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Mechanisms underlying metabolic shift in pluripotent stem cells: The potential role of sirtuins

Tese de Doutoramento em Biologia Experimental e Biomedicina, ramo de Biologia Molecular, Celular e do Desenvolvimento, orientada pelo Professor Doutor João Ramalho-Santos, e apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra .

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UNIVERSIDADE DE COIMBRA



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***MECHANISMS UNDERLYING METABOLIC SHIFT
IN PLURIPOTENT STEM CELLS:
THE POTENTIAL ROLE OF SIRTUINS***

Tese de doutoramento apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra (IIIUC), para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia Experimental e Biomedicina, ramo de Biologia Molecular, Celular e do Desenvolvimento, realizada sob a orientação científica do Professor Doutor João Ramalho-Santos (Faculdade de Ciências e Tecnologia e Centro de Neurociências e Biologia Celular da Universidade de Coimbra). Este trabalho foi desenvolvido no Centro de Neurociências e Biologia Celular da Universidade de Coimbra.

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Marcelo José Marques Correia

Universidade de Coimbra, Setembro de 2015

Cover:

The cover picture represents an overgrown cell culture of mouse embryonic stem cells with nuclei stained with Hoechst 33342 (blue).

The overgrown colonies of mouse embryonic stem cells seen in the picture parallels with a ridge of the highest mountains. Like both, life is fully of ups and downs. It is always necessary energy to climb the big obstacles to reach an end!

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Dedico esta tese

à minha confidente e amor **Patricia**
aos meus pais "**Zé**" e **Rosa Maria**,
aos meus avós da "rõla" **Licínio** e **Maria**
e à minha irmã Rute (, **Ana Rute!**).

Sem eles, sem o seu apoio e sem a sua paciência,
nunca teria chegado a este ponto

A todos eles o meu **muito obrigado!**

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RESUMO

De forma semelhante ao botão embrionário do blastocisto, as células estaminais embrionárias possuem a capacidade de contínua autorrenovação e de diferenciação nos três folhetos germinativos e em todos os tipos celulares constituintes de um indivíduo adulto. Por conseguinte, as células estaminais embrionárias possuem um futuro promissor a nível de medicina regenerativa, modelos de estudo de doença *in vitro*, assim como uma poderosa ferramenta para a descoberta de novos fármacos e estudos fármaco-toxicológicos.

Na última década, ocorreu um aumento exponencial do conhecimento relativo à biologia das células estaminais embrionárias, a nível epigenómico, transcriptómico, proteómico e metabolómico, estimulando um melhor e correto uso para estas células. No entanto, muitos elementos desta regulação são ainda desconhecidos, o que explica o porquê de todo o potencial associado às células estaminais embrionárias ainda não ter sido alcançado.

O estudo das sirtuínas ganhou um papel de destaque após a descoberta de que estas proteínas em leveduras, nemátodes e moscas têm um papel na longevidade e envelhecimento. Para além do seu papel na regulação da transcrição, as sirtuínas têm sido referidas como moduladores de muitos outros processos celulares, nomeadamente do metabolismo.

Nesta tese esperamos estudar o possível papel das sirtuínas na pluripotência das células estaminais embrionárias, assim como na sua capacidade de diferenciação, dando um foco especial na influência das sirtuínas no estado metabólico da célula.

Para desvendar o papel da sirtuina 1, tomamos partido de um modelo genético de células estaminais embrionárias com deleção da sirtuina 1. Comparadas às células da linha celular normal, as células estaminais embrionárias com deleção da sirtuina 1 estão mais predispostas à diferenciação espontânea, mesmo quando são cultivadas em condições de manutenção de pluripotência. No entanto, este potencial aparenta estar já enviesado, uma vez que quando sujeitas a um protocolo de diferenciação dirigido, estas células são menos eficientes a diferenciar em linhagens neuronais comparando com as células normais. Propomos que a capacidade metabólica das células poderá estar a ajudar a enviesar a capacidade de diferenciação, uma vez que as células estaminais embrionárias com deleção da sirtuina 1 têm um metabolismo glicolítico mais pronunciado em detrimento do metabolismo oxidativo comparando com as células normais. STAT3 (transdutor de sinal e ativador da transcrição 3) é um factor de transcrição necessário para as vias de sinalização em pluripotência, mas também uma peça necessária para o metabolismo mitocondrial, que poderá estar envolvido nestas transições pluripotência/diferenciação e glicólise/metabolismo oxidativo das células sem sirtuina 1.

Na segunda parte, foi usada uma abordagem farmacológica com o intuito de modular a expressão e atividade da Sirtuina 3. Com esse propósito, usamos o flavonoide natural kaempferol, que tem sido descrito como possuindo capacidades antioxidantes. Apesar de não ter sido possível modular a Sirtuina 3 no nosso modelo, observámos que o kaempferol poderá modular de forma distinta as propriedades das células estaminais embrionárias. Baixas concentrações aparentemente têm efeito potenciador de pluripotência, enquanto que altas concentrações não afetam a pluripotência, mas induzem morte celular, o que poderá estar associado a um aumento dos níveis da espécie reativa de oxigénio, anião superóxido. Esta parte do trabalho, embora demonstrando a falta de modulação da sirtuina 3, sugere que os compostos naturais, como o kaempferol, possam vir a ser usados em cultura para a modulação *in vitro* da pluripotência e do potencial de diferenciação, evitando o uso de compostos químicos não naturais nem fisiológicos.

No cômputo geral, com os estudos aqui apresentados nesta tese, esperamos estar a contribuir para o conhecimento atual no plano das células estaminais embrionárias. Ademais, este trabalho permitiu salientar novos indícios sobre o papel da sirtuina 1 na regulação da pluripotência através de uma modulação (também) metabólica.

PALAVRAS-CHAVE

Células estaminais embrionárias, sirtuinas, kaempferol, metabolismo, apoptose, pluripotência, diferenciação celular.

Similarly to the inner cell mass of a blastocyst, embryonic stem cells have the capacity of continuous self-renewal and to differentiate into all the three germ layers and all cell types that compose an adult individual. Thus, embryonic stem cells hold a promising future for regenerative medicine purposes, *in vitro* disease modeling, as well as a powerful tool for drug discovery and (pharmaco-) toxicological studies.

In the last decade, there was an exponential growth of knowledge regarding embryonic stem cell biology, such as in the fields of epigenomics, transcriptomics, proteomics and metabolomics, inciting a better and proper use of these cells. Nonetheless, many elements of regulation are still unclear which explains why the full potential of embryonic stem cells has not yet been reached.

A highlight on the sirtuins field arose after reports suggesting a role for these proteins in yeast, nematode and fly homologues in longevity and aging control. Besides their role on transcription control, sirtuins have been reported as modulators of several other cellular processes, namely metabolism.

In this thesis we aimed to study the potential role of sirtuins in embryonic stem cells pluripotency and their differentiation potential, with a particular focus on the potential influence of sirtuins on embryonic stem cell metabolic status.

To unveil the role of sirtuin 1, we took advantage of a genetic Sirt1 knockout embryonic stem cell line. Compared to the wild-type cell line, sirtuin knockout embryonic stem cells are more prone for spontaneous differentiation, even in pluripotency culture conditions. Nonetheless, this potential appears to be biased, given that when subjected to a lineage-directed differentiation protocol, they differentiate less efficiently in neuronal cell lineages than their wild-type counterparts. We reason

that the metabolic capacity of these cells may be driving this biased differentiation capacity, once sirtuin 1 knockout embryonic stem cells have a more pronounced glycolytic metabolism in detriment of oxidative metabolism when compared to wild-type cells. STAT3 (Signal Transducer and Activator of Transcription 3) is a transcription factor required for pluripotency signaling pathways, but also an instrumental player for mitochondrial metabolism, that could be involved in these pluripotency-differentiation and glycolysis-oxidative phosphorylation switches of cells lacking sirtuin 1.

In a second part of this thesis, a pharmacological approach was used with the intent to modulate sirtuin 3 expression and activity. For such purpose, we used the natural flavonoid kaempferol, which has been described to have anti-oxidant capacity. Although we were not able to modulate sirtuin 3 in our model, we observed that kaempferol could distinctly modulate embryonic stem cell properties. Lower concentrations of this flavonoid act as pluripotency enhancer, while higher concentrations do not affect pluripotency, but instead induce cell death, which can possibly be associated with increased levels of the mitochondrial reactive oxygen species superoxide anion. This part of the work, although ineffective in terms of sirtuin 3 modulation, suggests that natural compounds, such as kaempferol, could be used for *in vitro* modulation of stem cell pluripotency and differentiation potential, avoiding the use of non-natural and non-physiological chemical factors in culture conditions.

Overall, with the studies presented in this thesis we are confidently giving more insights for the current knowledge in the field of embryonic stem cells. Moreover, we reveal some new clues for the role of sirtuin 1 on the regulation of pluripotency through (also) metabolic modulation.

KEYWORDS

Embryonic stem cells, Sirtuins, Kaempferol, metabolism, apoptosis, pluripotency, cell differentiation.

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LIST OF ABBREVIATIONS

2-DG	2-deoxy-glucose
2i	stem cell culture medium with 2 inhibitors: PD184352 and CHIR99021
2OAADPR	2'-O-acetyl-ADP-ribose
Ac	acetyl
AceCS2	acetyl-CoA synthase 2
α FP	alpha FetoProtein
Akt	phosphoinositide-3-kinase/ protein kinase B – PKB
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AnnV	annexin V
ANT	adenosine nucleotide translocator
α SMA	alpha Smooth Muscle Actin
ATP	adenosine-5'-triphosphate
β -catenin	beta-catenin
bFGF	basic fibroblast growth factor
β III-tubulin	Beta III tubulin
BMP	bone morphogenetic protein
BSA	bovine serum albumine
cDNA	complementary DNA
CDX2	caudal type homeobox 2 transcription factor
CO ₂	carbon dioxide
CPS1	carbamoyl phosphate synthetase 1
CTR	control
DCA	dichloroacetate
DNA	deoxyribonucleic acid

E	embryonic day
EB	embryoid body
EC	embryonal carcinoma
ECAR	extracellular acidification rate
EpiSC	epiblast stem cells
Erk	extracellular-signal-regulated kinase
ERR α	estrogen-related receptor alpha
ESC	embryonic stem cells
ETC	electron transport chain
FGF	fibroblast growth factor
FOXO1	forkhead box O 1
GABP β 1	GA repeat binding protein, beta 1
galacDMEM	galactose supplemented DMEM medium
GDH	glutamate dehydrogenase
gluDMEM	glucose supplemented DMEM medium
gp130	glycoprotein-130
GSK3	glycogen synthase kinase 3
H ⁺	proton
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
hBM	human bone marrow
HDAC	histone deacetylases
hESC	human embryonic stem cells
hiPSC	human embryonic stem cells
HKII	hexokinase II
HMG2CS2	3-hydroxy-3-methylglutaryl CoA synthase 2
HSC	hematopoietic stem cells
ICC	immunocytochemistry
ICM	Inner cell mass
Id	inhibition of differentiation
IDH2	isocitrate dehydrogenase 2
IMM	inner mitochondrial membrane
iPSC	induced pluripotent stem cells

JAK	janus kinase
KO	knockout
LCAD	long chain acyl coenzyme A dehydrogenase
LDHA	lactate dehydrogenase A
LIF	leukemia inhibitory factor
LIF-R	LIF-receptor
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblasts
mESC	mouse embryonic stem cells
miPSC	mouse induced pluripotent stem cells
miR	microRNA
MMP	mitochondrial membrane potential
MSC	mesencymal stem cells
mtDNA	mitochondrial DNA
MTgreen	MitoTracker® Green FM
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay
NAD ⁺	nicotinamide adenine dinucleotide
NADH	reduced adenine dinucleotide
NAM	nicotinamide
NAMPT	nicotinamide phosphoryltransferase
nDNA	nuclear DNA
NF-κB	nuclear factor-κB
NSC	neuronal stem cells
O.D.	optical density
OCR	oxygen consumption rate
OCT4	octamer-binding transcription factor 3/4; Oct3/4 or Pouf5f1
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween-20
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PDH-E1α	pyruvate dehydrogenase subunit E1alpha

PFA	paraformaldehyde
PGC-1 α	proliferator-activated receptor gamma, coactivator 1 alpha
PI	propidium iodide
POLG	polymerase γ
Pou5f1	POU class 5 homeobox 1 transcription factor; Oct4
PSC	Pluripotent Stem Cells
qPCR	quantitative real time PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcriptase
SDH	succinate dehydrogenase
SDHA	Succinate dehydrogenase subunit A
Sir2 α	silent information regulator 2 alpha
SIRT	sirtuin
SMAD	"mothers against decapentaplegic" mammalian homolog
SOX2	SRY - type high mobility group box 2
SRB	Sulforhodamine B
SRY	sex determining region Y
STAT3	signal transducer and activator of transcription 3
TCA	tricarboxylic acid
TE	trophoectoderm
TGF β	transforming growth factor beta
TMRM	tetramethylrhodamine, methyl ester, perchlorate
TOM-20	translocase of outer membrane receptor subunit 20
tRNA	transfer RNA
TSA	trichostatin A
UCP2	uncoupler protein 2
WB	western blot
WT	wild-type
β -ME	beta-mercaptoethanol

General Introduction

Pluripotent Stem Cells

Mammalian development and embryonic stem cells

After fertilization of the oocyte with the spermatozoa, a unique cell – the zygote – is ready to develop into a complete new individual. The zygote is a totipotent cell, i.e. has the maximal potential to generate all new cells and tissues required for the generation of the new individual: the embryo itself and all the extra-embryonic tissues that are generated during embryogenesis. The zygote undergoes successive mitosis and generates the morula, a mass of cells at day 3-4 of embryogenesis, where totipotency is still present. Then, with subsequent divisions and initial cell organization, the blastocyst arises. Two main types of cells compose the blastocyst: the inner cell mass (ICM), which will form the embryo itself, and the trophoectoderm (TE) cells, which will form the placenta (Nichols and Smith, 2012). At this stage, totipotency has been eliminated rendering ICM cells pluripotent, i.e. ICM cells have the capacity to generate the three germ cell layers that will ultimately generate all the cells of a new organism (Nichols and Smith, 2012). Among other proteins, the ICM cells express the homeodomain-containing transcription factor NANOG and the octamer-binding transcription factor 3/4 – OCT3/4 (also known as the POU class 5 homeobox 1 transcription factor – Pou5f1; aforementioned will be referred as OCT4), while the TE cells express the caudal type homeobox 2 transcription factor – CDX2. It is believed that OCT4 and CDX2 are able to inhibit each other, allowing the specification and stabilization of the two cell lineages of the blastocyst: in the ICM, OCT4 is highly expressed leading to downregulation of CDX2, while in the TE the opposite regulation occurs (Nichols and Smith, 2012; Niwa et al., 2005; Strumpf et al., 2005).

