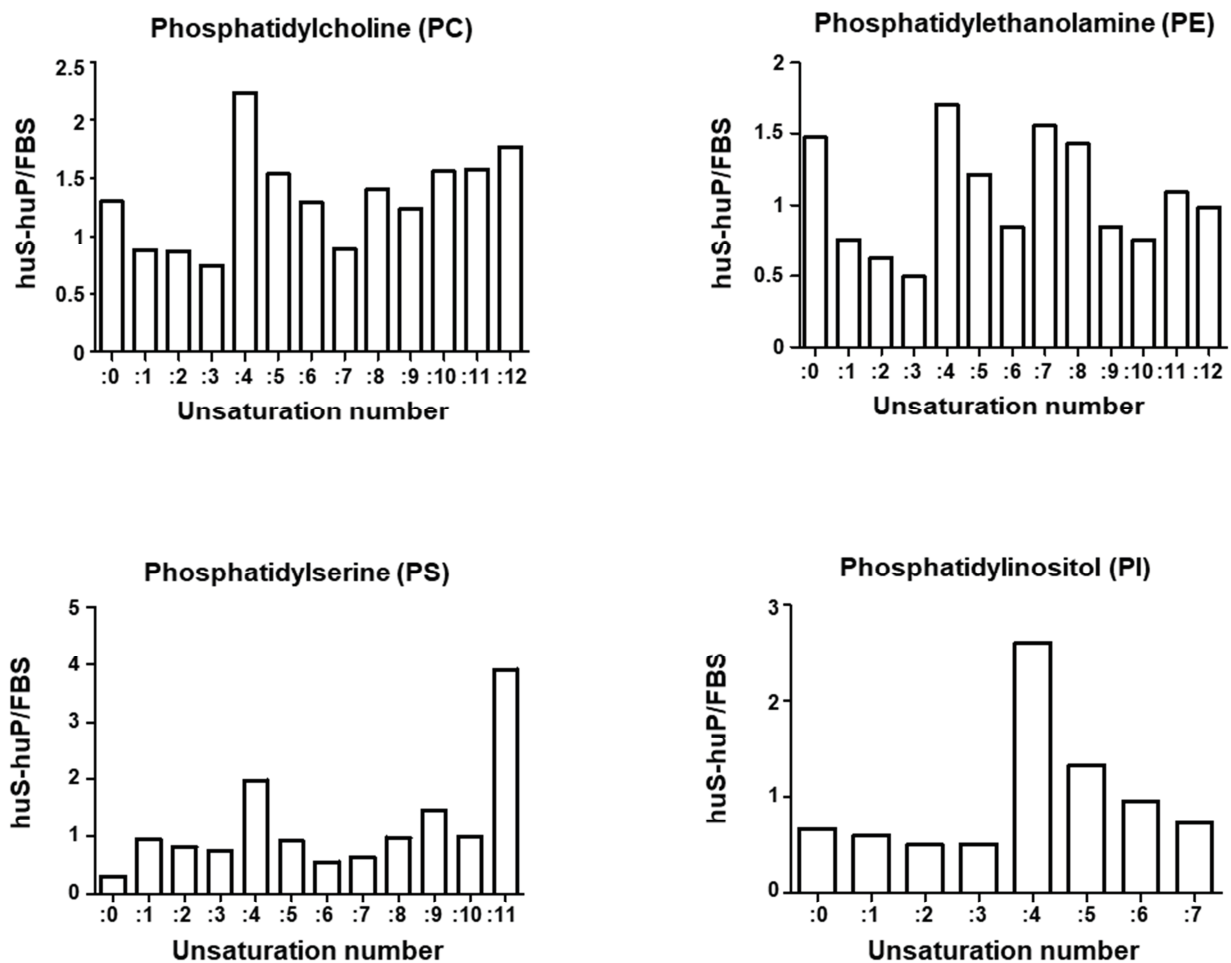


Figure 38. Membrane phospholipid profile in presence of FBS or human serum and plasma (huS-huP). CD34⁺ progenitors were stimulated with rEPO and cultured in presence of either 5% FBS or a combination of 3% AB human serum and 2% human plasma (huS-huP); membrane lipid composition has been assessed at day 10 of differentiation. Cell pellets containing 1x10⁶ cells have been stored at -80°C until analysis by LC-MS. Mean values of 3 independent experiments are expressed as a function of the fatty acid chain unsaturation degree (X axis, unsaturation number), in terms of ratio between samples cultured in presence of human serum and plasma (huS-huP) and in presence of FBS (Y axis, huS-huP/FBS).

