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Role of microRNAs and their machinery in hepatocytes and pancreatic beta cells

Gaia Fabris

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THÈSE DE DOCTORAT

Rôle des microARNs et de leur machinerie dans les hépatocytes et
les cellules béta pancréatiques

Role of microRNAs and their machinery in hepatocytes and pancreatic beta cells

Gaia FABRIS

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cellulaires

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Rôle des microARNs et de leur machinerie dans les hépatocytes et les cellules béta pancréatiques

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Résumé

Dans le foie normal de l'adulte les hépatocytes sont dans un état de quiescence, sauf lors d'une situation d'agression physique ou toxique. Dans ces cas-là, afin de pouvoir maintenir leur rôle dans l'homéostasie de l'organisme les hépatocytes endommagés ou sauvegardés vont proliférer. Un des facteurs limitants pour la prolifération est la présence d'acides aminés, qui sont nécessaires pour la synthèse des protéines au cours de la prolifération et division cellulaire, et pour l'activation de la voie mTOR, qui joue un rôle clef dans la régulation de la prolifération cellulaire.

Le but de mon projet de thèse était d'étudier dans des hépatocytes adultes le rôle des acides aminés dans la régulation de la transition entre l'état de quiescence et la croissance cellulaire. Pour cela nous avons utilisé comme modèle de foie partiellement reséqué des hépatocytes de rats adultes en culture primaire non-confluente. J'ai découvert que l'absence d'acides aminés induit l'entrée des hépatocytes dans un état de quiescence couplée à une augmentation de la protéine Drosha; cet accroissement étant indépendant de la voie mTOR. Inversement, la réduction de Drosha permet aux hépatocytes de proliférer, et cet effet semble indépendant des microARNs, qui sont les partenaires classiques de Drosha. L'ensemble de nos résultats révèlent dans les hépatocytes adultes l'existence d'effets non-classiques de Drosha dans le contrôle de la régulation de la croissance suite un stress nutritionnel. Un tel mécanisme pourrait être ciblé pour la prévention et/ou le traitement de l'insuffisance hépatique.

Le second projet de ma thèse concerne le rôle du microARN-375 dans la régulation de la sécrétion de l'insuline en réponse au glucose dans les îlots de Langerhans de rats et d'origine humaine. Le métabolisme glycolytique et oxydatif sont nécessaires pour la réponse sécrétoire au glucose. Alors que plusieurs microARNs sont connus pour moduler l'homéostasie des cellules bêta, le microARN-375 se démarque par sa forte expression dans les cellules bêta dans lesquelles il régule le fonctionnement, la prolifération et la différenciation. Sachant que le métabolisme du glucose est au centre de toutes les fonctions de la cellule bêta, nous avons étudié son impact sur le métabolisme dans des îlots humains et murins. Notre approche a fait appel soit à la surexpression du microARN-375, soit à sa réduction dans des îlots humains ou murins. Ensuite nous avons analysé la sécrétion de l'insuline et le métabolisme. De plus, nous

avons regardé l'expression du microARN-375 et la réponse sécrétoire au glucose dans des îlots de rats à différents stades de leur développement. Nous avons trouvé que la surexpression du microARN-375 dans les îlots murins et humains diminuait la sécrétion de l'insuline en réponse au glucose, mais pas celle au α -cétoisocaproate ou au KCl. De plus, le microARN-375 réduit la consommation d'O₂ engendrée par la glycolyse et le métabolisme du pyruvate, mais pas celle en réponse au α -cétoisocaproate. En même temps, la production de lactate est augmentée suggérant que le pyruvate est détourné des mitochondries. Enfin, la réduction du niveau du microARN-375 est associée à la maturation des cellules bêta de rat foetal et l'apparition d'une réponse sécrétoire au glucose. Pris dans leur ensemble nos résultats identifient le microARN-375 comme étant un régulateur central du métabolisme du glucose et de la sécrétion de l'insuline, et ainsi il pourrait être un rouage déterminant pour la maturation fonctionnelle de cellules bêta.

Mots clés : hépatocytes, prolifération cellulaire, acides aminés, Drosha; diabète, métabolisme du glucose, sécrétion de l'insuline, îlots pancréatiques, microARNs.

Abstract

In an adult healthy liver, hepatocytes are in a quiescent stage unless a physical injury, such as ablation, or a toxic attack occurs. However, to maintain their important organismal homeostatic role, the damaged or remaining hepatocytes will start proliferating to restore their functional mass. One of the limiting conditions for cell proliferation is amino acid (aa) availability, necessary both for the synthesis of proteins important for cell growth and division, and for the activation of the mTOR pathway, known for its considerable role in the regulation of cell proliferation. The aim of my main PhD project was to investigate the role of amino acids in the regulation of the switch between quiescence and growth of adult hepatocytes. To do so I used non-confluent primary adult rat hepatocytes as a model of partially ablated liver. I discovered that the absence of aa induces in primary rat hepatocytes the entrance in a quiescence state together with an increase in Drosha protein, which does not involve the mTOR pathway. Conversely, knockdown of Drosha allows the hepatocytes to proliferate and this effect appears to be independent of miRNAs, the canonical downstream partners of Drosha. Taken together, these observations reveal an intriguing non-canonical effect of Drosha in the control of growth regulation of adult hepatocytes responding to a nutritional strain, and they may help to design novel preventive and/or therapeutic approaches for hepatic failure.

A side project of my PhD involved the role of microRNA-375 in the regulation of glucose-induced insulin secretion in rat and human pancreatic islets. Enhanced β -cell glycolytic and oxidative metabolism are necessary for glucose-induced insulin secretion. While several microRNAs modulate β -cell homeostasis, microRNA-375 stands out as it is highly expressed in β -cells where it regulates β -cell function, proliferation and differentiation. As glucose metabolism is central in all aspects of β -cell functioning, we investigated the role of microRNA-375 in this process using human and rat islets; the latter being an appropriate model for in depth investigation. We used forced expression and repression of microRNA-375 in rat and human primary islet cells followed by analysis of insulin secretion and metabolism. Additionally, microRNA-375 expression and glucose-induced insulin secretion were compared in islets from rats at different developmental ages. We found that overexpressing of microRNA-375

in rat and human islet cells blunted insulin secretion in response to glucose, but not to α -ketoisocaproate or KCl. Further, microRNA-375 reduced O_2 consumption related to glycolysis and pyruvate metabolism, but not in response to α -ketoisocaproate. Concomitantly, lactate production was augmented suggesting that glucose-derived pyruvate is shifted away from mitochondria. Finally, reduced microRNA-375 expression was associated with maturation of fetal rat β -cells and acquisition of glucose-induced insulin secretion function. Altogether our findings identify microRNA-375 as an efficacious regulator of β -cell glucose metabolism and of insulin secretion and could be determinant to functional β -cell developmental maturation.

Keywords: hepatocytes, cell proliferation, amino acids, Drosha; diabetes, glucose metabolism, insulin secretion, pancreatic islets, microRNAs.

*“ While
the heart resounds and attracts
the music of the mandolin,
there, inside,
you filter
and distribute,
you separate
and divide,
you multiply
and lubricate,
you raise
and gather
the threads and the grams
of life, the final
distillate,
the intimate essences.
Submerged viscus,
measurer
of the blood,
you live
full of hands
and full of eyes,
measuring and transferring
in your hidden
alchemical
chamber. ”*

Ode to the liver – Pablo Neruda

*« Mientras
el corazón suena y atrae
la partitura de la mandolina,
allí adentro
tú filtras
y repartes,
separas
y divides,
multiplicas
y engrasas,
subes
y recoges
los hilos y los gramos
de la vida, los últimos
licores,
las íntimas esencias. Viscera
submarina,
medidor
de la sangre,
vives
lleno de manos
y de ojos,
midiendo y trasvasando
en tu escondida
cámara
de alquimista. »*

Oda al hígado – Pablo Neruda

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List of abbreviations

aa amino acids

ac-pre-mir-451 Ago-cleaved pre-mir-451

ADP adenosine diphosphate

Ago argonaute proteins

ANGPTL3 Angiopoietin-like 3

ALT alanine aminotransferase

ARNT hydrocarbon receptor nuclear translocator

ATG autophagy-related protein

ATP adenosine triphosphate

BCAA branched-chain amino acids

BCAT branched-chain-amino-acid aminotransferase

BCKA branched-chain keto acids

BCKD branched-chain α -keto acid dehydrogenase

cAMP 3',5'-cyclic adenosine monophosphate

CBP80 cap-binding complex 80

ChREBP carbohydrate response element binding protein

CoA Coenzyme A

CPT-1 carnitine palmitoyltransferase 1

DHAP dihydroxyacetone phosphate

DHCA 3 α ,7 α -dihydroxycholestanoic acid

DGCR8 DiGeorge syndrome critical region 8

DNL de novo lipogenesis

dsRBD dsRNA-binding domain

dsRNA double-stranded RNA

e.aa essential amino acids

EGF epidermal growth factor

EGFR epidermal growth factor receptor

eIF2A eukaryotic translation initiation factor 2-A

ER endoplasmic reticulum

ER α estrogen receptor alpha

EXP5 Exportin-5
F1,6P fructose 1,6-bisphosphate
F2,6P fructose 2,6-bisphosphate
F6P fructose-6-phosphate
FA fatty acids
FABP fatty acid binding protein
FAS fatty acid synthase
FFA free fatty acids
FIP200 focal adhesion kinase family interacting protein of 200 kDa
G3P glycerol-3-phosphate
G6P glucose-6-phosphate
G6Pase glucose-6-phosphatase
GCK glucokinase
GCN2 general control nonderepressible-2
GDH glutamate dehydrogenase
GKRP glucokinase regulatory protein
GLP-1 glucagon-like peptide-1
GP glycogen phosphorylase
GPAM glycerol-3-phosphate acyltransferase
GPCR G-protein-coupled receptor
GS glycogen synthase
GSK-3 glycogen synthase kinase 3
HB-EGF heparin-binding EGF-like growth factor
HBV hepatitis B virus
HCC hepatocellular carcinoma
HCV hepatitis C virus
HFD high-fat-diet
HGF hepatocyte growth factor
HGFR hepatocyte growth factor receptor
HNF3 β hepatocyte nuclear factor 3- β
HSC hepatic stellate cell
HTR2 hydroxytryptamine receptor 2

IgA immunoglobulin A
IGF-1 insulin-like growth factor-1
IL-6 interleukine-6
InsP3 inositol 1,4,5-trisphosphate
IR insulin resistance
IRS1 insulin receptor substrate 1
KapB Kaposin B
K_{ATP} channels ATP-sensitive K⁺ channels
KSHV Kaposi's sarcoma-associated herpesvirus
L-PK liver-type pyruvate kinase
LC3 microtubule-associated protein 1 light chain 3
LCFA long-chain fatty acids
LDH lactate dehydrogenase
lncRNA long non-coding RNA
LPS lipopolysaccharide
LSEC liver sinusoidal endothelial cell
LXRs liver X receptors
MID middle
miRISC miRNA-induced silencing complex
miRNA microRNA
mRNA messenger ribonucleic acids
mTORC1 mTOR Complex 1
mTORC2 mTOR Complex 2
NAFLD non-alcoholic fatty liver disease
NADPH nicotinamide adenine dinucleotide phosphate
NASH non-alcoholic steatohepatitis
ne.aa non-essential amino acids
NEFA non-esterified fatty acids
Ngn2 Neurogenin2
ORP8 oxysterol-binding-protein-related protein 8
PAZ Piwi–Argonaute–Zwille
PC pyruvate carboxylase

pdx1 pancreatic and duodenal homeobox 1
PEP phosphoenolpyruvate
PEPCK phosphoenolpyruvate carboxykinase
PFK-1 phosphofructokinase-1
PI3P phosphatidylinositol 3-phosphate
PKA protein kinase A
PKC protein kinase C
PH partial hepatectomy
piRNA PIWI-interacting RNA
pre-miRNA precursor microRNA
pri-miRNA primary microRNA
PP pancreatic polypeptide
PP1 protein phosphatase 1
PTEN phosphatase and tensin homolog
PTP1B protein tyrosine phosphatase 1B
RIIIDs RNase III domains
RER rough endoplasmic reticulum
RNA Pol II RNA polymerase II
rRNA ribosomal ribonucleic acids
RP reserve pools
RRP readily releasable pools
S1P sphingosine-1-phosphate
SER smooth endoplasmic reticulum
siRNA short interfering RNA
SIRT1 Sirtuin-1
SK2 sphingosine kinase 2
SREBP-1c sterol regulatory element binding protein 1c
ssRNA single-stranded RNA
T2D type two diabetes
TAG triacylglycerol
TCA tricarboxylic acid
TGF- α transforming growth factor- α

TGF- β transforming growth factor- β
TGF- β RI transforming growth factor- β type I receptor
TGF- β RII transforming growth factor- β type II receptor
TLR4 Toll-like receptor 4
tRNA transfer ribonucleic acids
TSC Tuberous Sclerosis Complex
ULK1 unc-51-like kinase 1
UTR untranslated region
VDCC voltage-dependent Ca^+ -channel
VEGF vascular endothelial growth factor
VLDL very low-density lipoprotein
VPS vacuolar protein sorting

Introduction

Liver

The liver is a key metabolic hub in our organism controlling life-critical tasks. Its functions range from the production of essential molecules such as proteins, lipids and carbohydrates, to the removal of both endogenous and exogenous products to maintain the whole-body homeostasis, the regulation of glycemia and the neutralization of toxic products. The multifunctional activities of the liver are possible due to the coordinated action of several highly differentiated cell types, arranged in a very efficient and specific architecture.

Anatomy

The liver is the largest solid organ and heaviest visceral tissue in the human body, with a weight of around 1,5 kg and approximately 15 cm diameter in adults. It is located on the right of the abdominal cavity, below the diaphragm, which separates it from the lungs, the heart, the stomach and the transverse colon. The liver presents two surfaces: the antero-posterior (or diaphragmatic) and the inferior (or visceral) surface. The diaphragmatic surface is attached to the diaphragm and to the anterior abdominal wall by the *falciform ligament*, which divides the organ in two uneven lobes, one bigger on the right and one smaller on the left. The visceral surface is characterized by three *fossæ* arranged in an H-shape, thus conferring to the inferior surface of the liver the appearance of 4 lobes. The left limb of the H is called *left sagittal fissure* and contains the ligamentum venosum and the round ligament of liver; the right limb of the H is known as *right sagittal fossa* and consists in a groove for inferior vena cava and for the gall bladder. Finally, the bar connecting the two limbs of the H is named *transverse fissure*, or porta hepatis, and hosts the nerves, the lymphatics and the portal vein, which lies behind and between the hepatic artery, on the left, and the hepatic duct on the right.

The liver is an organ of hemodynamic confluence, as it receives supply from two blood streams. The major source of blood (75%) is the portal vein, which delivers blood from the intestines, pancreas and spleen and it is rich in nutrients, hormones and toxins to eliminate. The 25% of blood supply comes from the hepatic artery, carrying oxygen from the *aorta*. The two circulation systems blend in the liver and the mixture of venous and arterial blood arrives to the hepatic microvessels at low pressure and oxygen tension. Therefore, an

exchange of nutrients and oxygen with the hepatic cells occurs and hepatic blood returns in the main circulation through the central vein.

From the macroscopic to the microscopic structure

The liver has the appearance of a solid organ and its main anatomical and functional unit is the hepatic lobule. All the lobules are separated by an areolar tissue in which the blood vessels, the nerves and lymphatics expand and ramify to provide the whole structure. A dense layer of connective tissue, the fibrous coat or *Glisson's capsule*, surrounds the entire surface of the organ, lying just beneath a serous investment derived from the peritoneum.

Each lobule has hexagonal shape and is formed by single layers of cells, the hepatocytes, organized in irregular radiating columns around the central vein. At each corner of the lobule is located one portal canal, making a total of six for each lobule (Figure 1 A). These portal canals, or *portal triads*, are composed by branches of portal vein, hepatic artery and bile ducts. Inside each lobule, a specific cellular organization and architecture guarantees the optimal liver functioning (Figure 1 B).

o *An overview on hepatic cell populations*

Hepatocytes, liver parenchymal cells, occupy about the 80% of the liver mass and constitute the liver metabolic units. They delimit a meshwork of capillaries (*hepatic sinusoids*) which convey the blood coming from the *portal triads* towards the center of the lobule, from where it drains into the vena cava. More in details, oxygen-rich blood from the hepatic artery mingles with nutrient-rich blood from the portal vein and, while progressing across the lobule through the sinusoids, it supplies the hepatic cells. This creates a gradient of oxygen, nutrients and waste products which results in a partitioning of functions called “metabolic zonation”, i.e. the localization of the hepatocytes typically in three different zones in the lobule, which influences their metabolic gene expression and functionality (Trefts et al., 2017).

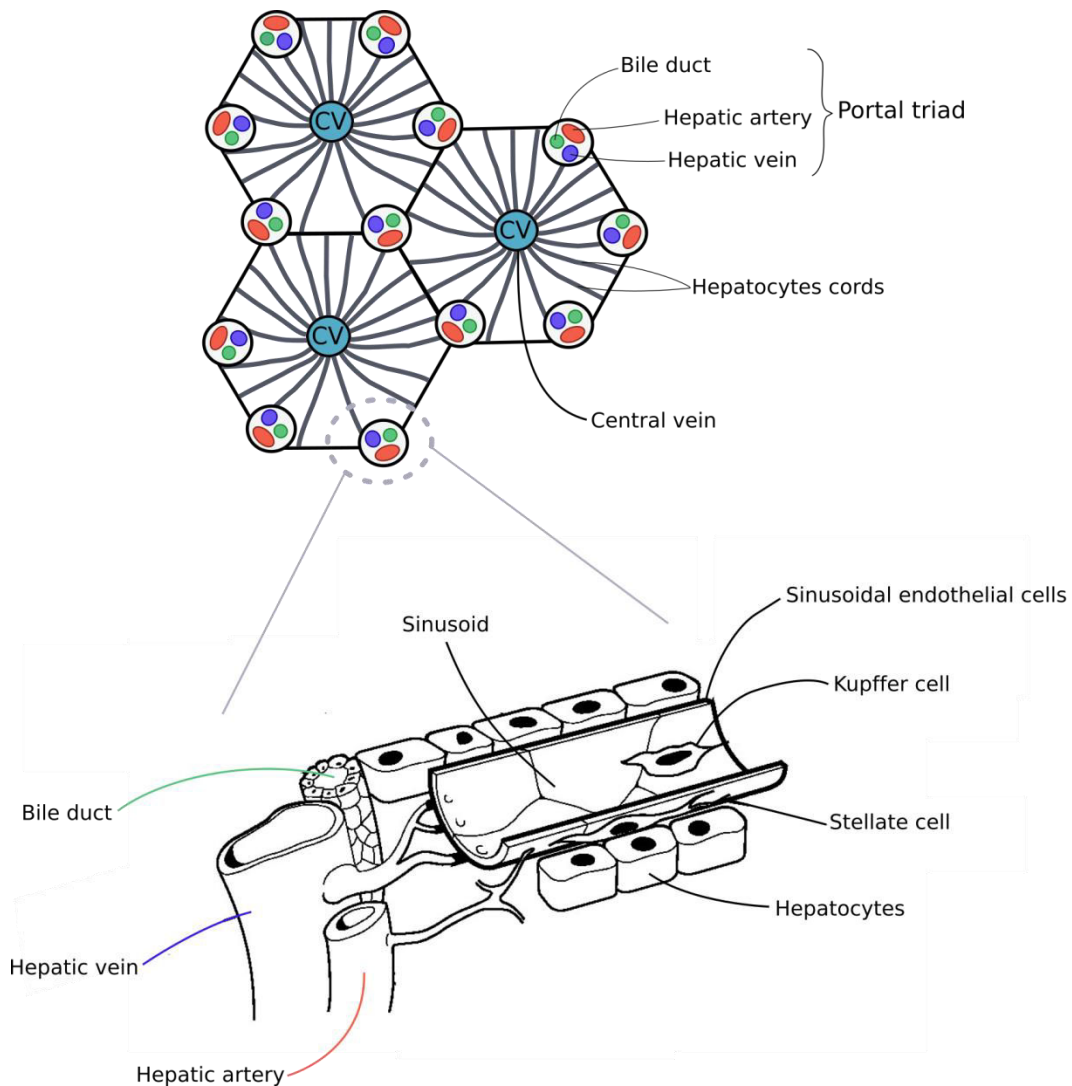


Figure 1 Schematic representation of the hepatic lobule and detail of the portal triad. (A) Each lobule, characterized by hexagonal shape, is composed by single layers of hepatocytes organized radially around the central vein (CV). At each corner of the lobule is a *portal triad* (B), composed by hepatic artery, hepatic vein and bile duct, and which is connected to the central vein through the sinusoid. Adapted from (Barritt et al., 2008)

The wall of the sinusoids is formed mainly by highly specialized endothelial cells (liver sinusoidal endothelial cells – LSECs), which encompass approximately 50% of nonparenchymal cells in the liver and have a specific morphology and function. These cells are provided with a multitude of fenestrations and their unique structure creates pores between 50-180 nm in humans - or 50-280 nm in mice and rats - thus forming a selective barrier between hepatocytes and sinusoidal blood which is permeable for fluids and

molecules, but not for cells (Trefts et al., 2017). Moreover, LSECs are the only endothelial cells in mammals missing a basal lamina, which facilitates the direct exchange of solutes and colloids with the hepatic parenchymal cells and makes the microcirculation of the liver the most porous of all endothelial barriers (Sørensen et al., 2015).

In addition, sinusoids contain a resident macrophage population called Kupffer cells. These cells are part of the sinusoids lining. Being normally located at their bifurcation and branching, the biggest part of their cellular surface faces the blood flow and their extracellular processes extend beneath the endothelial cells. They play an essential role in the clearance of both the hepatic artery and venous blood entering the liver, and they participate to the elimination of phagocytosed bacteria with other liver sinusoidal cell populations. Furthermore, they express immune sensory receptors, thus contributing to the innate immune defense of the liver (Abdullah and Knolle, 2017).

The sinusoidal endothelial cells and the hepatocytes are separated by the *space of Disse*, or perisinusoidal space, where the hepatic parenchymal cells extend their villi and microvilli. Here we find another hepatic nonparenchymal cell type called hepatic stellate cells (HSCs). Stellate cells are a dynamic cell population which exists mainly in a quiescent state, whose principal role is to stock vitamin A. Indeed, they are the main responsible of the regulation of retinoid homeostasis, being capable of accumulating the 80% of the entire body's retinoids in the form of retinyl palmitate in lipid droplets stored in their cytoplasm. In pathological condition, such as liver injury, they exit quiescence and pass in an activated state, in which they proliferate and gradually lose vitamin A stores. Upon activation, their main role resides in the synthesis, deposition and organization of extracellular matrix components including proteoglycan, adhesive glycoproteins and collagen (Senoo, 2004)(Trefts et al., 2017).

The second most abundant epithelial cell type of the liver, accounting for the 3-5% of the hepatic cell population, is the cholangiocyte. These cells form the walls of specialized vessels, the bile ducts. These ducts are in direct communication with the *bile canaliculi*, which are created by the membranes of adjacent parenchymal cells and receive the bile produced by the hepatocytes (Trefts et al., 2017). Interestingly, according to their morphology, cholangiocytes are shown to assess different functions. The large ones play a

pivotal role in the formation and transport of bile through transmembrane molecules expressed on their membranes and they also regulate both the volume and the composition of the biliary secretion. Small cholangiocytes, instead, participate in maintaining tissue homeostasis in the biliary system by controlling the expression of regulators of proliferation, senescence and apoptosis (Yoo et al., 2016).

Hepatocytes

Hepatic parenchymal cells - or hepatocytes - are large cells, often considered as specialized epithelial cells, with a characteristic polygonal shape. They are usually tetraploids and they represent the great majority of liver cells, comprising roughly the 80% of the hepatic population. Although liver activity and functions are the result of the mutual actions of the cells mentioned above, hepatocytes clearly play a pivotal role. Their functions include the metabolism of proteins, carbohydrates and lipids, the synthesis of bile acids and the transcellular movement of bile fluid, the storage of lipids, glycogen and vitamins, the processing of endogenous wastes and the detoxification of exogenous detrimental products and the synthesis and secretion of proteins. Protein synthesis requires a concomitant extensive transcriptional activity, as several messenger ribonucleic acids (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA) are needed to support a high rate protein translation. Therefore, multinucleate parenchymal cells are very common, and they also display singular or several large size nucleoli.

Being situated in the interface between two different physiological environments, the *bile canaliculi* dedicated to the secretion of bile by parenchymal cells, and the sinusoids, where the absorption of nutrients from the blood takes place, hepatocytes are highly polarized. This characteristic is primarily revealed by the polarization of their plasma membrane, defined by three distinct domains, but also by a polarized trafficking of molecules and cytoskeletal architecture (Treyer and Müsch, 2013). The apical surface in hepatic parenchymal cells lines the *bile canaliculi* and is characterized by the presence of tight junctions. The remaining cell membrane is called basolateral and it includes two different regions: the lateral membranes, large areas facing adjacent hepatocytes, which communicate through gap junctions; the basal membrane, which faces the *space of Disse* and whose surface is considerably increased by a multitude of microvilli dipped in the

plasma oozed from the sinusoids. The basal membrane is dedicated to the exchange between parenchymal cells and the sinusoids, as the absence of a basal lamina in both of them facilitates the entrance of several molecules carried by the blood in the hepatocytes and the release of modified products (Barritt et al., 2008)(Alberts et al., 2015) (Figure 2).

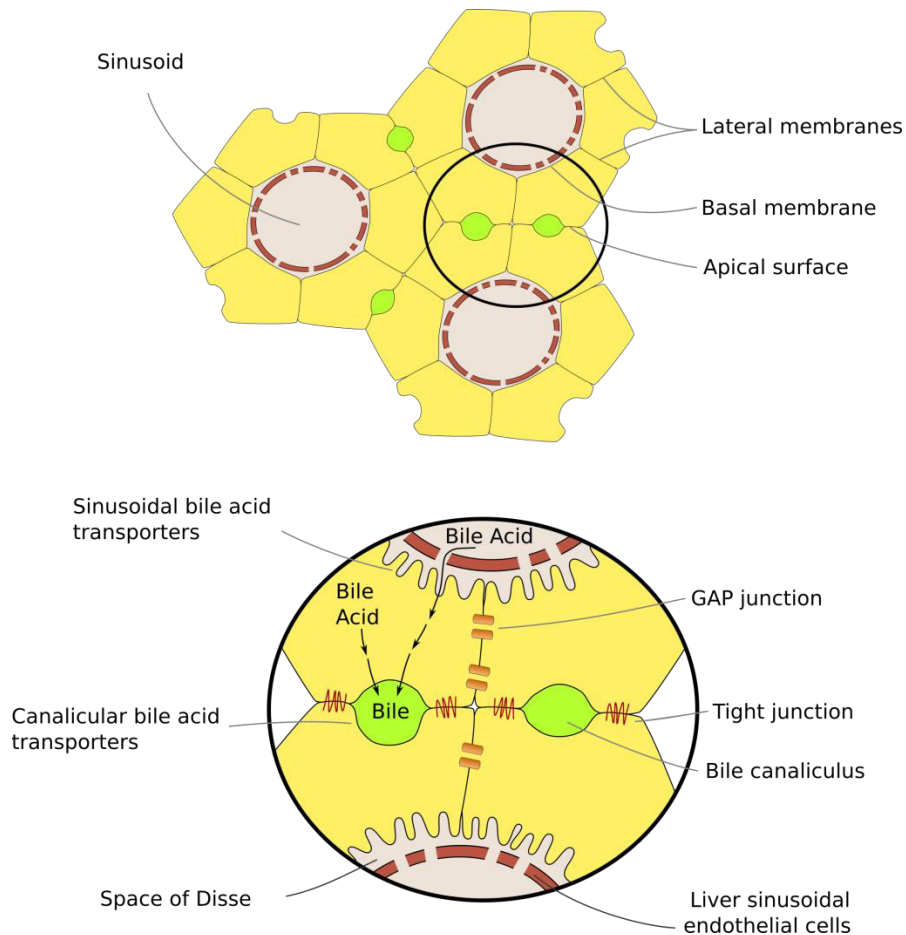


Figure 2 Schematic representation of the interconnection among hepatocytes. Each cell is located in the interface between bile canaliculi and sinusoids and, therefore, highly polarized. Lateral membranes line the connection between different hepatocytes, which communicate through gap junctions. The apical surfaces face the *bile canaliculi* and present tight junctions. The basal membranes allow direct contact with sinusoidal blood and their surface is increased by the presence of microvilli. Adapted from (Barritt et al., 2008)

The regulation of the substances entering and leaving the hepatocytes is regulated by the plasma membrane in many different ways. Its molecular structure includes membrane proteins that work as receptors for hormones, such as insulin and glucagon, or for other substances, for example the immunoglobulin A (IgA). When the access and exit from the parenchymal cells is not guaranteed by receptors, endocytosis or exocytosis, substances cross the membrane by taking advantage of carriers and protein channels. For example, glucose rapidly passes through the hepatocyte plasma membrane by means of transporters and, to prevent diffusion out of the cell, it is very efficiently converted in glucose-6-phosphate which can be used in glycogen synthesis. Whenever glycemia drops, parenchymal cell are able to break down the glycogen stored in the form of cytoplasmic granules and release glucose in the blood (Cardell and Cardell, 1990).

The role of Ca^{2+} -permeable channels present on the plasma membrane has been also extensively investigated, as intracellular Ca^{2+} concentration plays a pivotal role in the indirect regulation of a variety of hepatocyte functions. As a matter of fact, the metabolism of glucose, amino acids and fatty acids, the synthesis and secretion of proteins, the intracellular vesicular movement and even cell proliferation, apoptosis and necrosis depend on variations in the cytosolic concentration of Ca^{2+} (Barritt et al., 2008), which regulates also the excretion of bile and its movement along the *canaliculi* (Nieuwenhuijs et al., 2006). The movement of bile acids, Na^+ and other components of bile fluid across hepatocytes membrane changes its membrane potential and creates osmotic forces, which could detrimentally alter cellular volume if a control mechanism of the movement of ions did not take place. Remarkably, Ca^{2+} regulates the activity of a multitude of plasma membrane ion channels (Fitz and Sostman, 1994). According to the type of channel activated, the effects of changes in cytosolic Ca^{2+} on membrane potential can be variegated. For example, the activation of K^+ channels is fundamental to recover hepatocyte swelling upon nutrient uptake (Barfod et al., 2007). Therefore, the modulation of cytosolic Ca^{2+} concentration plays an important role in regulating hepatocyte membrane potential and cell volume.

Hepatocyte: a deeper look inside the cell

Hepatocyte main functions are performed by the concerted action of multiple cellular organelles highly abundant in the cytoplasm of hepatic parenchymal cells. Among them the ribosomes, which can be found both freely in the hepatocyte cytoplasm or associated to the rough endoplasmic reticulum (RER), the smooth endoplasmic reticulum (SER), without ribosomes, and the Golgi complex.

Free ribosomes and membrane-bound ribosomes are structurally and functionally identical, but they differentiate in the proteins they synthesize. The ribosomes associated to the endoplasmic reticulum are involved in all the proteins destined to be translocated through the ER and then exported, while the mature ribosomes free in the cytoplasm are implicated in the translation of all the other polypeptides, both secreted and destined to remain within the cell (Alberts et al., 2015).

Being highly specialized in protein synthesis and managing the translation of the 95% of the proteins secreted by the liver, hepatocytes have a well-developed RER where not only the synthesis of exported proteins takes place, but also the translation of lysosomal and membrane-associated enzymes (Devi, 2018).

The ER compartment lacking ribosomes, the SER, is implicated in carbohydrate metabolism and it also participates in the regulation of blood glucose levels. Indeed, the presence of glucose-6-phosphatases bound on its membrane enables the hepatocytes to release glucose in the blood upon gluconeogenesis or breakdown of the glycogen stored in the cytoplasm (glycogenolysis) (Bánhegyi and Mándl, 2001). Moreover, the SER is provided with enzymes involved in the synthesis of components of the bile, bilirubin glucuronide and bile acids, essential for a correct digestion of food, especially fat, for the neutralization of the food bolus in the stomach, for the elimination of metabolic wastes and for the control over excessive proliferation of exogenous bacteria in the gut (Bévalot et al., 2016). Most importantly, the SER hosts the cytochrome P450 enzymes involved in drugs and poison detoxification, and it is also the organelle where lipid and cholesterol synthesis take place, providing constituents for all the cell compartments, and where the proteins synthesized in the RER are assembled with lipids to originate the lipoproteins exported in the blood (Schwarz and Blower, 2016). Finally, the SER represents an intermediary compartment for

products that need to be further processed and modified in the Golgi complex, extensively developed in the hepatocytes due to the high rate of protein synthesis.

Similarly to the SER, peroxisomes also participate in detoxification, as they are equipped with enzymes involved in the metabolism of alcohol and toxic metabolic wastes. They constitute approximately 2% of the total hepatic proteins, making the liver the most peroxisome-rich organ in mammals (Leighton et al., 1968). They have been shown to participate in many critical metabolic processes such as fatty acid α - and β -oxidation, which leads to the production of acetyl coenzyme A available for biosynthetic reactions, to the degradation of purines, polyamines, amino acids and uric acid. Moreover, they are involved in lipid biosynthesis, containing enzymes necessary for the synthesis of plasmalogens (ether phospholipids important for the cellular membranes of some tissues) and for cholesterol-derived bile acids formation, which is a very unique function of hepatic peroxisomes (Wanders and Waterham, 2006)(Islinger et al., 2010).

Considering the extraordinary metabolic activity of the liver, it does not surprise that another abundant component of the hepatic parenchymal cells is constituted by mitochondria, which occupy over 20% of cellular volume. Mitochondria are the energetic factory of the cell, providing most of hepatocyte's ATP and also other essential resources for biosynthesis and cell growth. In fact, they contain a large number of enzymes involved in a variety of metabolic activities including ATP synthesis and transport, oxidative phosphorylation, tricarboxylic acid cycle (TCA or Krebs cycle) and pyruvate and fatty acid oxidation. One of their most remarkable hepatocyte-specific functions is to carry out two critical steps of the urea cycle, which is an essential mammal's metabolic pathway destined in eliminating the ammonia produced from nitrogen-containing compounds (for example amino acids) through the urine (Alberts et al., 2015). They are also involved in the first steps of bile synthesis by forming the bile acid intermediate DHCA ($3\alpha,7\alpha$ -dihydroxycholestanic acid) through a cytochrome P450 oxidase (specifically the sterol 27-hydroxylase) present in their inner membrane (Ferdinandusse and Houten, 2006).

The intracellular organization of hepatocytes, as well as their shape and movements, depends on a dynamic and adaptable system of filaments forming the cytoskeleton, composed essentially by microfilaments (made of actin), intermediate filaments and microtubules (made of tubulin). Both actin and tubulin are implicated in the intracellular motility. Microtubules are also described to participate in mitosis, cell shape determination,

intracellular transport of vesicles and secretion of albumin and lipoproteins. Microfilaments in hepatic parenchymal cells have been demonstrated to be very abundant around the *bile canaliculi* as they seem to play an active role in controlling their dilatation and contraction (Kawahara and French, 1990)(Watanabe et al., 1991).

Liver regeneration

A great emblem for regenerative medicine, as detailed by Carl Power and Rasko John (Power and Rasko, 2008) and others, is the Prometheus myth, a Greek ancient story telling about how Zeus punished Prometheus for having stolen the sacred fire from the gods and having donated it to the mankind. He was chained by the god on a rock in the Caucasus Mountains and every day an eagle would feed on his liver, organ which would have regrown overnight, condemning him to eternal punishment. It is hard to believe that ancient Greeks could actually know about this great self-repairing hepatic capacity, as it was firstly scientifically described not more than a century ago (Higgins and Anderson, 1931). Nevertheless, indeed one of the most remarkable characteristics of the liver is its ability to regenerate its volume upon tissue loss.

In physiological conditions adult hepatocytes rarely divide, being only 1-2% of them in the cell cycle at a given time, while the rest remains in a quiescent state (or phase G₀ of cell cycle), showing an estimated lifespan of approximately 400 days in adult rat liver (Grisham, 1962).

Quiescence is progressively acquired during neonatal hepatocyte maturation. However, a chemical injury to the liver or a partial hepatectomy (PH), i.e. the surgical removal of the 75% of total hepatic mass, is able to trigger their entrance in the cell cycle and the initiation of compensatory proliferation mechanisms (Berasain and Avila, 2015).

This peculiar biological process arose questions regarding the underpinnings of the maintenance of cellular homeostasis in the liver. The initial model proposed was named “streaming hypothesis” and described the liver as a “slowly renewing cell population”, where hepatocytes located at the portal space had the greatest proliferation capabilities and their progeny would gradually stream towards the hepatic vein, where they would be finally eliminated by apoptosis. Therefore the entire hepatic lobule would result from this periportal cell population (Zajicek et al., 1985). Although initially supported, this hypothesis

had been recently disputed (Stanger, 2015). It is now believed that in physiological conditions the replication of existing cells is responsible for the maintenance of hepatic cellular population, while progenitor cells show very little involvement in liver remodeling. Liver regeneration can happen following two different situations: (i) hepatic injury caused by toxins or viral infection, characterized by the damage of all the hepatocytes and the consequent differentiation of oval cells, considered as liver stem cells, in both parenchymal and biliary cells; (ii) PH, which triggers the entrance of all the remaining diploid hepatocytes into the cell cycle in a synchronized manner to replace the missing liver tissue (Sun and Irvine, 2014)(Tao et al., 2017). Upon liver resection, virtually all hepatocytes exit quiescence and divide until their original cell number in the hepatic tissue is restored. Interestingly, this regenerative process stops when the species-specific liver to body mass ratio is recovered (Michalopoulos and DeFrances, 1997). This leads to the hypothesis of the presence of a liver/body mass ratio master regulator, which has been called “hepatostat” and whose nature is still under investigation (Michalopoulos, 2010).