The blastocyst then suffers a series of morphological and molecular changes to give rise to the pre-implantation embryo where it is already possible to observe the core mass of pluripotent cells – epiblast – and an epithelial-like layer composed by primitive endoderm cells that already downregulated NANOG expression, separating the epiblast from the blastocyst cavity (Brons et al., 2007; Nichols and Smith, 2012; Tesar et al., 2007). From this time on, the blastocyst is ready to be implanted in the female uterus and undergo profound changes and development into a new organism with the entire somatic and germ cells. Although there are evidences that the epiblast cells could in certain conditions change into primitive endoderm cells, a recent report suggests that this phenomena can be a mere *in vitro* artifact and that, in fact, these two lineages are completely independent and have no capacity to change their already delineated fate commitment (Xenopoulos et al., 2015).

Pluripotent cell types

Pluripotency is the capacity to differentiate into all the cell types of a new organism presented by a restricted group of cells, the so-called Pluripotent Stem Cells (PSC). These cells have the ability to differentiate into the three germ cell layers (endo, meso and ectoderm) that will ultimately differentiate into all the new somatic cells of the organism. However, PSCs do not differentiate into the extraembryonic lineages, namely the trophoblast cells that are present at the blastocyst stage of development. Cells presenting such property are named totipotent.

Some of the most studied PSC are mouse and human embryonic stem cells (mESC and hESC) (Evans and Kaufman, 1981; Martin, 1981; Thomson et al. 1998), epiblast stem cells (EpiSC) (Brons et al. 2007; Tesar et al. 2007) and induced pluripotent stem cells (Takahashi and Yamanaka, 2006).

ESCs are isolated from the inner cell mass of the pre-implantation blastocyst. In the case of mESC, they are isolated at embryonic day E3.5 of mice development. mESC were primarily isolated in 1981 from the blastocyst and grown in culture dishes over feeder layers of mouse embryonic fibroblasts (MEF) by two distinct groups (Evans and Kaufman, 1981; Martin, 1981). mESC retain the pluripotency state of the ICM cells. As will be discussed later, mESC are dependent on leukemia inhibitory factor (LIF) (Smith et al., 1988) and bone morphogenetic protein (BMP) (Ying et al., 2003) as extrinsic factors in culture medium in order to be kept pluripotent. Moreover, mESC express OCT4, NANOG and SRY (sex determining region Y)-type high mobility group box 2 transcription factor (SOX2) pluripotency factors, required for pluripotency maintenance (Pereira et al., 2014).

ESCs were firstly derived from the human embryo in 1998 (Thomson et al., 1998). Although the molecular circuitry behind hESC pluripotency is similar to mESC, some differences exist concerning how these cells are routinely kept pluripotent *in vitro*, as will be explained later on.

In 2007, PSC were derived from the post-implantation epiblast and called EpiSC. These cells, as mESC, express OCT4, NANOG and SOX2 pluripotency factors and are capable to differentiate into the three germ layer lineages (Brons et al., 2007; Tesar et al., 2007). However, these cells already express some early differentiation markers, such as SOX17, T-Branchyury and fibroblast growth factor 5 (FGF5) (Brons et al., 2007; Tesar et al., 2007), suggesting that although they are in a pluripotent state, they are already in a committed state for differentiation.

A huge breakthrough in the stem cell and pluripotency field happened in 2006, when Takahashi and Yamanaka derived for the first time PSC, not from embryos in early stages of development, but from embryonic fibroblasts and, more than that, from adult fibroblasts (Takahashi and Yamanaka, 2006).

The derivation of iPSC was accomplished with forced retroviral expression of four factors: Oct4, Sox2, c-Myc, and Klf4, which were able to induce reorganization of the original epigenetic and molecular circuitry to a new one required during pluripotency (Takahashi and Yamanaka, 2006).

Pluripotency: a brief historical overview

Hints suggesting the existence of cells with pluripotent capacity were provided much earlier than ESC isolation. For example, one of the first clues for the existence of such cells appeared with the study of teratocarcinomas, a specific type of tumor with embryonal origin that present many cell types with a mixture of unorganized cells and completely differentiated bizarre structures such as teeth, hair follicles or even bones (Jackson and Brues, 1941). The presence of such distinct types of cells and tissues within the same tumor suggested that the seeding cells that gave rise to the tumor would have the capacity to generate all these different tissues within the same tumor. In fact, teratocarcinomas present a specific type of cells, denominated embryonal carcinoma (EC) cells, which are considered the stem cells within these tumors (Jackson and Brues, 1941). Kleinsmith and Pierce unsuccessfully attempted to *in vitro* culture EC cells. Nonetheless, they went further and isolated single EC cells from teratocarcinoma, propagated them and subsequently transplanted them into other animals (Kleinsmith and Pierce, 1964). These set of experiments showed that among a huge variability of cell lineages, there is a persistent pool of EC cells which are responsible for the generation of new teratocarcinomas with the whole cell lineages spectrum from the original tumor (Kleinsmith and Pierce, 1964). Posterior attempts to isolate and culture EC provided the basis for the establishment of new important culture and differentiation protocols, which would become

instrumental for posterior research with ESC and which further proved the extensive capability of EC cells to produce distinct cellular lineages (Kahan and Ephrussi, 1970; Martin and Evans, 1975; Rosenthal et al., 1970). Important was also the discovery that differentiating EC cells would form structures called embryoid bodies which resembled to some extent the mouse embryonic development process (Martin, 1980; Martin and Evans, 1975). Interestingly, early embryos when implanted into adult mice were also capable of generating teratocarcinomas, suggesting that EC may have an embryonic origin (Solter et al., 1970; Stevens, 1970). With subsequent studies, mESC were finally isolated, propagated and characterized (Evans and Kaufman, 1981; Martin, 1981).

The first mESC cultures took advantage of the plethora of protocols previously generated from EC cell culture. Hence, mESC were originally cultured on feeder cells composed by mitotically inactivated fibroblasts in a defined medium, which among other components presented relatively high concentrations of fetal calf serum (Evans and Kaufman, 1981; Martin, 1981) and 2-mercaptoethanol (Martin, 1981). Besides their similarities to ECs concerning their potential to differentiate into the three germ cell layers, mESC shared unquestionable morphological similarities with EC cells, either in the presence of a feeder cell layer or in its absence (Martin, 1981).

Establishment of mESC cell lines and optimization of *in vitro* culture procedures for these cells allied to their continuous self-renewal and pluripotency unraveled a complete new perspective in medicine with the promise to bring new cell regenerative applications into the clinic. However the capitalization of this promise has been hindered by many technical and ethical concerns, which become reflected, for example, in the extended period of time devoted for the proper comprehension of the molecular basis for pluripotency (which is still not yet completely disclosed). iPSC technology appeared more recently (Takahashi and Yamanaka, 2006) as a response to some of the major concerns and limitations attributed to embryonic stem cells. Although there is some debate regarding the complete regression or not to a complete pluripotent state, these cells overcome the major limitations and ethical concerns regarding the use of ESC for regenerative

medicine purposes (Carvalho and Ramalho-Santos, 2013; Ramalho-Santos, 2011). Moreover, this new technology offers new perspectives in terms of degenerative diseases, patient-customized regenerative therapy, personalized drug screen and disease modeling (Sousa et al., 2015).

In vitro signaling of PSC

mESC were first isolated onto a feeder cell layer that was believed to serve as support namely by releasing specific factors that would be required by mESC to retain their capacity to proliferate and keep pluripotent. Working with Buffalo rat liver cell-conditioned medium, Smith and colleagues (Smith et al., 1988) found that one of the most critical factors released by feeder cells was LIF. In the same year, other independent study confirmed LIF as a required factor to keep mESC pluripotent in culture (Williams et al., 1988). Even without a feeder cell layer, mESC cultured in gelatin-coated dishes in the presence of LIF are able to maintain their pluripotency (Smith et al., 1988; Williams et al., 1988). Although the majority of cells are kept pluripotent under these culture conditions, there are still remaining cells that tend to spontaneously differentiate in those dishes (Smith et al., 1988).

LIF is a cytokine belonging to the interleukine-6 group that has a great affinity for the LIF-receptor (LIF-R) (Gearing et al., 1991). Activated LIF-R recruits glycoprotein-130 (gp130) to form a heterodimer responsible for signal transduction (Onishi and Zandstra, 2015). LIF majorly signals through the JAK/STAT3 pathway. The LIF-R and gp130 heterodimer recruits and phosphorylates Janus Kinase (JAK) that in turn phosphorylates STAT3. This phosphorylation at tyrosine 705 induces STAT3 dimerization, triggering STAT3 shuttling into the nucleus, where it is responsible for the transcription control of genes related to self-renewal, pluripotency and survival (Matsuda et al., 1999; Niwa et al.,

1998). Together with JAK/STAT3, LIF also signals through two other pathways that are important to mention: phosphoinositide-3-kinase/ protein kinase B – PKB or Akt (PI3K/Akt) (Paling et al., 2004) and mitogen-activated protein kinase/ extracellular-signal-regulated kinase (MAPK/Erk) (Burdon et al., 1999). PI3K/Akt signalization acts partially by nuclear regulation of gene expression and also by glycogen synthase kinase 3 (GSK3) inhibition via its phosphorylation (Paling et al., 2004). Although still in debate, there are some reports that suggest that PI3K/Akt can also block the ERK pathway to maintain pluripotency (Graf et al., 2011). MAPK/Erk signaling pathway is also stimulated by LIF, but contrasting with the other pathways, this one is partly responsible for differentiation and inhibition of self-renewal (Burdon et al., 1999). Nonetheless, LIF supplementation maintains pluripotency suggesting that a fine-tuning is required and that JAK/STAT3 and PI3K/Akt pathways are sufficient to avoid differentiation through MAPK/ERK pathway. Nonetheless, MAPK/ERK may contribute to the residual amount of cells that tend to differentiate in the presence of LIF (Fig. 1.1).

Bone morphogenetic protein 4 (BMP4) is a serum protein that was found to be required for pluripotency regulation, acting together with LIF. BMP4 can activate the mammalian homologues of *Drosophila* “mothers against decapentaplegic” proteins 1, 4 and 8 proteins that translocate into the nucleus and are responsible for inhibition of differentiation (Id) genes (Ying et al., 2003). Additionally, BMP4 seems also to counteract differentiation through p38/MAPK inhibition (Qi et al., 2004).

In order to reduce the residual differentiated cells persistent in stem cell cultures maintained in the presence of LIF and BMP4 Ying and co-workers used a small inhibitor PD184352 to inhibit ERK pathway and observed that mESC were kept pluripotent with less spontaneous differentiation (Ying et al., 2008). Additionally, a GSK3 inhibitor - CHIR99021 – in combination with the previous molecule maintained the culture of mESC with almost all cells in a pluripotent status (Ying et al., 2008). GSK3 was already shown to be required for a more efficient derivation of ESCs from different mouse strains, which was believed to be due to its role in Wnt/beta-catenin (β -catenin) and Notch signaling regulation (Umehara et al., 2007). Indeed, more recently it was shown that inhibition of GSK3 acts in

part by de-repression of β -catenin, which in turn block the repressor of transcription – Tcf3 – of several pluripotency-related genes (Wray et al., 2011). A defined medium without serum combining these two inhibitors with LIF (which is commonly named as 2i medium) has become accepted as the better way to keep mouse embryonic stem cells at its best pluripotency state in culture.