To conclude, further investigation is still needed to understand how the regulation of lipid metabolism could eventually influence the membrane properties and consequently some phases of the erythroid differentiation process. Interestingly, it has been recently shown that CD36 deficiency is associated with alterations in insulin signaling as well as glucose metabolism, and that CD36-insulin receptor (IR) crosstalk is differently regulated by saturated vs unsaturated fatty acids (Samovski *et al.*, 2018). According to the role of CD36 in erythropoiesis, but also as fatty acid transporter in several cell types (Pohl *et al.*, 2005; Georgiou *et al.*, 2015; reviewed in Glatz and Luiken, 2015), interesting findings could be obtained by studying the contribution of fatty acid and lipid metabolism in human erythroid differentiation.

REVIEW:

**Metabolic regulation of hematopoietic stem cell commitment
and erythroid differentiation**



Metabolic regulation of hematopoietic stem cell commitment and erythroid differentiation

Leal Oburoglu, Manuela Romano, Naomi Taylor, and Sandrina Kinet

Purpose of review

Hematopoietic stem cell (HSC) renewal and lineage differentiation are finely tuned processes, regulated by cytokines, transcription factors and cell–cell contacts. However, recent studies have shown that fuel utilization also conditions HSC fate. This review focuses on our current understanding of the metabolic pathways that govern HSC self-renewal, commitment and specification to the erythroid lineage.

Recent findings

HSCs reside in a hypoxic bone marrow niche that favors anaerobic glycolysis. Although this metabolic pathway is required for stem cell maintenance, other pathways also play critical roles. Fatty acid oxidation preserves HSC self-renewal by promoting asymmetric division, whereas oxidative phosphorylation induces lineage commitment. Committed erythroid progenitors support the production of 2.4 million erythrocytes per second in human adults via a synchronized regulation of iron, amino acid and glucose metabolism. Iron is indispensable for heme biosynthesis in erythroblasts; a process finely coordinated by at least two hormones, hepcidin and erythropoietin, together with multiple cell surface iron transporters. Furthermore, hemoglobin production is promoted by amino acid-induced mTOR signaling. Erythropoiesis is also strictly dependent on glutamine metabolism; under conditions where glutaminolysis is inhibited, erythropoietin-signaled progenitors are diverted to a myelomonocytic fate. Indeed, the utilization of both glutamine and glucose in de-novo nucleotide biosynthesis is a *sine qua non* for erythroid differentiation.

Summary

Diverse metabolic networks function in concert with transcriptional, translational and epigenetic programs to regulate HSC potential and orient physiological as well as pathological erythroid differentiation.

Keywords

erythropoiesis, hematopoietic stem cell, lineage differentiation, metabolic reprogramming, self-renewal

INTRODUCTION

All living cells require energy to survive, proliferate and differentiate. In most mammalian cells, energy is supplied by the degradation of three major nutrients: glucose, glutamine and fatty acids. Even though nutrients are essential for all cells, the metabolic requirements of distinct cell lineages can be extremely divergent. Furthermore, even within the same lineage, cells that are quiescent, proliferating and differentiating often exhibit striking differences in their metabolism. In the context of hematopoietic stem cells (HSCs), where differentiation is highly regulated by cell–cell contacts and cytokine signals, recent studies have shown that cell metabolism is also a key player. Indeed, metabolic reprogramming conditions the relative levels of self-renewal versus lineage differentiation. Specifically, the balance between HSC maintenance

and generation of all blood lineages is modulated by metabolite entry and utilization, via mitochondrial-dependent as well as independent nutrient utilization pathways. This review focuses on recent discoveries in the metabolic features governing HSC fate and specifically addresses the role of nutrients in erythroid lineage specification.

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KEY POINTS

- Metabolic programs regulate the balance between HSC self-renewal and lineage differentiation.
- The hypoxic bone marrow niche and subsequent anaerobic glycolysis promote HSC maintenance.
- The production of 10^{11} – 10^{12} new blood cells per day in human adults is dependent on a complex regulation of iron and nutrient transport in erythroid progenitors.
- Erythroid specification requires glutamine- and glucose-dependent nucleotide biosynthesis.