Liver regeneration: the pathways involved

Liver regeneration is a complex biological mechanism primarily involving hepatic parenchymal cells, but which also needs the participation of other liver cell populations and at least three interconnected networks (cytokine, growth factor and metabolic) that act at different timings (Fausto et al., 2012). Three critical steps can be identified in this process: the *priming phase*, when cells exit the G0 phase to enter G1; the *proliferation phase*, during which hepatocytes progress towards the mitotic phase with the help of mitogens; the *termination phase*, when cell proliferation is blocked by negative factors (Tao et al., 2017). (Figure 3)

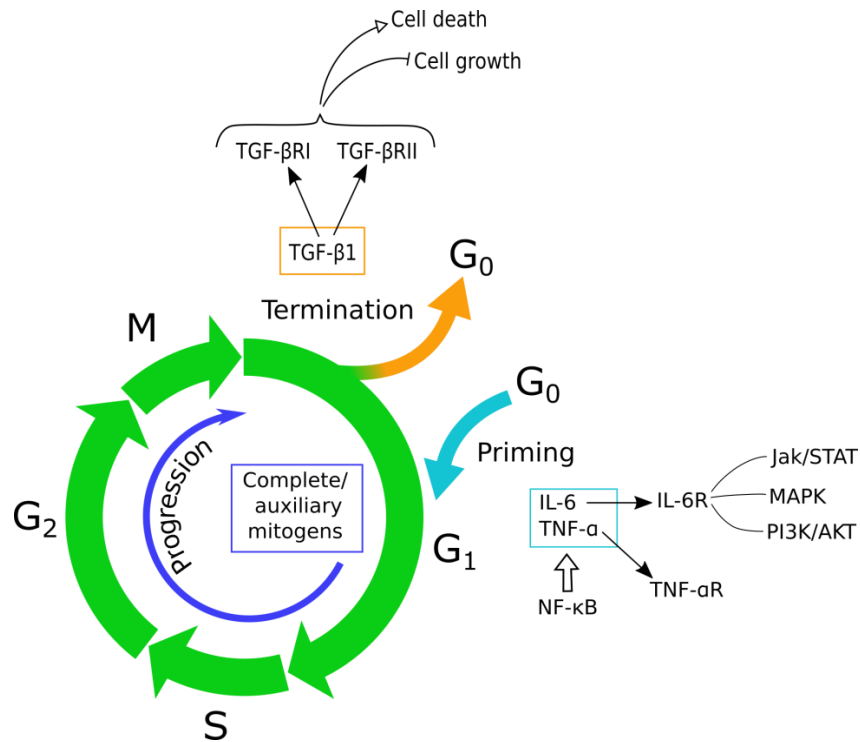


Figure 3 Liver regeneration is defined by three critical steps: 1) cells exit quiescence during the *priming phase*, triggered mostly by IL-6 and TNF- α ; 2) during the *progression phase*, cells transit from G₁ to mitosis thanks to the action of complete mitogens, especially HGF, EGF, TGF- α and HB-EGF, and the additional action of auxiliary mitogens, which increase or accelerate their effect; 3) the *termination phase* is induced once the liver/body ratio is restored and leads cells to re-enter quiescence especially through the action of TGF- β and other members of the same family. Adapted from (Tao et al., 2017)

o The priming phase

Liver damage and the loss of parenchymal cells initiate an inflammation response together with the release of cytokines and growth factors, especially tumor necrosis factor- α (TNF- α) and interleukine-6 (IL-6), by inflammatory cells. Kupffer cells, the hepatic macrophages, are essential actors in the priming of hepatocytes to re-enter the cell cycle. They are indeed the major source of these cytokines through the activation of the NF- κ B signaling pathway, which is initiated either by gut-derived factor lipopolysaccharide (LPS)/Toll-like receptor 4 (TLR4) signaling or by C3a and C5a, components of the complement system (Kang et al., 2012).

IL-6 is a pleiotropic cytokine secreted upon LPS stimulation and the binding to its receptor (IL-6R) leads to the Jak/STAT, MAPK, and PI3K/AKT activation. By assessing both cytoprotective and mitogenic functions, IL-6 expression is extremely important for the entire regenerative process, from its initiation to maintenance and progression (Fujiyoshi and Ozaki, 2011)(Schaper and Rose-John, 2015).

- The proliferation phase

This phase, also called *progression phase*, is characterized by the action of different molecules in the transition of the hepatocytes from the phase G1 of cell cycle to mitosis.

The molecules called *complete mitogens* show hepatotrophic effects, which means they are able to regulate specific genes' expression which stimulates DNA synthesis and cell proliferation. The most relevant are hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor- α (TGF- α) and heparin-binding EGF-like growth factor (HB-EGF). They control hepatocyte growth through the Ras-MAPK and the PI3K/AKT signaling pathways, renowned for their regulative role in cell cycle, by binding to their corresponding receptor c-Met or EGF receptor (EGFR) (Hong et al., 2000)(Mao et al., 2014). HGF was the first paracrine factor discovered to be implicated in hepatocyte proliferation through RAS activation, leading to prolonged MAPK activity, and PI3K/AKT activation (Nakamura et al., 1989). It is produced by stellate cells and released into the extracellular space, where it gets in contact with the hepatic parenchymal cells and its receptor c-Met, or hepatocyte growth factor receptor (HGFR), exposed on their membrane. The binding of c-Met with its ligand leads to the dimerization of the receptor and, subsequently, the activation of the aforementioned pathways, responsible for the increase in DNA synthesis and for cell cycle progression (García-Vilas and Medina, 2018).

The so-called *auxiliary mitogens* normally contribute to liver regeneration by increasing or accelerating the effects of the *complete mitogens*. Among them: (1) bile acids, which stimulate FoxM1b, a key modulator of cell cycle; (2) norepinephrine, which augments the effect of EGF and HGF and, through the activation of Smad7, it counteracts the growth inhibition of hepatocyte played by activin A; (3) vascular endothelial growth factor (VEGF), enhancer of sinusoidal endothelial cell and hepatocyte proliferation after PH; (4) insulin, which may contribute through the activation of inositol 1,4,5,-trisphosphate- (InsP3-)

dependent Ca^{2+} signaling pathways; (5) insulin-like growth factor-1 (IGF-1), which positively regulates HGF while downregulating TGF- β , an inhibitor of proliferation; (6) estrogen, which might sustain cell growth through the estrogen receptor alpha (ER α) and probably by a crosstalk with IL-6; (7) serotonin, contributing to liver regeneration via HT receptor 2 (HTR2) (Tao et al., 2017). Blood platelets have also been recently described to positively participate to hepatic re-growth by releasing HGF, VEGF, IGF-1 and serotonin (Meyer et al., 2015).

The Wnt/ β -catenin signaling pathway seems also to be involved the *proliferation phase* of liver regeneration. Wnt ligands, glycoproteins produced and secreted especially by Kupffer cells and endothelial non-parenchymal cells, lead to the expression of target genes important for the regulation of cell proliferation, such as c-myc and cyclinD1, through the classic wnt/ β -catenin signaling cascade (Monga, 2011).

Finally, the last category of *auxiliary mitogens* is constituted by exosomes, cell-derived nanovesicles produced in the cellular endosomal compartment and involved in intercellular communication. It has been recently described that hepatocyte-derived exosomes fuse with target hepatocytes and stimulate their proliferation. In fact their exosomes deliver sphingosine kinase 2 (SK2), which catalyzes the phosphorylation of sphingosine in target hepatocytes, thus forming sphingosine-1-phosphate (S1P) and promoting cell survival, growth and migration (Nojima et al., 2016).

o The termination phase

The hepatic regeneration process stops when the normal liver to body mass ratio of 2.5% is restored. At present, the most characterized antiproliferative factors are the transforming growth factor- β (TGF- β) and other members related to the same family.

TGF- β , specifically TGF- β 1, is active in the phase G1 of the cell cycle and, through the binding of its type I receptor (TGF- β RI) and type II receptor (TGF- β RII), it promotes both the block of cell growth and the induction of cell death both in vivo and in vitro (Romero-Gallo et al., 2005).

TGF- β 1 not only is expressed by hepatic cell populations, but also from extrahepatic tissues, for example by platelets (Katsuda et al., 2017) and spleen. The latter has also been proven to downregulate HGF and its receptor c-met, thus increasing its inhibitory effect on liver regeneration (Lee et al., 2015).

Finally, among the TGF- β family members, a relevant role is played by activin A, an activin subtype which was found to play as a negative regulator of hepatic regeneration by inducing cell growth arrest and apoptosis both in vitro and in vivo. It also decreases the production of fibronectin, a component of the extracellular matrix essential for liver regeneration (Date et al., 2000)(Takamura et al., 2005).

Liver-related diseases

Many liver-associated diseases had been described and their appearance and incidence depend on a variety of factors.

Non-alcoholic fatty liver disease (NAFLD), or hepatic steatosis, is one of the most studied and it is defined by the accumulation of lipids in the hepatocytes not due an excessive alcohol consumption. The presence of > 5% of *steatotic* hepatocytes, term referring to the accumulation of fat in the form of triglycerides, is considered as the minimum condition for the diagnosis of NAFLD and results in cellular inflammation, which can degenerate in worst forms of steatosis. Indeed, approximately the 10% of patients with NAFLD progress to non-alcoholic steatohepatitis (NASH), accompanied by hepatocellular ballooning, apoptosis and lytic necrosis. Chances are that the 25-30% of the those people further progress to liver fibrosis or even cirrhosis and, eventually, 10% of them will develop in several years hepatocellular carcinoma (HCC) (Ong and Younossi, 2007)(Farrell and Larter, 2006). (Figure 4)

The lipid accumulation typical of steatosis is considered to be mainly due to insulin resistance (IR) (Buzzetti et al., 2016). More specifically, IR would contribute to NAFLD development through two principal mechanisms: (i) the reduced suppression of the hormone-sensitive lipase (HSL) by insulin, characteristic in IR patients, would result in an increased triglycerides hydrolysis in the peripheral adipose tissues, leading to the augmentation of free fatty acids (FFAs) delivered to the liver and a subsequent massive esterification of FFA in triglycerides, thus causing steatosis; (ii) de novo lipogenesis (DNL), which accounts for 26.1% of the hepatic triglyceride formation in the livers of NAFLD subjects, against the 5% in healthy individuals, had been shown to be stimulated by IR through different pathways (El Idrissi et al., 2019).

The biological process responsible for the progression of NAFLD to NASH is still under investigation. An initial “two-hit” theory considered the increase in circulating FFAs, responsible for triglycerides accumulation in the hepatocytes, as the triggering event. The “second-hit” was a source of oxidative stress, such as mitochondrial or adipose tissue dysfunction, which would lead to lipid peroxidation, cell inflammation and the appearance of NASH (Day and James, 1998). However, currently the pathogenesis of NASH is considered as a complex, most likely “multi-hit”, process characterized by the additional effect of a multitude of events aside of the presence of classical steatosis (Buzzetti et al., 2016).

As mentioned before, the detrimental progression of NAFLD can ultimately result in cirrhosis. However, the strongest risk factor of hepatic cirrhosis is considered to be the abuse of alcohol, accompanied by hepatitis C virus (HCV) and hepatitis B virus (HBV) – respectively in developed countries, and in Asia and sub-Saharan Africa – infection (Detlef and Nezam, 2008). Cirrhosis is an advanced stage of liver fibrosis characterized by defects in the hepatic vascular system, leading to the funneling of the portal and arterial blood directly to the central veins, bypassing the exchange between sinusoids and hepatocytes. This particular condition is driven by the so-called sinusoidal capillarization, the transformation of the hepatic open circulation in a closed one: endothelial fenestration disappears and the *space of Disse* is substituted by scar tissue, resulting in fibrotic septa surrounding hepatocyte islands (Schaffner and Popper, 1963). The principal clinical consequences of cirrhosis are a malfunctioning of the hepatocytes, therefore impaired liver functions, portal hypertension and, in the worst circumstances, regenerative nodules resulting from an increased hepatocyte proliferation and that will eventually lead to HCC development (Detlef and Nezam, 2008).

HCC comprises the 70-90% of primary liver cancer, and it is associated with hepatic cirrhosis in more than 80% of the cases (Blachier et al., 2013). However, although quite unusual, its appearance can also take place in absence of advanced liver fibrosis or cirrhosis. The 15% of these events involve cases related to HBV infection (Kulik and El-Serag, 2019), while in the United States it seems to be mainly connected to the presence of liver steatosis (Mittal et al., 2016). Diabetes and obesity have been also associated to an increase in HCC incidence independently from NAFLD, being type 2 diabetes (T2D) coupled with a 2- to 3-fold increase risk of HCC (El-Serag et al., 2006).

A possible explanation for the involvement of NAFLD, obesity and T2D in HCC development, can be found in their close relationship with inflammation, increased oxidative stress and insulin resistance (Firneisz, 2014). The latter, in particular, can be easily linked to the uncontrolled cell growth and division characteristic of all the forms of cancer.

A chronic exposure of the hepatic tissue, adipose tissue and the infiltrated immune cells to an excess of lipids and other metabolites, causes a dysregulation in hormone and cytokine production, which can result in insulin resistance and lead to hyperinsulinemia. The latter two, in turn, are responsible for an increased expression of pro-inflammatory cytokines, such as TNF- α and IL-6, and are associated with an increase in IGF-1, already highlighted for its positive role in controlling “the proliferation phase” during liver regeneration. The resulting increase in cell proliferation and angiogenesis, and the reduction in apoptosis, is considered to strongly participate to the development of HCC and can thus explain the involvement of the pathological conditions aforementioned (Singh et al., 2018).

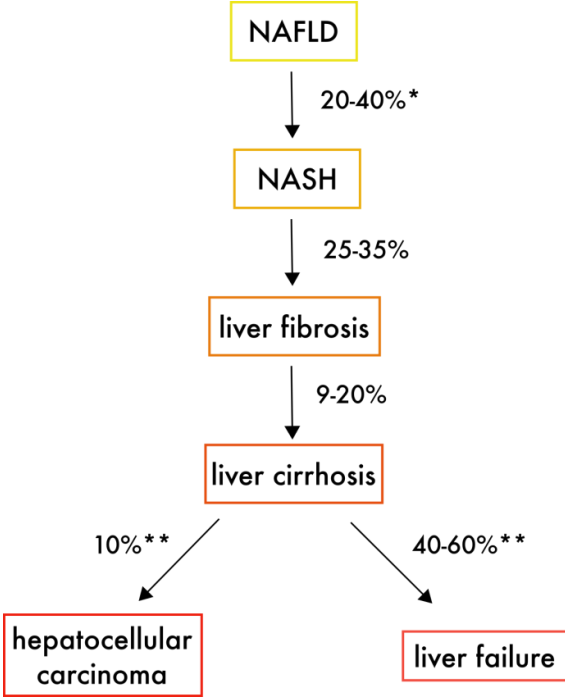


Figure 4 Schematic representation of the natural progression of non-alcoholic fatty liver disease (NAFLD). NASH non-alcoholic steatohepatitis. *In 3–6 years of follow-up **In 5–7 years of follow-up. Adapted from (El Idrissi et al., 2019)

Liver metabolism

As already mentioned at the beginning of this overview, the liver plays a crucial role in regulating the organismal homeostasis, as it represents the headquarters of most of the metabolic activities and it regulates the storage of nutrients, their breakdown, their synthesis and redistribution to other tissues.

Upon every meal, food is digested in the gastrointestinal tract, nutrients are adsorbed into the blood and transported to the liver through the portal vein. Glucose increase in the blood stimulates insulin secretion from the pancreas, which induces glucose storage in the liver through glycogenesis. Furthermore, in the hepatocytes glucose can be converted in amino acids (aa) or in fatty acids (FA). Aa are used as source of energy or to synthesize proteins and other bioactive molecules. FA and free fatty acids (FFAs) coming from the blood circulation are esterified in triacylglycerol (TAG) and either stored as small droplets or released in the bloodstream in the form of very low-density lipoproteins (VLDL).

When glycemia drops in the fasted state, glucagon is secreted from the pancreas and induces the hepatocytes to release glucose in the blood through the breakdown of glycogen (glycogenolysis). Moreover, lactate and alanine, produced respectively through glycogenolysis and protein catabolism in the muscle, and glycerol generated by adipose tissue through lipolysis of TAG, are delivered to the liver and are used as precursors for gluconeogenesis.

Therefore, we can also consider the liver as a hub metabolically connecting a multitude of important tissues, such as intestine, pancreas, adipose tissue and skeletal muscle, to guarantee a prompt management of nutrients requirement upon any condition.

Liver glucose metabolism

One of the main functions of the liver is to maintain a physiological glycemia by compensating its fluctuations in the fed and fasted state (Daly et al., 1998)(Klover and Mooney, 2004). This feature is possible thanks to the remarkable capacity of hepatocytes to balance between consumption and production of glucose (Mithieux, 2010). Glucose entrance inside the cell is mediated by members of the glucose transporter (GLUT) family,

transmembrane proteins encoded by the SLC2 gene and with a multiple substrate specificity which includes many hexoses. Each body cell type is provided with at least one member of the GLUT family, which in the hepatic parenchymal cells is mostly represented by GLUT2 (Mueckler and Thorens, 2013). GLUT2 has the very peculiar characteristic of showing low affinity for glucose but high capacity (Williams et al., 1968), thus guaranteeing glucose uptake at physiological conditions. Although GLUT2 is necessary for glucose entrance in the hepatocytes, the release of this monosaccharide does not rely only on its presence, but also on the existence of alternative glucose transporters and mechanisms (Guillam et al., 1998). Once inside the cell, glucose is converted in glucose-6-phosphate (G6P) by *glucokinase* (GCK) (Cárdenas et al., 1998), which cannot exit the hepatocytes, and as such takes part to different biochemical pathways according to the organismal needs. (Figure 5)

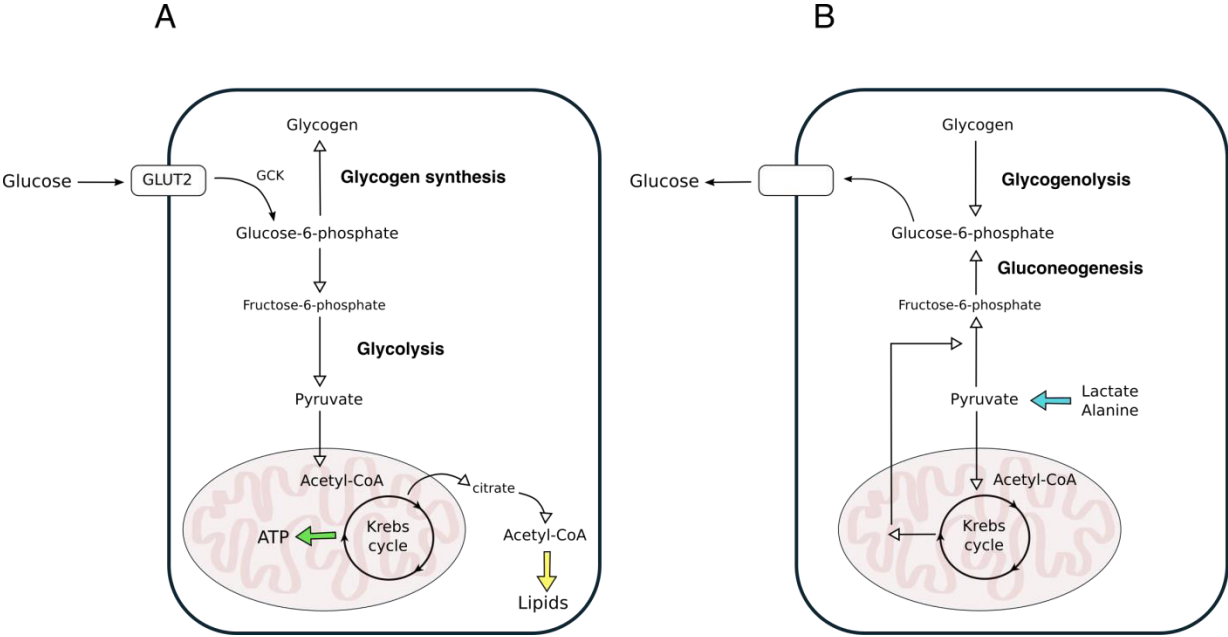


Figure 5 Simplified scheme of glucose metabolism. (A) Principal biochemical pathways acting in the postprandial phase to consume and store glucose; (B) Principal biochemical pathways in the postabsorptive glucose production: lactate and alanine are used as gluconeogenic substrates upon their conversion into pyruvate, while glycogen chains are broken down to release glucose units in the form of glucose-1-phosphate, to be then converted in glucose-6-phosphate. Adapted from (Oosterveer and Schoonjans, 2014)

Gluconeogenesis

Hepatic glucose production represents the 90% of the endogenous glucose synthesis and, in humans, half of it relies on gluconeogenesis (Petersen et al., 2017).

The principal gluconeogenesis precursors are lactate, glycerol and amino acids, which can be either acquired from extrahepatic tissues through the blood circulation or synthesized inside the liver. Lactate, originating upon intense exercise activity, derives from the muscle through enzymatic conversion of the pyruvate produced from glucose, and takes part to the Cori cycle (or lactic acid cycle). In a very simplified model, once in the liver lactate is converted in pyruvate first, and then back to glucose using the glycolytic enzymes in reverse except for three irreversible reactions, whose enzymes (*pyruvate kinase*, *phosphofructokinase-1* and *glucokinase*) are bypassed and replaced by others (*pyruvate carboxylase* and *phosphoenolpyruvate carboxykinase*, *fructose-1,6 bisphosphatase*, *glucose-6-phosphatase*). More in details, lactate is delivered to the hepatocytes and oxidized by *lactate dehydrogenase* (LDH) in the cytoplasm. The resulting pyruvate is then exported in the mitochondria and, through a sequence of biochemical reactions, returns to the cytosol when is finally converted in oxaloacetate. This can be then decarboxylated and phosphorylated by the sequential action of *pyruvate carboxylate* (PC) and *phosphoenolpyruvate carboxykinase* (PEPCK) to originate phosphoenolpyruvate (PEP), a crucial intermediate in biochemistry for its high-energy phosphate bond. PEP undergoes several biochemical reactions that lead to its conversion in fructose-6-phosphate (F6P) and finally in G6P, which can be dephosphorylated by *glucose-6-phosphatase* (G6Pase) to glucose in the ER (Rui, 2014).

The primary carbon skeleton used for gluconeogenesis can be also derived from glycerol, whose contribution for endogenous glucose production ranges between 3%-22%, depending on the increase in fasting duration (Consoli et al., 1987). Glycerol is released by adipose tissue following lipolysis of TAG and it enters the hepatocyte membrane through the aquaporin-9 (Jelen et al., 2012). Once inside the cell, it is phosphorylated in glycerol-3-phosphate (G3P) and it is finally converted in the gluconeogenic intermediate dihydroxyacetone phosphate (DHAP) (Han et al., 2016). In addition to glycerol, the increased lipolysis in white adipose tissue during fasting also produces non-esterified fatty acids (NEFAs). NEFAs cannot be used as gluconeogenic substrates, but the acetyl-CoA derived from their β -oxidation in the hepatocyte mitochondria is an allosteric activator of PC,

therefore it contributes to the increase in oxaloacetate production and the activation of gluconeogenesis (Adeva-Andany et al., 2016).

Amino acids also take part to gluconeogenesis as they can be easily converted in α -ketoacids through deamination reaction and then enter the Krebs cycle as intermediates (for example pyruvate, oxaloacetate, succinyl-CoA and α -ketoglutarate) (Rui, 2014). However, among healthy individuals, alanine is the principal source of gluconeogenic amino acid during the fasting state. Following the degradation of amino acids in the skeletal muscle, in case of energy needs, the resulting nitrogen is transaminated to pyruvate by *alanine aminotransferase* (ALT) and forms alanine, which is then released and reaches the hepatic parenchymal cells, taking part to the Cahill cycle (or glucose-alanine cycle) in a very similar way to the Cori cycle. Within the liver, alanine is in fact converted back to pyruvate and can be used as gluconeogenic substrate (Adeva-Andany et al., 2016).

Glycogen metabolism: glycogenesis and glycogenolysis

Glycogen is a large multi-branched polymer of glucose used as the main energy storage in the in the liver and muscle. Its concentration changes in response to the organismal energy demands and it is regulated by the action of two key enzymes: *glycogen synthase* (GS) controls glycogenesis by inducing the elongation of glycogen chains in the feeding state; *glycogen phosphorylase* (GP) breaks glycogen polysaccharide chain and releases glucose units in the form of glucose-1-phosphate during the fasting state (glycogenolysis) (Rui, 2014).

Both glycogenesis and glycogenolysis are tightly regulated by various complex mechanisms, included post-translational modifications. For example, both GS and GP are controlled through reversible protein phosphorylation: *glycogen synthase kinase 3* (GSK-3) inhibits GS by phosphorylation and, conversely, shows an opposite effect on GP activity. The action of GSK-3 can be reversed for both enzymes by *protein phosphatase 1* (PP1) (Rui, 2014). However, glycogen synthesis is commonly considered to be mainly suppressed by glucose and activated by insulin (Petersen et al., 1998). After a meal, the increase in glycemia stimulates insulin secretion, which induces glycogen synthesis through a double mechanism. On one side it activates Akt, which in turn phosphorylates and inactivates GSK-3,

thus preventing it to phosphorylate and inhibit GS. Additionally, it stimulates the acetylation of GP, hence favoring its dephosphorylation by PP1 and consequently suppressing its glycogenolytic activity (Zhang et al., 2012). Moreover, insulin stimulates GK expression, leading to an increase in G6P which, a part from being a precursor for glycogen synthesis, is also both an allosteric activator of GS and an allosteric inhibitor of GP (Agius, 2008). In fasting condition insulin is not secreted, GSK-3 is active and can exert its inhibitory role over GS activity. Instead, hypoglycemia stimulates the secretion of glucagon by pancreatic α cells. In the liver glucagon, together with epinephrine, regulates a cascade of events culminating in the conversion of GP in its phosphorylated active form. Glucagon can therefore enhance glycogen breakdown and promote glucose release in the blood (Han et al., 2016).

Glycolysis

Glycolysis, a crucial metabolic pathway for glucose catabolism and production of energy, is induced in the fed state, when glucose in the body is abundant.

The glycolytic process takes place in the cytoplasm and is governed by three critical rate-limiting enzymes, all regulated by allosteric mediators: GCK (also called *hexokinase IV*) converts glucose in G6P; *phosphofructokinase-1* (PFK-1) converts fructose 6-phosphate (F6P) in fructose 1,6-bisphosphate (F1,6P); *liver-type pyruvate kinase* (L-PK) converts PEP into pyruvate (Pilkis and Claus, 1991).

GCK, expressed both in the liver and in the pancreatic β cells, acts as a glucose sensor. Contrary to other hexokinase isotypes, its activity is not inhibited by its catalytic product, meaning that the liver is able to keep using glucose as the main source of energy independently on the increase of intracellular concentration of G6P, such as during feeding condition. GCK activity in the liver is controlled by glucokinase regulatory protein (GKRP), exclusively expressed in the hepatic tissue. In low glucose concentration, F6P enhances the binding between GKRP and GCK leading to its sequestration in the nucleus, thus inhibiting its action. When during the feeding state glucose concentration increases, it competes against F6P for the binding and induces the dissociation between GKRP and GCK, which can be therefore translocated in the cytoplasm and assess its function (Agius, 2008)(Brouwers et al., 2015).

PFK-1 can be considered as the enzyme that catalyzes the metabolic reaction responsible for the commitment of glucose to glycolysis. Its activity is allosterically inhibited by ATP and citrate, which are normally associated to energy abundance, while it is activated by fructose 2,6-bisphosphate (F2,6P).

L-PK, the third critical enzyme for hepatic glycolysis, is allosterically activated by the glycolytic intermediate F1,6P and by insulin-mediated dephosphorylation in the fed state. ATP, acetyl-CoA and long-chain fatty acids are, instead, allosteric inhibitors of this enzyme as they normally represent an index of energy abundance in the body. (Pilkis and Claus, 1991).

Apart from the tight regulation of the glycolytic key enzymes through post-translational modifications, glycolysis is also modulated at transcriptional level. Sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP) are the two main transcriptional factors positively regulating the expression of both glycolytic enzyme genes and genes involved in fatty acid biosynthesis, and their transcription is activated during the feeding state (Dentin et al., 2005).

Liver fatty acid metabolism: from lipogenesis to fatty acid oxidation

In the postprandial phase, when carbohydrates are abundant, hepatocytes are able to store energy not only in the form of the already discussed glycogen particles, but also as fatty acids, which are promptly esterified with G3P to generate triacylglycerol (TAG) and accumulated in the cytosol or released in the bloodstream in the form of VLDL. Fatty acids (FA) are used as fuel for muscular contraction, as components of phospholipids, which forms the cellular membranes, and for general metabolism, as their catabolism releases a large amount of energy expendable for a multitude of biochemical reactions. They can be either synthesized by the hepatocytes starting from glucose (*de novo* lipogenesis - DNL), they can originate from non-esterified fatty acids (NEFAs) released by adipose tissues in the blood (free fatty acids – FFA) or they can be separated from the glycerol molecule of the cytoplasmic storage of TAG.

DNL takes place in the cytoplasm of both adipocytes, where it directly contributes to long term fat storage, and hepatocytes, where fatty acids are mainly incorporated in VLDL and delivered to extrahepatic tissues to participate in building membranes by providing both structural components and energy source (Dumon et al., 2008). *De novo* synthesis of fatty

acids can be explained as a series of decarboxylative condensation reactions leading to the elongation of a fatty acid chain, whose synthesis is entirely governed by *fatty acid synthase* (FAS) (Smith et al., 2003). FAS activity is regulated through hormones and nutritional state: insulin and substrate availability activate the enzyme, while glucagon, catecholamines and fatty acids play a negative role on FAS activity (Clarke and Jump, 1996)(Sul et al., 2000)(Wiegman et al., 2003). More in detail, the regulation of lipogenesis by insulin and fatty acids is mediated by transcriptional factors, especially SREBPs, ChREBP and liver X receptors (LXRs). The first two had already been described above for their role in the regulation of glycolysis, in addition to lipogenesis.

NEFAs are taken up by hepatic cells in proportion to their concentration in the blood and can cross cellular membrane either by diffusion or through specific transporters identified as fatty acid transport protein, fatty acid translocase and CD36. Once inside the hepatocytes, long-chain fatty acids (LCFA), characterized by 14 carbons or more, covalently bind fatty acid binding protein (FABP) either directly or after being converted in LCFA-CoA. FABP act as intracellular carrier or chaperones and transport the fatty acids to other cellular compartments or inside the nucleus (Dumon et al., 2008). Mammalian liver expresses a single isoform of fatty acid binding protein, L-FABP, which delivers its load to the nucleus and activates the peroxisome proliferator-activated receptor- α (PPAR α). PPAR α is a specific nuclear receptor and, upon activation, promotes peroxisomal and mitochondrial fatty acid β oxidation (Martin et al., 2003)(Storey et al., 2012), thus contributing to keep intracellular concentration of NEFA and fatty acyl-CoA very low.

Hepatocytes induce fatty acid β -oxidation to produce a large amount of ATP when energy supply is low, for example in fasting conditions. The oxidation of non-esterified acyl-CoA can be induced in the peroxisomes or mitochondria and produces acetyl-CoA, which enters the Krebs cycle, together with NADH and FADH₂, co-enzymes used in the electron transport chain.

Peroxisomal oxidation slightly differentiates from the mitochondrial one: it is responsible for the metabolism of very long-chain fatty acids and its first reaction consists in the oxidation of fatty acid-CoA by *acyl-CoA oxidase*, rather than a dehydrogenation reaction, leading to the formation of H₂O₂ rather than reduced NAD⁺. Furthermore, peroxisomal oxidation results in a smaller amount of ATP produced compared to β mitochondrial oxidation because of the lack of an electron transport chain (Dumon et al., 2008). Regarding the

intramitochondrial oxidation of acyl-CoA, the very first step consists in the translocation of LCFA-CoA in the mitochondria. Since mitochondrial matrix does not contain any ACS enzyme to activate long chain fatty acids, this process is mediated by *carnitine palmitoyltransferase 1* (CPT-1). Medium or short chain fatty acids, on the contrary, can enter the mitochondria in a CPT-independent way and they are then activated by ACS inside the mitochondrial matrix (Kerner and Hoppel, 2000). Mitochondrial oxidation can be either complete, leading to the overall oxidation of acetyl-CoA in carbon dioxide in the TCA cycle, or incomplete, resulting in the formation of ketone bodies in a biochemical process called ketogenesis.

Ketogenesis is induced in the fasting state, when an increase in NEFAs uptake by the liver occurs or when the decrease in insulin levels leads to the upregulation of CPT-1 with a consequent substantial entrance of FA in the mitochondria. This reaction allows the liver to handle the increase in FA uptake as it permits the metabolism of five times more fatty acids than in complete oxidation, even if the quantity of ATP produced is the same. During ketogenesis acyl-CoA is converted in ketone bodies (acetoacetate and β -hydroxybutyrate) released from the liver in the blood circulation and exported to extrahepatic tissues, where acetoacetate and β -hydroxybutyrate can be converted into acetyl-CoA and used as fuel for citric acid cycle, thus providing an essential source of energy during starvation and exercise, especially for tissues such as heart, brain and muscle, but not the liver (Dumon et al., 2008).

Liver amino acid metabolism

The liver synthesizes and secretes the 85-90% of circulating protein bulk. Albumin, crucial for the maintenance of blood volume and for the transport of hormones and lipids, is the most abundant, accounting for the 55% of total plasma proteins. Additionally, the hepatic tissue is responsible for the secretion of acute-phase proteins, the majority of coagulation cascade and fibrinolytic pathway, growth factors and other peptides important for systemic regulation.

Liver also contributes to the whole-body protein homeostasis by participating to the dynamic equilibrium between protein synthesis and degradation. Indeed, it is highly efficient in protein break down and in the metabolism of the resulting amino acids. The latter can

provide substrate for gluconeogenesis by entering the TCA cycle or, coupled with the urea cycle, can generate energy for the hepatocytes to use (Trefts et al., 2017).

Amino acids

Amino acids (aa) are organic substances identified by both amino and acid groups. More than 300 aa exist in nature, but only 20 of them constitute the building blocks of proteins (proteinogenic aa) and are all the L-isomers. Some *non-protein* aa originates from post-translational modification of existing aa, for example ornithine, citrulline and homo-cysteine, and even if they are not incorporated in proteins, are nevertheless relevant for cell metabolism (Wu, 2009).

Depending on their dietary need, aa are traditionally divided in essential aa (e.aa) and non-essential aa (ne.aa). The e.aa cannot be synthesized by the organism or their *de novo* synthesis is not sufficient to meet the body demands, therefore they must be provided with the diet. They include phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine in adult humans, with the addition of arginine, cysteine and tyrosine essential only for infants and growing children. Ne.aa can be synthesized by the body in adequate quantity and in adults comprise arginine, cysteine, glycine, glutamine, proline, tyrosine, alanine, aspartic acid, asparagine, glutamate and serine. Glutamine is also considered to be conditionally essential for neonates in stressful conditions (Wu, 2009). The existence of a homeostatic pool of the 20 proteogenic aa inside the cell is necessary for the correct charge of tRNA molecules for protein biosynthesis, and it partially depends on the regulation of aa passage through the cellular membrane. To fulfill this role, human genome codify for around 50 different aa transporters belonging to at least 11 different families (Bröer and Palacín, 2011). Since the aa concentration in the blood is significantly lower than in the cytoplasm, aa transporters are mainly represented by exchangers and co-transporters instead of uniporters, which would rapidly decrease intracellular aa concentration (Bröer and Bröer, 2017).

In the body the principal storage of both peptide-bound and free aa residues is the skeletal muscle, as it occupies in humans the 40-45% of the entire organism (Davis and Fiorotto, 2009), but the liver remains the main actor in the biosynthesis and degradation of most of

the aa (Cynober, 2002). Ne.aa can be synthesized by a variety of cells mainly through a transamination reaction by *transaminases* (or *aminotransferases*), which catalyze the reversible exchange of an amino-group with a keto-group between an aa and a ketoacid. α -ketoglutarate is the one of the most represented amino-group acceptors and produces glutamate as the new amino acid. Glutamate can be then used either for the synthesis of glutamine through ammonia fixation, or for the synthesis of other aa by transaminase reaction (Spanaki and Plaitakis, 2012). Aa degradation happens instead through deamination, a biochemical reaction responsible for the catabolism of aa when there is an excess of protein intake. The aa whose degradation ultimately leads to the production of glucose are classified as glucogenic, while those directly converted in acetyl-coA are called ketogenic aa (Figure 6).

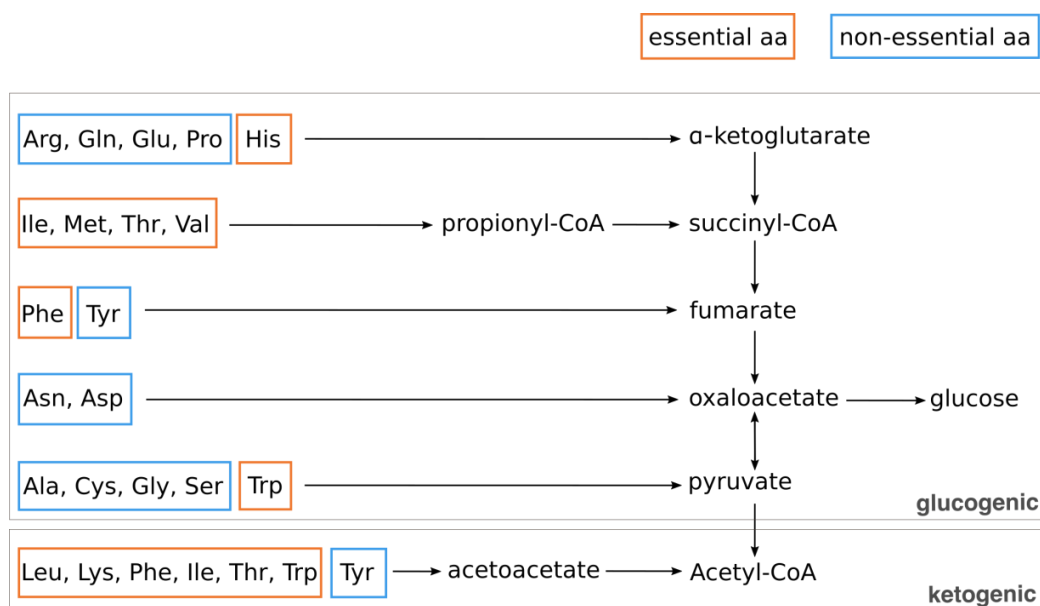


Figure 6 Scheme of essential and non-essential amino acids in human and their classification according to the energetic pathway in which they take part once degraded.