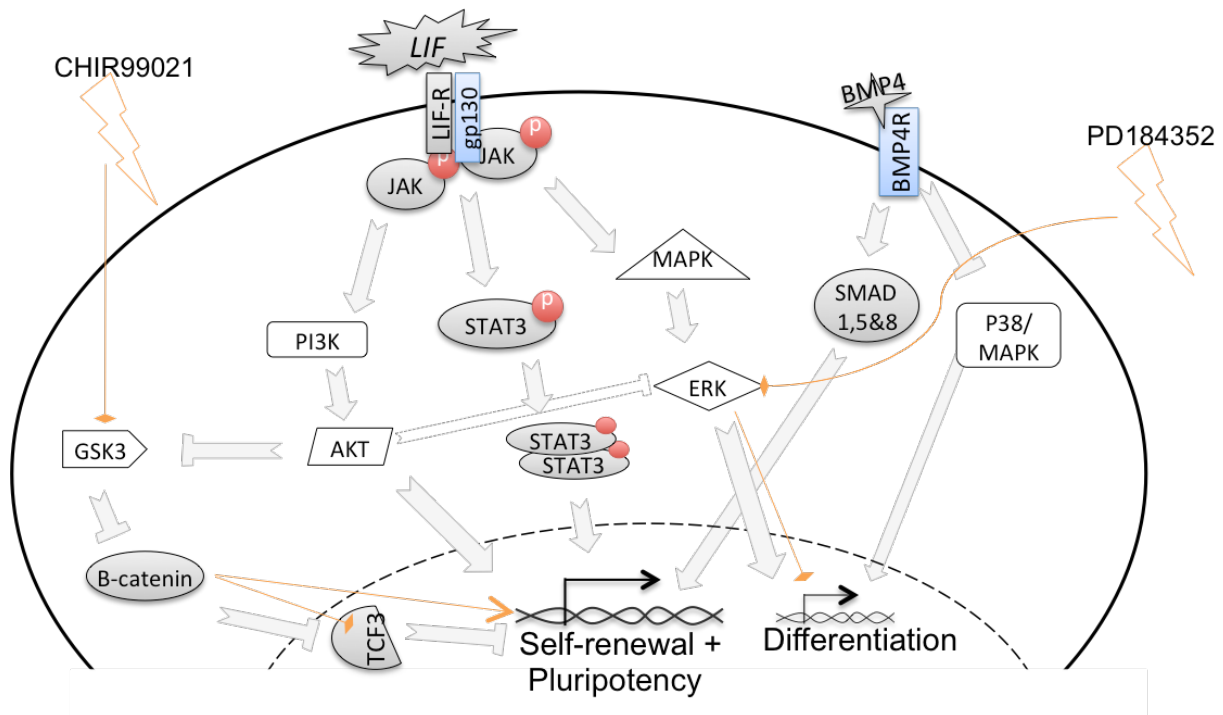


Figure 1.1 – Extrinsic signal and their signaling pathways controlling pluripotency of mESC.

LIF signaling activates JAK/STAT3 and PI3K/AKT pathways to support self-renewal and pluripotency of mESC. LIF also directly activates, and indirectly inactivates through PI3K/AKT, the MAPK/ERK pathway, which relates to differentiation. BMP4 signaling activates SMAD 1/5/8 and p38/MAPK also to support pluripotency and inhibit differentiation. The 2i inhibitors (CHIR99021 and PD184352) act inhibiting GSK3 and ERK, respectively, to increase self-renewal and pluripotency and inhibit differentiation of mESC.

In contrast to mESC, EpiSC cannot be propagated *in vitro* with LIF and/or 2i as mESC. Instead, EpiSC require Activin A and FGF2 supplemented medium to grow and maintain their pluripotency across several passages (Brons et al., 2007). As already mentioned, EpiSC already express several markers

of early differentiation. Thus, EpiSC pluripotent state is commonly called as “primed” pluripotency, contrasting to mESC ground state pluripotency which is called as “naïve” pluripotency (Nichols and Smith, 2009). Moreover, EpiSC can also be derived from mESC when cultured in Activin A and FGF2 (Guo et al., 2009), but the first attempts to achieve the opposite reversion culturing EpiSC in LIF and/or 2i failed, only being achieved by forced expression of some genes to revert them to ESC, responding to LIF (Bao et al., 2009; Guo et al., 2009). Nonetheless, latter attempts showed that this reversion from EpiSC to ESC is possible using LIF or 2i culture conditions, but it occurs in a very low rate and varies between different cell lines (Bernemann et al., 2011).

On the other hand, hESC rely on human recombinant basic FGF (bFGF) and Activin/Nodal to maintain pluripotency (Vallier et al., 2005), similarly to what is described for EpiSC, but are unable to respond to LIF (Daheron et al., 2004) or BMP4 (Xu et al., 2002), as mESC. Activin/Nodal signals through the transforming growth factor β superfamily (TGF β) (Vallier et al., 2005), activating in turn SMADs 2/3 that will actively and directly regulate Nanog expression (Xu et al., 2008). It is interesting to note that Activin/Nodal/TGF β can signal SMAD 2/3 during pluripotency (James et al., 2005; Xu et al., 2005), but upon differentiation, SMAD2/3 signaling decreases, while SMAD1/5 (targets of BMP4) increases (James et al., 2005). FGF2 acts, in part, in the feeder cells, stimulating the production of the required factors for hESC pluripotency, namely Activin A and others (Greber et al., 2007; Ohtsuka and Dalton, 2008).

Besides the similar molecular basis employed by hESC and EpiSC to sustain their pluripotency, these cells also share many other features such as the flat colony morphology, the low survival capacity when dissociated in single cells and the expression of some early differentiation genes even in pluripotent conditions (Nichols and Smith, 2009). Thus, it seems that hESC do not truly represent a homologue of mESC, but are rather in a more committed stage of pluripotency, and so coming closer to “primed” EpiSC. Hence, several studies were conducted trying to isolate a ground state hESCs (or resembling the pluripotency of naïve mESC), but some of the successful studies only arose very

recently. Indeed, this “naïve hESC” are dependent on LIF/STAT3 pathway and are more closely related to naïve mESC than mEpiSC (Chan et al., 2013), but further studies are needed in order to completely disclose the mechanisms and the pluripotency status behind each cell type.

Molecular axis of pluripotency

As stated before, similarly to ICM, ESCs highly express OCT4, NANOG and SOX2 transcription factors, which constitute the core pluripotency axis (Fig. 1.2).

OCT4 (also called as the POU class 5 homeobox 1 transcription factor – POU5F1) is a particular transcription factor that needs to be strictly maintained in a certain level range in cells in order to keep them pluripotent (Niwa et al., 2000). This means that OCT4 downregulation or overexpression will favor cells to differentiate preferably into trophoectoderm or endo- and mesoderm, respectively. In this way, OCT4 is required by ESC for pluripotency, but it is also instrumental in the process of differentiation/specification (Niwa et al., 2000). Moreover, this transcription factor can be found in two different subcellular compartments in ESCs. There are two different OCT4 spliced variant proteins: OCT4A and OCT4B (Guo et al., 2012; Liedtke et al., 2008). OCT4A, functions in the nucleus as a pluripotency transcription factor. OCT4B can be either present in nucleus or in the cytoplasm, but it is assumed to have other functions that are not related to pluripotency (Guo et al., 2012).

NANOG transcription factor is highly present in ESC, but absent in differentiated adult cells (Chambers et al., 2003). NANOG downregulation in ESC leads them to lose pluripotency and differentiate, preferentially into extraembryonic endoderm (Hyslop et al., 2005; Mitsui et al., 2003), which demonstrates the importance of NANOG to pluripotency. mESC cannot be derived from

NANOG Knockout (KO) embryos, but alternatively NANOG can be knocked out in established mES cell lines which are able to propagate *in vitro* but nevertheless present a high predisposition for differentiation (Mitsui et al., 2003). Moreover, overexpression of this transcription factor is sufficient to induce self-renew and to maintain pluripotency even in the absence of LIF (Chambers et al., 2003; Mitsui et al., 2003). However NANOG does not seem to be required for induction of pluripotency as it is not one of the cocktail factors commonly used for cell reprogramming (Takahashi and Yamanaka, 2006). On the other hand, it is required for final pluripotency establishment at the end stages of reprogramming (Silva et al., 2009).

SOX-2 is the third major pluripotency transcription factor. Sox2 is highly expressed at the first stages of development, namely in the morula, but becomes restricted to the ICM of the blastocyst. Along with germ cell layers differentiation, SOX2 remains present only in the neuroectodermal lineage cells (Avilion et al., 2003). Similarly to NANOG, mESC cannot be isolated and established in culture from SOX2 knockout embryos (Avilion et al., 2003). Moreover, SOX2 deletion in mESC induces their differentiation within the trophoectoderm lineage (Masui et al., 2007), reinforcing its role in pluripotency maintenance. Furthermore, OCT4 overexpression can rescue the differentiation capacity of these SOX2 KO cells, suggesting interplay between OCT4 and SOX2. Additionally, SOX2 can also modulate differentiation specification. SOX2 overexpression induces neural ectoderm differentiation while inhibiting mesodermal differentiation (Masui et al., 2007), while OCT4 overexpression does the opposite.

It is noteworthy to note the interplay between the three main pluripotency factors. When all three are expressed in ESC, they maintain pluripotency and self-renewal. However, upon differentiation triggers, NANOG seem to be rapidly downregulated, which allows OCT4 and SOX2 to differently induce exclusive specification in mesoderm or ectoderm, respectively (Masui et al., 2007).

There are other transcription factors involved in pluripotency maintenance that induce ESC differentiation if downregulated. These are: KLF2, KLF4, ESRRB, SALL4, TFCEP2/1, TBX3 among others.

Depletion of one of them is not sufficient to induce spontaneous differentiation of ESC, given that all the others can compensate its deficit in order to keep cells pluripotent. Taken together, all these factors, and many others that remain elusive, are intricate players in a related network with the pluripotency axis factors OCT4, SOX2 and NANOG (Fig. 1.2) (De Los Angeles et al., 2012; Kalkan and Smith, 2014; Martello and Smith, 2014).

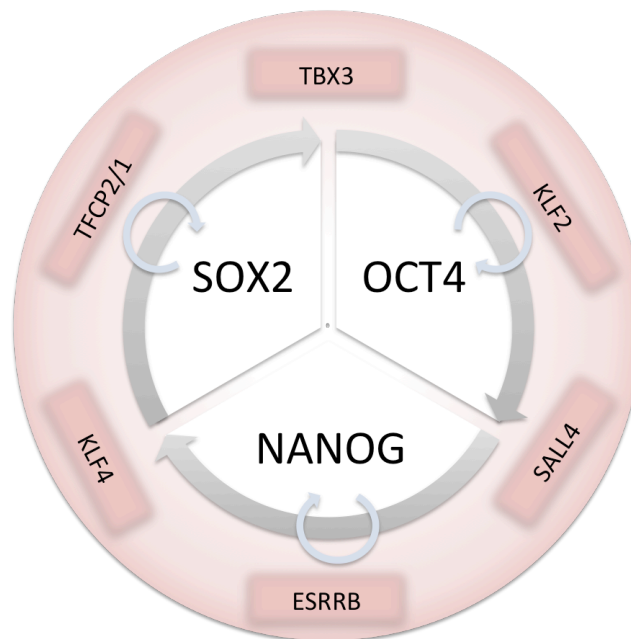


Figure 1.2 – Molecular control of pluripotency.

Major transcription factors responsible for pluripotency and self-renewal are represented. In the center, the axis of pluripotency is represented by the transcription factors OCT4, SOX2 and NANOG, which can auto-regulate and regulate each other. In the outside circle some of other transcription factors that are also responsible for pluripotency maintenance, in a lesser extent.

Mitochondria and metabolism

Mitochondrial overview

Benda introduced in 1898 the term “Mitochondrion”, originated from the Greek "mitos" (thread) and "chondros" (granule) (Benda, 1898; Ernster and Schatz, 1981). Although (still) debatable, it is believed that mitochondria arose from an endosymbiosis relationship between a glycolytic proto-eukaryotic cell and an oxidative bacterium (Vellai and Vida, 1999).

Although traditionally, these organelles are associated for their ability to generate energy by adenosine-5'-triphosphate (ATP) production via oxidative phosphorylation (OXPHOS), they are required for several other processes within the cell such as apoptosis control, calcium homeostasis, reactive oxygen species (ROS) and oxidative stress management, among others that are extensively described elsewhere (Pereira et al., 2014; Ramalho-Santos and Amaral, 2013; Ramalho-Santos et al., 2009).

In order to better understand this organelle, I will briefly focus on its composition/organization in the next few paragraphs. Mitochondria are composed by two membranes (inner and outer mitochondrial membranes) that delimitate the intermembrane space from the internal matrix of the mitochondria. Worthy to mention, the electron chain reaction (ETC) complexes and the ATP synthase (Fig. 1.3), responsible for the production of majority of the ATP in the cell, are embedded within the inner mitochondrial membrane (IMM) (Pereira et al., 2014; Ramalho-Santos and Amaral, 2013; Ramalho-Santos et al., 2009). The IMM is characterized by its cristae shape, which has a role in defining micro domains that enclose the complexes of the ETC and thus controlling the mitochondrial metabolic capacity of the cell (McBride et al., 2006). The outer mitochondrial membrane (OMM) contains

porins that forms large aqueous channels through the lipid bilayer, making the membrane permeable to solutes with a size up to 5,000 daltons. Such molecules can enter into the intermembrane space, but most of them cannot pass the impermeable IMM that presents selective transport proteins (Pereira et al., 2014).