TEXT OF REVIEW**Major metabolic pathways regulating cellular energetics in hematopoietic stem cells and progenitors**

Prior to reviewing recent advances in the metabolic regulation of HSCs and committed progenitors, the pathways contributing to cellular energetics will be presented. In the presence of oxygen, glucose-derived pyruvate and/or glutamine-derived α -ketoglutarate can enter into the mitochondrial tricarboxylic acid (TCA) cycle. Acetyl-coA, generated by β -oxidation of fatty acids (FAO) in the mitochondria, can also fuel the TCA cycle (Fig. 1). The NADH and FADH₂ that are generated undergo subsequent oxidative phosphorylation, driving ATP production. This pathway can generate up to 36 ATPs from one molecule of completely oxidized glucose, whereas the utilization of glucose via glycolysis provides only two ATPs. However, glycolysis may be advantageous to a cell as it provides a higher number of carbons and NADPH for the synthesis of macromolecules such as lipids, amino acids and nucleotides. Indeed, many tumor cells rely on glycolysis even when oxygen is present (aerobic glycolysis), a phenomenon called the Warburg effect [1]. Moreover, it is now known that this process also occurs in rapidly proliferating cells that are nonmalignant, likely because of their high metabolic demands [2]. Glucose metabolites can also be redirected into amino acid biosynthesis and nucleotide biosynthesis via the pentose phosphate pathway (PPP). Recent research has shown that this latter pathway can also optimize cell survival via a nucleotide-independent effect; the PPP-mediated production of NADPH efficiently protects cells from oxidative stress [3*]. Thus, the ensemble of these pathways promotes the generation of ATP, carbons and reducing equivalents that are required for cell survival and proliferation (Fig. 1). Notably though, differences in the metabolite intermediates that are

produced by each pathway have important metabolic consequences for HSCs, regulating their fate and lineage commitment.

Hematopoietic stem cell quiescence to commitment: from glycolysis to oxidative phosphorylation

Adult HSCs reside within a bone marrow niche where they are maintained in a quiescent state. The bone marrow environment has likely evolved to best facilitate the maintenance of HSCs throughout the life of the organism. In this regard, hypoxia is a critical parameter of the bone marrow niche [4–6]. Even *ex vivo*, HSCs that are maintained in hypoxic conditions exhibit a more efficient repopulating ability than HSCs that are cultured in atmospheric oxygen conditions [7,8,9**]. Notably, hypoxia favors anaerobic glycolysis, a metabolic state that has recently been shown to be required for stem cell maintenance [10,11].

Under conditions of hypoxia, the hypoxia inducible factor-1 (HIF1) is upregulated. Notably, this transcription factor as well as the upstream regulator Meis1 are highly expressed in dormant long-term HSC (LT-HSC) [4,5]. LT-HSC can be distinguished from short-term HSC, at least in part, by their rates of division; the former divide only twice a year, whereas the latter undergo approximately one division per month [12,13]. Consistent with a critical role for HIF1 in the quiescence and maintenance of LT-HSC, its deletion results in a severe loss of hematopoietic reconstitution potential in adult mice as well as during embryonic life [4,14]. Furthermore, the movement of HSCs from the more hypoxic endosteal area to the less hypoxic central marrow area also results in a loss of stem cell potential and this migration is prevented by HIF1 induction of the Cripto/GRP78 signaling axis, activating TGF β , Notch and PI3K pathways, amongst others [15]. HIF1 signaling directly interfaces with the metabolism of HSC; HIF1 promotes activation of pyruvate dehydrogenase kinases (Pdk), increasing anaerobic glycolysis and stem cell potential [4,11].

The anaerobic environment of the bone marrow also serves to preserve the genomic integrity of HSCs. This is because of, at least in part, low levels of reactive oxygen species (ROS). The ROS, produced by normal mitochondrial respiration, induce DNA damage and as such the low mitochondrial function of HSCs limits genotoxicity. Although there is an increase in DNA damage in aging HSCs [16], the mitochondria that are present in LT-HSCs are generally inactive or ‘nascent’ [17]. In agreement with this detrimental role for mitochondrial function and associated ROS production in HSC maintenance, it has been shown