Glucogenic aa: arginine (Arg), glutamine (Gln), glutamic acid (Glu), proline (Pro), histidine (His), methionine (Met), valine (Val), asparagine (Asn), aspartic acid (Asp), alanine (Ala), cysteine (Cys), Glycine (Gly), serine (Ser). Ketogenic aa: leucine (Leu), lysine (Lys). Ketogenic and glucogenic aa: phenylalanine (Phe), isoleucine (Ile), threonine (Thr), tryptophan (Trp), tyrosine (Tyr).

Deamination takes place primarily in the liver and allows the conversion of amino acids into usable resources, such as hydrogen and carbon, through the removal of amino-groups and their detoxification to urea (urea cycle). The catabolism of glutamine, histidine, arginine, ornithine and proline results in the production of glutamate which, in addition to its relevant role in aa synthesis, is also central for the whole-body nitrogen homeostasis as it serves both as nitrogen acceptor and donor. Indeed, in the deamination direction, *glutamate dehydrogenase* (GDH) catalyzes the conversion of glutamate to α -ketoglutarate and ammonia, which provides one of the 2 urea's nitrogen. The second nitrogen may be provided by the aspartate obtained through the action of *aspartate aminotransferase*, responsible for the transfer of an amino-group from glutamate to oxaloacetate. Both these enzymes are reversible, allowing the liver to adjust the relative quantities of ammonia and aspartate according to the urea cycle's needs (Brosnan and Brosnan, 2007). Furthermore, glutamate plays an additional key role in the regulation of the urea cycle as it is necessary for the hepatic production of a rate-controlling enzyme in urea synthesis (Bachmann et al., 1982). Valine, leucine and isoleucine are a specific class of e.aa called branched-chain aa (BCAA) whose catabolism, unlike most of the other aa, does not start in the liver due to the low hepatic activity of their first catabolic enzyme, the *branched-chain-amino-acid aminotransferase* (BCAT). BCAT activity is high in the skeletal muscle where it reversibly catalyzes the transfer of an amino group from the BCAA to α -ketoglutarate, thus originating glutamate and the corresponding branched-chain keto acids (BCKAs). Glutamate is then converted either in alanine or in glutamine and released in the blood together with BCKAs. BCKAs are irreversibly decarboxylated and converted to the corresponding branched-chain acyl-CoA esters by the *branched-chain α -keto acid dehydrogenase* (BCKD), an enzyme located on the inner mitochondrial membrane and highly abundant in the liver. Beyond the BCKD reaction, the metabolism of the BCAA originates both ketogenic and glucogenic products which can serve respectively for protein synthesis and energy production (Holeček, 2018). (Figure 7)

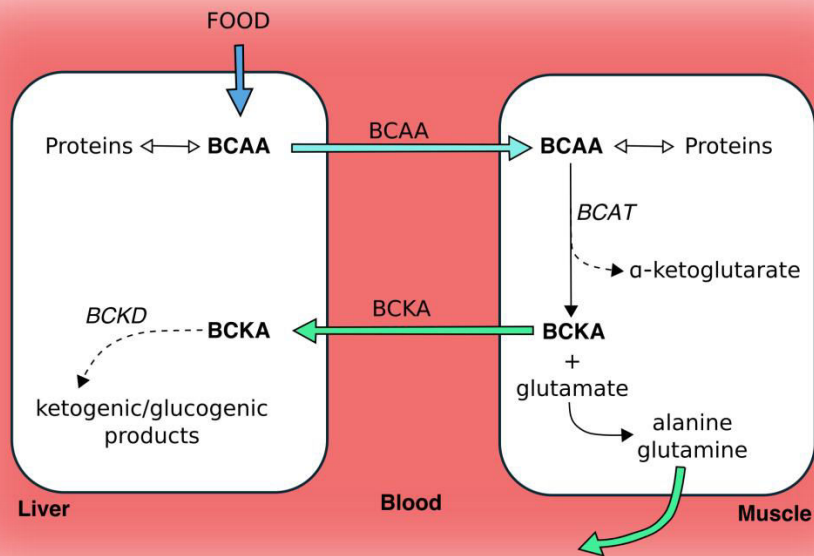


Figure 7 Simplified scheme of the cooperation between liver and skeletal muscle for the catabolism of branched-chain amino acids (BCAA). BCAA coming from food intake or from protein degradation in the liver are exported in the blood and reach the skeletal muscle. There, the *branched-chain-amino-acid aminotransferase* (BCAT) catalyzes the transfer of an amino-group from the BCAA to α -ketoglutarate, resulting in the production of glutamate and the corresponding branched-chain ketoacid (BCKA). The former is further converted to alanine or glutamine and released in the blood together with the BCKA. Back in the liver, the BCKA originates ketogenic or glucogenic products thanks to the action of the branched-chain α -keto acid dehydrogenase (BCKD). Adapted from (Holeček, 2018)

Amino acid regulatory mechanisms

Amino acids not only serve as building blocks for protein synthesis, but they also play a plethora of regulatory roles in cell function and metabolism, such as gene expression, cell signaling, protein synthesis, hormone secretion, oxidative stress and utilization of dietary nutrients, to name a few (Wu, 2009). Furthermore, they are essential actors in the regulation of cell proliferation through the activation of the “mechanistic target of rapamycin” (mTOR) pathway, one of the most developed and crucial nutrient sensing machineries in all eukaryotes. On the contrary, aa deprivation leads to an access of

uncharged aminoacyl-tRNA which activates the general control nonderepressible-2 (GCN2) kinase (Hinnebusch, 2005). GCN2 decreases global protein translation by phosphorylating eukaryotic translation initiation factor 2-A (eIF2A) and indirectly induces expression of stress-response genes through transcriptional factors such as ATF4 (Wek et al., 2006). To summarize, aa deprivation reduces general protein synthesis by activating GCN2 and inhibiting mTOR and these two mechanisms might be subject to cross-regulation (Novoa et al., 2001; Watanabe et al., 2007).

Intracellular amino acid sensing: the mTOR pathway

The TOR protein, originally identified in yeast and only few years later in mammals, is a serine/threonine kinase of around 280 kDa which is inhibited by the action of rapamycin (Koltin et al., 1991)(Sabatini et al., 1994). Since its discovery, the role of mTOR has been extensively studied and associated with the regulation of cell growth, function that is assessed principally through the promotion of anabolic processes, namely protein, lipid and nucleotide synthesis (Howell et al., 2013), while suppressing catabolic pathways such as autophagy (Meijer and Codogno, 2004).

Differently from the yeast, higher eukaryotes have just one *mtor* gene, which nevertheless originates two different complexes: mTOR Complex 1 (mTORC1) and 2 (mTORC2). These two variants only differ for two accessory proteins, respectively Raptor and Rictor, which confer each complex a specific function (Bhaskar and Hay, 2007).

mTORC1 is the most studied complex and it is the only one regulated by amino acid availability. It is generally accepted that the mTORC1 is activated by branched-chain amino acid (BCAA) (valine, leucine and isoleucine) (Hara et al., 1998)(Christie et al., 2002). A more recent paper showed that the activation of mTORC1 by amino acids is more complex. Schematically, some amino acids "prime" mTOR while others activate the "primed" mTOR (Dyachok et al., 2016). The "activators" (leucine, isoleucine, valine and methionine) are all essential amino acids and three of them are also branched amino acids. Among the "primers" (asparagine, glutamine, threonine, arginine, glycine, proline, serine, alanine and glutamic acid) only threonine is essential. Aa are necessary for the activation of mTORC1, but they are not sufficient. Indeed, this protein complex is a downstream mediator of many growth factors, including the insulin/IGF-1 pathway, which converge and inhibit the

Tuberous Sclerosis Complex (TSC), a negative regulator of mTORC1 signaling. TSC normally activates the small GTPase Rheb, which in turn directly activates mTORC1 (Saxton and Sabatini, 2017). More in detail, the presence of aa favors the heterodimerization and activation of Rag GTPases, RagA/B bound to GTP and RagC/D to GDP (Kim et al., 2008)(Sancak et al., 2008), which interact with the mTORC1 subunit Raptor, thus guaranteeing a functional subcellular localization for mTORC1 (Nojima et al., 2003)(Schalm et al., 2003). In fact, Raptor is responsible for the recruitment of mTORC1 on the lysosome membrane where its activator Rheb is also located, thus influencing Rheb's accessibility to the complex (Sancak et al., 2008). Once activated, mTORC1 is responsible of a signaling cascade resulting in the regulation of cell proliferation. In brief, it controls protein translation initiation through the phosphorylation of the kinase S6 and, subsequently, of the ribosomal protein S6 (Kuo et al., 1992)(Hentges et al., 2001) and of the translation initiation factor 4B (eIF4B) (Holz et al., 2005); through the phosphorylation of S6K1 it also activates the sterol regulatory element-binding protein (SREBP), which participates to cell growth as it is responsible for the *de novo* synthesis of lipids (Düvel et al., 2010); it inhibits autophagy by phosphorylating the autophagy-initiating kinase ULK1 (Hosokawa et al., 2009)(Jung et al., 2009)(Kim et al., 2011).

While mTORC1 is activated by aa and it is responsible for the regulation of cell growth and metabolism, mTORC2 mainly functions as an effector of the insulin-IGF1/PI3K signaling. It controls cell survival and proliferation through the phosphorylation of various members of the AGC family of protein kinases (PKA, PKG, PKC) and, most importantly, through the phosphorylation and activation of Akt, a key effector of insulin-IGF1/PI3K signaling (Sarbasov et al., 2005) (Figure 8). Akt also participates to the activation of mTORC2 through a positive-feedback loop. Indeed, a partial activation (phosphorylation on T308 by PDK1) of Akt induces the activation of mTORC2, which in turn fully activates Akt itself due to phosphorylation on S473 (Yang et al., 2015). Remarkably, mTORC2 signaling is also regulated through a negative feedback loop between mTORC1 and insulin-IGF1/PI3K signaling. In short, mTORC1 activates a negative regulator of insulin/IGF-1R signaling upstream of mTORC2 and Akt, while S6K1, indirectly activated by mTORC1, induces a phosphorylation-dependent degradation of insulin receptor substrate 1 (IRS1), thus inhibiting mTORC2 action (Saxton and Sabatini, 2017).

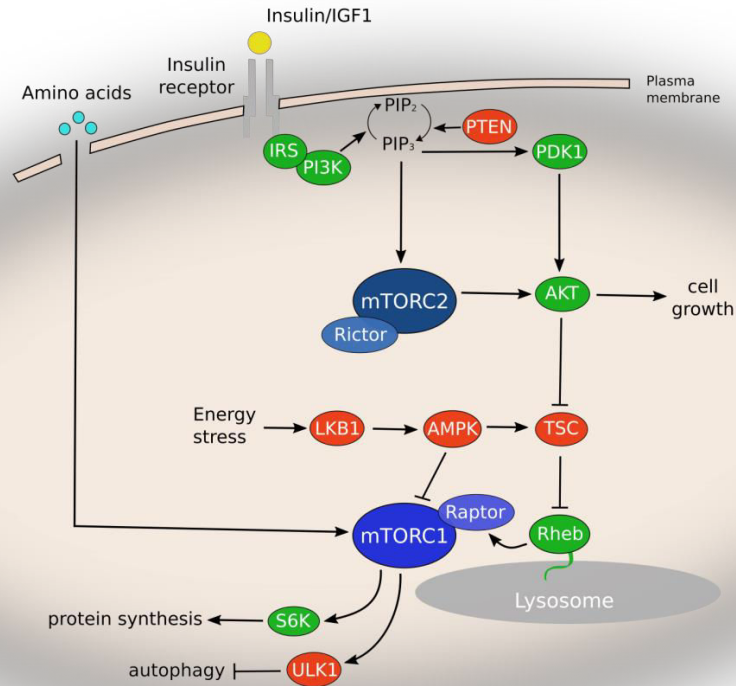


Figure 8 Simplified representation of mTOR signaling pathway in mammalian cells. mTORC1 is activated by growth factors through the PI3K-AKT pathway. PI3K catalyzes the phosphorylation of PIP₂ to PIP₃, which recruits PDK1 and AKT, resulting in the phosphorylation of AKT at T308 by PDK1. mTORC2 is also induced by PIP₃ and phosphorylates AKT at S473. Activated AKT then promotes mTORC1 activity by inhibiting TSC, thus activating Rheb. Energy stress also regulates mTORC1, acting via LKB1 and AMPK. Positive regulators of mTORC1 or mTORC2 are shown in green and negative regulators in red. AMPK, AMP-activated protein kinase; IGF1, insulin growth factor-1; IRS, insulin receptor substrate; LKB1, liver kinase B1; mTOR, mechanistic target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PDK1, 3'-phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3 kinase; PIP₂, phosphatidylinositol (4,5) bisphosphate; PIP₃, phosphatidylinositol (3,4,5) trisphosphate; PTEN, phosphatase and tensin homolog; Rheb, Ras homolog enriched in brain; S6K, S6 kinase; TSC, tuberous sclerosis complex. Adapted from (Meng et al., 2018)

Intracellular amino acid sensing: autophagy

Autophagy is a catabolic pathway characterized by the intracellular degradation of protein aggregates and organelles in order to, either eliminate damaged components in physiological condition, or provide a nutrient source and promote survival upon starvation. It involves the formation of a vesicle-like autophagosome to sequester cytoplasmic components and deliver them to the lysosome for recycling.

The formation of the autophagosome is a complex process coordinated mainly by three protein complexes. In mammals, the first one is the ULK1 complex, composed by unc-51-like kinase 1 (ULK1), autophagy-related protein 13 (ATG13), focal adhesion kinase family interacting protein of 200 kDa (FIP200) and ATG101. When autophagy is induced, this complex translocates to autophagy initiation sites where it recruits a second autophagic complex called vacuolar protein sorting 34 (VPS34). VPS34 complex leads to the production of phosphatidylinositol 3-phosphate (PI3P) at the phagophore, a cup-shaped double-membrane structure from where the autophagosome will originate, and the consequent recruitment of PI3P-binding proteins. These, together with other proteins, orchestrate the elongation of the phagophore and eventually its closure to form the complete autophagosome. This process involves a third protein complex (the ATG16L1-ATG5-ATG12 conjugation machinery) which catalyzes the conjugation of the ubiquitin-like ATG8 family (LC3A, B, C) to the lipid phosphatidylethanolamine. More specifically, LC3 (microtubule-associated protein 1 light chain 3) is cleaved by the protease Atg4, thus originating LC3-I and LC3-II. LC3-II is then conjugated with phosphatidylethanolamine and this lipidated form associates with the growing autophagosome membrane and it is believed to participate to its sealing. LC3-II remains on the autophagosome membrane until it fuses with the lysosome to form the autolysosome, whose cargo will be degraded by proteases, lipases, nucleases and glycosidases. Finally, specific lysosomal permeases release the resulting products in the cytosol where they serve as substrate for synthetic and metabolic pathways (Rabinowitz and White, 2010)(Zachari and Ganley, 2017) (Figure 9).

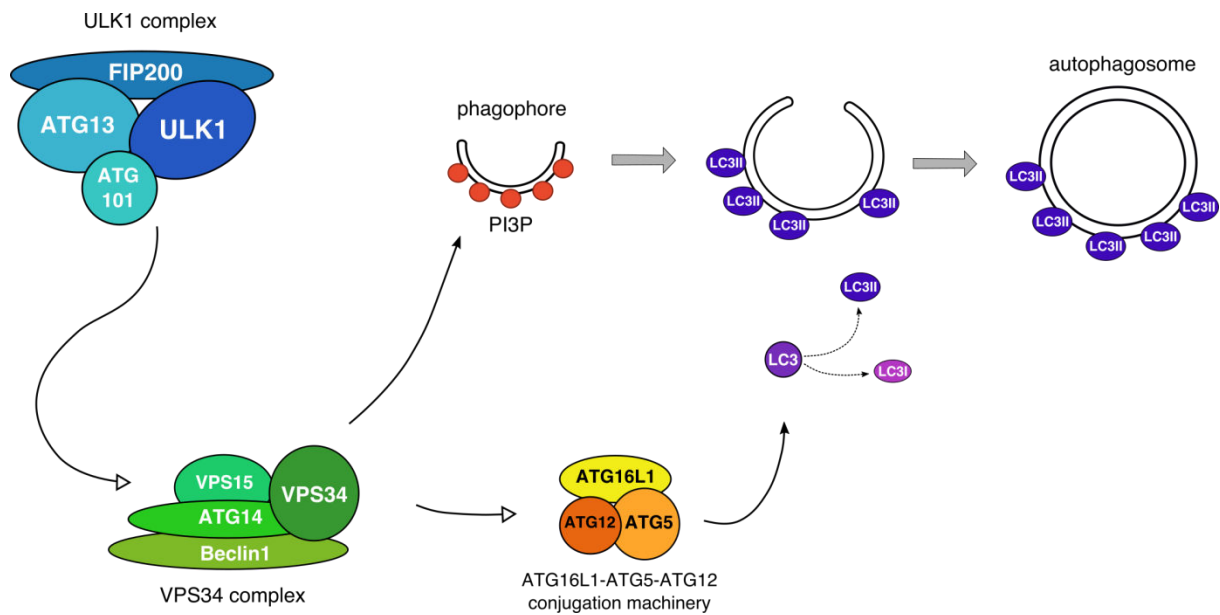


Figure 9 Simplified representation of autophagy induction in mammalian cells. Upon autophagy initiation, the ULK1 complex recruits the VPS34 complex, which leads to the production of phosphatidylinositol 3-phosphate (PI3P) at the phagophore. PI3P and other proteins coordinate the elongation of the phagophore. A third complex is then recruited, the ATG16L1-ATG5-ATG12 conjugation machinery, which induces the cleavage of LC3. LC3-II associates with the growing autophagosomal membrane, participates to its sealing and detaches only when the autophagosome fuses with the lysosome. Adapted from (Zachari and Ganley, 2017).

Although nutrient deprivation in general is known to induce autophagy, the mechanism of its activation dependent on amino acid deprivation is the most characterized. The connection between autophagy and rapamycin was first described in rat hepatocytes, thus unveiling the process responsible for the coordination between the catabolic process of autophagy and the anabolic process of protein synthesis (Blommaert et al., 1995). In aa abundance, mTORC1 is active and phosphorylates ULK1 and Atg13 in multiple sites, thus blocking both ULK1 catalytic activity and ULK1 complex translocation, therefore inhibiting autophagy initiation (Hosokawa et al., 2009)(Jung et al., 2009)(Puente et al., 2016). Under aa deprivation condition, however, mTORC1 is repressed and released from the lysosomal surface, resulting in the dephosphorylation of ULK1 and Atg13, in the activation of ULK1 kinase activity and in autophagy initiation (Zachari and Ganley, 2017).

Metabolism in proliferative and quiescent cells

High proliferative cells require nutrient and energy supply to be converted in biosynthetic precursors and thus adequately support cell growth. Healthy mammalian cells are normally stimulated to take up nutrients from the environment by growth factors, while cancer cells overcome this regulation mechanism leading to uncontrollable proliferation. In both cases, however, one of the major characteristics of proliferating cells is an increased glycolytic activity and glucose demand compared to cells displaying low rate proliferation (Vander Heiden et al., 2009). This is accompanied by a reduction in Krebs cycle, resulting in the production and excretion of an abundance of lactate. Although this phenomenon, the Warburg effect, was initially described in cancer cells (Warburg, 1956), it is also observed in highly proliferative healthy cells that switch their metabolism from oxidative phosphorylation to “aerobic glycolysis” regardless of the availability of oxygen and despite the decreased efficiency in ATP production (Lunt and Vander Heiden, 2011). Indeed, cytosolic glycolysis generates only 2 molecules of ATP per molecule of glucose, against the 36 ATPs produced by mitochondrial oxidative phosphorylation. However, it provides a variety of metabolic intermediates that can be diverted to fuel other anabolic pathways, thus supporting the synthesis of glycerol, ribose or non-essential amino acids, therefore the complex anabolic network in charge of synthesizing all the macromolecular components responsible for producing cell biomass. Moreover, ATP production happens at a faster rate during aerobic glycolysis than in oxidative phosphorylation and, in a condition of abundant glucose resources, this pathway may be preferred to meet the high demands of rapidly dividing cells (Vander Heiden et al., 2009)(Lunt and Vander Heiden, 2011).

In highly proliferative or cancer cells most of the glucose, after being phosphorylated, takes part to glycolysis, it is metabolized to pyruvate and converted to lactate. Another fraction of the monosaccharide is instead directed to pathways for the synthesis of nucleotides precursors, serine and glycine, phosphoglycerol and for the glycosylation of proteins. The minority quantity of pyruvate that is not converted to lactate, mostly enters the Krebs cycle to produce both ATP and biosynthetic intermediates, especially acetyl-CoA (DeNicola and Cantley, 2015). Acetyl-CoA, apart from being a key metabolite for the TCA cycle function, it is essential for cell proliferation as it represents the sole carbon source for cholesterol and

fatty acids biosynthesis, which in turn are required for the formation of cellular membranes. Furthermore, FA oxidation is an important source of ATP (DeNicola and Cantley, 2015). Amino acids, both essential and non-essential, are used principally for protein biosynthesis but, in case of highly proliferative cells, they participate to additional biosynthetic pathways. One of the most important amino acids for supporting cell proliferation, in synergy with glucose, is glutamine, whose metabolism provides almost all the carbon, nitrogen, energy and reducing equivalents necessary for cell growth and division (Vander Heiden et al., 2009). Glutamine is converted in glutamate via *glutaminase* action. Glutamate can be further converted in α -ketoglutarate, which provides carbons for Krebs cycle intermediates participating in the synthesis of various non-essential aa, or for the production of acetyl-CoA, therefore contributing to FA biosynthesis. Glutamine and glutamate also serve as nitrogen donors for nucleotides, amino acids (serine, alanine, asparagine, proline and arginine) and amino sugars. Remarkably, glutaminolysis has the capacity of generating more ATP than aerobic glycolysis, therefore glutamine may represent an important respiratory fuel for proliferating cells (Lunt and Vander Heiden, 2011).

Pancreas

The pancreas is an organ located transversely across the posterior wall of the abdomen, behind the stomach, and can be anatomically divided in three sections: head, body and tail. The exocrine part plays an essential role in macronutrients digestion, hence participating to energy/metabolism homeostasis, and the endocrine part is a key actor in the regulation of glycemia.

Both an exocrine and an endocrine organ

Similar to the liver, the pancreas shows both exocrine and endocrine functions. The exocrine components of this organ are organized in pancreatic acinus surrounded by a thin basal lamina, reticular stroma, and pancreatic stellate cells. This exocrine part of the organ occupies around the 90% of the pancreatic mass and comprises acinar, centroacinar and ductal cells arranged concentrically around the lumen of pancreatic ducts. These cells are

responsible for the involvement of the pancreas in the digestion process, as they secrete into the ducts the pancreatic juice, a mixture of digestive enzymes including amylase, pancreatic lipase and trypsinogen (Pandiri, 2014)(Röder et al., 2016).

The endocrine components, instead, are clustered together and form small island-like structures called *islets of Langerhans*, which only occupy the 1-2% of pancreatic volume, while the remainder is composed by lymphatics, nerves and fibrous connective tissue (Chandra and Liddle, 2009). Endocrine pancreatic cells release hormones in the circulation and are divided in five different cell types according to the specific kind of hormone secreted. Glucagon-producing α -cells and insulin-producing β -cells are the most abundant, accounting respectively for the 15-20% and the 65-80% of the total islet cells. In rodents, β -cells are located in the center while the other cell types in the periphery of the islets, whereas in humans α - and β -cells are more dispersed (Brissova et al., 2005)(Cabrera et al., 2006). The remaining mass of Langerhans' islets is composed for the 3-5% by pancreatic polypeptide (PP)-producing γ -cells, for the 3-10% by somatostatin-producing δ -cells and for less than 1% by ghrelin-producing ϵ -cells (Brissova et al., 2005)(Katsuura et al., 2002)(Wierup et al., 2002). Each hormone covers a specific physiological function in the body. Briefly, glucagon induces the increase in glycemia, whereas insulin shows an opposite effect. Additionally, glucagon stimulates insulin secretion. Somatostatin inhibits the release of both glucagon and insulin, while PP is responsible for the regulation of both endocrine and exocrine pancreatic functions (Röder et al., 2016). Ghrelin, instead, is believed to have a stimulatory effect on food intake, fat deposition and the release of growth hormone (Pradhan et al., 2013).

Through its different hormones, the pancreas maintains a fasting glycemia within a range of 4 to 5.6 mM, action accomplished especially thanks to the opposite action of glucagon and insulin. During the sleeping phase or between meals, glycemia drops, thus inducing glucagon secretion from α -cells and the consequent stimulation of hepatic glycogenolysis in order to rise glycemia. Moreover, during prolonged fasting, glucagon stimulates hepatic and renal gluconeogenesis hence increasing glycemia (Roden et al., 1996)(Petersen and Shulman, 2018). On the contrary, the high glycemia following a meal stimulates insulin secretion. Upon binding to its receptor, insulin enables the uptake of glucose into adipose tissue and muscle, hence leading to the removal of glucose from the blood stream. Furthermore, it

induces glycolysis, glycogenesis, lipogenesis and protein synthesis (Röder et al., 2016) (Petersen and Shulman, 2018).

The insulin secretion signaling pathway

The increase in glycemia is considered to be the main trigger of insulin secretion. Glucose circulating in the blood is taken up by the GLUT2 in rats/GLUT1 in humans glucose transporter located on β -cell external membrane and, once inside the cell, it is phosphorylated and subjected to glycolysis, thus producing pyruvate. The latter enters the mitochondria where it is metabolized by pyruvate carboxylase, associated with efflux of TCA cycle intermediates, and by pyruvate dehydrogenase, resulting in an increase in cytoplasmic ATP (or ATP/ADP – adenosine diphosphate – ratio). Because of the presence of ATP-sensitive K^+ channels (K_{ATP} channels), which close upon increase in ATP/ADP ratio, ATP serves as the main signaling molecule for insulin secretion. Indeed, these channels are responsible for the maintenance of β -cell membrane resting potential by transporting K^+ ions down their concentration gradient out of the cells in normal conditions. Hence, their closure causes membrane depolarization, which results in the opening of L-type voltage-dependent Ca^{2+} -channels (VDCCs) and the consequent increase in intracellular Ca^{2+} concentration. The elevation of cytosolic free Ca^{2+} triggers insulin-containing granules fusion with the cellular membrane and results in insulin exocytosis (Van Obberghen et al., 1973)(Henquin, 2000) (Figure 10).

β -cells secrete insulin in response to glucose in a biphasic manner, whose characteristics started to be studied more than fifty years ago (Curry et al., 1968). Originally it was believed that the first insulin spike, around 5/10 minutes from glucose stimulation and responsible for the fast release of most of the insulin quantity, accounted on a pool of insulin granules already close to the plasma membrane, and whose exocytosis depended on the contraction of microfilaments. The second phase of GSIS, lasting from 30 to 60 minutes, until restoring glycemia, was described to account mostly on insulin granules stored deeper in the cell and to involve the driving force of the microtubules (Van Obberghen et al., 1973)(Malaisse et al., 1974). Later studies on rat and mouse β -cells revealed that the second phase of GSIS could be induced independently from the block or the genetic removal of K_{ATP} channels (Sato et al.,

1992)(Gembal et al., 1992). This phase was therefore named K_{ATP} -independent GSIS, in contrast with the first phase K_{ATP} -dependent (Miki et al., 1998).

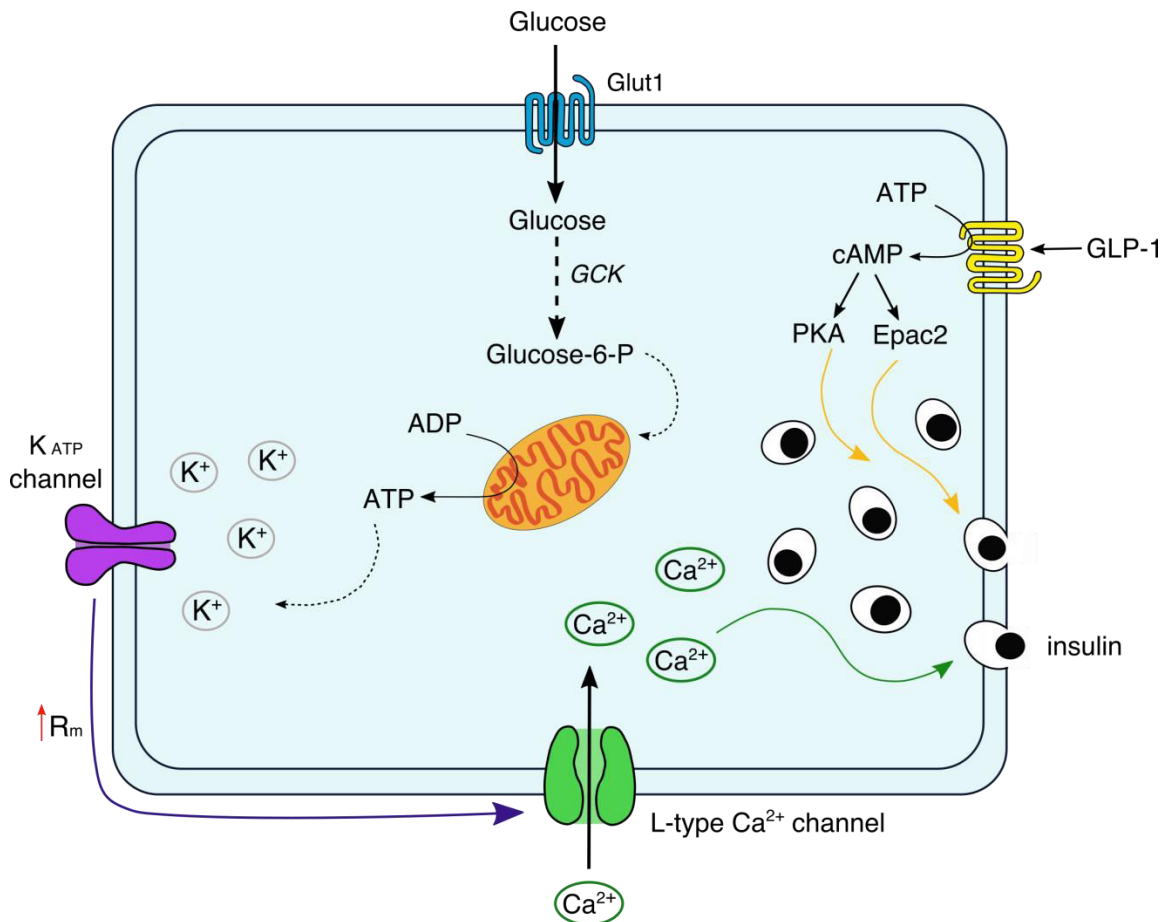


Figure 10 Schema of insulin secretion in response to glucose. Glucose enters the cell through the glucose transporter Glut1 (human) and is phosphorylated by glucokinase (GCK). Further cytosolic and mitochondrial metabolism leads to ATP production. The increase of ATP/ADP ratio results in the closure of the K_{ATP} channels, the consequent increase in membrane resistance ($R_m \uparrow$) and depolarization of the plasma membrane. Hence, the L-type Ca^{2+} channels open leading to Ca^{2+} influx which, in turn, trigger insulin exocytosis. Incretins such as GLP-1 potentiate exocytosis by both protein kinase A (PKA)-dependent and Epac2-dependent mechanisms. Adapted from (Ashcroft and Rorsman, 2012).

At present the first phase, to which we refer as the triggering pathway (Henquin, 2000), is believed to be at least partially regulated through the incretin hormone glucagon-like peptide-1 (GLP-1). The activation of the β -cell-enriched GLP-1 receptor stimulates both the cAMP-EPAC2 (Exchange Protein Activated by cAMP) branch, which mediates the positive effects of GLP-1 on GSIS first phase, and the cAMP-PKA branch, necessary for the former branch to be active (Hussain et al., 2012) (Figure 10). ERK2 was also proposed to be relevant for both insulin gene expression and the first phase of GSIS (Leduc et al., 2017). The second phase is instead known as the metabolic amplifying pathway, since it is not induced by membrane depolarization, but by additional signals following the metabolism of glucose (Henquin, 2011). Indeed, it was demonstrated to be induced by TCA intermediates and anaplerotic products, phospholipase C/protein kinase C (PKC) signaling, intracellular lipids alterations and/or cAMP increase, while it might be amplified by coupling factors such as NADPH, NADH, glutamate and malonyl-CoA (Newsholme et al., 2014). It is now accepted that there are two different pools of insulin granules in β -cells functionally connected to the biphasic GSIS and not so dependent on cytoskeleton remodeling: readily releasable pools (RRP), pre-docked within 100-200 nm from the plasma membrane, and reserve pools (RP). According to a newer model, the triggering phase implies a rapid insulin exocytosis from the RRP by a K_{ATP} -dependent mechanism. The second phase is characterized by a gradual increase in the K_{ATP} -independent signal through a phase of amplification/augmentation, and it is possible thanks to the replenishment of the RRP from the RP (Bratanova-Tochkova et al., 2002)(Aizawa and Komatsu, 2005). Although the triggering and amplifying pathways are commonly associated respectively to the first and second phase of GSIS, recent evidence demonstrates that amplification might be partially involved also in the triggering phase (Henquin, 2011).

The importance of amino acid in insulin secretion

Aside of being crucial for protein synthesis, aa are described to be also key modulators of insulin secretion. According to their specific type, the duration of their exposure and their concentration, they can indeed positively or negatively influence GSIS both *in vivo* and *in vitro* (Newsholme and Krause, 2012). More specifically, aa can regulate the triggering as well as the amplification of insulin secretion either by acting as Krebs cycle substrate, by causing

the depolarization of the plasma membrane thanks to their positive charge and specific aa transporters, or by indirectly depolarizing the membrane through the co-transport of Na^+ ions inside the cell (Nolan and Prentki, 2008)(Newsholme et al., 2010).

Glutamine, the most abundant aa in the blood and extracellular fluids, is not able to induce insulin exocytosis alone, but its combination with leucine results in allosteric GDH activation, thus leading to the entry of glutamine carbon in the Krebs cycle and ultimately stimulating insulin exocytosis. The production of glutamate from glutamine might also contribute to the direct induction of insulin secretion, but the exact molecular mechanism is still unclear (Newsholme et al., 2014). It was also shown that glutamate can accumulate within insulin vesicles (Høy et al., 2002) (Newsholme and Krause, 2012) and its release might both influence β -cell glutamate receptor activation, and regulate the secretion of glucagon by adjacent α -cells (Corless et al., 2006).

Alanine and arginine were also described to play an important role in insulin secretion regulation. Indeed, alanine-mediated Na^+ co-transport results in the induction of K_{ATP} -independent Ca^{2+} influx and, ultimately, in insulin exocytosis (McClenaghan et al., 1998)(Newsholme and Krause, 2012)(Salvucci et al., 2013). On the other hand, the entrance of the positively charged arginine in the β -cell leads to direct membrane depolarization, subsequent opening of the Ca^{2+} -dependent VDCCs and insulin exocytosis (McClenaghan et al., 1998) (Sener et al., 2000)(Newsholme and Krause, 2012).

Finally, another class of relevant aa for mediating insulin exocytosis are the BCAAs. Dietary products rich in BCAA and whey proteins have been associated to ameliorate fasting insulin levels, insulin release and glycemia both in *in vivo* animal models and in human subjects affected by obesity or T2D (Gaudel et al., 2013)(Jakubowicz and Froy, 2013). The exact mechanism of this effect is still not clear, but it has been suggested it might involve an increase in protein synthesis via the activation of mTOR signaling, the augmentation of anaplerosis and, thanks to the presence of leucine, the above-mentioned activation of GDH with consequent increase in Krebs cycle activity (Yang et al., 2006)(Newsholme et al., 2010)(Jakubowicz and Froy, 2013).

The liver-pancreatic islets axis

As already mentioned, liver plays a true central role in glucose homeostasis by storing or releasing this monosaccharide in response, respectively, to insulin or glucagon signaling. Therefore, the basis of the relationship between pancreatic islets and hepatic tissue is grounded on finely regulated mechanisms.

Insulin enhances glycolysis by promoting the expression of the hepatic GCK gene, the enzyme converting glucose into G6P. This action is mediated by SREBP-1c and requires, instead, the absence of cAMP (Sibrowski and Seitz, 1984)(Kim et al., 2004). Furthermore, insulin inhibits glycogen phosphorylase (GP) and GSK-3 through the PI3K/PKB pathway, which is responsible for the activation of glycogen synthase (Miller and Lerner, 1973)(Stalmans et al., 1974)(Aiston et al., 2003). On the other hand, the repressive effect of insulin on GSK-3 leads to a diminished expression of the forkhead transcription factor Foxo1, with consequent decrease in G6Pase (Schmoll et al., 2000)(Lochhead et al., 2001). Additionally, insulin inhibits the expression of PEPCCK gene in the liver by preventing the binding of the association of cAMP response element-binding protein to the RNA polymerase II on PEPCCK promoter (Nakae et al., 2001)(Duong et al., 2002).

In an opposite fashion compared to insulin, glucagon's binding to its G-protein-coupled receptors (GPCRs) in the liver results in a PKA-dependent stimulation of glycogenolysis and gluconeogenesis, but inhibition of glycolysis and glycogenesis (Jiang and Zhang, 2003). Moreover, glucagon was demonstrated to both inhibit pyruvate kinase gene expression and promote its mRNA degradation, thus preventing the formation of pyruvate, hence the last step of glycolysis (Decaux et al., 1989).

Aside from the direct effect of insulin and glucagon on the liver, hepatocyte-derived factors also have an influence on the pancreas and insulin secretion. Indeed, hepatocyte nuclear factor 3- β (HNF3 β) is crucial for the transcription of the pancreatic and duodenal homeobox 1 (pdx1), a transcriptional factor which regulates pancreas development (Wu et al., 1997). In addition, HNF1 α was found to be important for β -cell function as its absence results in impaired insulin secretion, most likely due to a diminished response to intracellular calcium (Pontoglio et al., 1998).