With their own genome, mitochondria presents 2 to 10 molecules of their mitochondrial DNA (mtDNA), encoding for some of the machinery required for their replication, transcription, translation and protein assembly (Carew and Huang, 2002). MtDNA is a 16.5 kb circular double-stranded molecule that encodes 22 transfer RNAs (tRNAs) and 12S and 16S ribosomal RNA (rRNA) and 13 proteins required for ETC and ATP synthase assembly (ND1, ND2, ND3, ND4, ND4L, ND5, ND6, CytB, COI, COII, COIII, ATPase6 and ATPase8) (Maximo et al., 2009; Pereira et al., 2014).

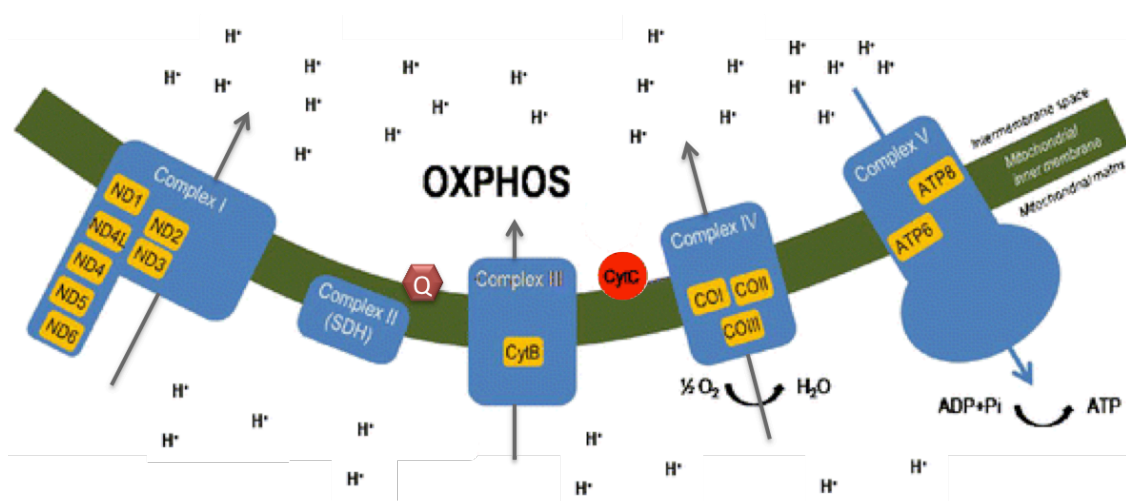


Figure 1.3 - The four electron transport chain complexes and ATP synthase.

Complexes I, III and IV of the ETC generate a proton gradient by pumping protons (H^+) from the mitochondrial matrix to the intermembrane space, which is then used by the ATP Synthase (sometimes referred as complex V) to generate energy in the form of ATP (adapted from (Maximo et al., 2009)).

Nevertheless, mitochondria are semi-autonomous organelles considering they are dependent on nuclear DNA (nDNA) which encodes a vast majority of the proteins of ETC and ATP synthase, as well as mtDNA polymerase γ (POLG), RNA polymerase, mtDNA transcription factors and ribosomal proteins required for mitochondrial biogenesis. All those proteins may be imported into the mitochondria (Ramalho-Santos et al., 2009; St John et al., 2010). This reveals that a well-orchestrated nucleus-mitochondria crosstalk must exist in order to cells can coordinate the transcription of mitochondrial genes (or its repression) and importing of all these proteins when responding to different stimuli, which ultimately lead to mitochondrial adjustment in its shape and metabolism rate.

Metabolism overview

Mitochondria are central organelles responsible for the majority of energy production by OXPHOS due to the activity of the four electron transport chain (ETC) complexes together with ATP synthase localized in the inner mitochondrial membrane, in an oxygen-dependent process.

Nevertheless, OXPHOS is not the only way cells rely in order to generate energy in the form of ATP. Glycolysis is one of the other possible ways cells use to produce ATP. When glucose is present, one molecule is converted in a first set of reactions (preparatory phase) in glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (that is thereafter converted in glyceraldehyde 3-phosphate) with a consumption of two ATP molecules. Subsequently, in the pay-off phase, the glyceraldehyde 3-phosphate is then converted into pyruvate by a series of reactions yielding four ATPs, 2 nicotinamide and reduced adenine dinucleotide (NADH) molecules.

Accordingly, in the end there is a gain production of 2 ATP and 2 NADH from the glycolytic process. Therefore, glycolysis constitutes an alternative to OXPHOS as a source for ATP. Indeed, in the absence of oxygen, which is required for OXPHOS, cells can survive in a first stage by generating energy by glycolysis. Moreover, there are cells that are mainly dependent on glycolysis over OXPHOS, even in the presence of oxygen, as for example cancer cells and stem cells. These glycolytic cells can then convert pyruvate into lactate, by the lactate dehydrogenase A (LDHA), in order to quickly recycle NAD⁺ and allow for the continuation of the glycolytic process (Pereira et al., 2014).

However, many cells preferentially use OXPHOS for energy production purposes, once this process can achieve a yield of 32-36 ATP molecules production per glucose molecule used (Pereira et al., 2014; Sousa et al., 2015). For that purpose, pyruvate instead of being converted into lactate is imported to the mitochondria where the highly controlled pyruvate dehydrogenase complex (PDH) converts it into acetyl-CoA (Rodrigues et al., 2015a). Acetyl-coA can then be oxidized in the tricarboxylic acid (TCA; also known as Krebs) cycle, leading to the production of the reducing agents succinate NADH required for OXPHOS. The ETC complexes can then acquire electrons from the oxidation of these reducing agents (those from succinate via FADH₂) produced from the TCA cycle (and from 2 additional NADH originated in glycolysis) and finally generate ATP through ATP synthase, as it will be subsequently explained.

Together, the ETC and ATP synthase, present in the IMM, are composed by approximately 90 known proteins. There are four ETC complexes: (1) Complex I (NADH: ubiquinone oxidoreductase); (2) Complex II (succinate: ubiquinone oxidoreductase); (3) Complex III (ubiquinone: ferricytochrome c oxidoreductase) and (4) Complex IV (cytochrome C: oxygen oxidoreductase (Eng et al., 2003; Pereira et al., 2014)

Additionally, there are also the two mobile electron transporters: coenzyme Q and cytochrome C (Fig. 1.3). The electrons are transported across the ETC complexes till being accepted by O₂ at the level of complex IV resulting in the production of water (H₂O). During this electron transport, the

generated energy is used to pump protons (H^+) from the mitochondrial matrix to the intermembrane space by the complexes I, III and IV, generating the proton gradient. Finally, the ATP synthase takes advantage of this proton gradient to generate ATP from ADP and inorganic phosphate, while driving H^+ back to the mitochondrial matrix (Carew and Huang, 2002; Pereira et al., 2014; Sousa et al., 2015). From an energy production efficiency point of view, OXPHOS has a yield of 32-36 ATP production compared to the 2 from glycolysis. Nonetheless, when glucose is not limited, as usually happens in *in vitro* cell cultures, glycolysis can generate ATP faster than OXPHOS, accomplishing the cell needs for energy. Moreover, several intermediate metabolites that occur during glycolysis can serve as precursors for other biosynthetic pathways, as the example of nucleotides (pentose phosphate pathway), serine/glycine and hexosamine synthesis pathways, as can be seen in figure 1.4 (Pereira et al., 2014).

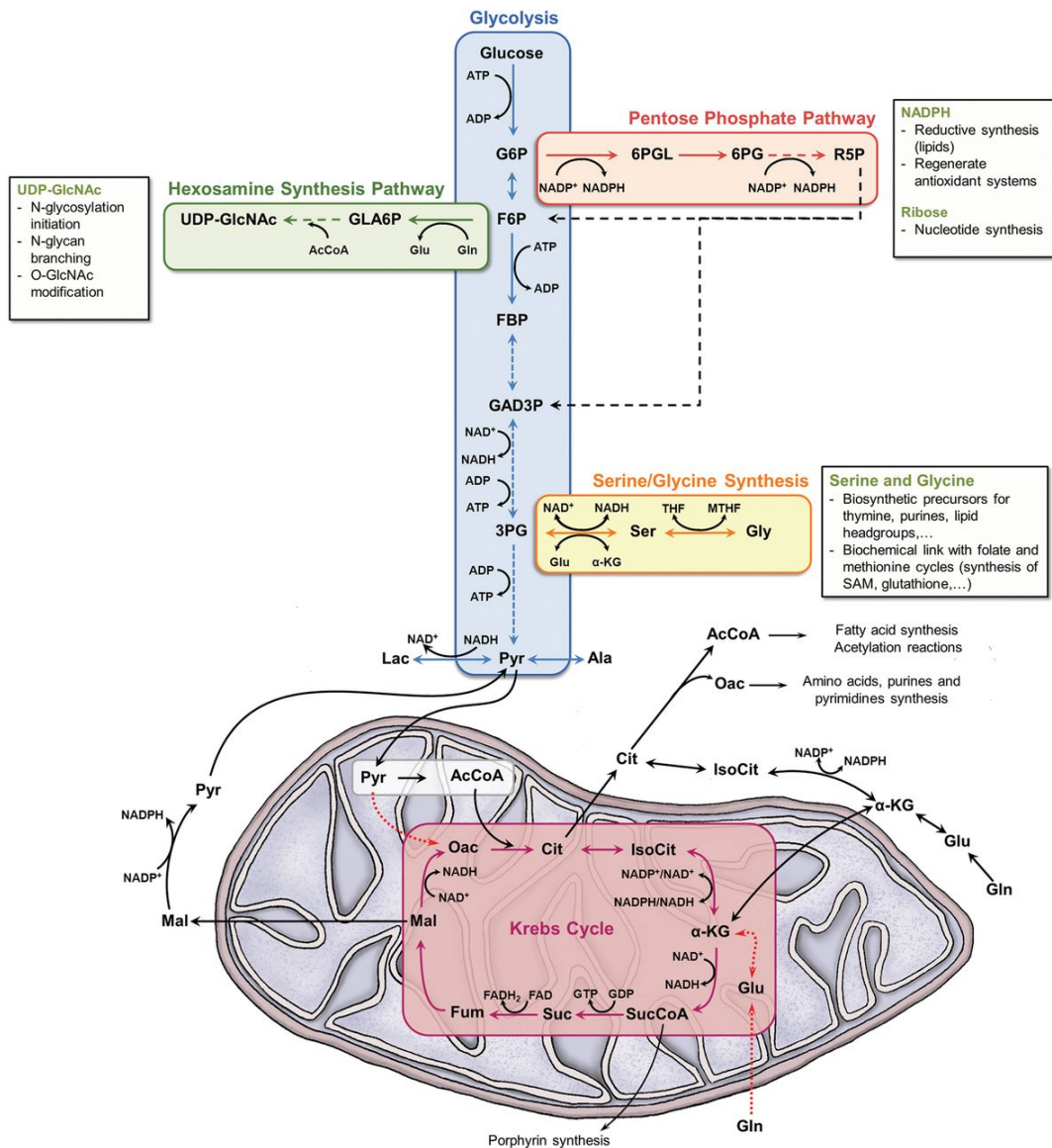


Figure 1.4 –Glycolysis and synthetic branching pathways.

Glycolysis *per se* does not only comprise straightforward metabolic pathways. Instead, the represented hexosamine synthesis, pentose phosphate, serine/glycine synthesis pathways as well as the Krebs cycle are important metabolic pathways that branch out from glycolysis, taking advantage of some glycolytic intermediate metabolites, and are important for generation of several other metabolites and intermediates required by cells (Pereira et al., 2014)

Reactive oxygen species (ROS), namely superoxide anion, can be an (un)desired product from OXPHOS. Some electrons transported through ETC complexes can outflow, usually at complexes I and III, reacting with oxygen causing its reduction to superoxide anion ($O_2^{\bullet-}$). ROS, in a physiological concentration range, are important to cells as they could act as secondary messengers in signaling pathways conferring some tolerance to mild stresses (Ramalho-Santos et al., 2009; Schieber and Chandel, 2014). However, when the physiological concentrations are passed, ROS accumulation can result in oxidative stress and cause lipid and protein peroxidation, DNA damage and even apoptosis induction. Thereby, ROS concentration within the cell has to be tightly regulated, either by decreasing its production (as example, decreasing OXPHOS and so decreasing electron leak) or by increasing the scavenging capacity of cells by increasing antioxidant defenses.

Mitochondria and Metabolism as regulators of Pluripotency and Differentiation

Recently, several studies were performed to unveil the mechanisms that control pluripotency and differentiation of ESCs. It is now clear that ESC and iPSCs rely mostly on glycolysis for energy production, while differentiated cells are biased towards OXPHOS as the main metabolic pathway to generate energy (Varum et al., 2011).