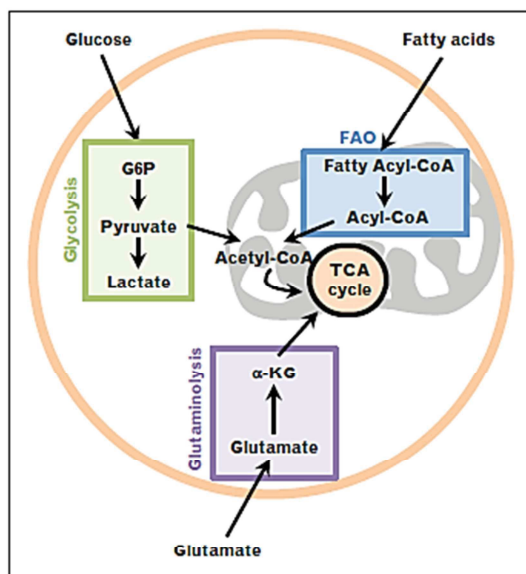


FIGURE 1. Schematic representation of major energy-producing metabolic pathways. Glucose, glutamine and fatty acids contribute to energy production. Following its transport into the cell, glucose gives rise to glucose-6-phosphate (G6P) and then pyruvate. Pyruvate can be metabolized to lactate in the cytoplasm or carried into the mitochondria where the acetyl-CoA derivative replenishes the tricarboxylic acid (TCA) cycle. Glutamine can undergo anaplerotic reactions following its conversion into glutamate to form alpha-ketoglutarate (α -KG) for use in the TCA cycle. Fatty acids are transported into the cells and are activated in the cytoplasm to acyl-CoA. Fatty acyl-CoA enters the mitochondria where it is a substrate for fatty acid β -oxidation (FAO), generating acetyl-CoA that, like glucose, fuels the TCA cycle.

that diverse mechanisms restricting mitochondrial biogenesis protect HSCs from ROS production; these include expression of noncoding RNAs from the Dkl1-Gtl2 locus, expression of the mitochondrial carrier homolog 2 and inhibition of the mTOR axis [18–20,21^{*},22,23]. As indicated earlier, directly suppressing the influx of glycolytic intermediates into the mitochondria by Pdk also augments stem cell potential [11]. Similarly, depletion of PTPMT1, a PTEN-like mitochondrial phosphatase that enhances glycolysis while attenuating oxidative phosphorylation, significantly increases HSC numbers because of a block in lineage differentiation [10]. Conversely, conditions that promote mitochondrial biogenesis and oxidative phosphorylation, at the expense of glycolysis, promote HSC differentiation [10,17,18,23]. Oxidative phosphorylation also results in increased HSC division, and this proliferation is coupled to stem cell exhaustion ([12,13] and reviewed in [24]).

Symmetric versus asymmetric hematopoietic stem cell division: regulation by fatty acid oxidation

As presented earlier, increases in mitochondrial biogenesis and oxidative phosphorylation promote HSC division and subsequent differentiation [10,17,18,23] but it is fatty acid oxidation (FAO) that regulates the ‘choice’ between HSC maintenance and commitment in dividing cells. By mechanisms that have not yet been completely elucidated, FAO promotes an asymmetric division that allows the generation of one committed daughter cell and one daughter cell with self-renewal potential. In contrast, under conditions where FAO is inhibited, HSCs undergo a symmetric division, producing two committed progenitors [25^{**},26] (Fig. 2). Interestingly, in one study, it was found that under ex-vivo conditions where FAO was neither inhibited nor induced, the proliferation of murine HSCs gave rise to 45% asymmetric divisions, 25% symmetric divisions inducing two daughter HSCs and 22% symmetric divisions producing two committed progenitors [25^{**}]. It will be important to elucidate how FAO, and/or intermediates of FAO, regulates these three types of cell division and assess whether other signaling pathways contribute to the decision between renewal and commitment.

HSC maintenance through FAO is promoted by the promyelocytic leukemia protein (PML) tumor suppressor; it activates the PML-peroxisome proliferator-activated nuclear receptor delta (PPAR δ) that is required for long-chain FAO. Loss of either PML or PPAR δ inhibits FAO, leading to symmetric division, increased HSC proliferation and lineage commitment [25^{**},27]. Similarly, blocking carnitine palmitoyltransferase 1 (CPT1), the first step in FAO, promotes HSC commitment [25^{**}]. The liver kinase B1 (LKB1) tumor suppressor, an upstream activator of AMPK, also regulates HSC commitment. In the absence of LKB1, HSC maintenance is severely impaired with progenitor commitment regulated by both AMPK-dependent as well as independent mechanisms [28^{**}]. LKB1 likely maintains stem cell potential by activating PPAR γ coactivator 1 (PGC-1), a transcriptional regulator of genes encoding FAO enzymes [29–32]. Thus, anaerobic glycolysis and FAO promote an asymmetric division that is critical for maintaining the stem cell potential of HSCs, whereas the transition of HSCs to committed progenitors occurs upon their symmetric division, a fate promoted by oxidative phosphorylation (Fig. 2).