MicroRNAs

MicroRNAs (miRNAs) constitute a highly conserved class of small non-coding RNAs of around 20-24 nucleotides in length, which plays a crucial role in post-transcriptional regulation, both in animals and plants, by silencing gene expression through translational repression and/or degradation of their target mRNAs. In mammals, a single miRNA negatively regulates hundreds of genes (Lim et al., 2005) and more than 60% of human coding RNAs contains at least one conserved miRNA-binding site plus additional non-conserved sites, meaning that the majority of proteins are most likely regulated by miRNAs (Friedman et al., 2008). As a consequence, a multitude of essential biological processes, such as pluripotency, cell proliferation, apoptosis and metabolism, are under the control of miRNAs and their dysregulation or malfunctioning is therefore often associated with human diseases (Ratnadiwakara et al., 2018). Additionally, increasing body of evidence showed that miRNAs also exist outside the cell, as *circulating miRNAs*, stored in membrane-derived vesicles or associated with the protein Argonaute 2, crucial for their inhibitory activity. They have been associated to a variety of diseases and pathologies, which make them interesting novel biomarkers for diagnosis and/or treatment, and they may serve as mediator of cell-to-cell communication (Mirra et al., 2015). MiRNA mechanism of action is assessed through the perfect or unperfect binding to the 3' untranslated region (UTR) of its complementary mRNA, which classically leads to the degradation of the mRNA itself or to the inhibition of its translation, respectively. The complementary region on the 5' of miRNAs, specifically the nucleotides between position 2 and 7, is called "miRNA seed" and is responsible for target recognition. Nevertheless, also the nucleotides in position 8 and 13-16 are relevant for a correct base pairing with the mRNAs target (Bartel, 2009).

MiRNAs in the liver

Most miRNAs are tissue-specific, meaning that their expression is restricted or enriched in certain cells, rather than others, where they are crucial for cellular identity and/or functioning.

MiR-122 is the most abundant liver-specific microRNA, accounting for 70% of the total liver miRNA population (Lagos-Quintana et al., 2002), and it directly or indirectly regulates the

expression of 24 hepatocyte-specific genes (Laudadio et al., 2012). For instance, it was demonstrated by Laudadio and colleagues that miR-122 and HNF6 directly stimulate their reciprocal expression through a positive feed-back loop (Laudadio et al., 2012). Furthermore, recent data showed that miR-122 regulates hepatocyte key metabolic features – such as ammonia elimination, CYP450 metabolism and albumin expression – and may promote hepatocyte maturation through another positive feed-back loop involving miR-122/FOXA1/HNF4 (Deng et al., 2014). Additional studies on human liver cancer cell line HepG2 and HCC cells highlighted the importance of miR-122 for the maintenance of a physiological balance between cell proliferation and differentiation, and for the suppression of tumor metastasis (Xu et al., 2010)(Deng et al., 2014). Another relevant microRNA promoting the hepatospecific phenotype was found to be miR-148a, which directly inhibits the expression of DNA (cytosine-5-)-methyltransferase 1 (DNMT1) and also serves as a tumor suppressor in HCC (Gailhouste et al., 2013), whereas *in situ* hybridization experiments revealed that miR-23b, which directly targets SMAD 3, 4 and 5, participates to the regulation of hepatocytes and cholangiocytes lineage specification through the TGF- β signalling pathway (Rogler et al., 2009).

As regeneration is one of the most remarkable liver capacities and needs to be tightly regulated, it does not come as a surprise that also miRNAs are actively involved in this process. A miRNA screening in rat regenerating liver, during the 72 hours following PH, showed the deregulation of 26 miRNAs including miR-20a/20b/93/106a, which target the VEGF pathway, hence possibly participating in the regulation of neoangiogenesis (Castro et al., 2010). A different kind of study by Jung and collaborators identified a group of miRNAs highly expressed in HCC and hESCs, whereas they appear downregulated in quiescent hepatocytes. These miRNAs, which include miR-106a/106b/17/93/301a/130b, were described to promote hepatocyte proliferation/transformation through the regulation of phosphatase and tensin homolog (PTEN) and TGF- β pathways (Jung et al., 2012). Some other miRNAs controlling hepatocyte proliferation during liver regeneration are miR-21/23b/221 (Chen and Verfaillie, 2014).

Even though a growing body of evidence pinpoints the importance of miRNAs for hepatocyte homeostasis, more studies will be needed to fully unveil these miRNA-mediated regulation mechanisms. (Figure 11)

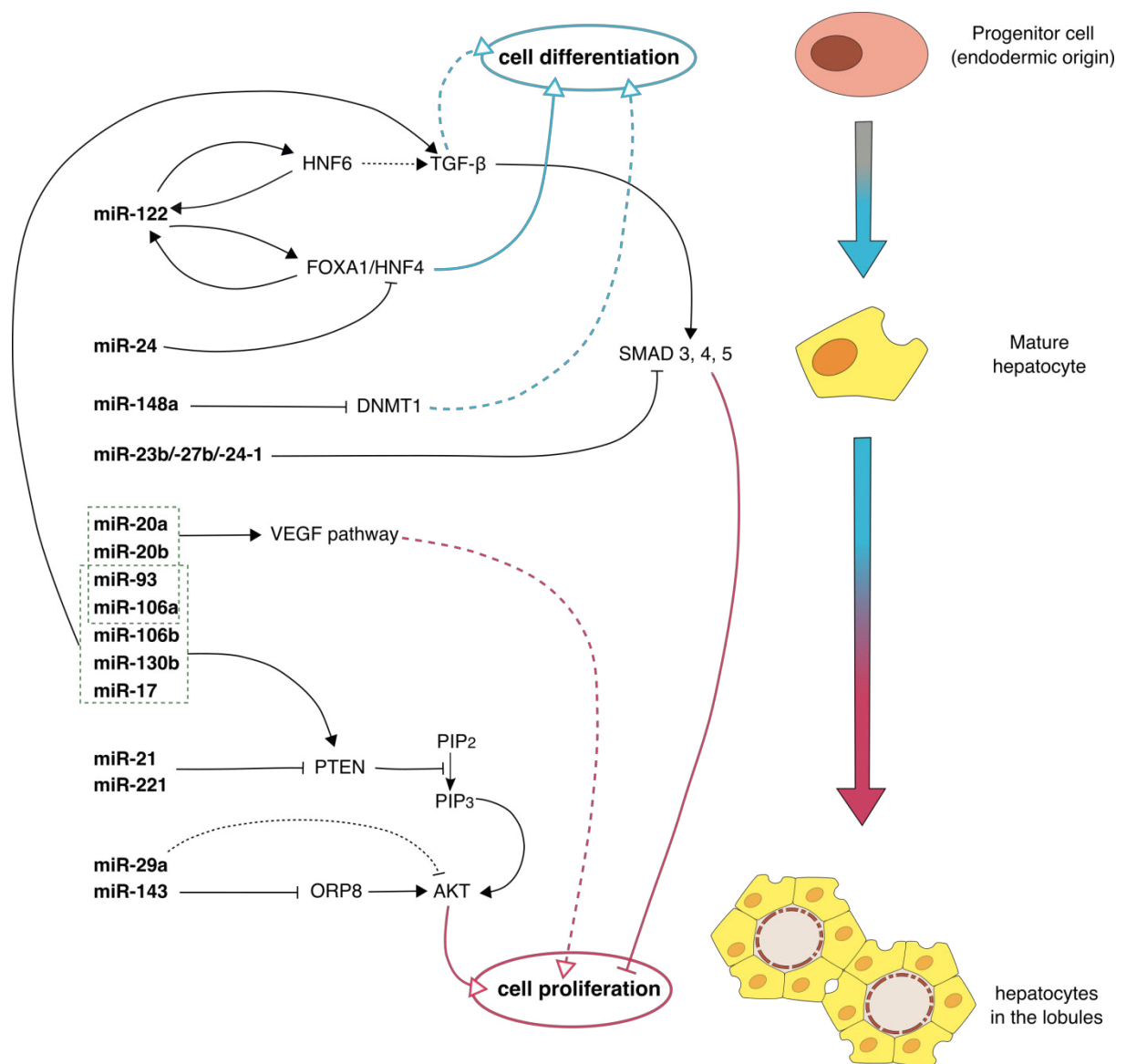


Figure 11 Key miRNAs regulating hepatocyte differentiation and proliferation. Blue color is associated with hepatocyte differentiation, while purple indicates hepatocyte proliferation. Full arrows are used to highlight a direct effect, broken arrows denote instead an indirect effect. More details about the miRNAs displayed can be found in the text.

MiRNAs and hepatocyte metabolism

Dicer1-knockout in the liver has been associated with a consistent decrease in miR-122/148a/192/194 expression and resulted in severe hypoglycaemia in fasted mice, suggesting the importance of miRNAs for liver glucose metabolism (Sekine et al., 2009). Later studies found that liver-specific miR-122 is downregulated in HFD-fed mice or in hepatocyte models of insulin resistance, and it targets protein tyrosine phosphatase 1B (PTP1B), an inhibitor of hepatocyte insulin signaling (Yang et al., 2012). Other miRNAs were demonstrated to differentially participate in the regulation of hepatic insulin signaling. Among them, miR-181a and miR-34a were found to bind the 3'-UTR of Sirtuin-1 (SIRT1), a positive regulator of insulin pathway in many cell types (Lee et al., 2010)(Zhou et al., 2012), while miR-144 and miR-33 have been shown to bind respectively IRS1 in a T2D rat model and IRS2 in HepG2 and Huh7 cells (Karolina et al., 2011)(Ramirez et al., 2013). Another study conducted on adipocytes and liver of diabetic rats showed that the upregulation of miR-29a/b led to the inhibition of AKT and the consequent mis regulation of glucose homeostasis (He et al., 2007). Later, miR-143 was demonstrated to directly inhibit oxysterol-binding-protein-related protein 8 (ORP8), an activator of AKT, thus contributing to the regulation of hepatic glucose metabolism (Jordan et al., 2011). Interestingly, miR-122 was firstly associated to the regulation of hepatic lipid metabolism through the indirect activation of SREBP1 in hepatic cells, hence positively regulating lipogenesis (Esau et al., 2006). However, miR-122 is not the only one being associated with lipid homeostasis. Vickers and colleagues investigated miR-27b in a mouse model of dyslipidemia, discovering that it directly inhibits the expression of Angiopoietin-like 3 (ANGPTL3) and glycerol-3-phosphate acyltransferase (GPAM), both implicated in lipid metabolism regulation (Vickers et al., 2013). Furthermore, additional studies identified miR-106b, miR-148a and miR-758 as direct targets of the ATP-binding cassette transporter ABCA1, so known as the cholesterol efflux regulatory protein CERP, thus playing a key role in cholesterol metabolism and secretion, whereas miR-107 was found to regulate the expression of FASN (FAS encoding gene) (Lauschke et al., 2017). Finally, some miRNAs have been suggested to participate to the regulation of liver drug metabolism by directly or indirectly targeting CYP genes. Studies on HepG2 cells proposed that miR-34a and miR-24 might inhibit the liver enriched nuclear factor HNF4 α , thus affecting the levels of CYP7A1 and CYP8B1 (Takagi et al., 2010). Further analysis by Oda and

colleagues demonstrated that mir-24 additionally regulates the activity of CYP1A1 through the inhibition of hydrocarbon receptor nuclear translocator (ARNT) at protein level in human liver (Oda et al., 2012). More recently, bioinformatic analysis proposed that miR-132/142-3p/21 might regulate CYP1A1 expression, CYP2A6 could be targeted by miR-142-3p and miR-21, CYP2C19 by miR-130b/185/34a, and miR-10a/200c/let-7g might regulate CYP2E1 (Rieger et al., 2013).

MiRNAs and pancreatic beta cells

From the review “Shaping and preserving β -cell identity with microRNAs” Dumortier O, **Fabris G**, Van Obberghen E (Dumortier et al., 2016)

miRNAs and β -cell differentiation/de-differentiation

Given the fact that the miRNA machinery is intimately implicated in regulating pancreas development and functional β -cell differentiation, it does not come as a surprise that many studies have attempted to pin down the precise contribution of specific miRNAs (Figure 12). One of the most relevant and widely explored miRNAs in β -cell development is miR-375. This miRNA is highly expressed both in human and mouse pancreatic islets. Remarkably, its expression increases during pancreas organogenesis together with insulin production and β -cell proliferation (Joglekar et al., 2009)(Poy et al., 2004). The relevance of miR-375 in pancreatic cell development was first investigated in zebrafish embryos (Kloosterman et al., 2007). In these pioneering studies using a loss of function approach, the authors demonstrated the decisive contribution of miR-375 to β -cell development. Two years later, miR-375 knockdown in mice confirmed its pivotal role in islet cell expansion (Poy et al., 2009). Subsequently, proof was provided that islet expression of miR-375 is transcriptionally regulated at different levels. Indeed, Avnit-Sagi and colleagues unveiled, in the miR-375 promoter region, consensus-binding sequences for important transcriptional factors involved in β -cell differentiation such as Pdx-1, Ngn3 and NeuroD1 (Avnit-Sagi et al., 2009)(Keller et al., 2007).

Taking into consideration the role of miR-375 in β -cell differentiation, it is reasonable to assume that its downregulation could enhance de-differentiation of insulin secreting cells. In fact, evidence is indeed being built highlighting an association between decreasing levels of miR-375 and β -cell de-differentiation in vitro (Nathan et al., 2015). Further, it has been recently found in vitro that overexpression of miR-375 can contribute to the differentiation of induced-pluripotent-stem cells into β -cell-like clusters (Shaer et al., 2014b). This overexpression is also sufficient to generate insulin-producing cells from mesenchymal stem cells coming from human placenta (Shaer et al., 2014a). However, a recent paper by Jafarian and colleagues (Jafarian et al., 2015) reveals that miR-375 does not suffice for the differentiation of hMSCs into functional β -cells due to the fact that the cells obtained could not respond to glucose in vitro. At the same time downregulation of miR-9, which is known to affect the β -cell secretory capacity (Plaisance et al., 2006) in islet-like clusters expressing miR-375, resulted in an obvious increase in glucose-stimulated responses. Viewed in aggregate, these results led the authors to suggest that overexpression of miR-375, coupled to simultaneous downregulation of miR-9, exerts synergistic effects on the differentiation of hMSCs into functional insulin-producing cells. In addition to miR-375, miR-7 has also been shown to participate in human embryonic stem cell differentiation in insulin-producing pancreatic cells (Wei et al., 2013). Indeed, proof was provided according to which expression of miR-7 is increased during human islet development and differentiation (Joglekar et al., 2009). Intriguingly, it has recently been found that this miRNA is also an inhibitor of β -cell proliferation through the mTOR signaling pathway (Wang et al., 2013).

MiRNAs and β -cell identity

The uniqueness of differentiated β -cells, whose metabolic properties define their “identity” as glucose sensor, results not only from cell-specific gene expression, but also from cell-selective repression of certain genes and hence their respective products. In this context the ‘disallowed genes’ hypothesis proposed by Rutter and Schuit (Pullen and Rutter, 2013)(Quintens et al., 2008) suggests that disallowed genes possess a deleterious potential and would be regulated by multiple independent mechanisms, including miRNAs. It is thought that miRNAs can repress unwanted genes whose activity may be harmful in a

particular cell type and/or context (Mendell and Olson, 2012). Indeed, Martinez-Sanchez et al. identified miRNAs as having a key role in the repression of such “forbidden” genes. In fact, the inactivation of the miRNA-processing enzyme, Dicer, specifically in adult β -cells in mice leads to a substantial reduction in miRNAs in these cells. Of the 14 disallowed genes studied, 6 genes were up-regulated after Dicer invalidation. In addition, Dicer silencing correlates with a loss of the secretory capacity of the islets (Martinez-Sanchez et al., 2015). Specific miRNAs have been shown to mediate repression of specific disallowed genes. For example, several miR-29 isoforms target MCT1 in β -cells, which ensures that muscle lactate or pyruvate, produced during exercise, is unable to stimulate inappropriately β -cell metabolism and hence prevents unwanted insulin secretion (Pullen et al., 2011). Indeed, forced expression of MCT-1 in β -cells provokes pyruvate-stimulated insulin release in isolated rat islets (Ishihara et al., 1999). Thus, miR-29 contributes to the β -cell-specific silencing of the MCT1 transporter and may thus contribute to β -cell specificity/identity. Other miRNAs are able to recognize the 3' UTR region of MCT1 mRNA. For example, miR-124a has multiple predicted target sites on the 3' UTR of MCT1 that are conserved among human, rat, mouse, dog and chicken. Downregulation of MCT1 by miR-124a has been experimentally validated (Lim et al., 2005)(Wang and Wang, 2006). Moreover, miR-27b, 506-3p are also predicted to recognize the MCT1 3'UTR. A challenging question arises as to whether members of the disallowed gene family other than MCT1 are regulated by miRNAs. In summary, several miRNAs appear to exert a determining role in the regulation of disallowed genes in β -cells, and as such provide evidence for a newly identified way through which miRNAs portray the functional identity of β -cells (Martinez-Sanchez et al., 2015).

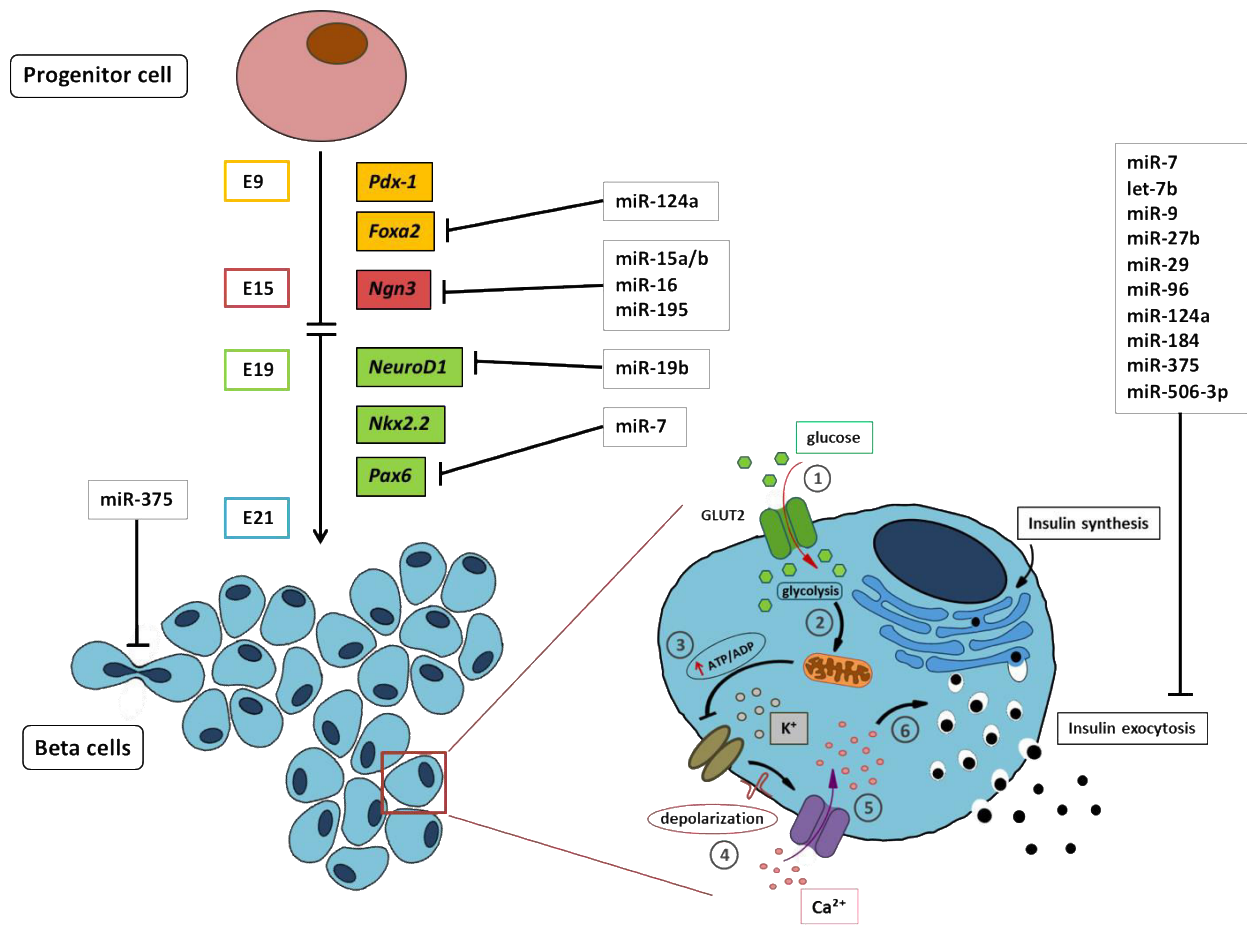


Figure 12 Examples of miRNAs impacting on β -cell differentiation and functioning. Some of the miRNAs discussed in the manuscript, and shown here, target transcription factors involved in β -cell development (the colors associated to each transcription factor on the arrow reflect the rat “embryonic age” of organogenesis they belong to). Other miRNAs affect β -cell functioning by directly or indirectly modulating insulin secretion. For example, let-7b is known to target myotrophin (MTPN), which is involved in insulin granule fusion with the plasma membrane, and thus directly inhibits insulin exocytosis. In contrast, miR-184 inhibits Ago2 expression affecting other miRNAs and hence interferes indirectly with the secretory pathway. Interestingly, some of the miRNAs listed exert a double role at different levels of β -cell functioning. For instance, miR-375 is able to interfere both with β -cell proliferation and with insulin exocytosis.

MiRNAs and β -cell function

The identity of β -cells does not originate only from its particular and streamlined metabolic machinery, but also from its unique feat to be able to provide insulin to the body.

MiR-375 is considered as the miRNA with the most robust expression in the endocrine pancreas. This miRNA was the first to be identified as being involved in insulin secretion (Poy et al., 2004). Indeed, forced expression of miR-375 in MIN6 insulinoma cells reduced glucose-induced insulin secretion by inhibiting exocytosis. Conversely, when miR-375 is decreased in mice, the cells secrete more insulin in response to glucose (Poy et al., 2004)(Dumortier et al., 2014). The effect of miR-375 on insulin exocytosis appears to be due to the binding of the miRNA to the 3' UTR of mRNA encoding for myotrophin (MTPN), which is known to be involved in insulin granule fusion with the plasma membrane. Further, our laboratory has shown that miR-375 downregulates insulin gene expression in INS1-E cells by acting on the mRNA of phosphoinositide-dependent kinase-1 (PDK-1), which was the second functionally identified miR-375 target. Remarkably, miR-375 expression is inhibited by glucose, attributing to miR-375 the status of a physiological regulator of β -cell functioning (El Ouaamari et al., 2008).

In the miRNA machinery, Argonaute2 (Ago2) exerts a central function (Liu et al., 2004b). Inhibition of Ago2 in pancreatic β -cells results in enhanced insulin release, suggesting that most of the miRNAs have a negative influence on β -cell physiology (Selbach et al., 2013)(Tattikota et al., 2014). As miR-375 constitutes the largest proportion of the total pool of miRNAs in islet cells, this observation may explain several similarities existing between models with a loss of Ago2 compared to a loss of miR-375. Indeed, the silencing of Ago2 leads to increased expression of miR-375 target mRNAs, including gephyrin and ywhaz. These targets positively contribute to exocytosis indicating that they may mediate the functional role of both miR-375 and Ago proteins in β -cells by modulating the secretory pathway (Selbach et al., 2013). Remarkably, in another study, Tattikota et al. showed that miR-184, highly conserved and abundantly expressed in β -cells, targets Ago2 (Tattikota et al., 2014). Reduction of miR-184 promotes the expression of its target Ago2 facilitating the function of miR-375. These observations illustrate perfectly well the complexity and the intricacy of the network involving small RNAs to maintain essential metabolic processes such as glucose-induced insulin secretion (Figure 12).

Likewise miR-124a has been found to act at different regulatory levels of insulin exocytosis by directly targeting MTPN (Krek et al., 2005) and GTPase Rab27 (Lovis et al., 2008). Other miRNAs are able to modulate insulin secretion such as let-7b also targeting MTPN (Krek et al., 2005), and miR-96, which regulate the mRNA and protein levels of synaptotagmin-like 4 (Sytl4, an inhibitor of insulin exocytosis (Lovis et al., 2008). Moreover, Sytl4 is antagonized by miR-9 via the interaction of the latter with the mRNA of the Onecut-2 transcription factor (one cut homeobox 2) (Plaisance et al., 2006). In addition, the members of the miR-29 family inhibit insulin secretion by decreasing the Onecut-2 protein in MIN6 cells and dispersed islet cells (Roggli et al., 2012).

Origins of miRNAs: hidden in the genome

MiRNAs were initially thought to be intergenic with an independent transcriptional activity, as they were found in non-coding regions between genes. However, studies in different species showed that their sequence can be intergenic, exonic or intronic. In mammals, most of them is located in the intronic sequence of both coding and non-coding genes, another 30% can be found in intergenic regions and the rest in the exons of genes (Ratnadiwakara et al., 2018). Depending on their reciprocal position on the genome, miRNA genes can be identified as monocistronic, expressed as independent transcripts, polycistronic, if they are clustered together and expressed as a singular primary transcript, which require an additional processing step to separate the different miRNAs, and sequences from which both a miRNA and a protein-coding mRNA originate (Lee et al., 2002). Even if for most miRNA genes the exact position of their promoter has not been mapped yet, intergenic miRNAs have been shown to be transcribed from their own promoter, whereas intronic miRNAs usually share the promoter of their host genes (Baskerville and Bartel, 2005)(Monteys et al., 2010). Nevertheless, a many miRNA genes possess multiple transcription start sites and it was recently demonstrated that almost 35% of the intronic miRNAs can be driven by independent promoters (Ozsolak et al., 2008)(Corcoran et al., 2009).

MicroRNA biogenesis: the canonical pathway

MiRNAs are generated as long transcripts of around 1 kb, called primary microRNAs (pri-miRNAs), by the RNA polymerase II (RNA Pol II) with the additional involvement of RNA Pol II-associated transcription factors and epigenetic regulators. They contain one or more characteristic hairpin structures in which the mature miRNAs are embedded and recognized by the nuclear microprocessor complex for further processing (Lee et al., 2004). More in details, a typical pri-miRNA is composed by a 33-35 bp stem connected to a terminal loop, and additional single-stranded RNA (ssRNA) extremities at both the 5' and 3' sides. The microprocessor complex precisely recognizes and cleaves this structure, thus originating a small hairpin-shaped RNA of 60-80 nucleotides in length, named precursor microRNA (pre-miRNA). The Microprocessor is a heterotrimeric complex formed by one molecule of an RNase III enzyme, called Drosha, and two molecules of a dsRNA-binding protein known as DiGeorge syndrome critical region 8 (DGCR8) and which represents an essential cofactor for Drosha enzymatic activity (Denli et al., 2004)(Gregory et al., 2004). The importance of Drosha and DGCR8, therefore of miRNAs, in development was demonstrated by a series of knockout experiments. Indeed, germline deficiency of Drosha resulted to be lethal already by embryonic day 7.5 in mice (Chong et al., 2010), DGCR8-knockout in mouse embryo caused early arrest in development and DGCR8-knockout embryonic stem cells showed impaired differentiation and proliferation (Wang et al., 2007). As demonstrated recently by Kim and colleagues' knockout experiments, Drosha's function is absolutely necessary for the canonical miRNA biogenesis (Young-Kook Kim, 2016). The catalytic activity of this RNase is associated to its carboxyl terminus, which hosts tandem RNase III domains (RIIIDs) and a dsRNA-binding domain (dsRBD). Two RIIIDs – RIIIDa and RIIIDb – dimerize intramolecularly thus forming the processing center of the Microprocessor, where the 3' and the 5' of the pri-miRNA is cleaved respectively by RIIIDa and RIIIDb. The dsRBD is necessary for substrate interaction, but it is not sufficient. Indeed, is the recruitment of DGCR8 at the processing center which provides the additional RNA-binding activity relevant for the correct and efficient functioning of the Microprocessor complex (Han et al., 2004a)(Kwon et al., 2016). Drosha cuts the hairpin at an 11 bp-distance from the connection site between ssRNA and dsRNA (basal junction), and approximately 22 bp away from the terminal loop (apical junction). In this process the basal junction covers the major role in determining the

cleavage site, whereas the apical junction's relevance resides in guaranteeing an efficient and accurate processing of the pri-miRNA (Ha and Kim, 2014).

The pre-miRNA originating from Drosha processing needs to be exported to the cytoplasm in order to complete its maturation. This is under the control of a specific protein called Exportin-5 (EXP5), which forms a transport complex with the GTP-binding nuclear protein Ran and a pre-miRNA (Yi et al., 2003)(Lund et al., 2004)(Bohnsack et al., 2004). After its translocation across the nuclear pore complex, GTP is hydrolyzed, causing the disassembly of the transport complex and the release of the pre-miRNA into the cytosol. This can be therefore cleaved by Dicer near the terminal loop, originating a small RNA duplex (Hutvagner et al., 2001)(Ketting et al., 2001)(Grishok et al., 2001).

Dicer is an RNase III-type endonuclease whose catalytic activity, similarly to Drosha, is defined by the intramolecular dimerization of tandem RIIIDs at the C-terminal (Zhang et al., 2004). The pre-miRNA recognition site resides, instead, at the amino-terminal helicase domain of the RNase, which interacts with the terminal loop of its target (Tsutsumi et al., 2011). Dicer crucial role in miRNA biogenesis and its importance for development was assessed through germline deficiency experiments in mice, which led to early embryonic lethality (Bernstein et al., 2003), and by knockout embryonic stem cells studies, resulting in defects in cell proliferation and differentiation (Kanellopoulou et al., 2005)(Murchison et al., 2005). Dicer binds to the pre-miRNA preferentially at the 3' protrusion generated by Drosha, as the cleavage sites are usually located at 21-25 nucleotides distance from the 3' end of dsRNAs (3'-counting rule) (MacRae et al., 2006). An additional mechanism has been recently identified in mammals and flies, which sees the binding of Dicer also to the phosphorylated 5' of pre-miRNAs and localizes the cleavage site at a 22-nucleotides distance from the 5' end (5'-counting rule) (Park et al., 2011). Dicer enzymatic activity generates a ~22 bp miRNA duplex, one of the two strands of which is loaded onto Argonaute (Ago) proteins to form a miRNA-induced silencing complex (miRISC), which is primarily responsible for the silencer activity of the miRNAs (Mourelatos et al., 2002)(Meister et al., 2004)(Liu et al., 2004a). (Figure 13)

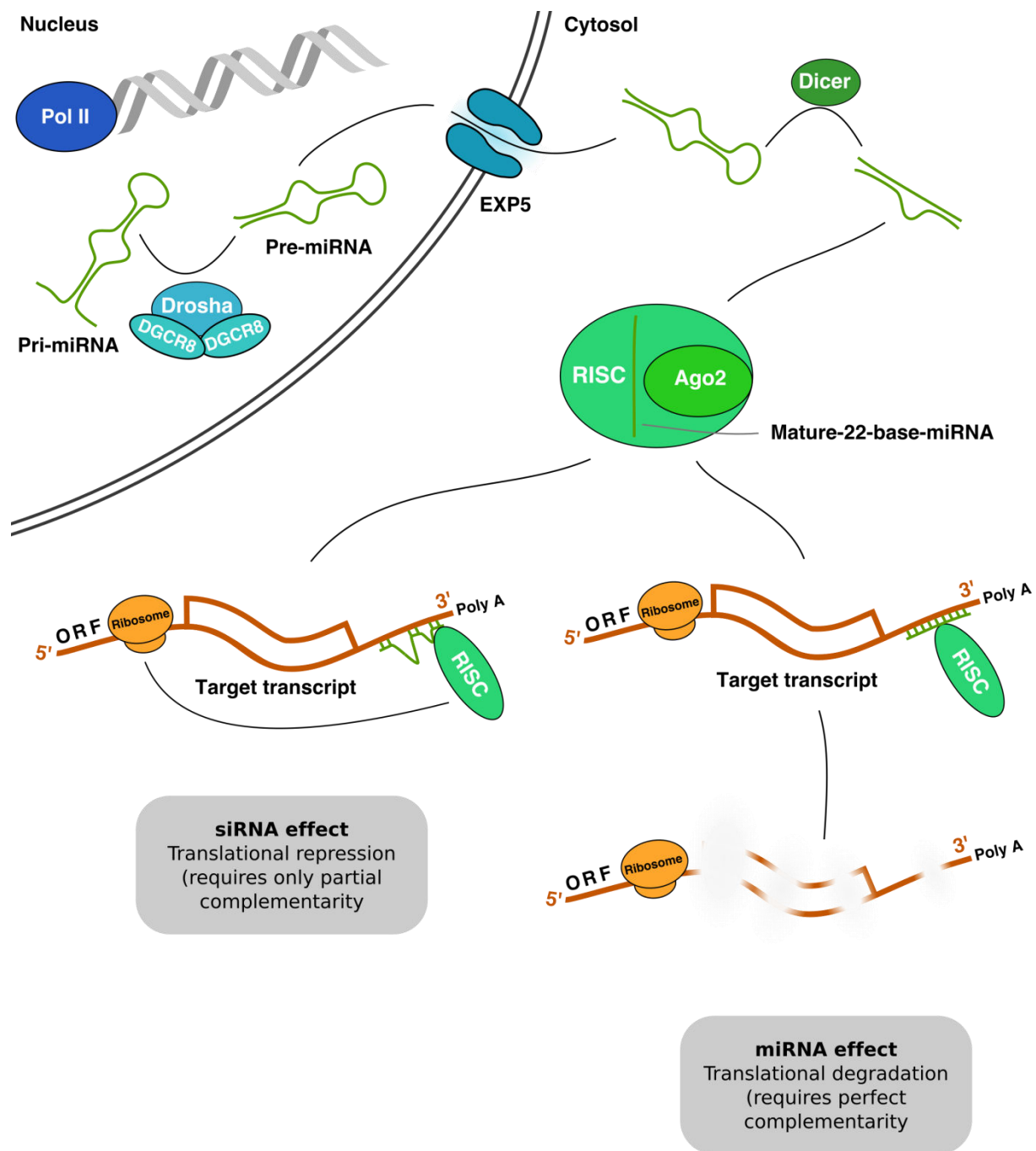


Figure 13 Schematic representation of miRNA biogenesis in mammalian cells. The primary microRNA (pri-miRNA) is transcribed by the RNA polymerase II (Pol II) and subsequently cleaved by the heterotrimeric complex formed by Drosha and DGCR8. The resulting pre-miRNA is exported from the nucleus by the Exportin 5 (EXP5). In the cytoplasm, Dicer cuts the loop of the pre-miRNA, thus originating a miRNA duplex. The association with argonaute 2 (Ago2) results in the degradation of one of the two miRNA strands and the formation of the RISC complex, which orchestrates miRNA silencing activity.

The eukaryotic Argonaute family comprises Ago proteins, which interact with miRNAs and short interfering RNAs (siRNAs) and are involved in post-transcriptional repression, and PIWI proteins, mostly expressed in germline cells, where they binds to PIWI-interacting RNAs (piRNAs) and silence the expression of transposable genetic elements (Meister, 2013). Both of them are characterized by specific domains from which the interaction with the miRNA duplex depends: amino-terminal (N), PAZ (Piwi-Argonaute-Zwille), MID (middle) and PIWI. More specifically, each Argonaute protein is organized in two lobes connected by a hinge, one containing the N-PAZ and the other the MID-PIWI domains. The N domain is important for the small RNA loading and unwinding, the MID domain anchors the 5', with a preference for U or A binding, and brings the small RNA to the PAZ domain, which binds the 3' and folds the RNA in a specific binding pocket (Jinek and Doudna, 2009). The PIWI domain has a similar structure than the RNase H, with a catalytic site which enables the slicing of target mRNAs between nucleotide positions 10 and 11, with respect to the 5' end (Parker et al., 2005). Concerning miRNA biogenesis, it has been described that mammals are equipped with 4 Argonaute proteins (Ago1-4) (Höck and Meister, 2008)(Benjamin Czech and Gregory J. Hannon, 2011). Although Burroughs and colleagues have reported that specific classes of miRNAs might have binding preferences (Burroughs et al., 2011), it has been shown that a random assignment of miRNAs to the different Argonautes takes also place (Dueck et al., 2012)(Wang et al., 2012). However, among the mammalian Argonautes, only Ago2 functions as an endonuclease (Meister et al., 2004)(Liu et al., 2004a) as Ago3, which has been recently discovered to have also a slicer ability, has very specific substrate requirements (Park et al., 2017). Ago2-knockout mice are embryonic lethal (Liu et al., 2004a), while mouse embryonic stem cells deficient in Ago1-4 result in defective miRNA silencing activity and undergo apoptosis (Su et al., 2009).

The association of Ago and RNA duplex forms a pre-RISC complex from which one RNA strand (the passenger strand) has to be separated from the guide strand and degraded in order to generate the mature and functional RISC. Usually the identification of the guide strand happens during the Ago loading phase, mostly according to the thermodynamically stability of the two 5' ends of the RNA duplex. In fact, the one showing the less stable 5' terminus is usually selected as guide strand and remains associated with the Ago protein (Schwarz et al., 2003)(Khvorova et al., 2003). An additional mechanism of selection is based

on the first nucleotide of the sequence, as Ago proteins tend to choose as guide strand the one with a U at nucleotide position 1 (Ha and Kim, 2014).

The exact molecular composition of the mature miRISC has not been completely resolved yet. Most likely the minimal RISC, composed by Ago and a miRNA, serves as a platform to recruit additional factors – both proteins required to build a functional RISC and Ago interacting proteins – which will define the specific action of the RISC on the mRNA target (Catalanotto et al., 2016). When the complementarity between the mRNA and the miRNA is very high or perfect, such as in plants, the mRNA is silenced by cleavage. On the contrary in mammals, where only an extremely low number of miRNAs have sufficient complementarity to trigger Ago2 endonucleolytic activity, the model suggested was a translational repression of the mRNA target (Bartel, 2004). However, a decade ago it has been proposed that animal miRNAs would instead induce the degradation of the great majority of mRNAs they bind, by a process distinct from endonucleolytic cleavage (Baek et al., 2008). Nevertheless, a small fraction of miRNA silencing activity (11-16%) has been still associated to a block in translation (Guo et al., 2010). As an explanation for these controversial data, it was recently suggested that the miRISC would inhibit mRNA translation first, and then induce its degradation probably independently from any specific property of Ago or of the miRNA itself, but most likely as a response to the block in the mRNA translation (Djuranovic et al., 2012)(Ameres and Zamore, 2013).