HESC and mESC present a low mtDNA copy number and their mitochondria show perinuclear localization (Chung et al., 2007; St John et al., 2005), allied to a globular shape and few and poorly defined cristae characterizing their mitochondrial immature state. Contrastingly, differentiated cells present an elaborate mitochondrial network, with elongated mitochondria and developed cristae (Facucho-Oliveira et al., 2007; St John et al., 2005; Varum et al., 2011). This mitochondrial structure

suggests that the metabolism may be different on both types of cells. Indeed, the immature mitochondrial state of ESC is accompanied with an increased glycolytic flux, a lower OXPHOS activity and a concomitant elevated lactate production and reduced O₂ consumption (Fig 1.5). During differentiation, an increase of mtDNA copy number is observed, a maturation of mitochondrial structure and cytoplasmic network, accompanied with increased OXPHOS and decreased glycolytic activity, increasing the ATP production and O₂ consumption (Chung et al., 2007; Facucho-Oliveira et al., 2007; St John et al., 2005). Moreover, hPSC have a reduced ratio between oxygen consumption rate (OCR) and extracellular acidification rate (ECAR – indirect measure for glycolysis), which is mainly due to higher ECAR rates in hPSCs, who are more dependent on glycolysis for ATP production (Zhang et al., 2011). It was also shown that glycolysis induction or OXPHOS inhibition can also promote stemness (Kondoh et al., 2007; Varum et al., 2009).

The reprogramming progression to hiPSC or miPSC is also accompanied by a metabolic transition from OXPHOS to a glycolytic metabolism (Folmes et al., 2011; Zhou et al., 2012), which can be enhanced by hypoxia or glycolysis induction [Fig 1.5; (Yoshida et al., 2009; Zhu et al., 2010)]. Although debatable what happens first and what can be the trigger: if nuclear reprogramming or metabolic shift, evidences shed some light over this topic. Folmes and coworkers showed that a metabolic reprogramming seem to occur at early stages of the reprogramming process till a pluripotent state, preceding the self-renewal and pluripotency gain (Folmes et al., 2011).

The differentiation process can also be manipulated and increased (or decreased, if desired) by metabolic modulation during the time course of the procedure. Our group showed recently that neuronal differentiation of mESC into a neuronal dopaminergic cell lineage can be blocked by antimycin A, an ETC complex III inhibitor. Indeed, antimycin A is not only capable of inhibiting differentiation, but it also helps to maintain pluripotency associated genes expression even when cells are subjected to differentiation conditions (Pereira et al., 2013). As mentioned before, the group also showed that Antimycin A elevates the pluripotent status of hESC (Varum et al., 2009). Cardiac

differentiation can also be modulated by metabolism interference, but also by mitochondrial dynamics modulation (Chung et al., 2007; Kasahara et al., 2013).

It is also interesting to note that more than OXPHOS modulation, modulation of enzymes responsible for glycolysis control, even in a pluripotent state, will target cells to eventually differentiate. As an example, pyruvate dehydrogenase (PDH), which converts pyruvate into acetyl-CoA, unless it is phosphorylated by pyruvate dehydrogenase kinases (PDHK) negatively regulating its function. Pharmacological inhibition of PDHK by dichloroacetate (DCA) induces loss of pluripotent markers in mESC, which is associated with glycolysis inhibition, similarly to what happens to mESC when LIF is removed from the medium (Rodrigues et al., 2015a). Interestingly, inhibition of the first step of glycolysis (the conversion of glucose into glucose-6-phosphate by Hexokinase) also primes mESC to differentiate (Rodrigues et al., 2015b). Indeed, glycolysis must be a key metabolic pathway for pluripotency maintenance. The glucose analog, 2-deoxy-glucose (2-DG) that mimics glucose insufficiency, induces death of mESC without affecting the differentiated feeder cells (Kondoh et al., 2007).

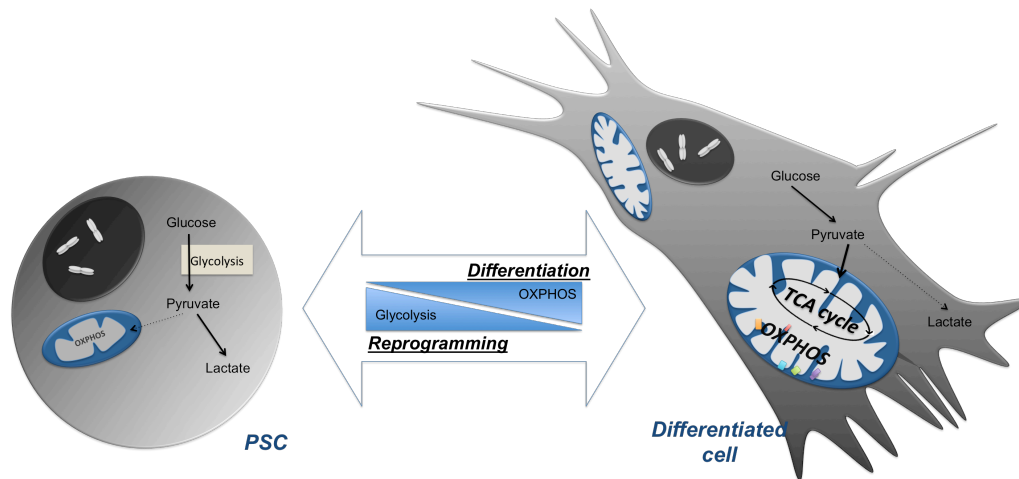


Figure 1.5 – Mitochondrial and metabolic differences between PSC and differentiated cells.

Pluripotent stem cells (PSC) rely mostly on glycolysis and present few, undeveloped and perinuclear localized mitochondria with reduced oxidative phosphorylation (OXPHOS) activity. Under differentiation, there is a metabolic transition towards an increased OXPHOS, concomitant with a development of mitochondrial cell number and highly developed mitochondrial organization. The opposite transition from a differentiated to a pluripotent states – reprogramming – is accompanied by the inverse remodeling of mitochondrial structure and metabolic reliance (adapted from (Sousa et al., 2015)).

Although this glycolytic phenotype is a characteristic of pluripotent stem cells, some disparities still occur between the different pluripotent stem cells. In accordance to some above-mentioned similarities between mEpiSC and hESC and their slight distance to mESC in terms of signaling pathways required for their pluripotency, the same is also observed in terms of their metabolism. Briefly, the pluripotent mEpiSC were also reported to present a similar metabolism to hESC given that both are more prone to glycolysis with low mitochondrial respiratory capability. However, when focusing in mESC, they were reported as being able to switch between OXPHOS and glycolysis (Zhou et al., 2012). With the generation of “naïve” hESC, it was shown that they are closer fits to mESC, with a less developed mitochondria matrix and higher respiratory capacity than the “primed” hESC counterparts (Ware et al., 2014).

Sirtuins

A general overview on Sirtuin functions and their impact on metabolism

Histone deacetylases (HDAC) are divided in two main groups: (1) the metal ion-dependent, considered the “classical” HDAC that can be inhibited by trichostatin A (TSA) and (2) the nicotinamide adenine dinucleotide (NAD⁺) dependent HDACs, usually known as Sirtuins that are not TSA inhibited (Cyr and Domann, 2011). Sirtuins (SIRT) are homologues of the silent information regulator 2 alpha (Sir2 α) yeast *Saccharomyces cerevisiae* protein (Frye, 1999). Mammalian sirtuins emerged as attractive targets for research after the discovery that their yeast homolog is able to increase lifespan (Kaeberlein et al., 1999) and that its activity is dependent on nicotinamide adenine dinucleotide – NAD⁺ [Fig. 1.6; (Imai et al., 2000)]. Since then, many efforts have been carried out to understand the role of sirtuins in mammalian organisms. Seven mammalian sirtuins were been described so far (SIRT1 – 7) (Frye, 2000).

Deacetylation is the most well described SIRT-mediated reaction, which involves the removal of acetyl groups from lysines of several proteins, namely histone tails, requiring NAD⁺ as a substrate for such reaction [Fig. 1.6; (Imai et al., 2000)]. The final end products are the deacetylated protein together with nicotinamide (NAM) and 2'-O-acetyl-ADP-ribose (2OAADPR) (Imai et al., 2000; Tanner et al., 2000). Nonetheless, other reactions mediated by sirtuins were already described: Sirt4 and 6 possess ADP-rybosyltransferase activity (Haigis et al., 2006; Liszt et al., 2005) and Sirt5 is capable of protein desuccinylation (Park et al., 2013) and demalonylation (Du et al., 2011).

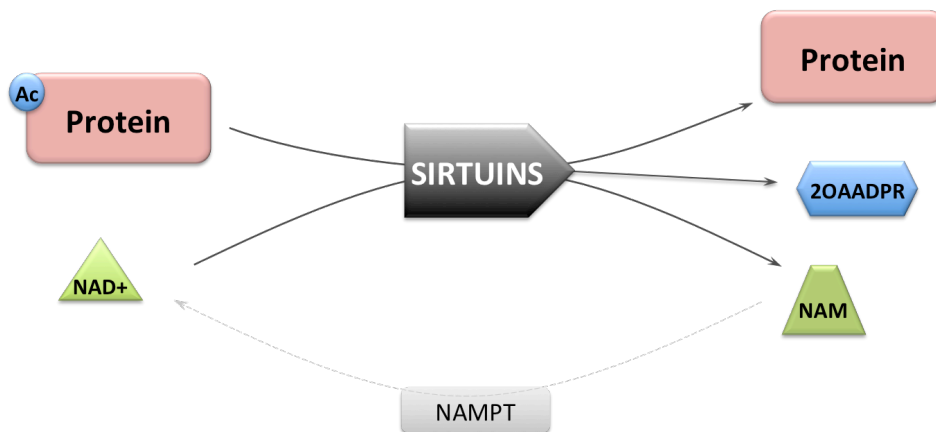


Figure 1.6 –Deacetylation reaction mediated by Sirtuins

Sirtuins deacetylate proteins requiring nicotinamide adenine dinucleotide (NAD^+) as a co-substrate for the reaction. 2'-O-acetyl-ADP-ribose (2OAADPR) and nicotinamide (NAM) are end-products generation from this reaction. Given that sirtuins are dependent on NAD^+ , there is an intricate and well-coordinated system for NAD^+ level regulation discussed in the text. Here the salvage pathway, i.e. the recycling of NAD^+ from NAM mediated by nicotinamide phosphoryltransferase (NAMPT) is represented.

Besides differences in activity, sirtuins also differ in terms of cellular localization. SIRT1, 6 and 7 are mainly localized in nucleus, yet SIRT7 is the only one that locates specifically in nucleolus (Haigis and Sinclair, 2010). Nonetheless, SIRT1 can also be present in the cytoplasm under specific conditions (Jin et al., 2007; Tanno et al., 2007). SIRT2 is the predominant cytoplasmatic sirtuin, thus it is not surprising that it has been already reported as being capable to control the cellular cytoskeleton and cell cycle dynamics (North et al., 2003). SIRT3, 4 and 5 are mainly localized in the mitochondria (Haigis and Sinclair, 2010; Pereira et al., 2012). Concerning Sirtuin 3, there is still some controversy regarding its localization. Some reports suggested that Sirtuin 3 is also present into the nucleus under specific conditions (Nakamura et al., 2008; Scher et al., 2007).

Sirtuins were initially defined as regulators of transcription mainly due to the major role associated till then for their yeast homologue (as its name denotes: **silent information regulator** 2 alpha - Sir2 α),

thereby classified as HDAC. Indeed, sirtuins have the capacity to deacetylate histone tails in their lysine residues, however several other proteins present in most cellular compartments can also be deacetylated by sirtuins. The abovementioned subcellular compartmentalization of sirtuins, particularly out of the nucleus, easily hints at this idea.

Sirtuins, as NAD^+ dependent enzymes, are considered cell metabolic sensors. Thus, it is not surprising that sirtuin activity is modulated by cell energy status as a way to compensate energy peaks or deficits. As an example, sirtuin activity is increased under caloric restriction (Lin et al., 2004; Lin et al., 2002) or exercise (Ferrara et al., 2008; Suwa et al., 2008) situations, which involve an increase in NAD^+ levels. Nonetheless, NAD^+ does not easily increase its concentration due to acute stresses. Although these stresses contribute for NAD^+ concentration regulation, a natural engineered cellular mechanism ultimately controls its availability, from its initial biosynthesis from tryptophan, its recycling process from nicotinamide (through nicotinamide phosphoryltransferase – NAMPT; Fig. 1.6), till its depletion by several other targets (see (Houtkooper et al., 2012) for a comprehensive review). Furthermore, AMP-activated protein kinase (AMPK), another metabolic sensor of AMP/ATP or ADP/ATP ratios, also regulates (and is regulated by) SIRT1 in order to (both) control mitochondrial function through peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1 α) (Canto et al., 2009; Price et al., 2012). The abovementioned articulated with the mitochondrial localization of SIRT3-5 reinforces the hypothesis that sirtuins must play a key role in metabolism.

Indeed, sirtuins do not only act as metabolic sensors but also as regulators (see table I for a summarized metabolic regulators that are directly regulated by sirtuins, as will be discussed next). As formerly mentioned, SIRT1 deacetylates and activates PGC-1 α , a transcription factor that stimulates transcription of genes related to mitochondrial biogenesis. SIRT1 deacetylation of PGC1 α could control oxidative phosphorylation (Lagouge et al., 2006), gluconeogenesis and glycolysis (Rodgers et al., 2005b), and fatty acid oxidation (Gerhart-Hines et al., 2007). In the same manner, SIRT1 deacetylates forkhead box O 1 (FOXO1) transcription factor (Brunet et al., 2004; Motta et al., 2004).