Metabolic regulation of erythropoiesis

The differentiation of HSCs to the erythroid lineage proceeds via a stepwise process; there is a first

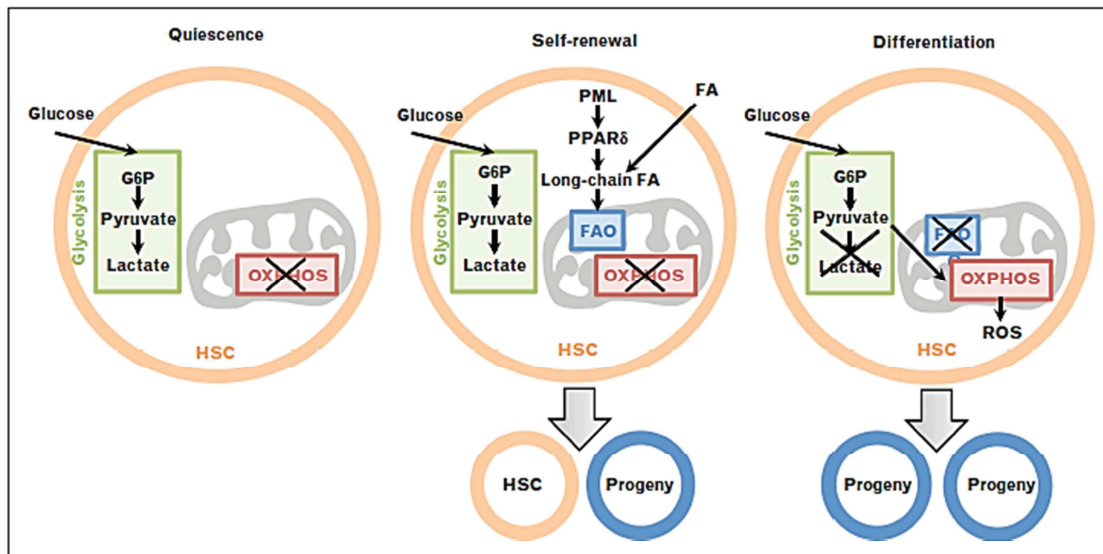


FIGURE 2. Metabolic pathways regulate the division dynamics and commitment of hematopoietic stem cells. The survival of quiescent hematopoietic stem cells (HSCs) in the anaerobic bone marrow niche is dependent on an active glycolysis and the suppression of oxidative phosphorylation (OXPHOS) (left). The self-renewal of HSCs is controlled by the promyelocytic leukemia (PML)-peroxisome proliferator-activated receptor delta (PPAR δ)-fatty acid oxidation (FAO) axis, which is required for the asymmetric division of HSCs, producing an HSC and a committed progeny (middle). However, under conditions where there is mitochondrial biogenesis with OXPHOS and subsequent generation of reactive oxygen species (ROS), HSCs undergo a symmetric division with the generation of committed progeny and subsequent exhaustion of the HSC pool (right). G6P, glucose-6-phosphate.

commitment to a burst-forming unit-erythroid cell, which then further differentiates to a colony-forming unit-erythroid cell. During terminal differentiation, a colony-forming unit-erythroid cell undergoes four to five divisions, regulated by Cyclin D3 levels [33]. This results in the sequential generation of proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts and orthochromatic erythroblasts. The latter expel their nuclei to generate enucleated reticulocytes. Notably, a distinguishing feature of erythropoiesis is that the daughter cells resulting from each division are morphologically and functionally different from their parent cell. These changes are associated with the gain of several plasma marker proteins including Glycophorin A, Glut1 and Band 3, whereas others such as CD36, CD71 and α_4 integrin exhibit early increases, but then diminish with further differentiation [34]. It is important to note though that the study of erythropoiesis has been confounded by significant differences in gene regulation during murine and human erythropoiesis [35^{***}].