MicroRNA biogenesis: the non-canonical pathways

In addition to the canonical miRNA biogenesis pathway just described, miRNAs and miRNA-like RNAs can also originate from alternative mechanisms which do not need the participation either of the Microprocessor complex or of Dicer, although in vertebrates they comprise only the 1% of conserved miRNAs (Ha and Kim, 2014). The first non-canonical pathway described was the mirtron production: a small RNA precursor is generated by mRNA alternative splicing and folds into a stem-loop structure which mimics a pre-miRNA, thus bypassing the Drosha-mediated processing step (Okamura et al., 2007)(Berezikov et al., 2007)(Ruby et al., 2007). Drosha activity is also unnecessary for small RNAs derived from the transcription of endogenous short hairpin RNAs (Babiarz et al., 2008)(Chong et al., 2010).

These specific RNAs were thought to be transcribed only by RNA Pol III but, it was recently showed, some of them are originated from the action of RNA Pol II (Xie et al., 2013). The biogenesis of Drosha-independent miRNAs still relies on Dicer. However at least one miRNA, miR-451, does not need Dicer processing, whose action is instead substituted by the catalytic activity of Ago2. Indeed, Drosha pri-miR-451 cleavage generates an ~18 bp hairpin which is too short to be processed by Dicer. Pre-miR-451 is therefore directly loaded into Ago2 protein which cuts it in the middle of its 3', thus originating a ~30 nucleotides-long intermediate identified as Ago-cleaved pre-mir-451 (ac-pre-mir-451). This ac-pre-mir-451 can already assess its silencing function or, alternatively, in can be further processed by a Poly(A)-specific ribonuclease to originate a shorter mature miR-451 of ~23 nucleotides (Ha and Kim, 2014).

Results

During my PhD fellowship I took part to two different research projects.

In my main project, which I conducted independently, I investigated the effects of the lack of amino acids on the regulation of cell growth in primary adult rat hepatocytes, and I found that the induction of quiescence following total amino acid deprivation is regulated through a new non-canonical role of the Drosha protein. This study, where I am first author, is currently under favorable revision in the journal "Cell Death and Disease".

I have been also involved in an additional research project about the role of microRNA-375 in the regulation of glucose-dependent insulin secretion in rat and human islets. The manuscript resulting from this investigation, where I am second author, has been submitted to "Journal of Endocrinology".

Amino acid-induced regulation of hepatocyte growth: possible role of Drosha

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Abstract

In an adult healthy liver, hepatocytes are in a quiescent stage unless a physical injury, such as ablation, or a toxic attack occur. Indeed, to maintain their important organismal homeostatic role, the damaged or remaining hepatocytes will start proliferating to restore their functional mass. One of the limiting conditions for cell proliferation is amino acid availability, necessary both for the synthesis of proteins important for cell growth and division, and for the activation of the mTOR pathway, known for its considerable role in the regulation of cell proliferation. The overarching aim of our present work was to investigate the role of amino acids in the regulation of the switch between quiescence and growth of adult hepatocytes. To do so we used non-confluent primary adult rat hepatocytes as a model of partially ablated liver. We discovered that the absence of amino acids induces in primary rat hepatocytes the entrance in a quiescence state together with an increase in Drosha protein, which does not involve the mTOR pathway. Conversely, Drosha knockdown allows the hepatocytes, quiescent after amino acid deprivation, to proliferate again. Further, hepatocyte proliferation appears to be independent of miRNAs, the canonical downstream partners of Drosha. Taken together, our observations reveal an intriguing non-canonical effect of Drosha in the control of growth regulation of adult hepatocytes responding to a nutritional strain, and they may help to design novel preventive and/or therapeutic approaches for hepatic failure.

Introduction

The liver is one of the eminently important organs in the regulation of organismal homeostasis in vertebrates, as it exerts and orchestrates multiple essential biological functions, including all fuel metabolism, detoxification of intrinsic and extrinsic substances and production of numerous proteins with diverse extra-hepatic actions. Proteins, representing around 15% of body mass in healthy adults ¹, have major roles not only as constituents of all cells, but also as molecules enabling cells to function, grow and differentiate. Therefore, the availability of amino acids (aa), the building blocks of proteins, is crucial for the existence of life itself. The liver plays a pivotal role in aa metabolism, being a chief actor in systemic protein synthesis and degradation. Indeed, 60% of the aa taken up by daily diet are destined to be catabolized and take part to the hepatic urea cycle ^{2,3}. The liver also performs gluconeogenesis of aa into glucose during starvation ⁴ and contributes to plasma aa homeostasis ⁵. One of the strikingly remarkable features of adult liver is its great regenerative capacities after partial ablation or injuries ^{6,7}. The liver is composed of parenchymal cells, i.e. hepatocytes, and non-parenchymal cells, including sinusoidal endothelial cells, cholangiocytes, Kupffer cells and stellate cells. Hepatocytes constitute the most abundant cell type in the liver, accounting for 80% of liver mass, and they perform the majority of the functions mentioned above ⁸. In an adult healthy liver, hepatocytes show an extremely low proliferation rate as they mainly remain in quiescence until an aggression occurs, which induces entrance in the cell cycle ^{9,10}. One of the conditions necessary for cell proliferation is an increment in protein synthesis, which allows cells to grow and divide. Aa availability, aside of being obviously indispensable for protein synthesis, plays also an essential role in regulating cell proliferation through the activation of mTOR and its downstream S6 kinase ^{11,12}.

Even though hepatocyte proliferation during liver regeneration is well characterized, yawning gaps exist concerning our understanding of its regulation by aa. The overarching aim of our work was to study how aa participate in the control of the switch between quiescence and growth of adult hepatocytes. By using non-confluent primary adult rat hepatocytes, we mimicked the condition of active proliferation after an injury, thus being able to investigate the regulation of hepatocyte growth capacity by aa. We show that the aa lack induces a quiescence state coupled to increased expression of Drosha, the major actor

in microRNA (miRNA) biogenesis. Furthermore, the siRNA-induced reduction of Drosha restores proliferation in aa absence, while the decrement in Ago2, necessary for miRNA functioning, does not. Collectively, our findings unveil a new non-canonical role of Drosha in regulating the response of adult hepatocytes to aa availability and could contribute to innovative approaches to prevent and/or treat hepatic failure.

Results

Amino acid deprivation blocks primary rat hepatocyte proliferation and induces a metabolic rest without affecting the mitochondria

To investigate the effect of total aa deprivation (aaD) on primary adult rat hepatocytes, we cultured them in aa presence or absence. The number of cells grown in a full-aa medium (DMEM) reaches a maximum within 24 h following cell isolation and remained constant thereafter up to 48h. In contrast, the number of hepatocytes cultured without aa was steady up to 48 h, suggesting that they do not grow (Fig. 1a). Furthermore, they maintained their original phenotype as we did not observe differences in the expression of key hepatocyte markers after 48 h aaD (data not shown). In addition, the aa-deprived hepatocyte population appears to be more stable after 48 h, as their number decreases slower compared to hepatocytes in complete DMEM. Indeed, after 72 h aaD the cell number decreases only by 30% against 70% in cells cultured in full-aa medium (Fig. S1a). To investigate whether this difference was due to changes in cell proliferation or in cell death, we analysed apoptosis after 48 h culture. We found that aaD does not increase apoptosis (Fig. 1b – S1b), suggesting that the stable cell number seen in aaD is likely due to proliferation arrest. To further explore the effect of aaD on cell metabolism, we measured the oxygen consumption rate (OCR) in aa presence or absence. As illustrated in Fig. 1c, the lack of aa leads to a 2-fold decrease in basal mitochondrial respiration and a 2.8-fold reduction in maximal mitochondrial respiration, but we observed no decrease in glycolysis in basal condition (Fig. 1d). Remarkably, this cannot be explained by differences in the protein level of the OXPHOS complexes (Fig. 1e). Further, both the network and the number of mitochondria per cell appear to be similar in both conditions (Fig. S1c-d). To decrypt in more detail the effect of aaD on primary rat hepatocytes, we investigated the implication of autophagy both in aa presence and absence. Predictably from the known effects of aa depletion on autophagy, a lack of aa leads to an increase in the LC3-II/LC3-I ratio, indicating augmented autophagy (Fig. 1f-g – S1e). To summarize, the data indicate that aa-deprived primary hepatocytes are growth-arrested and this is correlated to altered mitochondrial metabolism and increased autophagy.

Fig. 1:

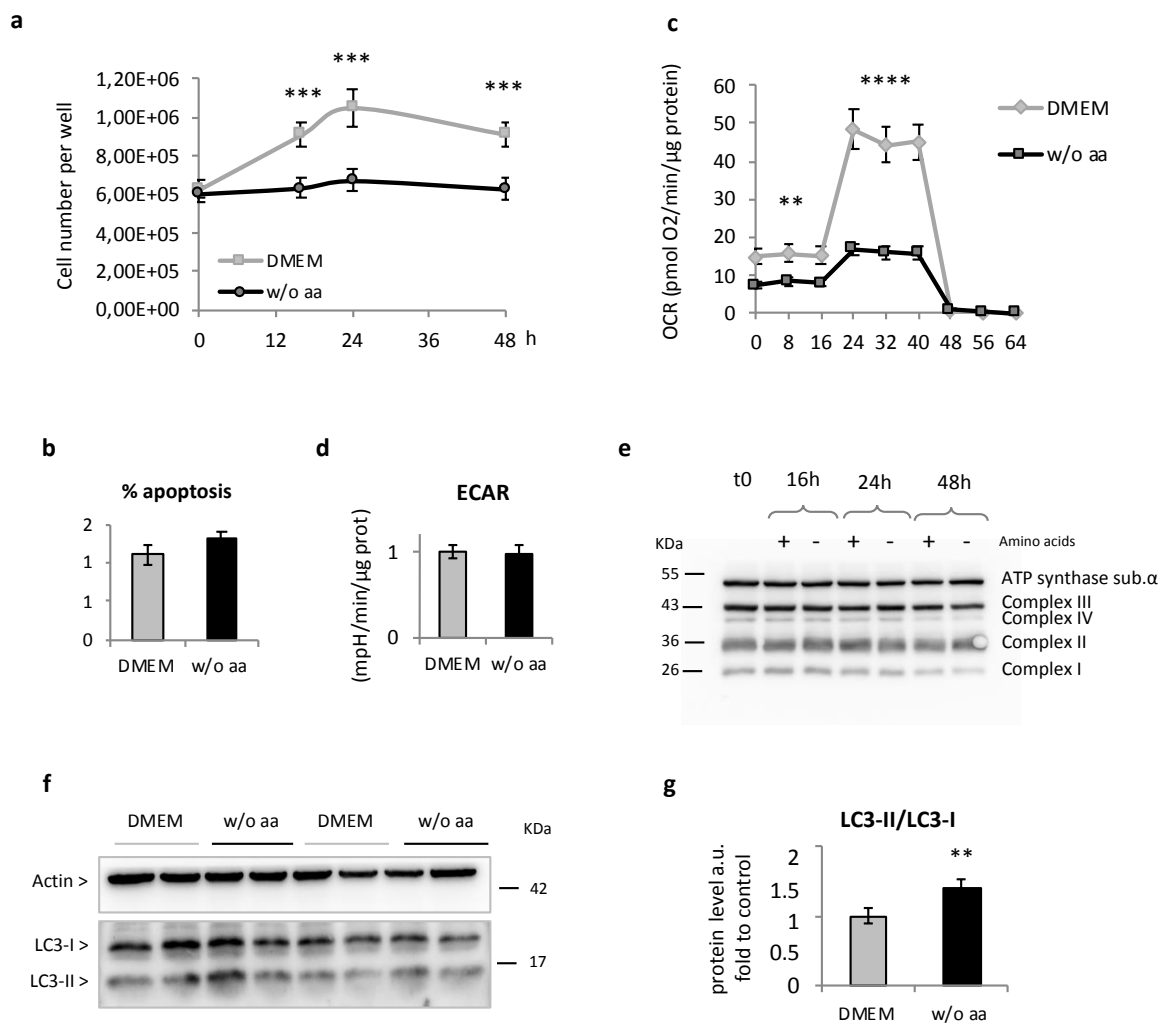


Figure 1. AaD in primary adult rat hepatocytes blocks cell proliferation and reduces mitochondrial metabolism without affecting either the oxphos complexes or the number of mitochondria. (a) Cell counting of primary adult rat hepatocytes in presence of aa (DMEM) and in absence of aa (w/o aa) at different time points. Values are means \pm SEM (n=3). ***p < 0.0005 (b) Quantification of primary adult rat hepatocyte's apoptotic rate in DMEM and after 48h aaD (w/o aa) by TUNEL assay. Results are expressed in percentage of apoptotic cells over total cell number. Values are means \pm SEM (n=3). (c) Mitochondrial oxygen consumption rate (OCR) from primary adult rat hepatocytes after 48h from plating in DMEM or w/o aa. Maximal OCR was determined using FCCP [2 μ M]. OCR is reported in the unit of picomoles per minute. Values are means \pm SEM (n= 16 replicates from 2 independent rats). **p < 0.005, ****p < 0.0001 (d) Extracellular acidification rate (ECAR) from primary adult rat hepatocytes after 48h from plating in DMEM or w/o aa. ECAR is reported in millipH units (mpH) per minute. Values are means \pm SEM (n= 16 replicates from 2 independent rats). (e) Western blot with antibody to the oxphos complexes of cell lysates obtained from primary rat hepatocytes after isolation (t0) and cultured for 16h, 24h and 48h either in DMEM or in medium w/o aa. (n=3). (f) Western blot analysis of LC3-I/II in primary adult rat hepatocytes after keeping them in culture for 48h in DMEM and in medium w/o aa; cells were treated with 25 μ M chloroquine for 1h before protein extraction. For quantification (g) each band was normalized by the actin band and the normalized values of LC3-II were then divided by LC3-I values. Values are means \pm SEM (n=3). **p < 0.01

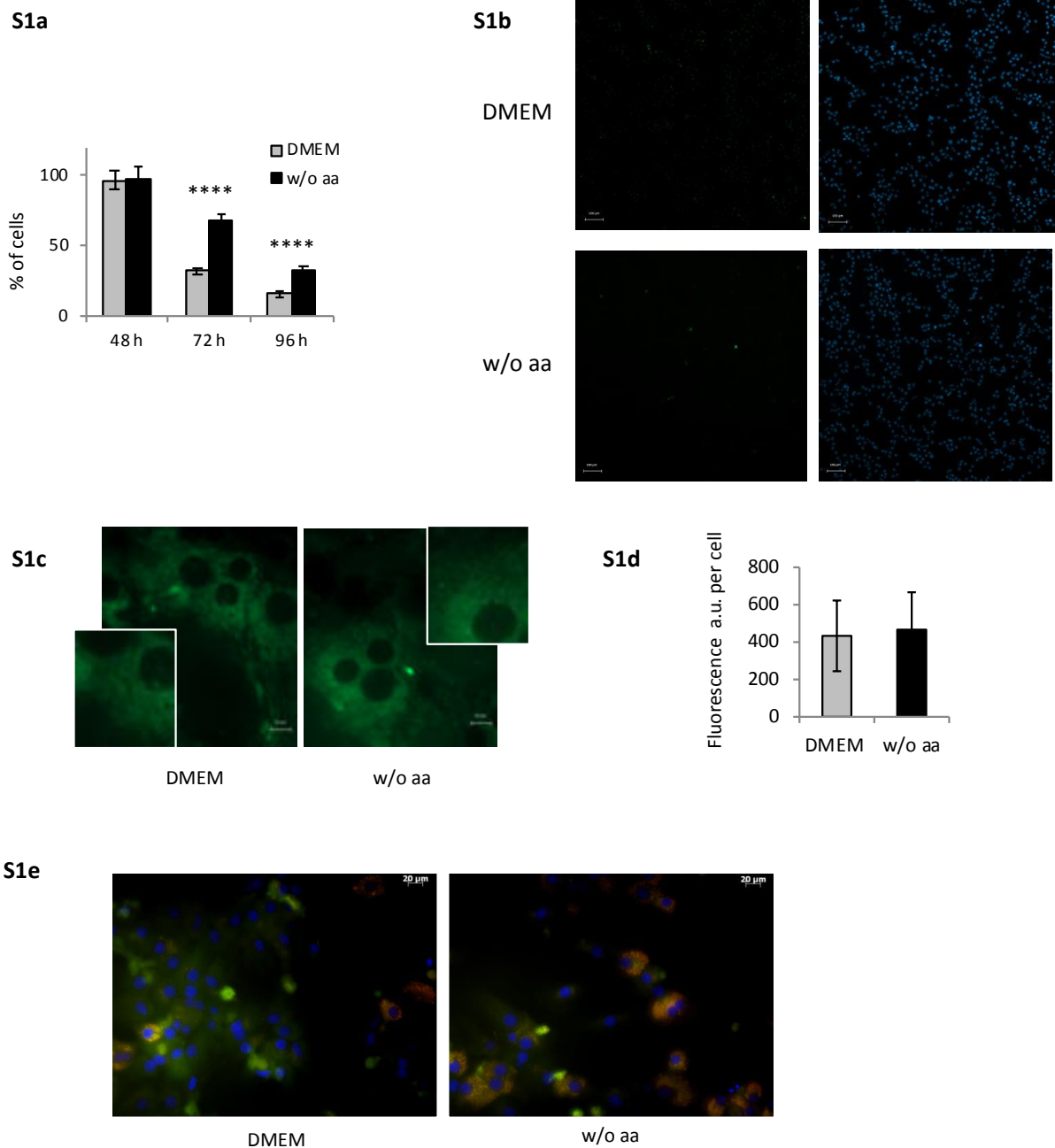


Figure S1. AaD in primary adult rat hepatocytes reduces the drop in cell number. (a) Cell counting of primary adult rat hepatocytes in full-aa medium (DMEM) and in absence of aa (w/o aa) at different time points. Results are normalized to values at 48h for both experimental conditions and expressed in percentage, being 48h-cell number 100%. Values are means \pm SEM ($n=3$). **** $p < 0.0001$. (b) Detection of apoptosis by TUNel assay: apoptotic cells (green) were counted together with DAPI (blue) in fixed primary adult rat hepatocytes after 48h in DMEM or in medium w/o aa. Scale bar = 100 μ m. (c) Mitochondrial network organization observed in control (DMEM) and aa-deprived (w/o aa) hepatocytes after 48h from plating using MitoTracker Green FM labeling. Scale bar = 10 μ m. (d) FACS analysis of the mean intensity of fluorescence per cell in primary adult rat hepatocytes after 48h in DMEM or w/o aa using MitoTracker Green FM labeling. Values are means \pm SEM ($n=3$). (e) Detection of LC3 puncta in the autophagosomes of primary adult rat hepatocytes after 48h aaD: cells in autophagy are characterized by co-localization of LC3 puncta (green) and lysosomal marker (red). Cell nuclei are marked with DAPI (blue). Scale bar = 20 μ m.

Amino acid-deprived hepatocytes are able to exit growth arrest after the addition of essential amino acids

Next, we asked whether the hepatocyte growth arrest was reversible or not. To this aim we cultured primary adult rat hepatocytes for a total of 48 h, the first 24 h in aa absence and the following 24 h with total aa restoration. Cell counting showed that the addition of a full supply of aa (essential and non-essential) to the medium allows hepatocytes to restart their proliferation and achieve the same level as hepatocytes which were not depleted (Fig. 2a). Interestingly, a similar experiment performed with the addition of solely essential aa (e.aa) gave partially different results as restoration of the level of cell number was not completely attained, showing a 10% deficit compared with non-depleted hepatocytes (Fig. 2b). Taken together, these findings suggest that aaD induces in primary adult rat hepatocytes a quiescence state which can be reversed by aa addition. However, e.aa apparently do not suffice to induce a full-blown restoration.

Fig. 2:

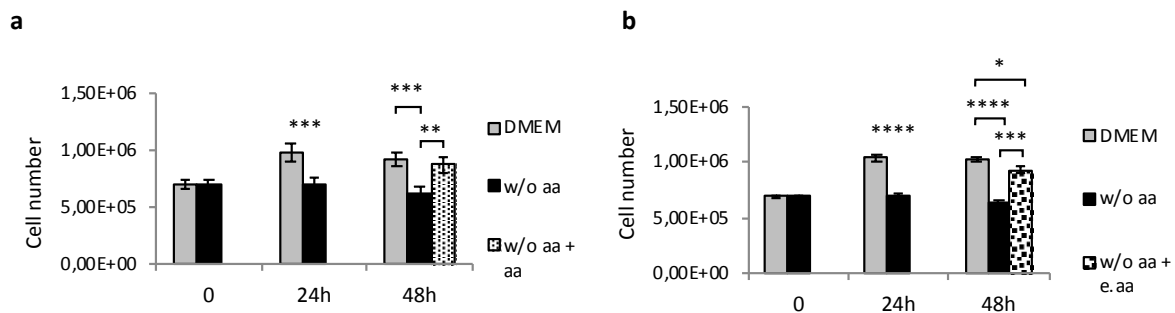


Figure 2. aaD in primary adult rat hepatocytes induces a quiescence state which can be reversed by aa addition. Cell counting of primary adult rat hepatocytes at t0, 24h and 48h after isolation. For the last timepoint, cells were kept in presence or absence of aa (DMEM; w/o aa) for 48h, or in medium w/o aa for 24h with the restoration of total aa (a) or essential aa (b) for the following 24h. Values are means \pm SEM (n=3). *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.

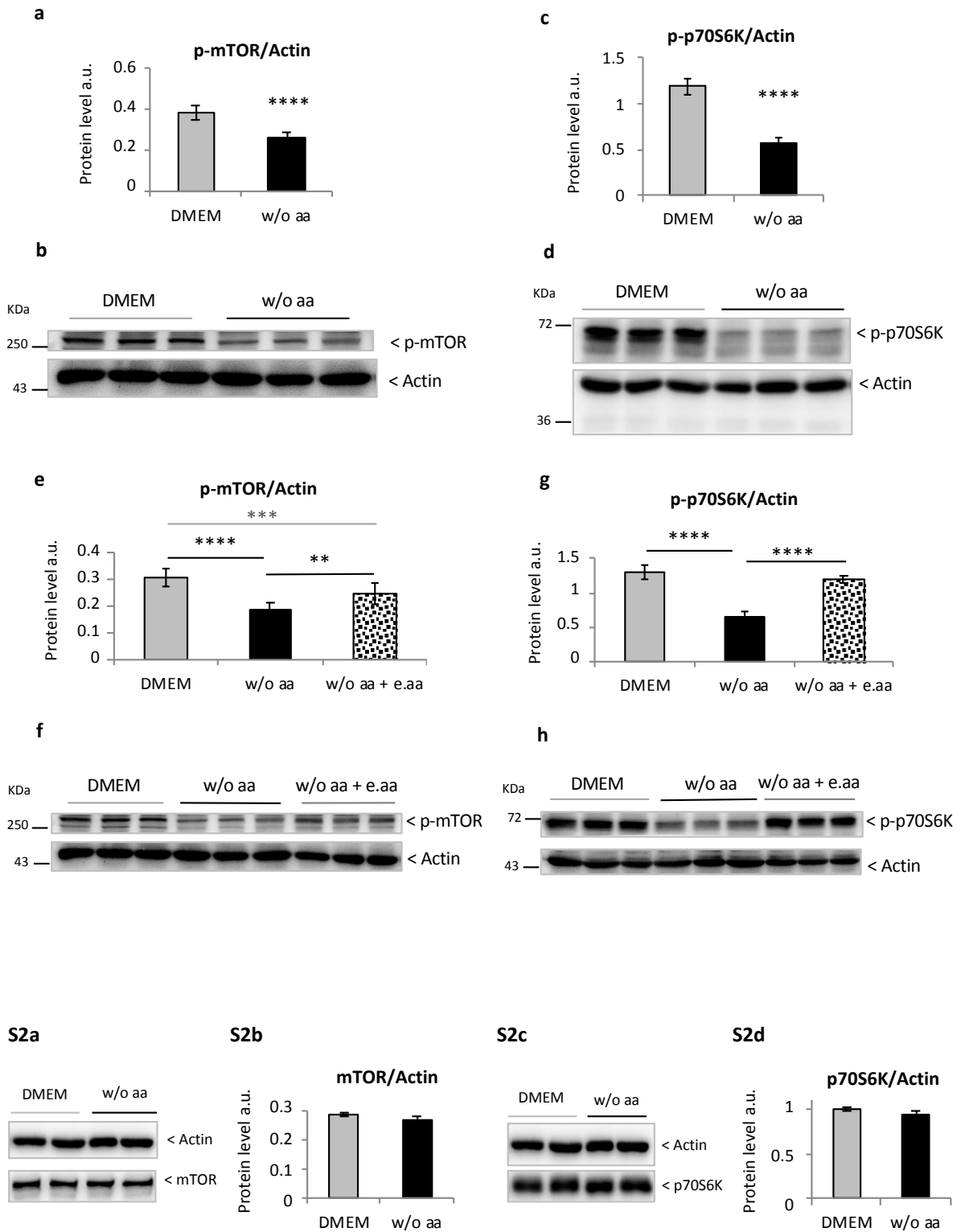
In aa deprived-growth arrested hepatocytes the activation of the mTOR pathway is repressed and this inhibition is partially reversed by essential aa

Considering the importance of aa in the regulation of the nutrient-sensing mechanistic target of rapamycin (mTOR) pathway^{13,14} and the role of this pathway in cell growth and division¹⁵, we investigated its activation in primary rat hepatocytes in aa presence or absence. Protein analysis showed that phosphorylation of both mTOR and its downstream kinase p70S6K are respectively reduced by 30% and 50% in aa-deprived cells (Fig. 3a-d). As the total protein level of both kinases is unchanged, this is compatible with the idea that activation of mTOR pathway is repressed by aaD (Fig. S2a-d). The inhibition of mTOR phosphorylation was not entirely due to e.aa deprivation, as e.aa addition is able to restore only partially mTOR phosphorylation. In fact, we observed an 18% phosphorylation decrease compared to levels obtained in hepatocytes cultured in DMEM (Fig. 3e-f). Intriguingly, the analysis of p70S6K in the same conditions gave a partially different outcome, as the presence of e.aa in the medium results in p70S6K phosphorylation levels comparable with those seen in the full-aa medium (Fig. 3g-h).

Figure 3. 48h aaD reduces mTOR and p70S6K phosphorylation, and the addition of e.aa to the medium is not sufficient to restore mTOR phosphorylation level. (a-b) Western blot analysis (a) and quantification (b) of mTOR phosphorylation after 48h in a full-aa medium (DMEM) or in medium w/o aa. Values are means \pm SEM (n=7). ****p < 0.0001 (c-d) Western blot analysis (c) and quantification (d) of p70S6K phosphorylation after 48h in a DMEM or w/o aa. Values are means \pm SEM (n=15). ****p < 0.0001 (e-f) Western blot analysis (e) and quantification (f) of mTOR phosphorylation after 48h in DMEM, w/o aa and in medium w/o aa supplemented with essential aa (w/o aa + e.aa). Values are means \pm SEM (n=4). **p < 0.01, ***p < 0.0005, ****p < 0.0001 (g-h) Western blot analysis (g) and quantification (h) of p70S6K phosphorylation after 48h in DMEM, w/o aa and w/o aa + e.aa. Values are means \pm SEM (n=7). ****p < 0.0001

Figure S2. AaD in primary adult rat hepatocytes does not affect the mTOR and the p70S6K total protein level. (a) Western blot analysis and (b) quantification of mTOR protein level after 48h in a full-aa medium (DMEM) or in medium w/o aa. Values are means \pm SEM (n=3). (c) Western blot analysis and (d) quantification of p70S6K protein level after 48h in DMEM or in medium w/o aa. Values are means \pm SEM (n=3).

Fig. 3:



Essential aa deprivation in primary adult rat hepatocytes leads to an increase in Drosha protein level

Ye et al. recently revealed the involvement of mTOR in miRNA biogenesis through the increase in Drosha after glucose deprivation¹⁶. To delve into the mechanisms implicated in the effects of aaD on hepatocyte metabolism, we investigated the effect of aa restriction on Drosha. Similarly to the aforementioned paper, we found that both 24 and 48 h aaD in primary rat hepatocytes lead to a 30% increase in Drosha protein (Fig. 4a-d) without affecting its mRNA level (Fig. S3a-b). Interestingly, the e.aa presence in the medium does not result in the same augmentation of Drosha protein as the one observed in aa absence, keeping it at the same level as in full-aa medium (Fig. 4e-f). These results suggest that, in addition to reversing the hepatocyte quiescence state, e.aa are able to prevent the increase in the Drosha protein.

Fig. 4:

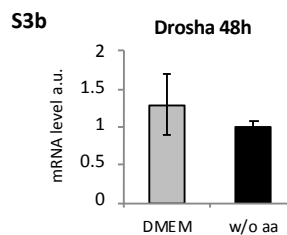
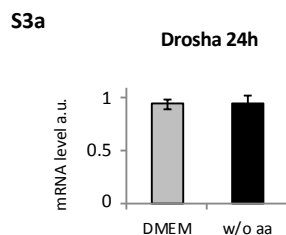
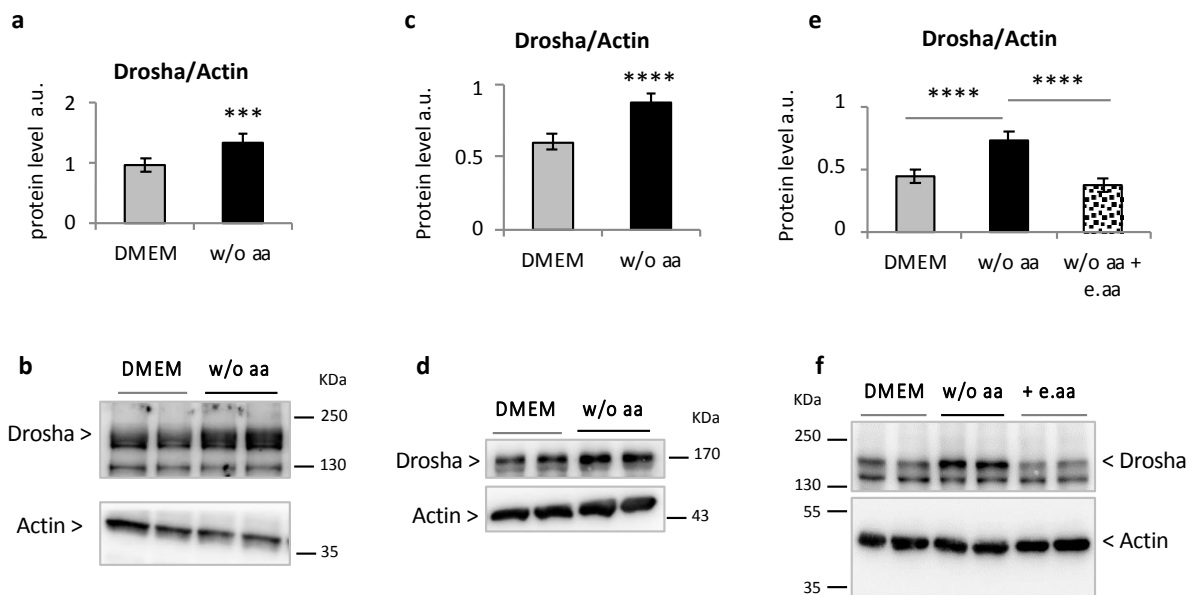


Figure 4. Essential aaD significantly increases Drosha protein in primary adult rat hepatocytes. (a-b) Western blot quantification (a) and analysis (b) of Drosha protein level after 24h in a full-aa medium (DMEM) or in medium w/o aa. Values are means \pm SEM (n=5). ***p < 0.0005 (c-d) Western blot quantification (c) and analysis (d) of Drosha protein level after 48h in DMEM or medium w/o aa. Values are means \pm SEM (n=16). ****p < 0.0001 (e-f) Western blot quantification (e) and analysis (f) of Drosha protein level after 48h in DMEM, w/o aa and in medium w/o aa supplemented with essential aa (w/o aa + e.aa). Values are means \pm SEM (n=7). ****p < 0.0001.

Figure S3. AaD in primary adult rat hepatocytes does not affect the Drosha mRNA level. Drosha mRNA measurement in primary adult rat hepatocytes: after 24h (a) or 48h (b) aaD, RNA was extracted, reverse transcribed and analyzed by quantitative PCR. Gene expression was normalized to the cyclophilin A (CyA) mRNA level. Values are means \pm SEM (n=3).

Neither short- nor long-term rapamycin treatment alters the Drosha protein level in primary adult rat hepatocytes

To assess the involvement of the mTOR pathway in Drosha modulation we treated primary adult rat hepatocytes with rapamycin. However, the specific inhibition of mTORC1 through a short-term treatment did not reveal an increment of Drosha (Fig. 5a-b). In contrast, an 8 h exposure resulted in a significant decrease in Drosha level (Fig. 5a-b). Similarly, 24 and 48 h treatment, likely responsible for the inhibition of both mTORC1 and mTORC2, did not lead to changes in Drosha protein (Fig. 5c-f). Taken together, our findings would indicate that in primary rat hepatocytes the mTOR pathway is not involved in the Drosha increase induced by aa depletion.

AaD in primary adult rat hepatocytes increases Drosha protein stability

To further untangle the mechanism underlying the Drosha increase, we investigated the half-life of the protein in aa presence or absence. As shown in Figure 6 a-b, the lack of aa in primary rat hepatocytes results in a slower degradation of Drosha compared to cells grown in a full-aa medium, the corresponding half-life of Drosha in medium w/o aa and in full-aa medium being respectively 1.9 hrs and 0.6 hrs. These results support the notion that the

aaD-dependent augmentation of Drosha is due, at least partly, to an increase in its protein stability compared to the control condition.

Fig. 5:

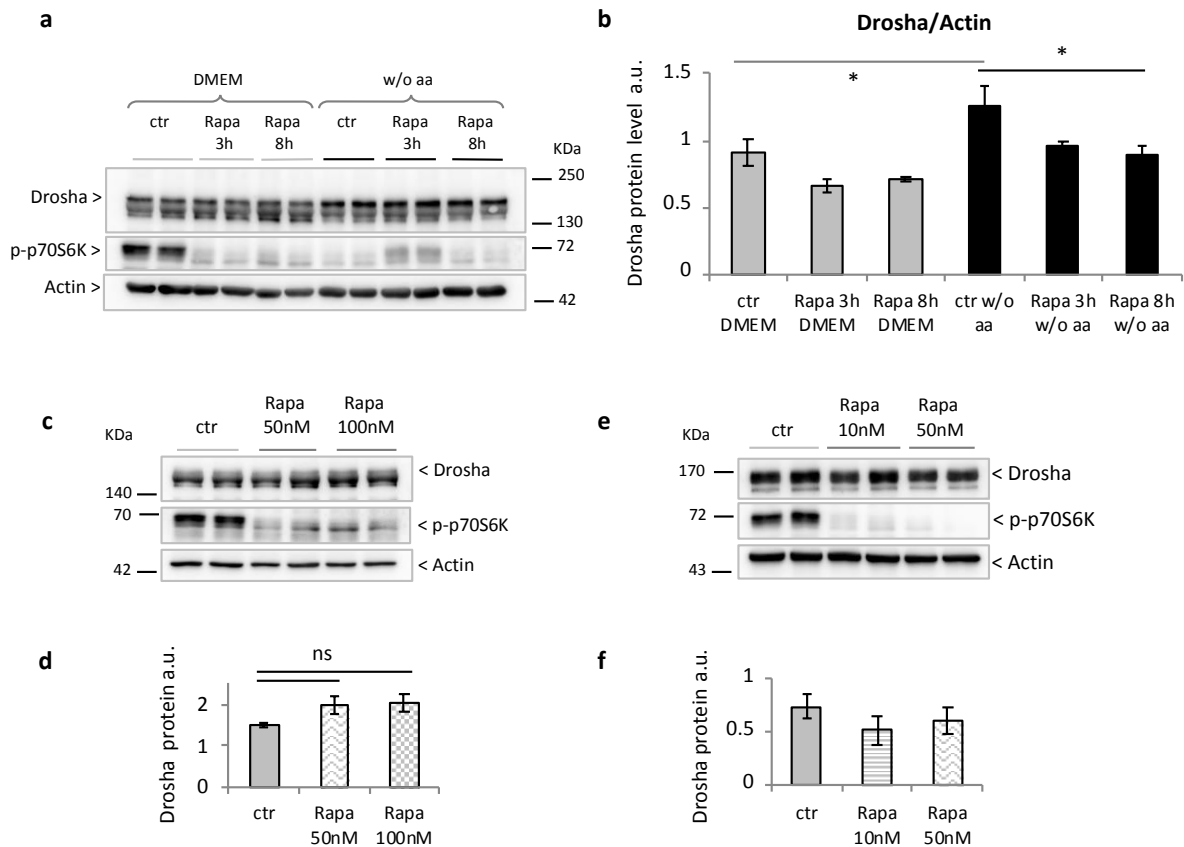


Figure 5. Neither short- nor long-term rapamycin treatments are able to reproduce the increase of Drosha observed in aa-deprived primary adult rat hepatocytes. (a) Western blot analysis of Drosha protein level and p70S6K phosphorylation and (b) Western blot quantification of Drosha protein in primary adult rat hepatocytes after 48h aaD without any treatment (Rapa ctr), after 3h and 8h rapamycin treatment [100 nM] (Rapa 3h, Rapa 8h). Note that p70S6K phosphorylation was measured as read out of rapamycin effect on mTOR activation. Values are means \pm SEM (n=3). *p < 0.05 (c) Western blot analysis of Drosha protein level and p70S6K phosphorylation and (d) Western blot quantification of Drosha protein in aa-deprived hepatocytes without any treatment (ctr) and rapamycin treatment for 24h. Values are means \pm SEM (n=3). (e) Western blot analysis of Drosha protein level and p70S6K phosphorylation and (f) Western blot quantification of Drosha protein in aa-deprived hepatocytes without any treatment (ctr) and rapamycin treatment for 48h. Values are means \pm SEM (n=5).