Similarly to PGC-1 α , FOXO1 regulates the expression of gluconeogenic genes (Frescas et al., 2005). Although not yet completely understood the underlying signaling pathways, the uncoupler protein 2 (UCP2) expression is also regulated by SIRT1, as its expression is elevated in SIRT1 KO animals. The exact role in cell metabolism is still unclear, but it is already known that pancreatic β -cells of SIRT1 KO animals present lower ATP levels and decreased insulin release (Bordone et al., 2006), suggesting that SIRT1 may also regulate metabolic processes by UCP2 expression regulation.

Nevertheless, the major metabolic regulation by sirtuins may be observed in mitochondria. Indeed, nearly 20% of known mitochondrial proteins can be acetylated (Kim et al., 2006), some of them on more than a single aminoacid residue (277 acetylation sites were found for 133 mitochondrial proteins). Moreover, about 65% of those acetylated proteins have a role in mitochondrial metabolism (95 out of 133), which demonstrates that acetylation and deacetylation mechanism may have an important role in mitochondrial function (Kim et al., 2006).

The major mitochondrial regulator seems to be SIRT3, given that it has the highest deacetylase activity within mitochondria (Lombard et al., 2007). All mitochondrial complexes, as well as ATP synthase are (in)directly regulated by SIRT3. Complex I NDUFA9 (Ahn et al., 2008) and complex II succinate dehydrogenase A (SDHA) (Cimen et al., 2010) subunits are directly deacetylated and inhibited by SIRT3. Although there specific target subunits for complexes III (Bao et al., 2010; Kendrick et al., 2011; Kim et al., 2010), IV (Bao et al., 2010; Kendrick et al., 2011) and ATP synthase (Bao et al., 2010), were not identified, studies where SIRT3 was removed or downregulated demonstrated that these complexes present higher acetylation, which associates with decreased function. Those data suggests that SIRT3 has a broad regulation control in oxidative phosphorylation capacity. Moreover, Krebs cycle is also regulated. Besides the discussed SDHA subunit of succinate dehydrogenase complex, SIRT3 also deacetylates the Krebs cycle enzyme isocitrate dehydrogenase 2 (IDH2) that is responsible for conversion of isocitrate to α -ketoglutarate (Schlicker et al., 2008). Indirectly related to Krebs cycle, glutamate dehydrogenase (GDH) (Schlicker et al., 2008) that is

responsible for glutamate to α -ketoglutarate conversion and acetyl-CoA synthase 2 (AceCS2)(Hallows et al., 2006) that generates acetyl-CoA from acetate are also directly deacetylated by SIRT3, contributing to their activation.

SIRT3 is also related with glycolysis. Pyruvate dehydrogenase subunit E1 (PDH-E1 α) is deacetylated by SIRT3 contributing to its activity. In line with this, SIRT3 knockdown in myoblasts induces glycolytic activity as measured by extracellular acidification (ECAR) analysis (Jing et al., 2013) SIRT3 also deacetylates cyclophilin D promoting its detachment from its complex with adenosine nucleotide translocator (ANT) and thereby contributing to hexokinase II (HKII) detachment from mitochondria (Shulga et al., 2010). This reaction is required for enhancement of oxidative phosphorylation mediated by galactose (Shulga et al., 2010).

Fatty acid oxidation, via long chain acyl coenzyme A dehydrogenase (LCAD) deacetylation (Hirschey et al., 2010), and ketone body production, via 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2) deacetylation (Shimazu et al., 2010), are other metabolic pathways regulated by SIRT3. Both studies present two alternative ways how SIRT3 is involved in fasting mechanisms, but the understanding of these metabolic pathways regulation by deacetylation is still limited.

Much less is known about metabolic regulation by other sirtuins. SIRT2, the predominant sirtuin in cytoplasm deacetylates α -tubulin (North et al., 2003). Moreover, it controls cell cycle (Dryden et al., 2003), which can be mediated by this tubulin deacetylation reaction (Inoue et al., 2007). No reports till date appeared suggesting metabolic regulation by SIRT2. Nonetheless, due to its localization, cytoplasmatic metabolic reactions could be regulated by SIRT2.

Interestingly, SIRT4 seem to ADP-ribosilate GDH, prompting its inactivation (Haigis et al., 2006). Contrarily to SIRT3, under caloric restriction conditions, SIRT4 expression and consequently its activity is reduced (Haigis et al., 2006), suggesting that there is an intricate and coordinated control between sirtuins to maintain cell homeostasis. Moreover, it raises the hypothesis that more proteins could be regulated by different sirtuins under different physiological situations. Although the

underlying mechanisms are still unclear, SIRT4 regulates glutamine metabolism and the TCA cycle in response to DNA damage stresses (Jeong et al., 2013).

SIRT5 was implicated in urea cycle through direct deacetylation and activation of carbamoyl phosphate synthetase 1 (CPS1) (Nakagawa et al., 2009). Nonetheless, SIRT5 plays an important role in succinylation of mitochondrial proteins. The majority of the mitochondrial proteins found to be possible targets for succinylation are responsible for regulation of ketone body production and fatty acid oxidation (Rardin et al., 2013). Thus, further studies that hopefully will arise will implicate SIRT5 in mitochondrial regulation via this novel post-translational modification within mitochondria.

The only report regarding SIRT6, implicated it in cancer proliferation via glycolytic regulation. SIRT6 is downregulated in several human cancers, which may be in basis for increased aerobic glycolysis in these cancers, similarly to what is seen in SIRT6 depleted MEFs (Sebastian et al., 2012).

Some reports also implicate SIRT7 in metabolism regulation by direct deacetylation of the regulator of transcription GA repeat binding protein, beta 1 (GABP β 1) (Ryu et al., 2014) and by increasing expression of the nuclear orphan receptor TR4/TAK1 (Yoshizawa et al., 2014). Oxidative phosphorylation may be positively regulated by SIRT7, once mitochondrial genes expression is increased, which is probably due to facilitated heterodimerization and activation of GABP β 1 by its deacetylation (Ryu et al., 2014). TR4/TAK1 is involved in hepatic lipid metabolism. SIRT7 indirectly stabilizes and increases its expression via regulation of ubiquitine-proteossomal DCAF1 and DDB1 proteins (Yoshizawa et al., 2014).

Table I – Metabolic regulators directly modulated by sirtuins

Compilation of major sirtuin targets that are directly or indirectly responsible for metabolic processes.

	Target	Target function	Functional result	References
SIRT1	PGC-1α - Peroxisome proliferator-activated receptor- γ co-activator 1 α	Key factor that mediates mitochondrial biogenesis and functioning	Activation	(Nemoto et al., 2005; Rodgers et al., 2005a)
	p53	Protection of the organism under DNA damage	Induction of apoptosis	(Vaziri et al., 2001)
	FOXO1 - Forkhead box O	Capability to induce apoptosis and to induce resistance to stress; gene expression regulation	Induction of gluconeogenic genes expression	(Brunet et al., 2004; Motta et al., 2004)
SIRT3	OXPHOS	Complex I – NDUFA9	Electron transport chain of the mitochondria	(Ahn et al., 2008)
		Complex II - SDHA		(Cimen et al., 2010; Finley et al., 2011)
	GDH - Glutamate dehydrogenase	Glutamate conversion into α -ketoglutarate	Activation	(Lombard et al., 2007)
	IDH2 - Isocitrate dehydrogenase 2	Conversion of isocitrate to α -ketoglutarate using NADP ⁺ as the electron acceptor	Activation	(Someya et al., 2010)
	SDHA - Succinate dehydrogenase	Oxidation of succinate to fumarate	Activation	(Cimen et al., 2010; Finley et al., 2011)
	Cyclophilin D	Regulation of the permeability transition pore	Promotes HKII (hexokinase II) detachment from mitochondria	(Shulga et al., 2010)
	PDH-E1α - Pyruvate dehydrogenase subunit E1	Conversion of pyruvate into acetyl-CoA	PDH activity activation	(Jing et al., 2013)
SIRT4	GDH	Glutamate conversion into α -ketoglutarate	Inhibition by ADP-rybosilation	(Haigis et al., 2006)
SIRT5	CPS1 - carbamoyl phosphate synthetase 1	Detoxification of excess ammonia and urea production	Upregulated activity	(Nakagawa et al., 2009)
SIRT7	GABPβ1 - GA repeat binding protein, beta 1	Regulation of transcription of mitochondrial genes	Enables heterodimerization and consequent activation of transcription	(Ryu et al., 2014)

All the above-mentioned studies clearly show that sirtuins have a wide range role in metabolic regulation. Nonetheless, this broad regulation may be well coordinated by cells, but there is still a lack of knowledge regarding this aspect. Similarly to what happens to GDH (Haigis et al., 2006; Lombard et al., 2007), probably several proteins can be differently regulated by singular sirtuins under distinctive conditions. Moreover, sirtuin functions may be well coordinated, but it is still unclear how. One of the recent insights that indirectly corroborate this hypothesis is a recent report showing that Sirtuin 3 is a target of PGC-1 α , itself a known target of Sirtuin1 (Kong et al., 2010). In this report, induction of PGC-1 α expression in mouse primary hepatocytes and in differentiated C2C12 myotubes led to an increase in Sirt3 expression. Silencing PGC-1 α in the same cells was able to significantly reduce Sirt3 expression (Kong et al., 2010). Although any report directly relates SIRT1 and SIRT3 cooperation or feedback regulation, this hypothesis was already raised (Bell and Guarente, 2011).

The roles of Sirtuin 1 in embryos and Pluripotent Stem Cells

In 2003, SIRT1 was firstly implicated in development and embryogenesis in mice. Heterozygous animals were crossed in order to obtain SIRT1 KO newborns and their proportion obey Mendelian ratios, however a great amount of them died on late prenatal or early postnatal period, usually within the first week (Cheng et al., 2003; McBurney et al., 2003b). Embryos and newborn SIRT1 KO animals present developmental defects that should be on the basis for their mortality. SIRT1 KO embryos and mice presented smaller size and smaller organs when compared to WT embryos (Cheng et al., 2003; McBurney et al., 2003b). Moreover, their lung, pancreas (McBurney et al., 2003b), heart

(Cheng et al., 2003) and eye (Cheng et al., 2003; McBurney et al., 2003b) present several abnormalities.

The SIRT1 KO animals that survived until maturity are sterile (McBurney et al., 2003b). On one side, males present impaired spermatogenesis: sperm are produced in reduced amounts (Coussens et al., 2008; McBurney et al., 2003b) and possess abnormal morphology (McBurney et al., 2003b) as well increased DNA damage (Coussens et al., 2008); on the other side, although females retain a normal oogenic progression (Coussens et al., 2008), abnormal (and almost absent) ovulation capacity happen probably due to hormonal issues (McBurney et al., 2003b). High expression of SIRT1 in the ovaries and testes of normal animals (Sakamoto et al., 2004) together with its role in fertility highlights the importance of SIRT1 in gamete production.

Moreover, SIRT1 is suggested as one important regulator of normal embryonic development. Indeed, SIRT1 is highly expressed in a early morula and blastocyst embryo stages (McBurney et al., 2003b) and decreases along with the embryonic development (Sakamoto et al., 2004) as is graphically represented in figure 1.7 [generated in the Genevestigator bioinformatic tool – <https://www.genevestigator.ethz.ch/> (Laule et al., 2006)].

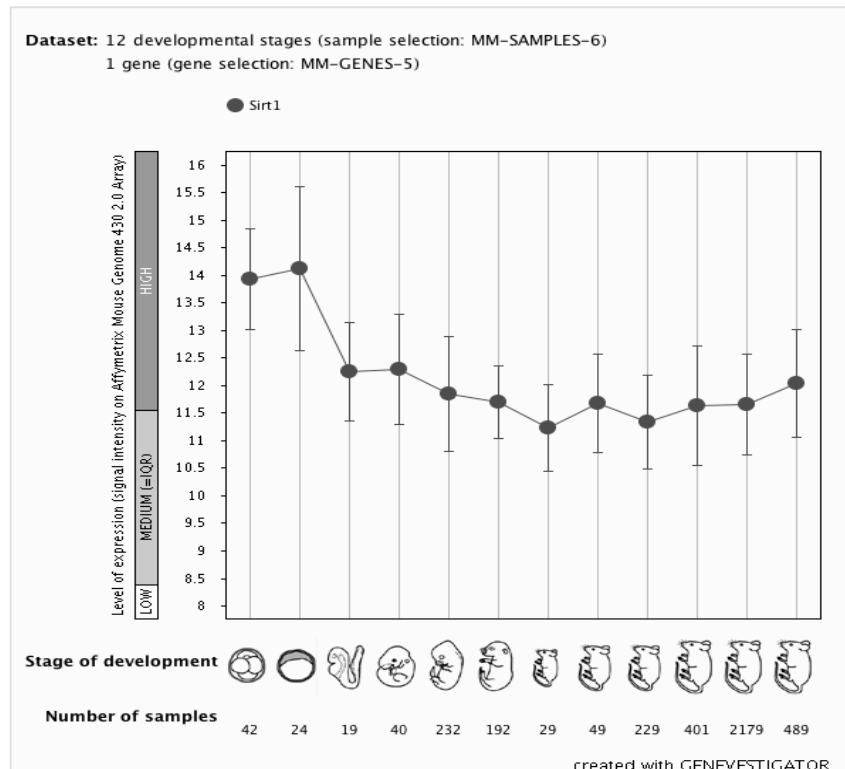


Figure 1.7 –Sirtuin 1 expression during murine embryonic development

SIRT1 mRNA expression peaks at the very first stages of mice development (morula and blastocyst stages). Throughout development there is a decrease in SIRT1 expression, suggesting that SIRT may be important for the developmental process. This image was generated in Genevestigator bioinformatic tool – <https://www.genevestigator.ethz.ch/> (Laule et al., 2006) in August, 2015 and represent the average SIRT1 expression at the different development stages from a specified number of samples analyzed by microarray technology (Affymetrix).