Within the different hematopoietic cell lineages, the first metabolism-related studies likely focused on iron-dependent hemoglobin synthesis in erythroid cells. Seventy percent of the iron in the

body is found in the form of heme in red blood cells and iron is indispensable for the production of 10^{11} – 10^{12} new blood cells day in human adults. Iron is exported from enterocytes, hepatocytes and macrophages via ferroportin (SLC40A1), increasing iron levels in the plasma. Conversely, under conditions of high iron levels, ferroportin activity is inhibited by hepcidin, a liver-derived hormone [36,37]. Furthermore, there is a multilevel regulation as a hormone termed erythroferrone (previously referred to as Fam132b or myonectin), secreted by erythroblasts in response to anemia, directly limits the expression of hepcidin in the liver [38]. The transfer of iron into the erythroblast is positively regulated by the transferrin receptor (TfR1, CD71) whereas TfR2, an actual component of the erythropoietin receptor complex [39], negatively impacts on erythroid differentiation by decreasing sensitivity to erythropoietin [40,41]. Erythroblastic island macrophages can directly supply iron to erythroblasts in the form of ferritin and play a critical role in stress-induced erythropoiesis [42–44]. Erythroblasts have also evolved mechanisms to dispose of excess heme, exporting heme through cell membrane and mitochondrial feline leukemia virus C receptor heme transporters; in

their absence, high heme levels lead to severe anemia [45–47].

As regards the energy sources used to fuel erythroid differentiation, it is interesting to note that the human erythrocyte expresses more Glut1 than any other cell in the body, with more than 300 000 copies of Glut1 per cell (representing more than 10% of the total protein mass of the membrane) [48]. Notably though, Glut1 is only upregulated at late stages of erythroid differentiation, after a progenitor has already undergone most of its division cycles. Furthermore, in the vast majority of species, Glut1 expression characterizes neonatal erythrocytes and is rapidly lost with age, with the subsequent induction of Glut4 [49–51]; Glut1 expression is a specific feature of the few ascorbic acid-defective species (higher primates, fruit bats and guinea pigs). In these deficient mammals, we and others have found that Glut1 promotes the uptake of the oxidized form of vitamin C (DHA) [49–55].

On the basis of these data, it was tempting to hypothesize that nutrients other than glucose contribute to erythroid differentiation and commitment. We focused on glutamine because although Glut1 is not detected at the cell surface of human HSCs, the ASCT2 glutamine transporter is highly expressed and expression continues throughout erythropoiesis [56^{***}]. Blocking either ASCT2-mediated entry of glutamine into progenitors or glutaminolysis diverts erythropoietin-stimulated progenitors to a myelomonocytic fate. Notably, erythroid specification of HSCs is not contingent on glutamine-derived ATP production, but rather on

glutamine-dependent nucleotide biosynthesis. In line with the critical nature of nucleotides, shunting of glucose through the PPP, resulting in the synthesis of five-carbon sugars used for nucleotide biosynthesis, enhances the magnitude and kinetics of HSC erythroid commitment [56^{***}] (Fig. 3).

The precise mechanisms forcing the diversion of erythropoietin-stimulated hematopoietic progenitors to a myelomonocytic fate under nucleotide-scarce conditions have not yet been elucidated. However, it is important to note that amino acids such as glutamine are more than just actors in the TCA cycle and nucleotide generation. Together with leucine and arginine, glutamine activates the mTOR (mammalian target of rapamycin) pathway, a signaling cascade regulating multiple cell processes [57]. Indeed, blocking the LAT3-mediated entry of leucine into zebrafish and murine erythroid progenitors inhibits the mTORC1 complex, specifically impacting on erythropoiesis via a reduction of hemoglobin production [58^{***}] (Fig. 3). mTORC1 activity in erythroid cells is also regulated by dietary iron [59^{***}], showing the close interplay between these distinct metabolites in conditioning erythroid differentiation. Nevertheless, mTOR inhibition by rapamycin has also been reported to enhance murine erythroid differentiation [60^{***}]. It will be interesting to determine whether some of the reported experimental differences may be because of changes in the interactions between mTOR signaling components and the mitophagy/autophagy processes that are required for erythropoiesis [61,62]. The latter play a critical role in the elimination of