Fig. 6:

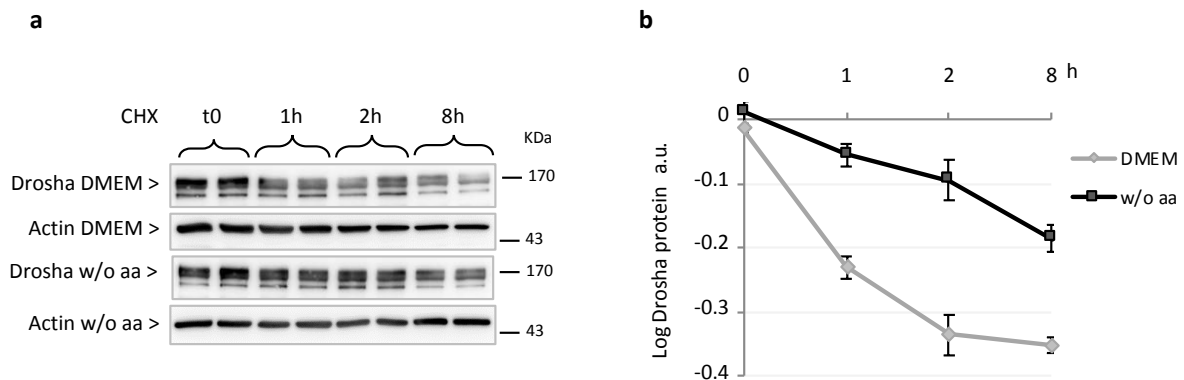


Figure 6. 48h aaD in primary adult rat hepatocytes increases Drosha protein stability. (a) Western blot analysis and (b) quantification of Drosha protein level in primary adult rat hepatocytes cultured in full-aa medium (DMEM) or in absence of aa (w/o aa): the protein content of cells isolated from the same liver was analyzed just before treatment (t0) or after 1h, 2h and 8h from the addition of cycloheximide (CHX) [50 μ M]. Values are means \pm SEM (n=3). *p < 0.05, ***p < 0.0005.

Drosha knockdown allows cell proliferation in aa-deprived hepatocytes without affecting mitochondrial metabolism and autophagy

To clarify Drosha's role in aa-deprived hepatocytes we knocked down its expression using siRNA. As illustrated in Figure 7a, Drosha knockdown in primary adult rat hepatocytes allows proliferation already after 24 h aaD. Similarly, hepatocytes exposed to 48 h aaD and having an induced reduction of Drosha, display normal growth (Fig. 7b). Taking into consideration the proliferating capability of Drosha-knocked down hepatocytes in aa absence, we wondered whether Drosha could also affect cell metabolism. However, the analysis of OCR in presence or absence of Drosha did not show any change either in basal and maximal mitochondrial respiration (Fig. 7c), or in glycolysis in basal conditions (Fig. 7d). Therefore, Drosha appears to be involved in the regulation of primary adult rat hepatocyte proliferation but exerts no detectable effect on their oxygen consumption in presence or absence of aa. We revealed earlier that, in absence of Drosha, aa-deprived hepatocytes are able to grow. It is accepted that autophagy supports cell survival under nutrient and energy deprivation and its activation is classically regulated through the inhibition of the mTOR pathway. In light of

this we investigated an alternative regulation mechanism for this process through increased Drosha. The analysis of LC3 cleavage demonstrated that Drosha knockdown has no effect on autophagy after 24 h aaD (Fig. 7e-f) and that the increase observed after 48 h aaD does not depend on Drosha (Fig. 7g-h). Together, our results do not support Drosha's involvement in autophagy regulation.

Fig. 7:

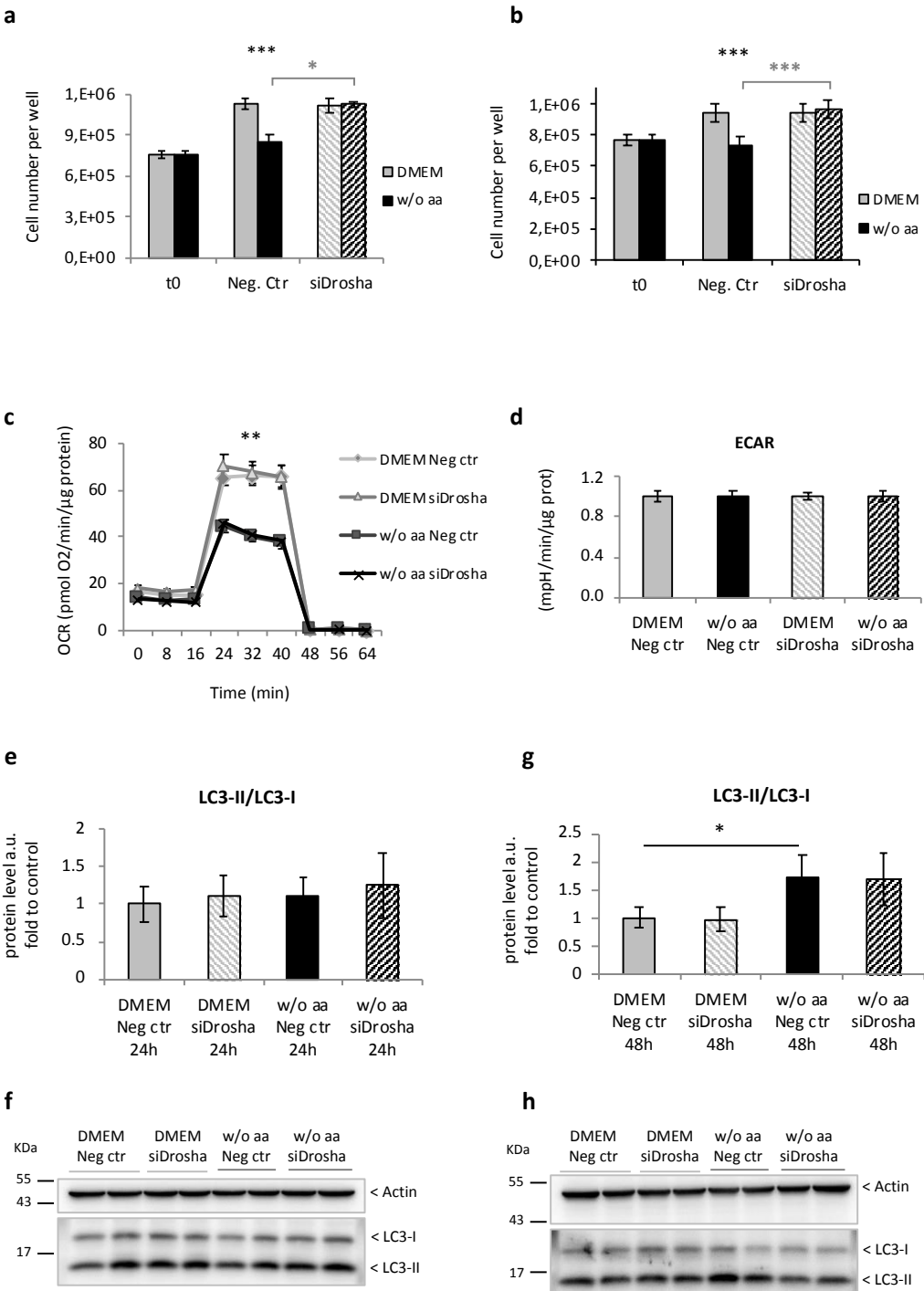


Figure 7. Drosha knockdown restores cell proliferation in aa-deprived primary adult hepatocytes without affecting oxygen consumption and autophagy. (a-b) Cell counting of primary rat hepatocytes transfected with 50 nmol/L negative control-siRNA (Neg. Ctr) or Drosha-siRNA (siDrosha) for 24h (a) and 48h (b), in full-aa medium (DMEM) or in absence of aa (w/o aa). Values are means \pm SEM (n=3). *p < 0.05, ***p < 0.0005 (c-d) Mitochondrial oxygen consumption rate (OCR) (c) and extracellular acidification rate (ECAR) (d) from primary rat hepatocytes cultured in DMEM or w/o aa and transfected with 50 nmol/L negative control-siRNA (Neg. Ctr) or Drosha-siRNA (siDrosha) for 48h. Maximal OCR was determined using FCCP [2 μ M]. OCR is reported in the unit of picomoles per minute and ECAR is reported in milli-pH units (mpH) per minute. Values are means \pm SEM (n=11 replicates from 2 independent rats). **p < 0.005 (e-h) Western blot quantification and analysis of LC3-I/II in primary adult rat hepatocytes transfected with 50 nmol/L negative control-siRNA (Neg. Ctr) or Drosha-siRNA (siDrosha) and kept in culture for 24h (e-f) or 48h (g-h) either in DMEM or in medium w/o aa; cells were treated with 25 μ M chloroquine for 1h or 4h before protein extraction. Values are means \pm SEM (n=3). *p < 0.05.

In primary adult rat hepatocytes deprived of aa the expression of miR-23a/b and miR-27b is increased, but Ago2 knockdown does not allow cell proliferation

Drosha is one of the main actors in the biogenesis of miRNAs, which function as post-transcriptional regulators in many important biological processes. MiRNA expression is often modulated by changes in the cellular environment. Having observed an increase in Drosha in aa-deprived hepatocytes, we evaluated whether aaD could be linked to a general miRNA upregulation. Therefore, we analyzed the expression of a subset of liver specific and/or enriched miRNAs in aa presence or absence. Among the miRNAs studied, after 24h aaD only miR-23a-3p appeared to be significantly upregulated, with a 1.3-fold increase compared to control cells (Fig. 8a). Similarly, 48h aaD significantly augmented only the expression of miR-23b-3p and miR-27b-3p, showing respectively a 1.3 and 1.8-fold increment (Fig. 8b).

To approach a possible involvement of miRNAs in the adaptation of hepatocytes to aa restriction, we investigated cell proliferation after blocking miRNA activity. Argonaute proteins are the main components of the RNA-induced silencing complex (RISC) needed for miRNA functioning. Since Ago2 is the only member of the family characterized by catalytic activity¹⁷, we decided to knock it down using siRNA. We found that an approximately 70% Ago2 reduction in primary adult rat hepatocytes did not impact cell proliferation, either in aa presence or absence (Fig. 8c). These results suggest that Drosha's role in the adaptation of hepatocytes to aa restriction is most likely not dependent on miRNA biogenesis.

Fig. 8:

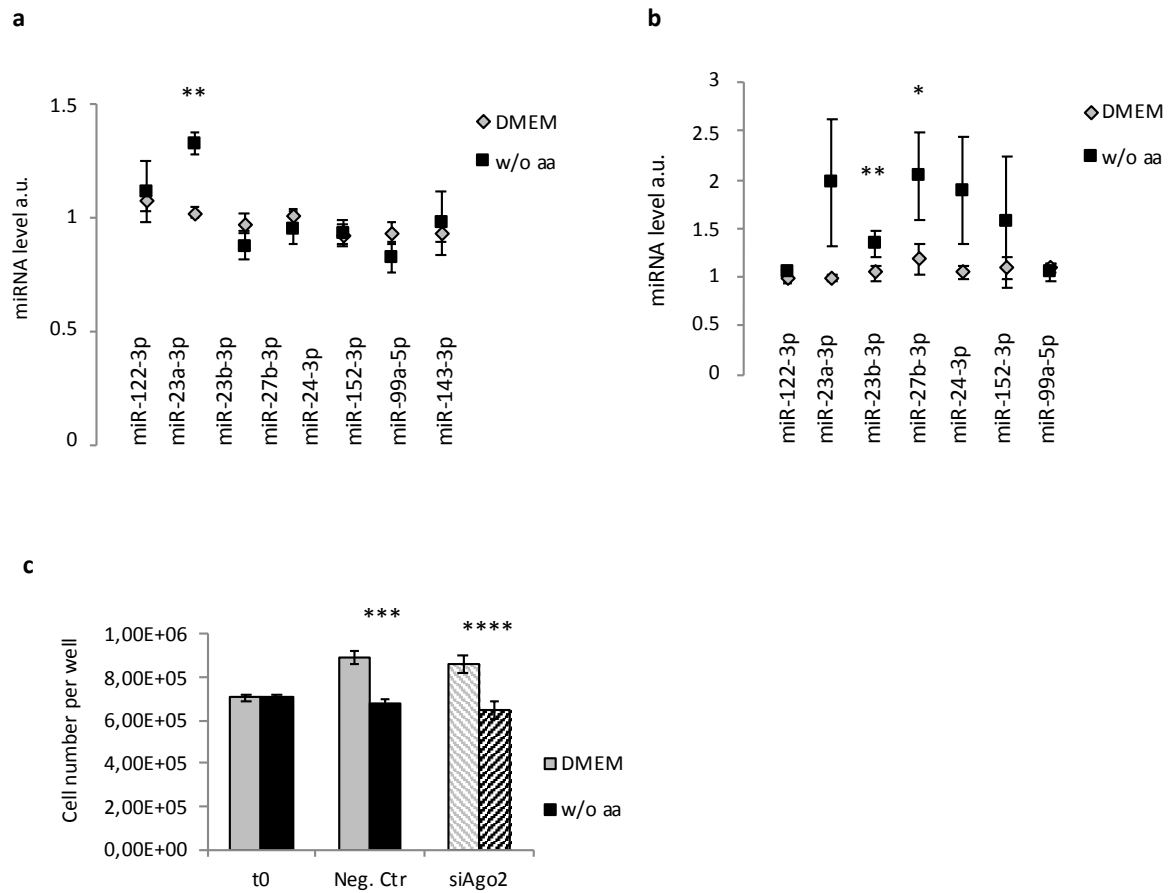


Figure 8. AaD in primary adult rat hepatocytes increases the expression of miR-23a/b and miR-27b, but Ago2 knockdown has no effect on cell proliferation. Analysis of miRNA expression level in primary adult rat hepatocytes after 24h (a) and 48h (b) either in full-aa medium (DMEM) or medium w/o aa. RNA was extracted, reverse transcribed and analyzed by Real Time PCR. The expression of miR-23a-3p, -23b-3p, -27b-3p, -24-3p, -152-3p, -99a-5p, -143-3p was normalized to the miR-122-3p transcript level, while the expression of miR-122-3p was normalized to miR-99a-5p. Values are means \pm SEM (n=3). *p < 0.05, **p < 0.005 (c) Cell counting of primary adult rat hepatocytes transfected with 50 nmol/L negative control-siRNA (Neg. Ctr) or Ago2-siRNA (siAgo2) for 48h in full-aa medium (DMEM) or in absence of aa (w/o aa). Values are means \pm SEM (n=3). ***p < 0.0001, ****p < 0.00001.

Discussion

The effect of nutrient deprivation has been extensively investigated both *in vivo* and *ex vivo* as it affects organismal development and all aspects of mature cell physiology and well-being, and as it induces profound changes in the organs involved in fuel homeostasis in adult life. The liver is a key organ in organismal homeostasis as it impacts on essential functions such as fuel metabolism, production of a myriad of biologically important molecules, and detoxification of harmful intrinsic and foreign substances. It is established that to maintain its multiple vital functions the liver is capable to start growing after partial ablation⁶. As expected, this ability strictly depends on the efficiency of the remaining healthy hepatocytes to regenerate and this is influenced by availability of nutrients. Among those, an increase in the demand of aa, as building blocks of proteins, has been described¹⁸. A recent study showed that protein restriction in mice decreases the volume of both the liver and hepatocytes and these defects were not completely reversed by aa restoration in the diet¹⁹. Hence, we can speculate that a deficiency in dietary protein intake could have a strong and lasting impact on the capacity of recovery in cases of hepatocellular deterioration. Therefore, a deeper understanding of the effect of aa availability on the liver is needed.

To delve into the mechanistic underpinnings of adaptation of the liver to aa scarcity we investigated the effect of aa deprivation (aaD) on primary adult rat hepatocytes. We showed that a complete removal of aa from the hepatocyte culture media arrests cell proliferation without increasing the apoptotic rate but leading to the entrance in a quiescence state. This reversible state is likely to protect and preserve the starved hepatocytes, as quiescence often appears when cells are facing a stress which would undermine their homeostatic integrity and survival^{20,21}. In a healthy adult liver, normally all the hepatocytes show a very low proliferation rate as the great majority of them are blocked in quiescence, or G0²². In contrast, when an aggression occurs, healthy hepatocytes exit the resting state and rapidly enter in the cell cycle in a synchronized manner to attempt to replace the part of the liver damaged or lost^{9,10}. However, the process of hepatocyte isolation, through collagenase perfusion, can induce the “priming” of quiescent hepatocytes, i.e. the G0/G1 transition necessary for initiating cell proliferation. After attachment, overexpression of D-type cyclins could be sufficient for the entrance in the S phase, allowing hepatocyte proliferation in the absence of mitogens²³. In full-aa medium, our primary rat hepatocytes are indeed able to

proliferate until they arrive to confluence, between 24 h and 48 h from isolation. In contrast, when cultured in aa absence, they are not capable to start proliferation. Hematopoietic stem cells, known to exist mostly in a quiescent state ^{24,25}, are characterized by low levels of mitochondrial activity ^{26,27}. Similarly, we observed that aaD of primary hepatocytes leads to decreased oxygen consumption compatible with quiescence. This drop does not appear to correlate with a switch in cell metabolism, as no change in glycolysis was observed. Moreover, cells are capable to exit growth arrest when essential aa are again available and this ability of restarted proliferation is specific of cells in a quiescence state ^{20,21}. One of the main regulators of cell proliferation is the mTOR pathway, which is controlled by multiple stimuli including aa. As expected, we found an inhibition of this pathway when cells were cultured in aa absence. Surprisingly, we observed that the addition of solely e.aa was not entirely sufficient for mTOR phosphorylation restoration, while it completely suffices for p70S6K activation. We also showed that aaD, and specifically e.aa deprivation, leads to increased Drosha protein. A similar picture was observed by Ye et al., according to whom glucose deprivation in murine embryonic fibroblasts induces an increment in Drosha through the mTOR pathway. This subsequently results in increased miRNA expression and in protection against apoptosis ¹⁶. Similarly, we found that deprivation of nutrients, in our case aa, results in increased Drosha. However, at variance with the embryonic fibroblast situation, we did not detect in our hepatocytes an involvement of the mTOR pathway in the Drosha increase.

Our major finding is that the augmentation in Drosha protein is sufficient to regulate the entrance of adult hepatocytes in a quiescence state when a lack of aa occurs. Indeed, we found that Drosha knockdown counteracts the entrance in quiescence, re-starting cell proliferation. How the inhibition of Drosha allows cells proliferating in aa absence remains unclear. We hypothesize that, considering the high glucose concentration and the presence of serum in the culture media, proliferation could be supported by alternative energy sources after that Drosha inhibition eliminated hepatocytes growth arrest. We can also imagine that macropinocytosis could occur, providing building blocks for proliferation from the collagen coating on the dishes in which the hepatocytes are cultured. In fact it was recently shown that lung fibroblast are able to increase the internalization of nanoparticles through macropinocytosis when a collagen extracellular matrix is present ²⁹. It was reported in primary human foreskin fibroblasts that quiescence, induced by serum starvation, is

related to enhanced expression of a series of miRNAs, but also to the downregulation of main actors of miRNA biogenesis through augmented autophagy³⁰. Among them, Drosha is decreased after aaD of fibroblasts, which differs from what we observed in primary rat hepatocytes. Moreover, while we also show an increase in autophagy following aaD, this did not appear to be linked to the regulation of Drosha. Concerning the mechanism underlying the aaD-induced increment in Drosha protein, it is likely due to post-transcriptional effects as its mRNA is apparently not augmented, while we found a greater protein stability. A plausible scenario could be an enhanced Drosha acetylation, which prevents Drosha's ubiquitination and subsequent degradation³¹. Indeed, an increase in the acetylation of enzymes controlling hepatic metabolism is seen in the context of high fat diet³², which is associated also with a decrease in aa content in livers³³. The most urgent question to be addressed concerning our observations is to understand by which mechanistic underpinnings Drosha is able to regulate hepatocyte proliferation. Drosha's canonical role is to participate to miRNA biogenesis through its RNase activity by recognizing specific motifs on double stranded RNA. MiRNA expression is modulated by environmental signals, thus allowing a finely adapted regulation of gene expression and the adjustment of the cells to specific conditions^{34,35,36,37}. Nutrient deprivation in murine embryonic fibroblasts increases miRNA biogenesis to protect against apoptosis¹⁶ and in serum-starved primary human fibroblasts an alternative miRNA biogenesis pathway increases to regulate cell growth-related functions³⁰. However, in our hepatocytes we did not observe a general effect of aaD on miRNA expression as, among the miRNAs studied, only miR-23a appeared to be augmented after 24h aaD, and miR-23b and miR-27b upregulated after 48h. Moreover, the inhibition of miRNA activity through Ago2-knockdown did not affect the hepatocyte proliferation ability, as aa-deprived cells were not capable of growing. Mammals have 4 Argonaute proteins (Ago1 to 4) participating in miRNA biogenesis^{38,39} and, although Burroughs and colleagues reported that specific classes of miRNAs might have preferences in binding Argonaute proteins⁴⁰, a random assignment of miRNAs to the different Argonautes takes also place^{41,42}. However, among the mammalian Argonautes, the most relevant one with catalytic activity is Ago2^{17,43} as Ago3, which has been discovered to have also a slicer ability, has highly specific substrate requirements⁴⁴. Therefore, we do not favor the idea that a compensatory mechanism of the other Argonautes could counteract Ago2 inhibition and maintain miRNA functioning in aaD.

To summarize, our key finding is that Drosha participates in the control of the growth of cultured adult hepatocytes and that this action does not appear to be mediated by miRNAs, the canonical partners of Drosha. An urgent challenge is to parse out how this non-canonical action of Drosha is initiated by aa deprivation and how it takes place. Be that as it may, altogether, our observations revealing a novel role of Drosha improve our comprehension of the complex intertwined multifactor and multipath network controlling hepatocyte proliferation. Importantly, all advance in the understanding of hepatocyte growth and hence liver regeneration is extremely invaluable as it may contribute to the design of innovative means to protect the liver and/or to regenerate it in disease situations.

Materials and methods

Cell culture

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Nice-Sophia Antipolis, Nice, FR (CIEPAL-NCE 00500.02). Hepatocytes were isolated from adult male Wistar rats (200–250 g), from Elevage Janvier, Le Genest St. Isle, France) by collagenase dissociation of the liver as described previously (Fehlmann et al., 1979). After isolation, the liver was perfused with 0.15mg/mL Collagenase Clostridium histolyticum Type IV (Sigma) through the portal vein. Cells were mechanically isolated, centrifuged at 670 g for 1 min and re-suspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 1% (w/v) BSA. Hepatocytes were separated from the rest of the cells by gradient centrifugation with Percoll solution (GE Healthcare) diluted with 1/10 of HBSS (Gibco) at 50 g for 5 min. Then the obtained pellet was finally washed three times with DMEM supplemented by 0.2% (w/v) BSA and 1% (v/v) penicillin streptomycin mixture (Gibco) and centrifuged at 170g for 30 sec. The cells were plated in Corning Biocoat 6-well or 12-well plates respectively at a final concentration of 10^6 cells/well and 350 000 cells/well in Waymouth's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Sigma) and 1% (v/v) penicillin streptomycin mixture, and maintained at 37 °C, 5% CO₂. After 4h, the medium was replaced with DMEM w/o aa (Genaxxon bioscience) supplemented with 3.5g/L D-glucose, 10% (v/v) fetal bovine serum and 1% (v/v) penicillin streptomycin mixture,

with or without the addition of L-glutamine (Lonza), MEM Non-essential Amino Acid (100x) solution (Sigma) and MEM Amino Acids (50x) solution (Sigma). Medium was changed every 24h.

Cell counting

Primary rat hepatocytes were seeded at 10^6 cells per well in a six-well plate. 4h after plating, cells were detached by addition of 500 μ L trypsin-EDTA (0.05%) (Gibco) per well at different time points (0, 16h, 24h and 48h). Detached hepatocytes were then collected, centrifuged at 670 g for 1 min and the pellet resuspended in 1 mL fresh medium. 200 μ L of cell suspension was finally diluted in 20 mL PBS and cells were manually counted by using a Nageotte chamber.

Transfection

Primary rat hepatocytes were seeded at 3.5×10^4 cells per well in a twelve-well plate. 4 h after plating, cells were transfected using Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer's guide. Each well was transfected with 50 nmol/L of Drosha-siRNA (ON-TARGETplus Rat Drosha siRNA – SMARTpool, Dharmacon), Ago2-siRNA (ON-TARGETplus Rat Ago2 siRNA – SMARTpool, Dharmacon) or negative control-siRNA (ON-TARGETplus Non-targeting siRNA, Dharmacon). Medium was replaced after 24 h and cells were analyzed 48h after transfection.

Autophagy analysis

Primary rat hepatocytes were seeded at 3.5×10^4 cells per well in a twelve-well plate and transfected as described before. After 48 h from transfection, hepatocytes were treated with chloroquine [25 μ M] (Sigma) for 4 or 1 h to inhibit the autophagosome-lysosome fusion. Cells were then washed with ice cold PBS 1X, followed by protein extraction and analysis by Western blot.

Rapamycin treatment

Primary rat hepatocytes were seeded at 10^6 cells per well in a six-well plate. For short term treatment, [100 nM] rapamycin (Calbiochem) was added to each well after 40 h from hepatocytes isolation and kept for a total of 3 h and 8 h. For long term treatment, [10 nM], [50 nM] or [100 nM] rapamycin was added to each well just after hepatocyte isolation, for a total of 24 h or 48 h treatment. Cells were then washed with ice cold phosphate-buffered saline or PBS 1X (Dulbecco's Phosphate Buffered Saline 10x, Gibco) before performing total protein extraction for analysis.

Analysis of protein stability

Primary rat hepatocytes were seeded at 10^6 cells per well in a six-well plate. After 40 h from isolation, cells were treated with cycloheximide (CHX) [50 μ M] (Sigma) for 1, 2 and 8 h before being washed with ice cold PBS 1X and snap frozen for further protein analysis.

Protein lysate and quantification

Hepatocytes were washed with ice cold PBS 1X and scraped from dishes in 200 μ L (for 6-well plates) or in 100 μ L (for 12-well plates) of lysis buffer (Hepes 50 mM, NaCl 150 mM, NaF 100 mM, EDTA 10 mM, $\text{Na}_4\text{P}_2\text{O}_7$ 10 mM) supplemented with the protease inhibitors aprotinin 0.02 mg/mL (Euromedex), AEBSF 2 mM (Euromedex) and leupeptin 0.01 mg/mL (Euromedex), the phosphatase inhibitor Vanadate 2 mM and 1% Nonidet P-40. The cell suspension was then homogenized for 20 min at 4°C and centrifuged at 14000 g for 20 min at 4°C. The supernatants were quantified with BCA (Interchim; Serva) and used for the experiments described below.

Western blot analysis

Protein expression was analyzed by Western blotting using the following antibodies: anti-Drosha, Cell Signaling, dilution 1:1000; anti-p70 S6K, Cell Signaling, dilution 1:1000; anti-p-

p70 S6K (Thr389), Cell Signaling, dilution 1:1000; anti-mTOR, Cell Signaling, dilution 1:1000; anti-p-mTOR (Ser2448), Cell Signaling, dilution 1:1000; anti- β -Actin, Sigma-Aldrich, dilution 1:5000; anti-LC3, Novus Biological, dilution 1:200; MitoProfile Total OXPHOS Antibody Cocktail, MitoSciences, dilution 1:1000. An equal amount of total proteins (12-20 μ g) extracted from primary rat hepatocytes were separated by electrophoresis and transferred to polyvinylidene difluoride membranes (Membrane PVDF 0.45 μ m, Immobilon-P). Primary antibody incubation was performed overnight at 4°C and then detected with HRP-conjugated anti-rabbit or anti-mouse immunoglobulins (Vector Laboratories). Immunoreactive proteins were revealed by enhanced chemiluminescence (Luminata Crescendo WB HRP Substrate, Millipore) and the intensity of each band quantified with ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

RNA extraction and quantitative PCR

RNA from primary hepatocytes was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 1 μ g or 10 ng of total RNA was reversetranscribed into complementary DNA (cDNA) by using respectively QuantiTect Reverse Transcription Kit (Qiagen) and Universal cDNA Synthesis Kit II (Exiqon). It was then analyzed using SYBR Green (ABI PRISM 7000 Sequence Detector System). The amount of cDNA used in each reaction was normalized to the housekeeping gene cyclophilin A or miR-122-3p. The primers and real time PCR assay conditions are available upon request.

Oxygen consumption analysis

For metabolic analysis, primary rat hepatocytes were seeded at 5×10^4 cells per well in a 24 multi-well Seahorse plate (Seahorse, Agilent, Santa Clara, CA, USA). 48 h after seeding, the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) were determined using an XF24 Extracellular Flux Seahorse Analyzer (Agilent). Maximal OCR was determined using FCCP (2 μ M), while rotenone and antimycin-A (2 μ M each) were used to inhibit mitochondrial respiration. All parameters were calculated as described previously (Brand and Nicholls, 2011).

FACS analysis

Cells were detached by addition of trypsin-EDTA (0.05%), collected and incubated for 30 min at 37°C, 5% CO₂ with 200 nM MitoTracker Green FM (Invitrogen), according to the manufacturer's instructions. Flow cytometric analysis was performed by FACS Calibur and analyzed by CellQuest Pro (BD).

Immunofluorescence

Primary rat hepatocytes were seeded at 10⁶ cells per well in a six-well plate. After 48 h, cells were incubated with [50 nM] LysoTracker (Life Technologies) for 1 h at 37°C, 5% CO₂ and then fixed with 4% (v/v) paraformaldehyde. Incubation with anti-LC3 (Novus Biological) was performed overnight at 4°C and detected with secondary anti-mouse (Fluo Anti-Mouse FITC, Jackson ImmunoResearch). LC3 puncta in the autophagosomes were identified by co-localization of fluorescently labeled LC3 and lysosomal markers. Images were acquired on an Axiovert microscope (Carl Zeiss, Le Pecq, France) and pictures were captured and treated with AxioVision software (Carl Zeiss).

Mitochondrial staining

Primary rat hepatocytes were seeded at 10⁶ cells per dish in a 35 mm-diameter petri-dish coated with collagen. After 48 h, medium was replaced and cells incubated at 37°C, 5% CO₂ with 200 nM MitoTracker Green FM (Invitrogen). After incubation the bottom of each petri-dish was cut and reversed in a glass petri-dish. Fresh medium was added and mitochondria visualized by excitation with 516 nm wavelength. Images were acquired with an observer D1 microscope (Carl Zeiss GmbH, Jena, Germany) under oil immersion and pictures were captured and treated with Zen software (Carl Zeiss).

Terminal deoxynucleotidyl transferase mediated dUPT nick-end labeling (TUNEL) assay

The apoptotic rate of primary rat hepatocytes was detected by using the In Situ Cell Death Detection Kit, Fluorescein (Roche). Cells were fixed by incubation with ice-cold MetOH 100% for 1h at 4°C. Afterwards, they were washed twice with PBS and incubated with TUNEL reaction mixture for 1 h at 37°C, as described by the manufacturer. Positive cells were visualized by excitation with 515-565 nm wavelength and quantified with ImageJ software. Images were acquired on an Observer D1 microscope (Carl Zeiss GmbH, Jena, Germany) using an objective A-Plan 10x/0.25 Ph 1.

Statistical analysis

Data are presented as mean \pm SEM; *n* represents the number of independent rats. Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California USA). Paired two-tail Student's *t* test was used to compare two conditions. Differences between more than two conditions were analyzed by one-way ANOVA (paired or unpaired) followed by Sidak's multiple comparison test. A *p* value < 0.05 was considered significant.

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Author contributions

G.F. and O.D. designed the study, researched data, and contributed to discussions and the manuscript. N.G and D.F.P. researched data and contributed to discussion. E.V.O. designed the research project, contributed to the discussions and the manuscript. GF and EVO wrote the manuscript.

Declaration of interests

The authors declare that there is no duality of interest associated with the manuscript.

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Confidential

MicroRNA-375 regulates glucose metabolism-related signaling for insulin secretion in rat and human islets

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Declaration of interests: None.

Abstract

Enhanced beta cell glycolytic and oxidative metabolism are necessary for glucose-induced insulin secretion. While several microRNAs modulate beta cell homeostasis, microRNA-375 stands out as it is highly expressed in beta cells where it regulates beta cell function, proliferation and differentiation. As glucose metabolism is central in all aspects of beta cell functioning, we investigated the role of microRNA-375 in this process using human and rat islets; the latter being an appropriate model for in depth investigation.

We used forced expression and repression of microRNA-375 in rat and human primary islet cells followed by analysis of insulin secretion and metabolism. Additionally, microRNA-375 expression and glucose-induced insulin secretion were compared in islets from rats at different developmental ages.

We found that overexpressing of microRNA-375 in rat and human islet cells blunted insulin secretion in response to glucose ($6.47 \pm 0.61\%$ vs $3.04 \pm 0.46\%$ and $3.77 \pm 0.72\%$ vs $1.39 \pm 0.18\%$, respectively; fractional insulin release) but not to α -ketoisocaproate or KCl. Further, microRNA-375 reduced O₂ consumption related to glycolysis ($112 \pm 5\%$ vs $156 \pm 12\%$ of basal respiration) and pyruvate metabolism ($147 \pm 5\%$ vs $179 \pm 8\%$ of basal respiration), but not in response to α -ketoisocaproate. Concomitantly, lactate production was augmented (0.27 ± 0.02 vs 0.13 ± 0.04 mmol/l) suggesting that glucose-derived pyruvate is shifted away from mitochondria. Finally, reduced microRNA-375 expression was associated with maturation of fetal rat beta cells and acquisition of glucose-induced insulin secretion function.

Altogether our findings identify microRNA-375 as an efficacious regulator of beta cell glucose metabolism and of insulin secretion and could be determinant to functional beta cell developmental maturation.

Keywords

diabetes, glucose metabolism, insulin secretion, microRNAs, pancreatic islets.

Abbreviations

GSIS, Glucose-stimulated insulin secretion; KIC, alpha-ketoisocaproate; KRBH, Krebs-Ringer Bicarbonate-Hepes buffer; Ldh, lactate dehydrogenase; LP, low protein diet; microRNA, miR; miR-375, microRNA-375; TCA cycle, tricarboxylic acid cycle.

Introduction

The main beta cell mission consists in its ability to secrete insulin for maintenance of organismal glucose homeostasis. This hallmark requires expression of a set of genes (allowed genes) and the concomitant repression of others (disallowed genes)[1, 2]. Indeed, beta cells have a dedicated metabolism comprised of a tightly regulated chain of elements from glucose sensors to a specialized exocytosis machinery. Glucose metabolism in beta cells linked to insulin secretion has several exclusive features. First, glucose transport is not limiting for its metabolism, and it results in a rapid equilibrium between intracellular and extracellular glucose at all glycemic levels. The second particular trait is that glycolysis is initiated by glucokinase having a high K_m for glucose associated to an elevated V_{max} , and being devoid of feedback control[3]. As beta cells express low levels of plasma membrane monocarboxylate transporters, little or no monocarboxylates, including lactate, enter the cells. Further, the low lactate dehydrogenase (LDH) activity of the beta cells accounts for the fact that a major fraction of the glucose entering the beta cells is oxidized by the mitochondria[4, 5]. These distinctive beta cell properties must be maintained to achieve efficient oxidative phosphorylation, lipid metabolism and signaling[3], thus making optimal glucose sensing possible. It is thought that beta cell dysfunction in type 2 diabetes could result from a reduced expression of key genes, triggering the loss of beta cell identity and hence promoting dedifferentiation[4, 6, 7]. Importantly, grounds have been gleaned in favor of beta cell dedifferentiation in human type 2 diabetes. Indeed, dedifferentiated cells account for almost 40% of the beta cells in type 2 diabetes patients compared to 8% in controls[8]. Thus, it appears that the maintenance of beta cell identity throughout life is crucial for glucose homeostasis and organismal physiology.

The biological importance of microRNAs is substantiated by the diverse and profound phenotypic consequences occurring upon changes in their expression. These modifications are associated with physiological modulations, but also with perturbed development and pathological situations. Thus, microRNAs have emerged as major modulators of gene expression in many biological programs including organ development and metabolism[9].

One of the most relevant and widely explored microRNAs in beta cells is miR-375[10-12], which is preferentially expressed in islets and is the most abundant microRNAs in adult beta cells[13]. We and others demonstrated that this microRNA is a chief regulator of beta cell

mass and functions through incompletely understood mechanisms[14-17]. In addition, miR-375 expression is upregulated in islets from type 2 diabetics[18-20]. Similarly, we observed in a murine model of predisposition to type 2 diabetes following a low protein diet (LP) during pregnancy, that miR-375 expression is increased in the endocrine pancreas of the progeny[14]. This is accompanied by a reduction in both glucose-stimulated insulin secretion (GSIS) and beta cell proliferation. Anti-miR-induced normalization of miR-375 expression levels in the islets of LP descendants partially restored insulin secretion and cell proliferation to levels of control animals. While it is generally accepted that miR-375 is essential for the development of the endocrine pancreas[21-23], facts in favor of additional roles are appearing. Indeed, evidence is being built highlighting an association between low miR-375 levels and beta cell dedifferentiation[24]. Further, it has been recently found *in vitro* that miR-375 overexpression is sufficient to generate insulin-producing cells from pluripotent stem cells or from human mesenchymal stem cells[25, 26]. Taken together the striking features of miR-375 action in beta cells, *i.e.* inhibition of GSIS and promotion of beta cell differentiation, reveal at first glance a potentially discordant image.

Beta cell differentiation is a complex multistep process finely orchestrated by numerous transcription factors. The ultimate beta cell maturation step is thought to occur around weaning. It consists in the acquisition of glucose stimulus-secretion coupling which depends on the occurrence of glucose metabolism dedicated to insulin secretion[27]. As glucose metabolism governs insulin secretion and is inherent to mature beta cells, we posit here that the miR-375 effects on beta cell function could be due to glucose metabolism alterations.

Materials and methods

Reagents

Culture media and buffer solutions were from Gibco (ThermoFisher Scientific, Gometz le Châtel, France), FBS and trypsin from Invitrogen (Cergy Pontoise, France). Other reagents were from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France).

Human islets

Islets were provided by the Islets for Research distribution program through the European Consortium for Islet Transplantation under the supervision of the Juvenile Diabetes Research Foundation (31-2012-783). Human pancreases were harvested from 5 adult brain-dead individuals (males=2, females=3; age, mean \pm SEM: 44.0 \pm 10.0 years; BMI, 21.0 \pm 1.0 kg/m²; HbA1c, 36 \pm 1 mmol/mol (5.4 \pm 0.1%)) in agreement with French regulations and Lille University Ethical Committee. Experiments used islets with >90% viability and >80% purity (endocrine versus exocrine tissue).

Animals and diets

All procedures followed ARRIVE guidelines, were conducted in accordance with EU directives for animal experiments (2010/63/EU) and were approved by the French Research Ministry (MESR 00500.02). Three month-old Wistar rats (Janvier; Le Genest Saint Isle, France) were kept under conventional conditions, with free access to water and food. Nulliparous female rats (200–250 g) were mated with male rats and used at day 21 of gestation.