Recent studies also pointed to Sirt1 as necessary to normal differentiation potential of PSC, namely mESC (Ou et al., 2011; Saunders et al., 2010; Tang et al., 2014), hESC (Calvanese et al., 2010) and iPSCs (Ou et al., 2011; Yu et al., 2015). Moreover, its maximal expression at E4.5 of embryo development, followed by a decline over development (McBurney et al., 2003a; McBurney et al., 2003b; Sakamoto et al., 2004; Saunders et al., 2010), reinforces its importance in pluripotency regulation and developmental processes.

Corroborating this notion, SIRT1 is highly expressed in embryonic stem cells (Calvanese et al., 2010; McBurney et al., 2003a; McBurney et al., 2003b). For example, in hESC Shef-1 and mESC R1 cell lines, SIRT1 is highly expressed in the nucleus, as expected (Calvanese et al., 2010; McBurney et al., 2003a). In figure 1.8 [generated in Genevestigator bioinformatic tool – <https://www.genevestigator.ethz.ch/> (Laule et al., 2006)], a panel of murine cell lines and their Sirt1 mRNA expression is represented. ESCs present the highest Sirt1 expression (16 first represented cell lines; Fig. 1.8).

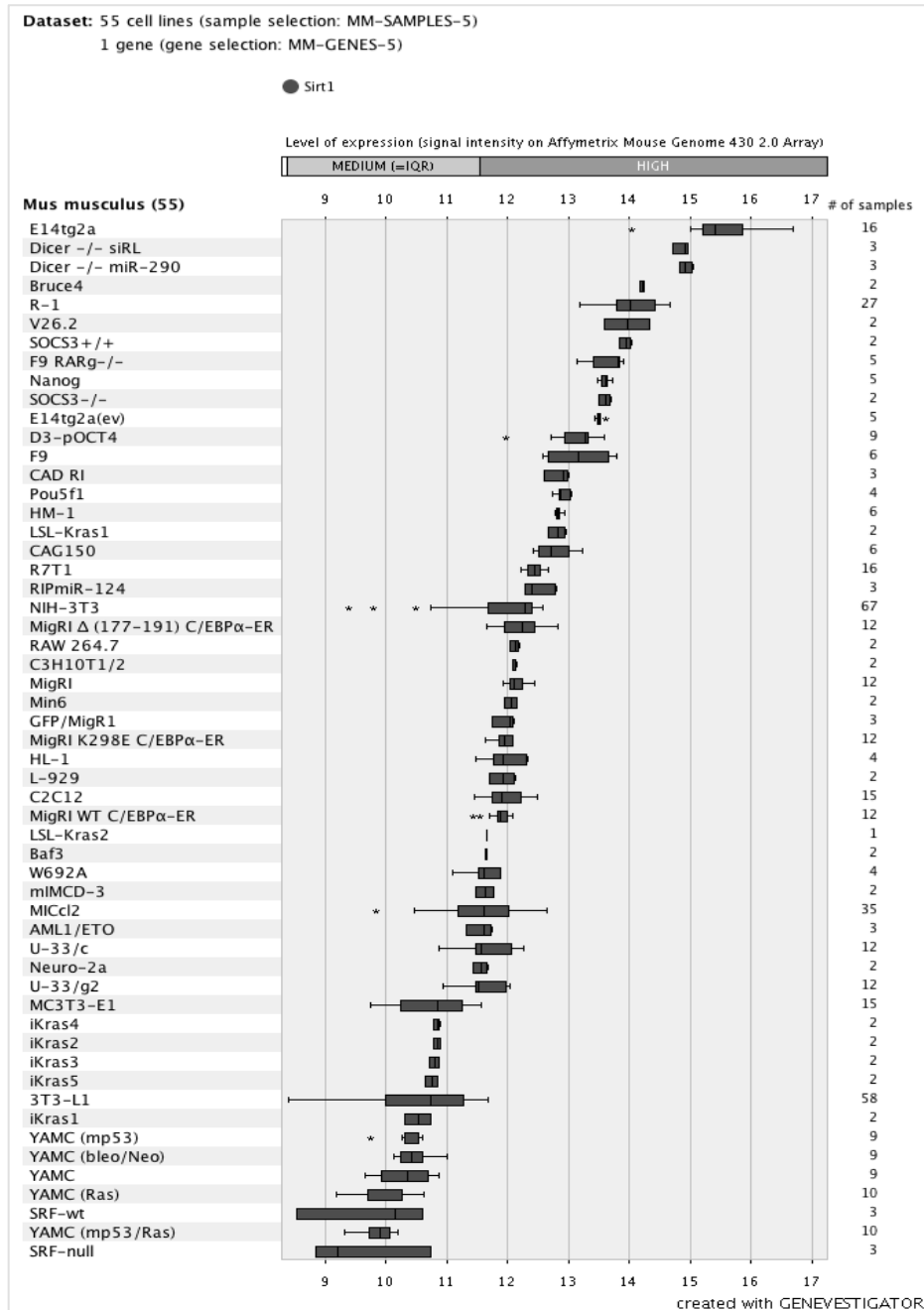


Figure 1.8 –Comparative expression of Sirtuin 1 in different mouse cell lines

The average SIRT1 mRNA expression is represented for 55 mouse cell lines. It is worth noting that the highest mean expression is observed for different mouse embryonic stem cells lines. This image was generated in Genevestigator bioinformatic tool – <https://www.genevestigator.ethz.ch/> (Laule et al., 2006) in August, 2015 and represent the average SIRT1 expression from different mouse cell lines from a specified number of samples analyzed by microarray technology (Affymetrix).

The so-called tumor suppressor p53 is a pluripotency regulator that can regulate Nanog expression (Li et al., 2012; Lin et al., 2005) and potentially other pluripotency transcription factors. When ESC are subjected to DNA damage, p53 binds to the Nanog promoter inhibiting its expression (Lin et al., 2005) and ESC tend to lose pluripotency and differentiate, which does not happen when p53 is silenced (Li et al., 2012). Additionally, when ROS increases by beta-mercaptoethanol (β -ME) removal from medium, in a wild-type situation, apoptosis is induced in mESC due to p53 translocation into the mitochondria. However, under the same conditions apoptosis is not induced for SIRT1 KO cells. Instead, p53 translocates to the nucleus where it represses Nanog expression (Han et al., 2008). Although somehow contradictory, it should be noted that ROS induction in the last study does not necessarily mean that DNA damage was induced, which was not addressed. Indeed, in a similar study, increase of ROS by β -ME removal and direct DNA damage approach using mitomycin-C induces different post-translational phosphorylation changes of p53 (Chae and Broxmeyer, 2011). For instance, after β -ME removal there was enhanced serine 389 phosphorylation of p53 in WT cells, which did not occur in Sirt1 KO cells, while after mitomycin-C treatment both WT and Sirt1^{-/-} mESC increased the phosphorylation in the same residue suggesting that Sirt1 must be involved in different regulatory mechanisms accordingly to different stimuli that cells are subjected to (Chae and Broxmeyer, 2011). Nonetheless, other interplays are implicated in SIRT1 modulation of pluripotency by p53. In OCT4 KO hESC, SIRT1 overexpression is sufficient to partially prevent differentiation of these cells, which is partially due to p53 deacetylation at Lysines 120 and 164 and consequent inactivation (Zhang et al., 2014b). However, it was not addressed if this is due to specific Nanog modulation. Nevertheless, several pluripotency factors were affected (Zhang et al., 2014b). Not surprisingly, SIRT1 is also important for induction of pluripotency given that its overexpression increases miPSC generation, while its knockdown does the opposite (Lee et al., 2012; Mu et al., 2015). microRNA-34a (miR-34a) modulates SIRT1 expression and iPSC generation (Lee et al., 2012). Moreover, SIRT1 deacetylates SOX2 at lysine 72, in an OCT4 dependent way, improving the

reprogramming process. SIRT1 silencing and/or SOX2 lysine 75 mutated into a glutamine 75 (mimicking acetylation) significantly decreased the efficiency of reprogramming (Mu et al., 2015). On the other hand, NANOG expression in iPSC is highly expressed when SIRT1 was overexpressed (Lee et al., 2012) and declined when Sirtuin 1 was absent (Mu et al., 2015). Still, the role of SIRT1 in reprogramming is debatable. A recent study showed that SIRT1 KO or overexpression in MEFs resulted in similar iPSC generation efficiency, despite the fact that *Sirt1*^{-/-} iPSCs had short telomeres (De Bonis et al., 2014). The distinct conclusions may result from different methodologies, reagents and culture conditions. Further studies must be conducted to completely disclose the exact role of Sirtuin 1 in reprogramming.

SIRT1 also regulates autophagy, apoptosis and genomic stability in ESC. As referred above, β -ME removal from the medium induces apoptosis in mESC, which is mediated by two different but interconnected ways: (1) p53 deacetylation and concomitant translocation to mitochondria (Han et al., 2008) and (2) deacetylation and activation of JNK and PTEN that activate FOXO1. Thus, FOXO1 translocate into the nucleus, inducing Bim1 and Puma pro-apoptotic proteins expression (Chae and Broxmeyer, 2011). In both cases, SIRT1 KO mESC were unable to induce apoptosis in response to β -ME withdrawal. Apoptosis can also be induced by hydrogen peroxide (H₂O₂) in mESC, in a SIRT1-independent way (Ou et al., 2014). Nevertheless, autophagy is induced by H₂O₂ in WT mESC, increasing LC3 I/II and Beclin-1 expression through mTOR pathway inhibition. SIRT1 KO mESC do not possess that capacity (Ou et al., 2014). Additionally, mESC exposure to H₂O₂ induces SIRT1 dissociation from the loci of genes that are commonly repressed, and relocation within DNA breaks where appropriate repair mechanisms are required. This can be a mechanism how SIRT1 supports chromosome stability. Under H₂O₂ exposure and SIRT1 inhibition there is an evident increase in chromosomal fusion aberrations in mESC (Oberdoerffer et al., 2008). Similar role of SIRT1 in chromosome stability was seen in MEFs and their iPSC counterparts (De Bonis et al., 2014). *Sirt1*^{-/-} MEFs and iPSC present higher amount of chromosome abnormalities when compared to *Sirt1*^{+/+}

controls (De Bonis et al., 2014). During the reprogramming process, there is a decrease of chromosome breaks in both cell types, still *Sirt1*^{-/-} iPSCs present a higher degree of breaks than WT iPSCs (De Bonis et al., 2014).

Sirtuin 1 and cell differentiation

A tight control is mandatory to keep in balance pluripotency and differentiation. As seen above, SIRT1 is closely related to pluripotency and survival. In the same way, Sirtuin 1 is required for proper differentiation. Firstly, SIRT1 is quickly downregulated upon differentiation (Calvanese et al., 2010; Saunders et al., 2010). Secondly, its importance for development and differentiation is also highlighted by several developmental abnormalities that are observed in SIRT1 KO animals (Cheng et al., 2003; McBurney et al., 2003b). Thereby, many studies showed direct implication of SIRT1 in differentiation.

SIRT1 binds to promoter regions of some hESC differentiation-related genes, such as PAX6, WNT6 and DLL4 among others, in pluripotency conditions. Under differentiation, their expression is up-regulated (Calvanese et al., 2010), suggesting that SIRT1 is a candidate target for a proper differentiation trigger. Indeed, SIRT1 knockdown or knockout in mESC modulates early differentiation. SIRT1 KO mESC present lower alkaline phosphatase activity under pluripotent conditions, which is lowered by RA-induced differentiation (Tang et al., 2014). Moreover, as will be discussed next, PSC and adult stem cells differentiation is impacted by SIRT1 (table II – summarizing data on SIRT1 implications in PSCs differentiation).