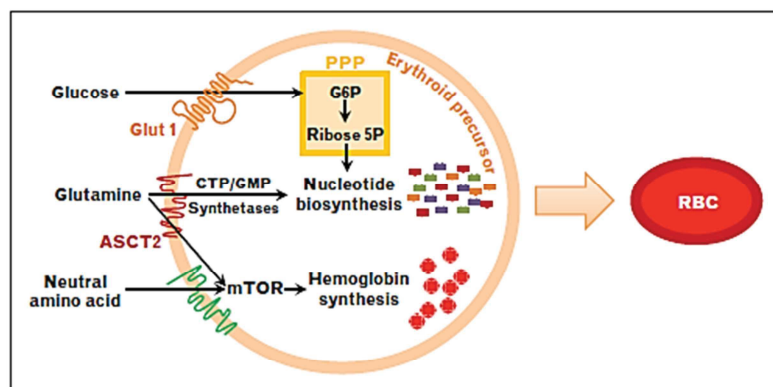


FIGURE 3. Glucose, glutamine and neutral amino acids regulate erythroid differentiation via induction of biosynthetic pathways. Erythroid precursors express Glut1 and ASCT2 at their surface, allowing the transport of glucose and glutamine, respectively. These two metabolites are crucial for nucleotide biosynthesis. Shunting of glucose through the pentose phosphate pathway (PPP) gives rise to ribose-5-phosphates, one of the building blocks of nucleotides, whereas glutamine donates its amino group for the synthesis of nucleotides by cytidine triphosphate/guanosine monophosphate (CTP/GMP) synthetases. Both the PPP and glutamine-regulated nucleotide synthesis are required for erythroid differentiation. Glutamine and other amino acids such as leucine and arginine can activate the mTOR pathway, essential for hemoglobin synthesis. G6P, glucose-6-phosphate.

mitochondria, ribosomes and other organelles during erythroid terminal differentiation (reviewed in [62]). However, in contrast with mTOR activity, autophagy is generally induced under conditions of limiting amino acid/nutrient metabolism. Research focusing on the precise connections between nutrient utilization, mTOR activation and autophagy in erythroid lineage specification are ongoing.

New perspectives

Cell metabolism, often defined by biochemistry students as the pathways via which a cell produces ATP, is clearly a much more complex process. Furthermore, our initial definitions of the term 'nutrient' have been broadened to encompass a wide range of metabolites as well as the intermediates of those metabolites. Metabolites are more than a source of 'nutrition,' playing active regulatory roles in a wide range of developmental cascades. For example, succinate, which accumulates under conditions of impaired TCA cycle progression, stabilizes HIF1 levels, inducing HIF1-dependent transcripts such as the proinflammatory IL-1b cytokine as well as high ROS levels [63,64,65]. Conversely, increasing alpha-ketoglutarate, a cofactor in HIF α prolyl hydroxylase activity, inhibits HIF1 [66]. Furthermore, the glycolytic metabolite phosphoenolpyruvate has recently been found to activate NFAT signaling by repressing sarcoendoplasmic reticulum calcium transport ATPase, a pump that transports calcium ions from the cytoplasm into the sarcoendoplasmic reticulum [67]. Thus, metabolites function as key regulators of several signaling cascades that may directly impact on HSC maintenance and commitment.

Metabolites also play key roles in regulating the epigenetic state of stem cells and their progeny [68]. Alpha-ketoglutarate is a required cofactor of JmjC domain-containing histone demethylases and the Tet family of DNA dioxygenases, thereby directly affecting the methylation state of the cell [69]. Indeed, glutamine-derived alpha-ketoglutarate is required for the histone/DNA demethylation of embryonic stem cells, maintaining their pluripotency, while conversely, decreases in alpha-ketoglutarate drive differentiation [70]. Similarly, the acetyl-CoA that is produced by glycolysis is required to maintain the high level of histone acetylation that conditions embryonic stem cell pluripotency [71]. As regards the specificities of erythroid differentiation, it is notable that mutations in Tet2 are one of the most common causes of myelodysplastic syndrome and dys-regulated erythropoiesis [72]. Furthermore, the SetD8 histone methyl-transferase positively regulates erythroid cell maturation [73,74].

CONCLUSION

Future studies focused on metabolite-induced epigenetic modifications, transcription regulation and the redox state of HSC and erythroid progenitors promise to bring novel developments to our understanding of physiological and pathological erythropoiesis.

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Conflicts of interest

There are no conflicts of interest.

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