Islet isolation

3 month-old rat islets were obtained after density gradient centrifugation in histopaque-1077 (Sigma) as previously described[28]. Hand-picked islets were cultured in RPMI-1640 medium supplemented with FBS (10%, v/v) and antibiotics. Neoformed fetal rat islets were obtained as described[29].

Culture and transfection of dissociated islet cells

Rat or human islets were dissociated by trypsinization, and seeded at a density of 25 \times 10³ cells/cm² in dishes coated with 804G-ECM[14, 30]. 48 h after plating, cells were transfected using Lipofectamine 2000 (Invitrogen) and with double-stranded RNA oligonucleotides corresponding to mature miR-375 or control microRNA (ThermoFisher) at a concentration of

100 nmol/l. Single-stranded miRCURY LNA inhibitor (Exiqon, Vedbaek, Denmark) specifically blocking endogenous miR-375 was used at a concentration of 100 nmol/l. All analyses were performed 72 h after transfection.

RNA extraction and RT-qPCR

RNAs were isolated using TRIzol reagent (Invitrogen). For miRNA analysis, 10 ng of total RNA was reverse transcribed using Universal cDNA synthesis kit (Exiqon). qPCR and data evaluation were performed using StepOnePlus apparatus and associated software (Applied Biosystem). U6 and 5S were used as endogenous controls. The primers are from Exiqon.

Lactate determination

Lactate concentration was determined by ionic chromatography using a Dionex DX600 device equipped with an ion Pac AS11 column (ThermoFisher). Sequential anion elution including lactate was obtained at 1 ml/min by a KOH gradient generated by the EG40 eluent generator according to the manufacturer's instructions.

Insulin secretion

All experiments were performed using Krebs-Ringer Bicarbonate-Hepes buffer (KRBH) containing 3 mmol/l glucose and 0.1% BSA (w/v). Dissociated islet cells were pre-incubated for 1h at 37 °C before incubation in fresh medium containing secretagogues. After 1h, media and cell homogenates obtained by sonification were analysed for insulin content using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden). To eliminate variations due to differences in cell number, insulin secretion was expressed as the percentage of the cellular insulin content, which is referred to as fractional insulin release.

Glucose uptake

Islet cells were preincubated for 45 min at 37°C in 3 mmol/l glucose KRBH and then 10 min in KRBH containing 20 mmol/l glucose and 0.5 uCi of [³H] deoxyglucose (Amersham Pharmacia Biotech, Uppsala, Sweden). Glucose incorporation was stopped on ice and cells were lysed using NaOH 0.2N and neutralized with HCl 0.2N. Radioactivity was measured and the total protein amount recorded using BCA method. Results are expressed in cpm/mg protein/min.

Mitochondrial metabolism

For metabolic analysis, dissociated cells were seeded in a 24 multi-well plate (Seahorse, Agilent, Santa Clara, CA, USA) and cultured/transfected as described above. The oxygen consumption rate (OCR) was determined using an XF24 Extracellular Flux Seahorse Analyzer (Agilent). Rotenone and Antimycin-A (2 µmol/l each) were used to inhibit mitochondrial respiration.

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using InStat3 software (Graphpad, San Diego, CA, USA). Paired two-tailed Student's t tests were used to compare differences between samples from paired experiments. Differences between all other datasets were analysed by one-way ANOVA with Student-Newman-Keuls post-hoc test. p-values <0.05 were considered significant.

Results

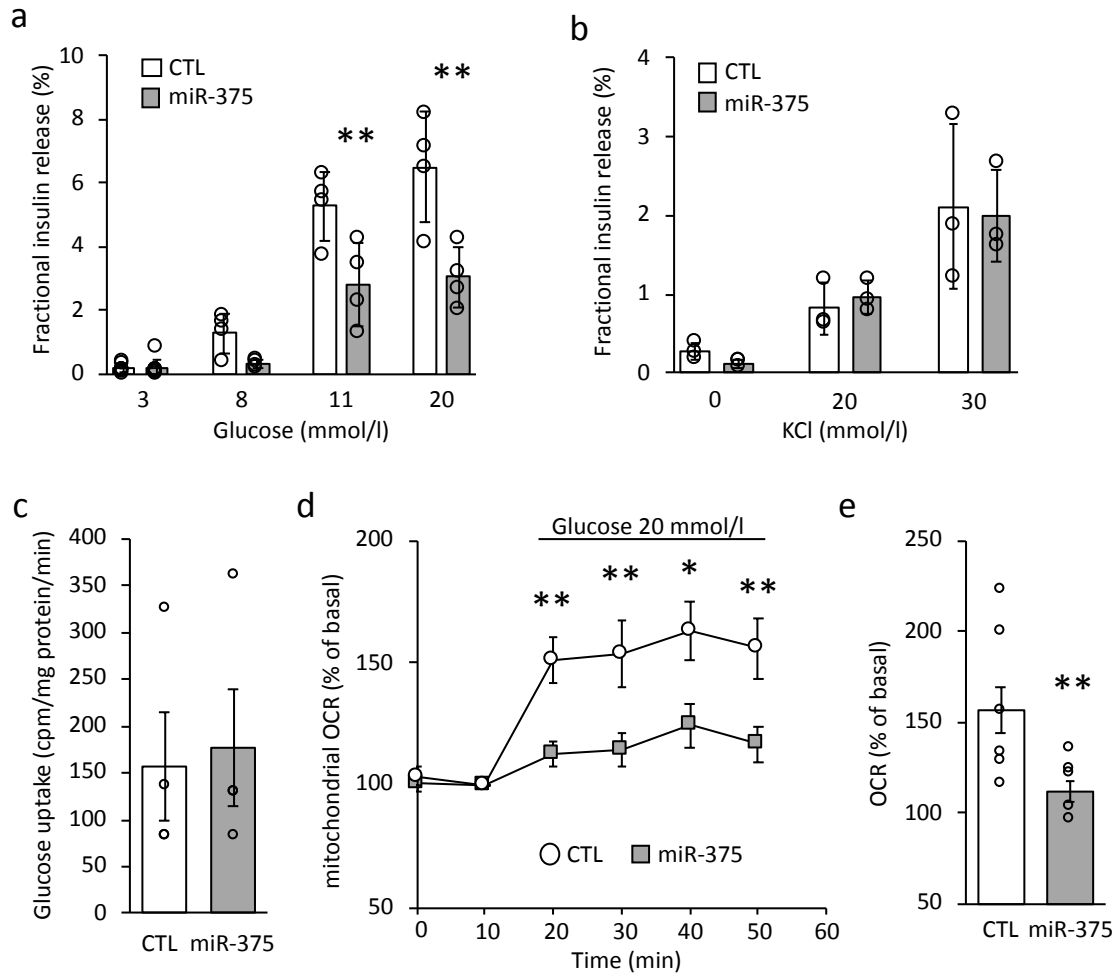
miR-375 overexpression in islet cells impairs insulin secretion and the Ca^{2+} rise induced by glucose but not by KCl

We investigated the miR-375 impact on beta cell function by comparing dissociated primary adult rat islet cells transfected with miR-375 or control microRNA (CTL). Double-stranded RNA oligonucleotides corresponding to mature miR-375 or control microRNA were first tested in a dose-response experiment from 50 to 200 nmol/l (ESM Fig. 1a-c). Augmenting the amount of transfected miR-375 resulted in an increased miR-375 level in the cell (ESM Fig. 1a) and in a decreased insulin secretion in response to 20 mmol/l glucose (ESM Fig. 1b), without affecting the insulin cell content (ESM Fig. 1c). We choose to use transfection with 100 nmol/l of miR-375 which resulted in an approximately 25-fold increase in miR-375 level and a reduced GSIS by approximately 50% at all glucose concentrations (Fig. 1a). To evaluate whether this could be attributed to altered exocytosis, we measured insulin secretion at low glucose in the presence of depolarizing KCl concentrations. High miR-375 levels did not modify insulin secretion at the KCl concentrations tested (Fig. 1b). Together the data are compatible with the view that miR-375 interferes with GSIS by altering beta cell metabolism and metabolic signaling for secretion rather than by hampering late steps in regulation of the exocytosis apparatus.

miR-375 impacts glucose-stimulated respiration

To investigate whether the dampened insulinotropic action of glucose could be due to a miR-375-induced inhibition of glucose uptake, we compared glucose transport using 3H -2-deoxyglucose in primary islet cells transfected with control microRNA or with miR-375. As shown (Fig. 1c), no difference in 2-deoxyglucose uptake was observed at 20 mmol/l glucose. To decrypt miR-375 effects on intermediate metabolism, O_2 consumption in response to glucose was evaluated. It was profoundly reduced by miR-375 (Fig. 1d-e) to a similar extent as the GSIS inhibition.

Figure 1.



ESM Figure 1.

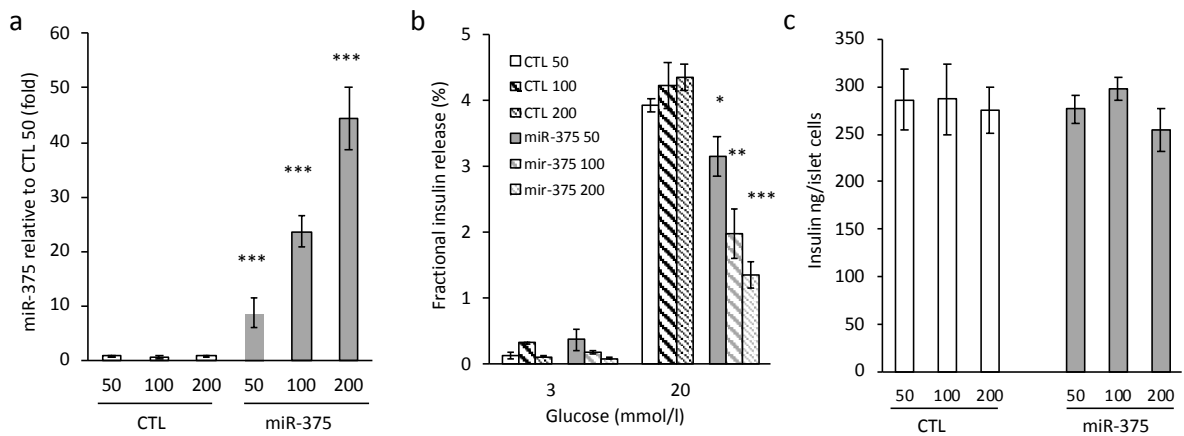


Figure 1. Forced miR-375 expression in adult rat primary pancreatic islet cells impairs glucose metabolism and insulin secretion induced by glucose but not by KCl. (a-b) 72 h post-transfection with miR-375, dissociated islet cells were assayed for insulin secretion using various glucose and KCl concentrations. To eliminate variations due to differences in cell number, insulin secretion is expressed as the percentage of the islet cell insulin content, which is referred to as fractional insulin release. (c) Glucose transport evaluation in dissociated islet cells 72 h post transfection. The results are expressed in cpm/mg protein/min. (d-e) Mitochondrial O₂ consumption and averages of O₂ consumption determinations made in the absence or presence of 20 mmol/l glucose. Means ± SEM, n = 4 (a-c) or 3 (d-e). *P < 0.05, **P < 0.01, miR-375 versus control (CTL).

ESM Figure 1. Forced miR-375 expression in dissociated primary adult rat islet cells does not impact cell differentiation. 48 h after plating and cell culture, dissociated primary rat islet cells were transfected with 50, 100 or 200 nmol/l of double-stranded RNA oligonucleotides corresponding to the mature miR-375 sequence or to a scrambled control miR (CTL). 72h after transfection cells were harvested for RNA extraction or used for insulin secretion experiments. (A) Measurement of miR-375 content by RT-qPCR. (B-C). For secretion experiments, dissociated islet cells were preincubated for 60 min in modified Krebs-Ringer buffer (KRBH) containing 0.1% BSA (w/v) and 3 mmol/l glucose. Thereafter, they were incubated during 1 h with various glucose concentrations (3 or 20 mmol/l). To eliminate variations due to differences in cell number, insulin secretion (displayed in B) is expressed as the percentage of the islet cell insulin content (displayed in C), which is referred to as fractional insulin release. Means ± SEM; n=3. *P < 0.05, miR-375 versus CTL; **P < 0.01, miR-375 versus CTL; ***P < 0.001, miR-375 versus CTL.

Reduced endogenous miR-375 levels in primary islet cells increases glucose metabolism and insulin secretion

To confirm the repressive miR-375 action on insulin secretion, we decreased the endogenous miR-375 level with its antisense. miR-375 reduction in islet cells increased GSIS with no impact on insulin content (Fig. 2a-b). Remarkably, the diminished miR-375 level augmented glucose-induced O₂ consumption (Fig. 2c-d). Together, our gain and loss of function approach identifies miR-375 as a robust insulin secretion modulator which impacts glucose-induced mitochondrial metabolism.

Figure 2.

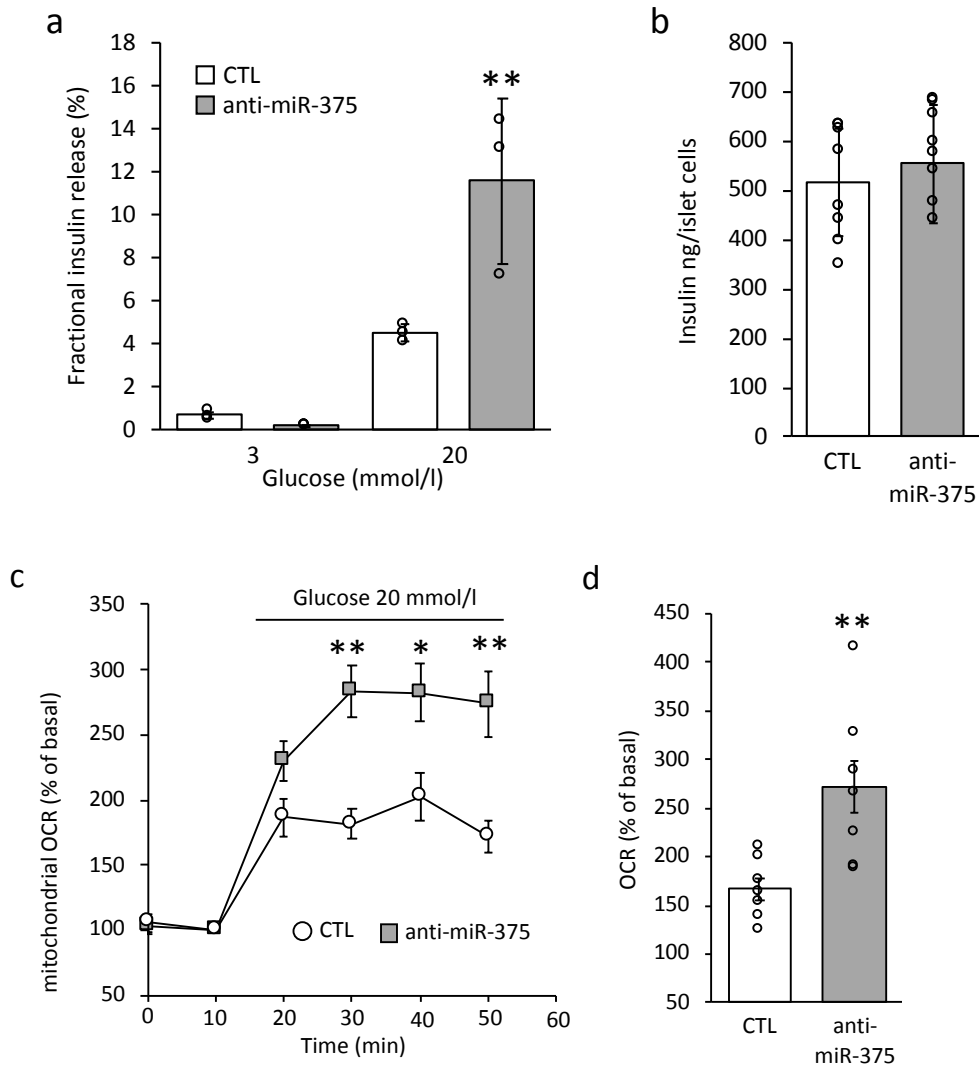


Figure 2. Reduced miR-375 expression in adult rat primary pancreatic islet cells increases glucose metabolism and insulin secretion induced by glucose. Glucose-induced insulin secretion (a), insulin content (b) and mitochondrial O₂ consumption (c) were evaluated 72 h after transfection with anti-miR-375 or with anti-CTL. Panel D shows the averages of O₂ consumption. Means ± SEM, n = 3. **P < 0.01, anti-miR-375 versus anti-CTL.

miR-375 does not affect the action of a fuel stimulus enhancing mitochondrial metabolism independently of glycolysis

To further explore miR-375 action on cell metabolism, we used α -ketoisocaproate (KIC), which induces insulin release by directly augmenting the tricarboxylic acid (TCA) cycle intermediate α -ketoglutarate, and also via acetyl-CoA and acetoacetate production. As illustrated in Fig. 3a, KIC stimulated insulin secretion in a dose-dependent manner comparably in control and miR-375 overexpressing islet cells. Further, mitochondrial metabolism remained unaltered in miR-375-transfected islets in response to KIC, as reflected by O₂ consumption determinations (Fig. 3b-c).

miR-375 modifies pyruvate metabolism

The above results suggest that miR-375 could perturb insulin secretion by interfering with the production of glycolysis metabolites, in particular the last one in the pathway, pyruvate. To examine if miR-375 controls pyruvate mitochondrial metabolism we tested the effect of high pyruvate (50 mmol/l) concentrations on insulin secretion and O₂ consumption. We used an elevated concentration of pyruvate, as it is not an efficacious secretagogue because the plasma membrane monocarboxylate transporter-1 is poorly expressed in adult islets [31]. While pyruvate induced insulin secretion in control islets, its insulinotropic effect in miR-375 transfected islets was diminished (Fig. 3d) to a similar extent as GSIS (Fig. 1a). Forced miR-375 expression also reduced O₂ consumption in response to pyruvate (Fig. 3e). In differentiated beta cells, pyruvate is slightly reduced to lactate and hence most of the glucose-derived pyruvate is metabolized in the mitochondria via pyruvate dehydrogenase and anaplerotic reactions[32]. However, islet cells overexpressing miR-375 showed a 2-fold increase in lactate release into the medium (Fig. 3f). This suggests that enhanced miR-375 expression partially redirects pyruvate metabolism from mitochondrial oxidation to anaerobic glycolysis and lactate production.

Figure 3.

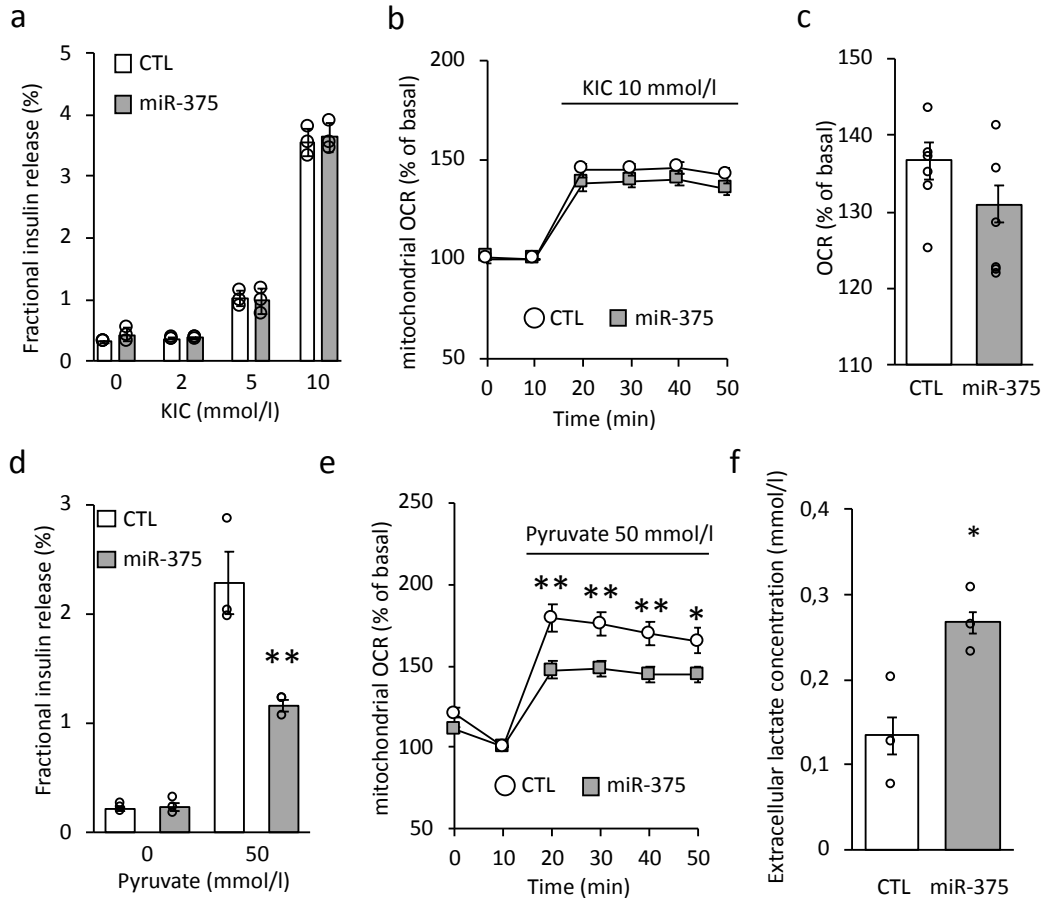


Figure 3. Forced miR-375 expression in adult rat primary pancreatic islet cells impairs pyruvate-induced insulin secretion and pyruvate metabolism with a shift toward lactate production but does not affect KIC effects. Insulin secretion measured 72 h post-transfection in dissociated islet cells incubated with various concentrations of (a) α -ketoisocaproate (KIC) or (d) pyruvate. O_2 consumption was recorded from cells in presence of KIC (b-c) or pyruvate (e). (f) Lactate measurement in the incubation medium of transfected dissociated islets 4h after glucose addition (11 mmol/l). Means \pm SEM of 3 (a-e) or 4 (f) independent experiments. * $P < 0.05$, ** $P < 0.01$, miR-375 versus CTL.

Forced miR-375 expression in human islet cells reduces insulin secretion in response to glucose, but not KCl or to KIC

Next, we addressed in human beta cells the role of miR-375 as regulator of glucose metabolism and GSIS. We measured insulin secretion in islets from 5 nondiabetic donors transfected or not with miR-375. A 12-fold increased miR-375 level 72 h post transfection (Fig. 4a) markedly reduced GSIS, without affecting the KIC or KCl response (Fig. 4b). Hence, the miR-375 overexpression data on human islet cells reproduce the results obtained with rat islet cells in terms of insulin secretion in response to glucose, KIC and KCl.

Figure 4.

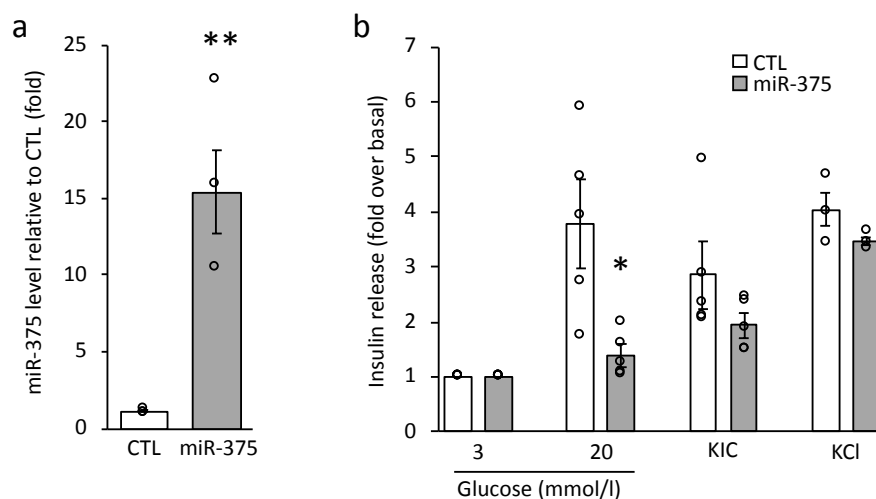


Figure 4. Effect of miR-375 on insulin secretion in human islet cells. (a) miR-375 expression analyzed by qPCR in dissociated human islet cells 72 h after miR-375 transfection. (b) Insulin secretion measured in dissociated human islets 72 h after transfection and using various secretagogues. Values are means \pm SEM, $n = 5$ human islet isolations. * $P < 0.05$, ** $P < 0.01$, versus CTL.

Reduced miR-375 expression is associated with in vitro glucose responsiveness and maturation of fetal rat beta cells

We next explored the possibility that miR-375 could be involved in beta cell metabolic maturation for GSIS and thus interfere with the acquisition of glucose-responsiveness in the transition from fetal to neonatal beta cells. We took advantage of the finding that functional maturation of glucose stimulus-secretion coupling of fetal rat islets can be accelerated *in vitro* by glucose [33, 34]. Thus, we measured the expression of miR-375 in untreated fetal islets compared with fetal islets after *in vitro* maturation by glucose (neonatal islets). As illustrated in Fig. 5a, untreated fetal islets did not respond to 20 mmol/l glucose, but they released insulin when stimulated with KIC. However, after *in vitro* maturation, they secreted insulin appropriately in response to high glucose (Fig. 5b). Remarkably, lower glucose responsiveness of insulin release by untreated fetal beta cells was associated with high miR-375 expression compared to neonatal islets (Fig. 5c). Collectively, these results suggest that repression of miR-375 expression participates to the acquisition of glucose stimulus-secretion coupling.

Figure 5.

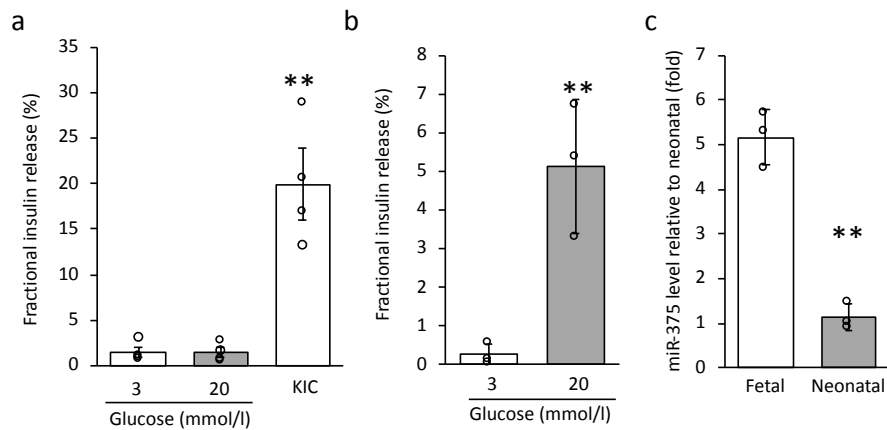


Figure 5. The repression of miR-375 expression is required for the acquisition of the competence for glucose stimulus-secretion coupling. After their digestion, 21-day-old fetal pancreases were either cultured for 7 days in 11 mmol/l glucose for the obtention of “neonatal” islets or purified with density-gradient for fetal islet isolation. (a) Insulin secretion analysis of fetal islets in response to glucose or α -ketoisocaproate (KIC). Means \pm SEM, n = 3 independent experiments. **P<0.01, KIC versus glucose 3 mmol/l. (b) Insulin secretion analysis of neonatal islets in response to glucose. Values are means \pm SEM, n = 3. **P<0.01, glucose 20 mmol/l vs 3 mmol/l. (c) miR-375 expression analysed by RT-qPCR in fetal and neonatal islets. Means \pm SEM, n=4 islet preparations. ** p<0.01.

Discussion

Since their discovery, several microRNAs have been implicated in the development and function of beta cells[10, 11]. Amongst these, miR-375 stands out as it is highly expressed and regulates not only beta cell development and proliferation, but also its function[13, 15, 16, 23, 24, 35]. However, while it is accepted that miR-375 is essential for endocrine pancreas development[21-23], its inhibitory action on insulin secretion reveals its multiple facets in beta cell physiology. Thus, deciphering miR-375 precise functions and mode of action is of considerable interest. To this end, using primary rat and human islets, we delved into the miR-375 effects on glucose metabolism, that governs insulin secretion and modulates beta cell characteristics.

Insulin secretion in immature beta cells is characterized by a poor responsiveness to glucose but a mature-like sensitivity to amino acids[36, 37]. In addition, immature islets consume more oxygen at low glucose compared with mature islets but their ability to increase oxygen consumption in response to high glucose is considerably lesser compared with mature islets. This clearly suggests that beta cell maturation is associated with profound metabolic changes[27]. Transcriptomic analyses to determine the basis of poor glucose responsiveness of neonatal beta cells, compared with adult beta cells, revealed that many genes are differentially expressed, including those encoding enzymes of mitochondrial shuttles, *Pc* and *carnitine palmitoyl-transferase 2*[38]. In addition, *Ldh* was significantly elevated in neonatal beta cells, which is particularly interesting as efficient *Ldh* expression would divert pyruvate away from the mitochondria towards lactate production. This scenario is compatible with the idea that metabolic specialization of adult beta cells for generating ATP and additional anaplerosis-derived metabolic coupling factors from aerobic glycolysis is deficient in immature neonatal beta cells[38]. However, glucose exposure of immature beta cells fosters their metabolic maturation. Indeed, nutrient shifts at weaning, induced by replacement of fat-enriched maternal milk with a carbohydrate-rich diet, drive postnatal beta cell maturation via islet-specific microRNAs[39]. While in the latter report no alterations in miR-375 expression were observed, the entirely different developmental stages examined and the experimental conditions used in our study very likely account for the divergence in microRNA expression. It is well known that fetal rat islets cultured with 11 mmol/l glucose acquired near-adult levels of insulin secretion in response to high glucose[40]. Here we

confirmed this and further demonstrated such decreased miR-375 expression in fetal rat islets matured in 11 mmol/l glucose. By contrast, forced miR-375 expression in mature rat and human beta cells resulted in mirror effects on GSIS. Further, we found that upon miR-375 overexpression mature beta cells display enhanced lactate production. At variance with earlier reports focused on mice beta cells *in vivo* and *in vitro*[13, 16, 17], in our dissociated rat or human islets miR-375 overexpression has no impact on the insulin exocytosis machinery as KIC- and KCl-induced secretion were preserved. The reason for this discrepancy is unclear, but one possible explanation could be animal species. Together our findings strongly support the view that robust miR-375 expression in human or rat beta cells favors an immature metabolic phenotype with low glucose-responsiveness rather than impacting on the exocytosis machinery *per se*.

Recently, the concept emerged that the reduction in beta cell mass in type 2 diabetes could reflect a loss/fading of their crucial attributes rather than increased apoptosis[6, 41, 42]. Considering the increased miR-375 expression in islets from animals and humans with type 2 diabetes[16, 18-20, 43], and the current study showing that miR-375 overexpression results in reduced GSIS in human and rat islets, miR-375 appears to be a likely contributor to the fading of essential characteristics of beta cells in type 2 diabetes pathogenesis.

Conclusion

This study enhances our understanding of the diverse roles whereby miR-375 modulates beta cell function. First, we report that miR-375 modulates key beta cell metabolic pathways specifically for GSIS, but not in response to other secretagogues (fuel and non-fuel stimuli). Second, our work, by focusing on metabolism, unearths a novel mode of action of this microRNA. Third, it provides a plausible mechanism to explain how miR-375 modulates GSIS by redirecting glucose carbons from mitochondrial metabolism to lactate formation, and thus inhibiting glucose-induced ATP generation and the production of additional anaplerosis-derived regulatory metabolites. Finally, our findings uncover relevant links between epigenetic regulators, key beta cell traits and type 2 diabetes pathogenesis. Considering that the beta cell demise is a most challenging conundrum in the diabetes field, understanding

the safeguarding of beta cell fundamental properties will be of major importance for leveraging strategies to preserve it in pathophysiological conditions.

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Contribution statement

OD and DFP designed the study, researched data, and contributed to the manuscript. GF, VC, NG, PL, CH, CD, SD, FP and JKC researched and analyzed data. EVO and MP designed the research project, contributed to the manuscript. All authors contributed to the discussion on the manuscript and approve its final version. E.V.O is the guarantor of this work.

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Discussion

General discussion

Both projects of my PhD research are connected to the miRNAs and their machinery, highlighting the importance of this system in two key organs involved in metabolism, the liver and the pancreatic beta-cells. Indeed, a singular miRNA, miR-375, is crucial not only for the development of the endocrine pancreas, but also for glucose-induced insulin secretion. At the same time, the main protein involved in miRNA biogenesis, Drosha, appears to play a pivotal role in the regulation of cell growth in response to amino acid (aa) deprived-hepatocytes. While non-canonical roles of Drosha have been revealed during the last decade, its newly identified miRNA-independent action has not been described to the best of our knowledge.

More insights about Drosha and its regulation

Our data on aa-deprived hepatocytes showed an increase in Drosha protein, but no change in its mRNA expression. Since this increment correlates with augmented protein stability, we speculate that Drosha undergoes post-translational modifications, which protects it from degradation. It is well known that Drosha most relevant regulatory component is its amino-terminal region, which is characterized predominantly by proline-rich (220 amino acids) and serine/arginine-rich (250 amino acids) domains (Wu et al., 2000)(Filippov et al., 2000). Although the role of Drosha N-terminal region has been shown not to be necessary for miRNA processing *in vitro* (Han et al., 2004b), its post-translational modification have been found to influence both Drosha subcellular localization and stability, suggesting that this region may function as a regulatory platform (Lee and Shin, 2018). More in detail, recent studies by Tang and colleagues showed that GSK3 β phosphorylation of Drosha on Ser 300/302 residues is necessary for a correct localization of the RNase III in the nucleus (Tang et al., 2010)(Tang et al., 2011). Furthermore, its N-terminal lysines serve as substrate for the regulation of Drosha ubiquitin-proteasome induced degradation. Indeed, those lysines are both binding sites for activated ubiquitin molecules, which address Drosha to the proteasome, or for a variety of acetyl transferases – such as p300, CBP and GCN5 – whose action prevents Drosha ubiquitination (Tang et al., 2013). We therefore posit that, under aa

scarcity, Drosha's stability could be enhanced by an increase in its acetylation state, differently from what would happen in full-aa medium.

Drosha's non-canonical roles and hypothesis

In the present study, our main discovery is that Drosha appears to be a crucial actor in the regulation of aa-dependent cell proliferation in primary rat hepatocytes, independently from its role in miRNA biogenesis. Drosha is a 160 kDa nuclear protein belonging to the family of RNase III-type endonucleases, which act on dsRNAs. Drosha-mediated cleavage of pri-miRNAs is necessary for miRNA biogenesis, but it is also associated to pri-miRNA destabilization (Lee and Shin, 2018). Considering this additional feature, a question arose regarding the possible involvement of Drosha in growth regulation through the interaction with alternative substrates.

During one of the first characterizations of Drosha, some evidences suggested a putative role of this protein in ribosomal RNA (rRNA) biogenesis. Indeed, a modest quantity of Drosha during the S phase of cell cycle is translocated in the nucleolus and the antisense inhibition of this RNase III correlates with the accumulation of precursors of 5.8 S rRNA (Wu et al., 2000) (Liang and Crooke, 2011). Additionally, more recent studies highlighted the implication of Drosha-mediated rRNA processing in cell cycle progression in human multi-potent stromal cells (Oskowitz et al., 2011). Nevertheless, there are some discrepancies among the different reports aiming to investigate this alternative role of Drosha. For instance, Lee and colleagues' Drosha-knockdown experiments did show a block in miRNA biogenesis, but no effect on rRNA biogenesis was observed (Lee et al., 2003). Similarly, Drosha mutagenesis in mice did not affect the overall levels of mature rRNAs (Chong et al., 2008). Taken together, these studies indicate that Drosha might only have a marginal role, if any, in rRNA biogenesis, therefore we do not favour this mechanism as the major regulator of hepatocyte quiescence.

Transcriptomic analysis upon Drosha deficiency identified a certain amount of downregulated mRNAs, all characterized by hairpin-structures resembling those of pri-miRNAs. This suggests that Drosha could be involved in the post-transcriptional

destabilization of mRNAs through the cleavage of pri-miRNA-like hairpins embedded within their sequence (Kadener et al., 2009)(Chong et al., 2010). Drosha first direct target to be identified was DGCR8 mRNA, whose sequence contains two hairpin structures evolutionary conserved which are recognized and cut by Drosha. This mechanism of DGCR8 gene repression is proposed to serve as a means of autoregulation for the microprocessor complex (Han et al., 2009) (Triboulet et al., 2009). Drosha-mediated cleavage also plays an important role in fate determination and differentiation of progenitor cells. A plethora of studies indeed demonstrated the involvement of Drosha in the differentiation of neuronal progenitors (Knuckles et al., 2012)(Di Carlo et al., 2013) (Marinero et al., 2017), while it was shown in hematopoietic cells that Drosha-mediated cleavage of *Myl9* and *Todr1* mRNAs is necessary for dendritic cell development and myelopoiesis (Johanson et al., 2015). Besides these biologically relevant mRNAs, many others are cleaved by Drosha even though with less efficiency and much more variability among different cell types and experimental approaches. It is however certain that Drosha processing of miRNA's stem-loop is far more efficient than for mRNA's one, whose structures and sequences appear to be suboptimal (Lee and Shin, 2018). Acknowledging that our data do not explain the molecular mechanism responsible for Drosha-dependent regulation of hepatocyte proliferation, it stands to reason that the use of RNase inhibitors would help to establish whether Drosha's catalytic activity is necessary for cell quiescence or not.

This being said, we do not exclude that the involvement of this protein in the regulation of cell growth in primary hepatocytes could be independent from its RNase activity. Indeed, it recently emerged that Drosha is able to transcriptionally activate many human genes by binding short hairpins within their promoter-proximal regions, and this function appeared to be independent of Drosha's catalytic activity. More precisely, it is arbitrated through its N-terminal RS domain, which mediates the interaction with RNA Pol II and RNA-binding protein cap-binding complex 80 (CBP80 (Gromak et al., 2013). Another RNase-independent action of Drosha consists in the positive regulation of alternative splicing probably through interactions between the microprocessor complex and spliceosome components, although the specific mechanism is still poorly understood (Havens et al., 2014). Taken into consideration these data, we suggest Drosha's regulatory activity in cell growth might be assessed through the modulation of the expression of specific genes involved in the cell

cycle pathway. Transcriptional and proteomic analysis could shed light on this hypothesis by putting in evidence Drosha's possible partners.

Drosha's role in the regulation of quiescence: exploring possibilities

At least another question remains unsolved regarding how the inhibition of Drosha can allow hepatocytes growth despite the lack of aa. Cell proliferation requires in fact the provision of macronutrients essential both for building new cellular material and to provide fuel sources for energetic needs (Bohnsack and Hirschi, 2004). A possible explanation comes from the fact that both the full-aa and the aa-deprived medium are rich in glucose and contain serum, which might support hepatocyte proliferation in aa scarcity. Indeed, it has been described that most mammalian cells in culture principally use glucose and glutamine to sustain their growth and division (Vander Heiden et al., 2009). In this scenario, we speculate that Drosha might be only implicated in the removal of a hypothetical block which would impede hepatocytes to proliferate when exposed to nutrient shortage.

It is worth also mentioning that Drosha protein is expressed in all tissues (Dai et al., 2016), therefore its non-canonical function in primary rat hepatocyte growth regulation could possibly represent a general mechanism used by proliferative cells to control their entrance or exit from the cell cycle, according to amino acid availability. A correlation with the mTOR pathway in this context would explain Drosha's role in cell proliferation, since mTORC1 is considered the main aa-dependent regulator of cell growth (Howell et al., 2013). Furthermore, a paper recently published by Ye and colleagues highlighted a mTOR-dependent increase in Drosha protein following glucose deprivation in murine embryonic fibroblasts, with a consequent effect on miRNA expression (Ye et al., 2015). Interestingly, beside the augmentation of Drosha induced by aa deprivation, our data are at variance with the aforementioned study as we did not detect an involvement of mTOR pathway in Drosha's regulation. In addition, we did not find a general miRNA upregulation in aa-deprived hepatocytes compared to control, and the analysis of cell proliferation in presence or absence of aa after blocking miRNA activity, through Ago2 silencing, does not favour the hypothesis of a miRNA-dependent regulation of cell proliferation.

A very powerful microRNA in pancreas

Our study on miR-375 in pancreatic β -cells represents a valuable contribution to the increasing body of evidence directed to characterize the incredible range of functions this islet-enriched miRNA covers in the pancreatic development and identity. The role of miR-375 in β -cell differentiation is well characterized and widely described (Avnit-Sagi et al., 2009)(Joglekar et al., 2009)(Kloosterman et al., 2007). On the other hand, its implication in the regulation of insulin secretion, firstly discovered by Poy and colleagues (Poy et al., 2004), still needs to be fully clarified. β -cell identity is defined by its ability to secrete insulin in response to glucose (glucose-stimulated insulin secretion - GSIS) (Gembal et al., 1992). However only mature β -cells were demonstrated to be highly responsive to glucose concentration. Indeed, this ability is gradually acquired by neonatal β -cells during post-natal life through a process of functional maturation triggered by glucose exposure, i.e. by the change in diet from high-fat milk to high-carbohydrate chow during the weaning (Otonkoski et al., 1988)(Otonkoski et al., 1991). In the present paper we confirmed the involvement of miR-375 in the regulation of GSIS, as its overexpression in rat pancreatic islet altered insulin secretion in response to glucose stimulation. Conversely, we found that the upregulation of this miRNA impacted the metabolic signaling of insulin secretion rather than its exocytosis per se. Additionally, we observed a decrease in O_2 consumption in response to glucose, while the inhibition of miR-375 augmented glucose-induced O_2 consumption, suggesting miR-375 at least partially regulates glucose-induced mitochondrial metabolism. It was recently described by Dor's group that the quantity of oxygen consumed at low glucose is higher in immature islets than in mature ones, although their capacity of increasing O_2 consumption in response to increasing concentration of glucose is considerably lower (Stolovich-Rain et al., 2015). These data are compatible with the results obtained by Jermendy and colleagues (Jermendy et al., 2011), who showed that immature β -cells lack the metabolic capacity of mature islets to produce ATP and anaplerosis-derived products from aerobic glycolysis. Taking into consideration the data collected by Regazzi's group showing that the diet shift at weaning is also accompanied by changes in islet-specific microRNAs (Jacovetti et al., 2015), we wondered if a variation in miR-375 expression could also be observed between immature and mature rat β -cells, thus possibly highlighting a role for this miRNA in the metabolic maturation of fetal β -cells and in the acquisition of the GSIS capacity. Interestingly, control

fetal rat islets showed low GSIS but no defects in insulin exocytosis, together with an upregulation in miR-375. On the contrary, fetal rat islet treated with 11 mmol/l glucose – known to induce the functional maturation of pancreatic islets *in vitro* – showed an appropriate insulin response to glucose and reduced expression of miR-375. These findings further support our hypothesis of miR-375 affecting β -cell glucose response rather than the insulin exocytosis machinery. It must be noted that our results are in contrast with previous findings of Stoffel and colleagues, who found that the overexpression of miR-375 in mice β -cells impairs GSIS without changes in glucose metabolism (Poy et al., 2004). Conversely, miR-375KO mice were described to exhibit defects in β -cell phenotype, restored by the re-expression of miR-375 in β -cells of the same animals, while a 2-fold increase in miR-375 expression in mice did not have any effect on β -cell mass and functions (Latreille et al., 2015). The origins of these incongruences might be related to differences in the animal species (rats and human versus mice) used as a model. Indeed, our results were additionally corroborated by experiments showing that the effect of pyruvate on insulin secretion was very similar to that of GSIS in rat islets overexpressing miR-375, compared to control islets. Moreover, the forced expression of miR-375 showed an augmented lactate release, suggesting that miR-375 might affect GSIS by promoting pyruvate reduction instead of its mitochondrial metabolism, hence inhibiting both glucose-induced ATP generation and the production of anaplerosis-derived metabolites.

[Additional question regarding miR-375: possible targets](#)

Considering the biological actions of miRNAs, the question regarding their targets naturally arises. Previous studies from our laboratory identified PDK1 as a direct target of miR-375 (El Ouaamari et al., 2008). This investigation, performed in INS-1E pancreatic cell line, showed that miR-375 directly binds *pdk1* mRNA, thus inhibiting its protein product and resulting in the inhibition of PI3-kinase signaling cascade, in insulin mRNA and cell proliferation. Also, glucose was demonstrated to decrease miR-375 expression, resulting in the increase of PDK1. To this of our knowledge this was the first demonstration that a nutrient could regulate the expression of a microRNA. In addition, this effect of glucose is particularly relevant for beta cells as it could explain, at least in part, the effect of glucose on insulin gene expression and secretion. Note that, a recent work on β -cell derived cells from adult

human pancreatic islets found the overexpression of miR-375 increases insulin mRNA level through effects on the same pathway (Nathan et al., 2015). We suggest these discrepancies might be due to the origin of the cells used (primary cells versus cell line) and to eventual species-specific differences. Interestingly, we have recently identified by qPCR analysis two potential miR-375 targets crucial for mitochondrial metabolism, as the overexpression of miR-375 in rat β -cells resulted in a decrease of their mRNA level. However, proteomic analysis performed by our collaborators in Montreal (IRCM Institut de la Recherche Clinique Montreal) on rat islets transfected with control miR or miR-375, did not confirm our previous results. Indeed, considering that in mammalian systems the binding of miRNAs to their mRNA targets results in the decrease the level of the corresponding translated product, we were expecting to observe downregulation of these two proteins but instead they were not listed among those displaying differential expression. Nevertheless, it should be taken into consideration that the repressive effect of a miRNA on its target is typically modest, often close to 20% (Selbach et al., 2008)(Baek et al., 2008), which led us not to exclude the possibility of having missed the effect of miR-375 on our proteins. In addition, as in the proteomic analysis a single time-point was studied, additional experiments are required to integrate the global action of miR375. Given those limitations, we decided to change the experimental approach and additionally measure their expression by western blot analysis. Since we did not observe any difference between rat pancreatic islets overexpressing miR-375 and control islets, we suppose that the two proteins we identified do not constitute a direct target of miR-375, assumption that was supported by miRNA target prediction software. Thus, we hypothesize that miR-375 might target one or several transcripts upstream of these key metabolic enzymes, hypothesis that we are planning to further investigate.

In summary, our work contributes to the understanding of the diverse roles played by miR-375 in the modulation of β -cell functions and provides additional evidence of its regulation mechanism on GSIS.

Annex

REVIEW ARTICLE

Shaping and preserving β -cell identity with microRNAsO. Dumortier PhD¹ | G. Fabris MSc¹ | E. Van Obberghen MD, PhD²¹University Côte d'Azur, Inserm, CNRS, IRCAN, France²University Côte d'Azur, CHU, Inserm, CNRS, IRCAN, France**Conflict of interests:** The authors declare no conflict of interest.**Corresponding Author:** Emmanuel Van Obberghen, Medical School, University Côte d'Azur, IRCAN, 7th Floor, 28 av. de Valombrose, 06107 Nice Cedex 2, France (vanobbeg@unice.fr).

The highly sophisticated identity of pancreatic β -cells is geared to accomplish its unique feat of providing insulin for organismal glucose and lipid homeostasis. This requires a particular and streamlined fuel metabolism which defines mature β -cells as glucose sensors linked to an insulin exocytosis machinery. The establishment of an appropriate β -cell mass and function during development as well as the maintenance of their identity throughout life are necessary for energy homeostasis. The small non-coding RNAs, microRNAs (miRNAs), are now well-recognized regulators of gene transcripts, which in general are negatively affected by them. Convincing evidence exists to view miRNAs as major actors in β -cell development and function, suggesting an important role for them in the distinctive β -cell 'identity card'. Here, we summarize key features that associate miRNAs and the establishment of the appropriate β -cell identity and its necessary maintenance during their 'long life'.

KEYWORDS β -cell development, β -cell identity, insulin secretion, microRNAs, type 2 diabetes**1 | INTRODUCTION**

The identity of cells is defined by the expression of a particular set of genes and the resulting production of specific proteins and constituents depending on them. These expression patterns follow a complex scenario during cell development, where timing and quantity of the proteins produced are essential and finely regulated. The maintenance of these distinctive cell identities throughout life is required for normal physiology and health of the organism.

In mammals, β -cells are the major cell type within the pancreatic islets. In humans, they represent nearly 60% of the islet cells.¹ They are the unique source of circulating insulin, the chief anabolic hormone. Indeed, insulin is critically important for fuel homeostasis and energy storage. Concerning glucose homeostasis, β -cells serve as a glucostat in a highly sophisticated homeostatic feedback system.² Schematically, the glucose-sensing mechanism of β -cells can be divided into two components: (1) the proximal events of glucose entry and metabolism, and (2) the distal mechanism of insulin secretion, going from mitochondrial signal generation and initiation of cell membrane depolarization to exocytosis.³ Defects in insulin secretion lead to chronic hyperglycaemia, the hallmark of diabetes mellitus.⁴ As in humans the replication capacity of β -cells declines dramatically with age,⁵ the establishment of an appropriate β -cell number with

proper functioning and the maintenance of the identity of these long-living cells are necessary for organismal homeostasis.

MicroRNAs (miRNAs) are short, single-stranded non-coding gene products, classically 20-22 nucleotides long that in general are derived from endogenous hairpin transcripts. The human genome codes for several thousand miRNAs that regulate gene expression through modulation of target messenger RNA.⁶ miRNA biogenesis consists in a series of consecutive steps that convert primary miRNAs into biologically mature and active miRNAs.^{7,8} The miRNA maturation is driven by two RNase III enzymes, the nuclear Drosha and the cytosolic Dicer. After being processed, the mature miRNA is anchored in the cytosol to an Argonaute (Ago) protein, building the core of the RNA-induced-silencing-complex (RISC). Most of the small single-stranded RNAs interact with specific sequences in the 3' untranslated region (UTR) of mRNA, thereby inducing mRNA degradation and/or translation inhibition. While initial observations have shown that miRNAs act in the cytosol, evidence is being gathered illustrating that they can in addition regulate gene expression in the nucleus.⁹ Since their discovery in the early 1990s, grounds are expanding in favour of a key role for non-coding RNAs in a growing list of biological programs including cell differentiation, proliferation and metabolism.¹⁰⁻¹² Computational predictions of miRNA targets estimate that a single miRNA can regulate hundreds of different mRNAs, suggesting that a

large proportion of the transcriptome is subjected to miRNA regulation. Hence, miRNAs may modulate up to 60% of all human genes.¹³ A further intriguing feature of miRNAs is that multiple miRNAs can impact on a single mRNA which creates a combinatorial rheostat allowing finely regulated mRNA functioning. In the present review, we will summarize recent key advances in our understanding of miRNAs as having a determining role in the establishment and the maintaining of β -cell identity and its unique characteristics. Finally, we will briefly discuss how miRNAs could be involved in the loss of β -cell singularity in diabetes.

“miRNAs are small non-coding RNAs affecting gene expression through interaction with the 3'UTR of their target mRNA, leading to reduced levels of the encoded protein.”

2 | miRNAs AND THE DEVELOPMENT OF β -CELLS

2.1 | miRNAs as general modulators of pancreas development

To the best of our knowledge, the essential role of miRNAs in development was first investigated by Bernstein et al.¹⁴ In their groundbreaking experiments, they showed that a whole body knockdown of *Dicer1* in mice leads to early embryonic lethality. The importance of *Dicer1* was later investigated specifically in pancreas by the generation of a *Pdx1-Cre* transgene to delete the second RNase III domain of *Dicer1* in all the pancreatic lineages of embryonic mice. In this model, the formation of every miRNA in the embryonic pancreas of the mice is expected to be blocked. Interestingly, the transgenic mice managed to survive until birth, but died after 3 days, suffering from defects in development and differentiation of pancreatic cell lineages.¹⁵

Another group recently addressed the consequences of *Dicer1* ablation by means of a *Cre*-transgene under the influence of the *Ngn3* promoter,¹⁶ thus blocking miRNA maturation only in the endocrine progenitor cells. At birth, no morphological defects in the islets of the mutant mice were observed. However, already at 1-2 weeks of age, altered islet organization and diminished endocrine cell mass, as well as decreased insulin and glucagon secretory capacity, were found.

Finally, the role of *Dicer* was also investigated in differentiated β -cells using *RIP-Cre* transgene to invalidate the enzyme. Interestingly, Kalis et al did not notice changes in either β -cell mass or number in the mice, but only limited effects on cell size and pancreas architecture.¹⁷ Taken together, these data support the view of an essential contribution of *Dicer1* only at the very first steps of pancreas development, whereas *Dicer1*-dependent miRNA processing does not appear to be required for the final strides of β -cell differentiation (for review see Ref. 18).

2.2 | miRNAs and β -cell dedifferentiation

Given the fact that the miRNA machinery is intimately implicated in regulating pancreas development and functional β -cell differentiation,

it does not come as a surprise that many studies have attempted to pin down the precise contribution of specific miRNAs (Figure 1). One of the most relevant and widely explored miRNAs in β -cell development is miR-375. This miRNA is highly expressed both in human and mouse pancreatic islets. Remarkably, its expression increases during pancreas organogenesis together with insulin production and β -cell proliferation.^{19,20} The relevance of miR-375 in pancreatic cell development was first investigated in zebrafish embryos.²¹ In these pioneering studies using a loss of function approach, the authors demonstrated the decisive contribution of miR-375 to β -cell development. Two years later, miR-375 knockdown in mice confirmed its pivotal role in islet cell expansion.²² Subsequently, proof was provided that islet expression of miR-375 is transcriptionally regulated at different levels. Indeed, Avnit-Sagi et al. unveiled, in the miR-375 promoter region, consensus-binding sequences for important transcriptional factors involved in β -cell differentiation such as *Pdx-1*, *Ngn3* and *NeuroD1*.^{23,24}

“miRNAs appear as major actors in the genesis and life of cells as they affect β -cell differentiation, proliferation and hormone secretion”

Taking into consideration the role of miR-375 in β -cell differentiation, it is reasonable to assume that its downregulation could enhance dedifferentiation of insulin secreting cells. In fact, evidence is indeed being built highlighting an association between decreasing levels of miR-375 and β -cell dedifferentiation *in vitro*.²⁵ Further, it has been recently found *in vitro* that overexpression of miR-375 can contribute to the differentiation of induced pluripotent stem cells into β -cell-like clusters.²⁶ This overexpression is also sufficient to generate insulin-producing cells from mesenchymal stem cells coming from human placenta.²⁷ However, a recent paper by Jafarian et al²⁸ reveals that miR-375 does not suffice for the differentiation of human mesenchymal stem cells (hMSCs) into functional β -cells due to the fact that the cells obtained could not respond to glucose *in vitro*. At the same time, downregulation of miR-9, which is known to affect the β -cell secretory capacity²⁹ in islet-like clusters expressing miR-375, resulted in an obvious increase in glucose-stimulated responses. Viewed in aggregate, these results led the authors to suggest that overexpression of miR-375, coupled to simultaneous downregulation of miR-9, exerts synergistic effects on the differentiation of hMSCs into functional insulin-producing cells. In addition to miR-375, miR-7 has also been shown to participate in human embryonic stem cell differentiation in insulin-producing pancreatic cells.³⁰ Indeed, proof was provided according to which expression of miR-7 is increased during human islet development and differentiation.¹⁹ Intriguingly, it has recently been found that this miRNA is also an inhibitor of β -cell proliferation through the mTOR signalling (mammalian target of rapamycin) pathway.³¹

2.3 | miRNAs and the pancreatic transcriptional network

While approaching the role of miR-7 in β -cell differentiation, a few years ago the group of Hornstein revealed that this miRNA modulates endocrine cell development by targeting the transcriptional

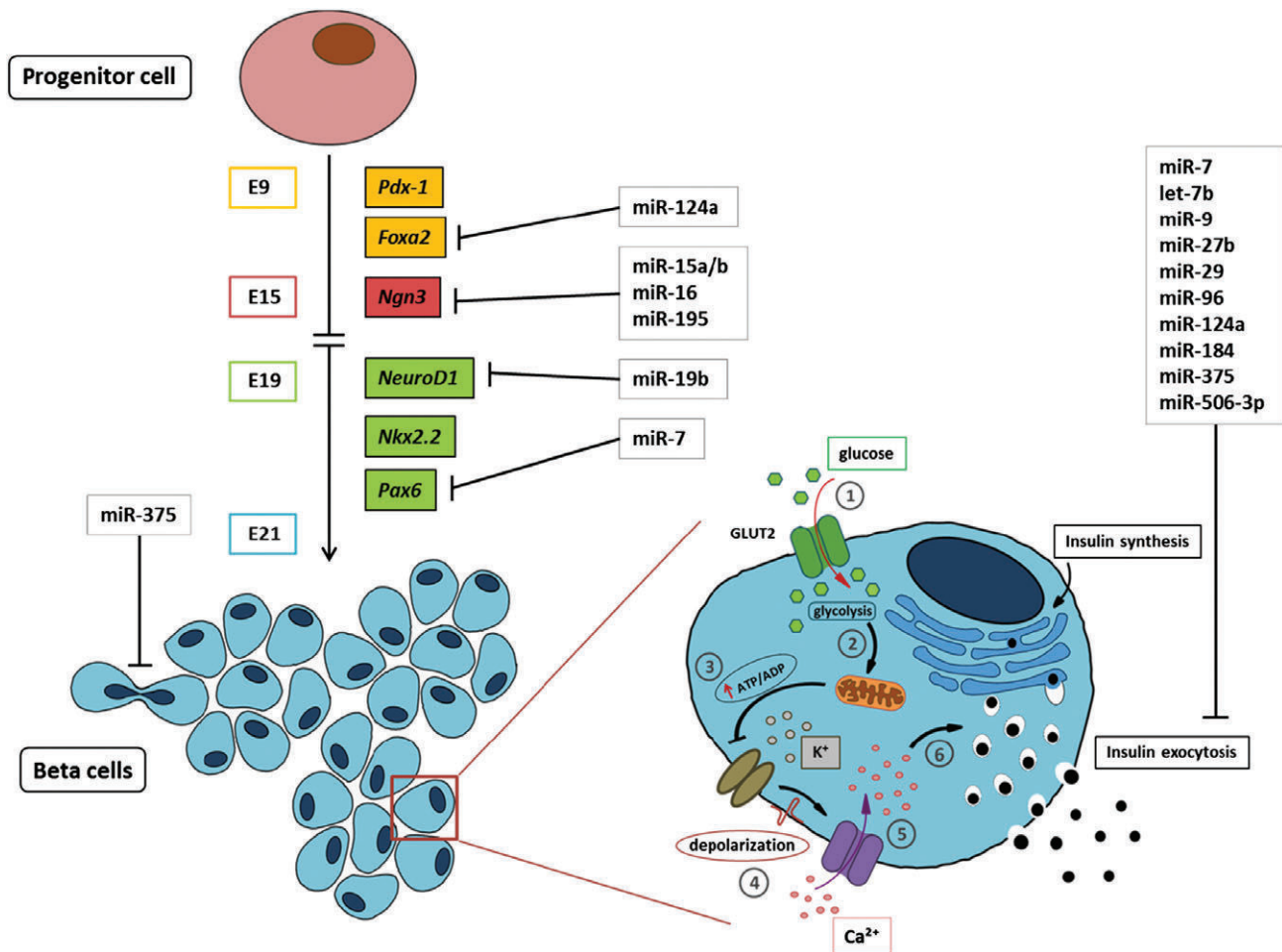


FIGURE 1 Examples of microRNAs (miRNAs) impacting on β -cell differentiation and functioning. Some of the miRNAs discussed in the article, and shown here, target transcription factors involved in β -cell development (the colours associated with each transcription factor on the arrow reflect the rat 'embryonic age' of organogenesis they belong to). Other miRNAs affect β -cell functioning by directly or indirectly modulating insulin secretion. For example, let-7b is known to target myotrophin (MTPN), which is involved in insulin granule fusion with the plasma membrane, and thus directly inhibits insulin exocytosis. In contrast, miR-184 inhibits Argonaute2 (Ago2) expression affecting other miRNAs and hence interferes indirectly with the secretory pathway. Interestingly, some of the miRNAs listed exert a double role at different levels of β -cell functioning. For instance, miR-375 is able to interfere both with β -cell proliferation and with insulin exocytosis.

factor Pax6, which belongs to the key factors regulating β -cell specification during development. Furthermore, the authors demonstrated that the overexpression of miR-7 in pancreatic explants of mouse embryos results in a decrease in Pax6 expression, with a concomitant reduction in insulin content. Conversely, miR-7 knockdown correlates with increased β -cell number.³² The list of miRNAs involved in pancreas development by targeting its transcriptional network is still growing as now miR-15a/b, miR-16 and miR-195 are similarly implicated. Data published by Joglekar et al report that inhibition of these miRNAs in regenerating mouse pancreatic cells augments Ngn3 expression, together with its downstream partners Nkx2.2 and NeuroD1. Furthermore, overexpression of the same miRNAs in mouse embryonic pancreatic explants leads to a decrease in the hormone-producing cell number, confirming the key role of these miRNAs in β -cell development and specification.³³ miR-124a is another miRNA which participates in regulating pancreas development at early stages of organogenesis. Indeed, in 2007, we demonstrated that this miRNA modulates the protein level of Foxa2 by binding to the 3'UTR of its mRNA. Foxa2 is known to be definitely required for differentiation as

it controls the expression of many genes involved in pancreas development. In point of fact, we found that augmented miR-124a expression negatively regulates Foxa2 protein level, thus leading to a decrease in several target genes such as Pdx-1, as well as Kir6.2 and Sur1, involved in glucose metabolism and insulin secretion.³⁴

The member of the miR-17-92 cluster, miR-19b, by interacting with NeuroD1, is an additional example of miRNAs implicated in the transcriptional network of mice pancreatic endocrine progenitors.³⁵ The authors discovered that miR-19b directly binds the 3'UTR of NeuroD1 mRNA, thus leading to a decrease in mRNA level and its corresponding protein. While further investigations will be necessary to clarify the exact molecular mechanism, they suggest that miR-19b actually downregulates the insulin 1 gene, by targeting NeuroD1, and consequently alters β -cell differentiation and functioning.

2.4 | miRNAs and β -cell development

Recent emerging evidence identified additional miRNAs not directly involved in silencing transcriptional factors as described above, but

which could potentially impact on pancreas development by other means. For example, miR-24 could be such an actor. Indeed, this miRNA has been shown to target menin, a nuclear protein implicated in the cell cycle,³⁶ but which has been connected also to pancreas development.³⁷ Remarkably, Vijayaraghavan and colleagues observed that in MIN6 and betaTox5 cells, miR-24 binding to menin mRNA leads to cellular proliferation and improves cell viability.³⁸ In addition, growing grounds demonstrate that menin plays a decisive role in the processing of certain miRNAs.³⁹ Taken as a whole, these data point to the existence of a bidirectional crosstalk between miRNA processing and β -cell development, and further highlight the complexity of the miRNA network involved in pancreas development. The list of miRNAs incriminated in pancreas genesis is already long, but is definitely expected to stretch.

3 | miRNAs AND β -CELL IDENTITY

Generally speaking the metabolic properties of β -cells define the 'identity' of the mature β -cells as a glucose sensor. At the granular level, the β -cell metabolic machinery is designed to sense small fluctuations in blood glucose level and to supply insulin accordingly to the needs of the body. Central to glucose sensing by β -cells is the stimulation of glycolytic and oxidative metabolism, ultimately causing enhanced mitochondrial ATP synthesis. Glucose metabolism in β -cells has several exclusive features. First, glucose transport into β -cells is not limiting. Indeed, Glut-1 and Glut-2 have a uniquely high K_m for glucose, and are expressed at an elevated level in human or rodents β -cells, respectively. This ensures a rapid equalization of glucose between the extracellular space and the cytosol at all glycaemic levels.⁴⁰ The second particular trait of β -cell metabolism is that glycolysis is controlled by glucokinase, which has low affinity for glucose and appears to lack any form of feedback regulation. Since β -cell express low levels of membrane monocarboxylate transporters (MCT1) and low lactate dehydrogenase (LDH) activity, over 80% of the glucose carbons within β -cells are oxidized in the mitochondria.⁴¹ Thus, the uniqueness of differentiated β -cells results not only from cell-specific gene expression but also from cell-selective repression of certain genes and hence their respective products.

In this context, the 'disallowed genes' hypothesis proposed by Rutter and Schuit^{42,43} suggests that disallowed genes possess a deleterious potential and would be regulated by multiple independent mechanisms, including miRNAs. Their view stems from the identification of more than 60 genes that are selectively silenced in adult β -cells. These genes are strongly expressed in most other mammalian tissues, and code for enzymes and mitochondrial proteins, which are not geared for optimal mitochondrial metabolism. Therefore, in the β -cell, their repression is needed for efficient oxidative phosphorylation allowing optimized sensing of glucose. However, the mechanisms responsible for the control of the disallowed genes are still elusive.

It is thought that miRNAs can repress unwanted genes whose activity may be harmful in a particular cell type and/or context.¹² Indeed, Martinez-Sanchez et al identified miRNAs as having a key role in the repression of such 'forbidden' genes. In fact, the inactivation of the miRNA-processing enzyme, Dicer, specifically in adult

β -cells in mice leads to a substantial reduction in miRNAs in these cells. Of the 14 disallowed genes studied, 6 genes were upregulated after Dicer invalidation. In addition, Dicer silencing correlates with a loss of the secretory capacity of the islets.⁴⁴

Specific miRNAs have been shown to mediate repression of specific disallowed genes. For example, several miR-29 isoforms target MCT1 in β -cells, which ensures that muscle lactate or pyruvate, produced during exercise, is unable to stimulate inappropriately β -cell metabolism and hence prevents unwanted insulin secretion.⁴⁵ Indeed, forced expression of MCT1 in β -cells provokes pyruvate-stimulated insulin release in isolated rat islets.⁴¹ Thus, miR-29 contributes to the β -cell-specific silencing of the MCT1 transporter and may thus contribute to β -cell specificity/identity. Other miRNAs are able to recognize the 3'UTR region of MCT1 mRNA. For example, miR-124a has multiple predicted target sites on the 3'UTR of MCT1 that are conserved among human, rat, mouse, dog and chicken. Downregulation of MCT1 by miR-124a has been experimentally validated.^{46,47} Moreover, miR-27b, 506-3p are also predicted to recognize the MCT1 3'UTR. A challenging question arises as to whether members of the disallowed gene family other than MCT1 are regulated by miRNAs.

In summary, several miRNAs appear to exert a determining role in the regulation of disallowed genes in β -cells, and as such provide evidence for a newly identified way through which miRNAs portray the functional identity of β -cells.⁴⁴

4 | miRNAs AND β -CELL FUNCTION

The identity of β -cells does not originate only from its particular and streamlined metabolic machinery, but also from its unique feat to be able to provide insulin to the body. Insulin secretion is a complex process with two well-orchestrated parts. In a first phase, glucose enters in β -cells and is metabolized. In the second phase, its metabolism leads to calcium entry and insulin granule exocytosis.³ Schematically, when glucose increases in the blood it is taken up by β -cells through glucose transporters. Once inside, glucose is converted to glucose-6-phosphate by glucokinase, and, after glycolysis, to pyruvate which enters the mitochondria. The enriched pyruvate supply to the mitochondria stimulates ATP synthesis.⁴⁸ The increased ATP/ADP ratio induces the closure of KATP channels, leading to membrane depolarization with subsequent calcium entry and eventuating in exocytosis of insulin granules.⁴⁸

As mentioned earlier, miR-375 (see Section 2.4) is considered as the miRNA with the most robust expression in the endocrine pancreas.²⁰ This miRNA was the first to be identified as being involved in insulin secretion.²⁰ Indeed, forced expression of miR-375 in MIN6 insulinoma cells reduced glucose-induced insulin secretion by inhibiting exocytosis. Conversely, when miR-375 is decreased in mice, the cells secrete more insulin in response to glucose.^{20,49} The effect of miR-375 on insulin exocytosis appears to be due to the binding of the miRNA to the 3'UTR of mRNA encoding for myotrophin (MTPN), which is known to be involved in insulin granule fusion with the plasma membrane. Further, our laboratory has shown that miR-375 downregulates insulin gene expression in INS1-E cells by acting on the mRNA of phosphoinositide-dependent kinase-1 (PKD-1), which was

the second functionally identified miR-375 target. Remarkably, miR-375 expression is inhibited by glucose, attributing to miR-375 the status of a physiological regulator of β -cell functioning.⁵⁰

In the miRNA machinery, Argonaute2 (Ago2) exerts a central function.⁵¹ Inhibition of Ago2 in pancreatic β -cells results in enhanced insulin release, suggesting that most of the miRNAs have a negative influence on β -cell physiology.^{52,53} As miR-375 constitutes the largest proportion of the total pool of miRNAs in islet cells, this observation may explain several similarities existing between models with a loss of Ago2 compared to a loss of miR-375. Indeed, the silencing of Ago2 leads to increased expression of miR-375 target mRNAs, including *gephyrin* and *ywhaz*. These targets positively contribute to exocytosis indicating that they may mediate the functional role of both miR-375 and Ago proteins in β -cells by modulating the secretory pathway.⁵³ Remarkably, in another study, Tattikota et al showed that miR-184, highly conserved and abundantly expressed in β -cells, targets Ago2.⁵² Reduction of miR-184 promotes the expression of its target Ago2 facilitating the function of miR-375. These observations illustrate perfectly well the complexity and the intricacy of the network involving small RNAs to maintain essential metabolic processes such as glucose-induced insulin secretion (Figure 1).

Likewise, miR-124a has been found to act at different regulatory levels of insulin exocytosis by directly targeting MTPN⁵⁴ and GTPase Rab27.⁵⁵ Other miRNAs are able to modulate insulin secretion such as *let-7b* also targeting MTPN,⁵⁴ and miR-96, which regulate the mRNA and protein levels of synaptotagmin-like 4 (*Sytl4*, an inhibitor of insulin exocytosis).⁵⁵ Moreover, *Sytl4* is antagonized by miR-9 via the interaction of the latter with the mRNA of the *Onecut-2* transcription factor (one cut homeobox 2).²⁹ In addition, the members of the miR-29 family inhibit insulin secretion by decreasing the *Onecut-2* protein in MIN6 cells and dispersed islet cells.⁵⁶

5 | miRNAs AND LOSS OF β -CELL IDENTITY IN DIABETES

Type 2 diabetes (T2D) constitutes the vast majority (85–95%) of all diabetes cases in most countries. The disease is characterized by a combination of insulin resistance and a dysfunction of insulin producing β -cells. While during the disease process there is a functional decline of β -cells, it would appear that in T2D there is in addition a decrease in β -cell number.⁵⁷ Although the participation of a β -cell identity crisis during T2D pathogenesis is still controversial,⁵⁸ the loss of β -cell identity as an alternative explanation for β -cell failure is starting to gain momentum. According to this concept, β -cell insufficiency during T2D is not due to cell death, but rather to reduced expression of key β -cell genes, triggering the loss of β -cell identity and thus favouring β -cell dedifferentiation. This hypothesis stems from early metabolic observations^{59,60} and a more recent mechanistic study showing that ablation of *FoxO1* in adult β -cells in mice induces their reversion to a progenitor phenotype.⁶¹ It remains an open question whether dedifferentiation is primarily involved in diabetes progression or rather in β -cell failure with the transition from pre-diabetes to diabetes. Recently, Accili's group provided evidence of β -cell dedifferentiation in human T2D. Importantly, they demonstrated that dedifferentiated

cells account for almost 40% of the β -cells in T2D patients compared to 8% in controls.⁶² In addition, β -cell-specific transcription factors were ectopically found in glucagon- and somatostatin-producing cells of diabetic subjects, supporting the view that β -cells become dedifferentiated and convert to other endocrine-like cells.

To assess the involvement of miRNAs in diabetes, most research efforts have aimed at establishing the expression profiles of animal models. In fact, several miRNAs show indeed aberrant expression in islets of animal models of T2D. The majority of these studies focus on miRNAs having a potential impact on the life, death and function of β -cells.⁶³ For example miR-34a, miR-146, miR-21, miR-29 and, more recently, miR-200a have been shown to be involved in β -cell apoptosis, while miR-375, miR-7, miR-124a, miR-152 or miR-184 modulate insulin secretion.^{53,64,65} However, while changes in miRNA expression in human islets from T2D donors exist, the limited available data do not permit to draw a clear picture. Important possible pitfalls exist due to the small cohort size, the lack of uniformization in miRNA measurement techniques and confounding factors related to age, body mass index and sex differences.⁶⁶

“miRNA expression can be regulated by both friends (eg, glucose) and foes (eg, diabetic environment) of β -cells, making them versatile modulators”

To the best of our knowledge, no particular miRNA has been unambiguously identified as modulator of β -cell identity during the pathogenesis of T2D. However, provocative studies suggest that this could be the case. Indeed, using tamoxifen-inducible disruption of *Dicer1* specifically in adult mouse β -cells, Melkman-Zehavi et al showed that 3 weeks after tamoxifen treatment a dramatic reduction occurs in the pancreatic insulin content with no differences in β -cell apoptosis. Immunohistochemical analysis of insulin showed a uniform and strong expression in the controls, while the insulin immunostaining in *Dicer1* transgenic animals was weak and varied considerably from cell to cell. Cells that lost *Dicer1* activity conserved the expression of the secretory vesicle protein, synaptophysin, and did not express alternative hormone markers such as somatostatin, pancreatic polypeptide, glucagon or ghrelin. This suggests that β -cells maintain at least some endocrine features of mature β -cells. However, no difference in the expression of *Ngn3*, *Pdx-1*, *MafA*, *Nkx6.1* and *Pax6* was found.⁶⁷ Using permanent *Dicer* deletion with the *RIP-Cre* transgene, Kalis et al obtained similar results characterized by a progressive decrease in insulin producing cells and in total β -cell mass without evidence for altered proliferation or apoptosis. However, the expression of β -cell markers was not investigated.¹⁷ In addition, it has to be stressed that in the above-mentioned study (see Section 3) *Dicer1* inactivation in adult β -cells increased the expression of 'disallowed' genes.⁴⁴ Taken together, these findings open new perspectives on the potential role of miRNAs in the β -cell identity crisis during T2D pathogenesis.

6 | CONCLUSIONS AND PERSPECTIVES

In adult mice, β -cells appear to belong to a group of tissues that could be maintained by self-duplication of differentiated cells.^{68,69}

However, as β -cell turnover in human adults is very limited, the maintenance of the genuine β -cell 'identity card' is crucial for the preservation of fuel homeostasis. We have attempted here to summarize key features which associate miRNAs and the establishment of the appropriate β -cell identity, which has to be maintained during their 'long-life' for organismal homeostasis.

Numerous observations have deciphered the essential roles of miRNAs in establishing and/or maintaining cell identity. Maybe the strongest advocate fact is that mice lacking one of the key miRNA-processing genes fall short in the establishment of β -cell lineage during organogenesis or in the maintenance of their personality throughout adult life. While thousands of miRNAs have been identified in humans, the dearth of information concerning their precise roles is striking and should urgently be addressed. Identification of miRNA targets is one of the most challenging tasks because a single miRNA has hundreds of potential mRNA targets. Conversely, several different miRNAs can control a single mRNA target. To render the situation even more multifaceted, it is now established that miRNAs can play both positive and negative roles in gene expression and that their area of intervention on gene expression is not limited to the cytosol, but expands to the nucleus. These thought-provoking features of the miRNA world are adding new layers of complexity to the regulatory dynamics of β -cell gene expression by miRNAs.

The relationship between miRNAs and β -cell physiology appears to operate as a bidirectional link. Indeed, miRNAs impact on β -cells by interacting with key players involved in their function/identity. By the same token, miRNA expression and processing in β -cells are modulated by major metabolic stimuli such as glucose.^{10,70,71} This mutually enriching relationship between miRNAs and metabolism is likely to participate in the complex regulation of glucose homeostasis. This aspect is particularly relevant considering the fact that hyperglycaemia, β -cell failure and associated aberrant expression of miRNAs are underlying defects of type 1 diabetes, and in many cases also of T2D. Improving the preservation of β -cell identity could be a leap forward in the treatment of diabetes and may contribute to therapeutic approaches based on expanding the functional β -cell mass obtained from scarce donor islet tissue for transplantation into multiple recipients.

While yawning gaps exist in our understanding of the precise impact of miRNAs in the pathogenesis of diabetes, our current global picture is certainly fragmented as several other members of the non-coding RNA family are likely to play an important role. To meet this unmet challenge, concerted efforts of numerous laboratories are needed.

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