Table II – Implications of Sirtuin 1 deregulation in ESC differentiation

Compilation of major findings concerning sirtuin1 expression or activity modulation and its implication for embryonic stem cell (ESC) differentiation potential

Specie/ Cell line	Sirtuin 1 interference approach	Differentiation implications	Reference
Human / Shef1	↓ Sirt1 siRNA Knockdown	More than 50% developmental gene markers present more than 2-fold expression difference to CTR after EB induction	(Calvanese et al., 2010)
Mouse / TC1	↓ Sirt1 knockout	More than 60% developmental gene markers present more than 2-fold expression difference to CTR after EB induction	
Mouse / R1	↓ Sirt1 knockout	Defective and delayed hematopoietic differentiation	(Ou et al., 2011)
Mouse / R1	↓ Sirt1 Knockout	Increased expression of Hcrt gene, suggesting an increase of orexin neurons	(Hayakawa et al., 2013)
Mouse / iPSC-S ₁₀ 5F9	↓ NAM pharmacological inhibition	Increased neuronal stem cells formation from iPSC differentiation	(Hu et al., 2014)
Mouse / D3	↑ Resveratrol SIRT1 activation	Increased osteoblastic differentiation Inhibited adipocyte differentiation	(Srivastava et al., 2012)
	↓ NAM inhibition	Abrogated osteoblastic differentiation Upregulated adipocyte formation	
Mouse / ES-D3	↑ Sirt1 overexpression Sirt1 upregulation through miR-34a overexpression	Increased Smooth muscle cell markers expression	(Yu et al., 2015)
	↓ Sirt1 pharmacological inhibition Sirt1 siRNA silencing	Decreased smooth muscle cell markers expression	
Mouse / R1	↓ Sirt1 Knockout	Increased RARβ ₂ , CRAB-P2 and Cdx1 differentiation markers expression upon retinoic acid (RA) differentiation	(Tang et al., 2014)
Mouse /E14Tg2.a	↓ Sirt1 shRNA downregulation		

mESC hematopoietic differentiation is negatively impacted by SIRT1 absence. Delayed downregulation of pluripotency factors is observed across differentiation, associated with delayed upregulation of hematopoietic markers, resulting in deficient hematopoietic competent cells (Ou et al., 2011). Reinforcing this, isolated murine bone marrow progenitors increase their hematopoietic differentiation when the SIRT1 activator resveratrol is used, but inhibited by the SIRT1 inhibitor nicotinamide (Matsui et al., 2012). Moreover, SIRT1 KO murine bone marrow progenitors present less hematopoietic differentiation potential than WT cells (Matsui et al., 2012). Hematopoietic stem cell (HSC) homeostasis is also modulated by SIRT1 in an aging-related process. Transcription factor FOXO3 remains in nuclei when deacetylated, which is crucial for HSC homeostasis and differentiation potential. SIRT1 KO compromises their differentiation potential, similarly to what happens in aged WT HSC (Rimmele et al., 2014). Nonetheless, if HSC pool maintenance in mice is affected by SIRT1 is still controversial. SIRT1 absence was demonstrated to do not affect the pool of HSC in adult mice by (Leko et al., 2012), but others (Peled et al., 2012) showed that SIRT1 inhibition by NAM enriches the HSC population, while decreasing their differentiated cell population (Peled et al., 2012). NAM effects should be carefully interpreted as SIRT1 direct effects given that it is not specific for SIRT1, but rather common to the activity of all sirtuins.

miR-34a regulates Sirt1 expression in pluripotent (Saunders et al., 2010), induced pluripotent (Lee et al., 2012) and differentiating cells (Saunders et al., 2010; Yu et al., 2015). Thus, SIRT1 and miR-34a show interconnected regulation of mESC and hPSC differentiation. Smooth muscle differentiation from PSC requires increased levels of SIRT1, which is modulated by miR-34a (Yu et al., 2015). Tight control of SIRT1 levels is also observed for skeletal muscle differentiation from myoblasts. SIRT1 overexpression negatively regulates skeletal muscle differentiation through acetyltransferase PCAF and MyoD deacetylation (Fulco et al., 2003). Skeletal muscle, as well adipocyte, differentiation is also affected by caloric restriction in an AMPK and SIRT1 dependent way (Fulco et al., 2008; Lai et al., 2012). Moreover, mESC adipocyte differentiation is inhibited, while osteoblastic differentiation by

resveratrol is increased, in a very similar way as insulin (Srivastava et al., 2012). Indeed, SIRT1 and insulin can both stimulate β -catenin translocation within the nuclei where it acts modulating differentiation (Srivastava et al., 2012). Similarly, β -catenin translocation to the nucleus controls mesenchymal differentiation through regulation of differentiation related genes in mesenchymal stem cells (MSC), in a SIRT1 mediated fashion (Simic et al., 2013). In line with this, Sirt1 activation by resveratrol increases, while SIRT1 downregulation decreases differentiation potential of human bone marrow-MSC (hBM-MSC). Moreover, SIRT1 enhances hBM-MSC pool, by SOX2 deacetylation (Yoon et al., 2014).

Although the exact role of SIRT1 in ectodermal lineage differentiation is still debatable some involvement is certain. SIRT1-deficient mice present exencephaly, eye abnormalities and retinal defects (Cheng et al., 2003) and PSC differentiation within neuronal cell lineages is affected by SIRT1 perturbation. SIRT1 NAM-inhibition facilitates the generation of neuronal progenitor cells from miPSCs (Hu et al., 2014). Moreover, SIRT1^{-/-} mESCs differentiate more efficiently in orexigenic type neurons than WT mESC (Hayakawa et al., 2013). Still, neuronal differentiation may be SIRT1-regulated in a ROS-dependent manner. Mouse neural stem cells (NSC) cultured in the presence of pro-oxidative molecules present decreased proliferation and biased differentiation into astrocyte cells, while the opposite happens under anti-oxidative conditions (Prozorovski et al., 2008). Oxidative conditions induce SIRT1 upregulation in NSC differentiation into astrocytes, but SIRT1 downregulation restores neuronal differentiation in these conditions (Prozorovski et al., 2008). The nuclear localization of SIRT1 is an important aspect for proper NSC differentiation. In NSC, SIRT1 is localized in cytoplasm, rapidly shuttling into the nucleus under differentiation conditions (Hisahara et al., 2008). The same study shows that pharmacological or genetic downregulation of SIRT1 significantly reduces NSC differentiation into neurons favoring astrocyte differentiation, while its overexpression increases neuronal differentiation. In line with this, overexpression of a cytoplasmic restricted SIRT1 protein resembles the phenotype of SIRT1-downregulated cells (Hisahara et al.,

2008). *In vivo* studies also show that SIRT1 lack is beneficial for NSC proliferation, yet not disclosing its implication in cell specialization (Ma et al., 2014). SIRT1 knockdown was also shown to induce spontaneous neurite development in Neuro-2a neuroprogenitor cell line, while its overexpression inhibits differentiation, even in differentiating conditions (Liu et al., 2014). Resveratrol efficiently enhance the neuronal-like cells differentiation outcome from bone marrow mesenchymal stem cells (Joe et al., 2015). Different cell lines, cell culture conditions, SIRT1 interference mechanisms and differentiation protocols from the above-mentioned studies may be the basis for such conflicting results concerning SIRT1 role in neuronal differentiation. Nonetheless, it is clear that SIRT1 is involved in ectodermal differentiation potential, but its beneficial or detrimental role must be clarified.

Possible roles of other sirtuins in pluripotency and differentiation

Much less attention was paid to the possible roles of other sirtuins in pluripotency and differentiation. No obvious developmental defects are detected in knockout animals for those sirtuins (Finkel et al., 2009; Kim et al., 2011), which could dissuade several studies. Nonetheless, some reports suggested implication of other sirtuins in differentiation.

SIRT2 negatively contributes for adipocyte differentiation primarily by deacetylation of FOXO1 (Jing et al., 2007; Wang and Tong, 2009). During normal adipocyte differentiation, SIRT2 levels tend to decrease with time, but forced expression of SIRT2 attenuates this differentiation capacity (Jing et al., 2007). Additionally, SIRT2 also regulates adipocyte differentiation in a nutrient and energy

dependent manner. Food deprivation, as well cold stimulation, stimulates SIRT2 expression, favoring lipolysis in detriment of adipocyte differentiation (Wang and Tong, 2009).

SIRT3 appears to be involved in brown adipocyte differentiation (Giralt et al., 2011), as well in myoblastic differentiation (Abdel Khalek et al., 2014). SIRT3 involvement in brown adipocyte differentiation is regulated by its increased expression mediated by PGC-1 α and activation of its downstream target estrogen-related receptor α (ERR α), which results in SIRT3 upregulation concomitant with increased differentiation potential (Giralt et al., 2011). Although upstream SIRT3 signaling pathways were elucidated, the direct SIRT3 downstream action is still unclear. Concerning myoblastic differentiation, SIRT3 expression increases at maximal levels at the beginning of differentiation and its downregulation is responsible for defective differentiation, increased ROS levels, defective increase of basal respiration as well as decreased citrate synthase, complex II and cytochrome C oxidase activities (Abdel Khalek et al., 2014). Interesting, cells where SIRT3 was downregulated show decreased PGC-1 α expression (Abdel Khalek et al., 2014), suggesting that SIRT3, besides being regulated by PGC-1 α , is also its regulator.

SIRT6 is the only sirtuin than SIRT1 that was implicated in ESC differentiation. A recent study shows that SIRT6 is responsible for proper downregulation of the pluripotency genes Nanog, Oct4 and Sox2 during differentiation via deacetylation of histone lysines H3K56 and H3K9 at their promoter regions (Etchegaray et al., 2015). OCT4 and SOX2 are positive transcription enhancers of TET enzymes. Via OCT4 and SOX2 downregulation upon differentiation, TET enzymes are no longer transcriptionally enhanced. Phenotypically, ESC derived from mouse SIRT6 KO embryos present a biased differentiation towards neuroectodermal cell lineages (Etchegaray et al., 2015). SIRT6 downregulation also inhibits adult rat bone marrow mesenchymal stem cell differentiation, which is mediated by lack of inhibition by deacetylation of the nuclear factor- κ B (NF- κ B) (Sun et al., 2014).

As a general overview, sirtuins seem to be important in differentiation-related mechanisms. SIRT1 is the most well studied sirtuin possibly due to its major homology with the yeast SIR2 α , thus tempting

to skew many studies towards the unraveling of its function due to its possible role in aging and longevity. Nonetheless, in last couple of years there was a significant increase in the number of studies disclosing the roles of other sirtuins in animal and cell physiology. Thus, similarly to the recent study implying a role for SIRT6 in ESC differentiation (Sun et al., 2014), I expect that many more will arise in the next months/years implying other sirtuins role in differentiation mechanisms.

Background and Aims

The main goal for this thesis is to understand the role of sirtuins in mouse embryonic stem cell pluripotency and differentiations potential, with a particular focus on possible effects through metabolic changes. Thus, a special focus will be put on metabolic clues that might be on the basis of the sirtuin action on embryonic stem cells. To do that, we will investigate two specific goals that are then presented on chapter II and chapter III.

On **chapter II**, we aim to disclose possible implications of Sirtuin 1 in embryonic stem cells. Sirtuin 1 has already been implicated in pluripotency (Calvanese et al., 2010; McBurney et al., 2003a; Tang et al., 2014) and differentiation control of embryonic stem cells (Hayakawa et al., 2013; Hu et al., 2014; Ou et al., 2011; Srivastava et al., 2012; Yu et al., 2015) and adult stem cells (Fulco et al., 2003; Hisahara et al., 2008; Ma et al., 2014; Yoon et al., 2014). Moreover, Sirtuin 1 is a candidate metabolic modulator mainly acting through deacetylation and control of some transcription factors responsible for metabolic-related gene expression, such as PGC1 α (Nemoto et al., 2005; Rodgers et al., 2005b) or FOXO1 (Motta et al., 2004). Nonetheless, there was not reported until date any possible metabolic modulation by Sirtuin 1 in embryonic stem cells. Thus, we aim to study possible effects of Sirtuin 1 in embryonic stem cell metabolism.

In this chapter, our aim will be achieved taking advantage on a Sirtuin 1 Knockout R1 mouse embryonic cell line. After confirmation of SIRT1 absence, we aim to compare pluripotency as well as differentiation potential of these cells into neuronal cell lineages. In a second part of this aim, we

will evaluate metabolic clues on mESC that can be affected by Sirtuin 1 and how it can affect pluripotency.

On **chapter III** we aim to perform a pharmacological approach, using kaempferol, in an attempt to manipulate Sirtuin 3, and understand its effects on mouse embryonic stem cells. This approach was selected as an easily and fast way to address possible roles for SIRT3 in mESC.

Kaempferol is a natural flavonoid widely present in plants (Calderon-Montano et al., 2011) and has been used in cancer studies where it was found to induce apoptosis, cell cycle arrest in disease mimic-conditions (Kang et al., 2009; Lee et al., 2014; Rajendran et al., 2014). Moreover, kaempferol was recently suggested as a potential activator (Cimen et al., 2010; Marfe et al., 2009) and inducer of Sirtuin 3 expression (Marfe et al., 2009). SIRT3 is the main mitochondrial deacetylase, regulating a great amount of metabolic pathways (Lombard et al. 2007).

Thus, kaempferol will be used to positively affect Sirtuin 3 activity and expression in mouse embryonic stem cells, in order to disclose a role for SIRT3 in metabolism of these cells. Moreover, we aim to evaluate possible implications of kaempferol in mESC, such as pluripotency and differentiation potential and other mechanisms that could be affected by kaempferol such as proliferation, cell cycle and apoptosis.

***Sirtuin 1: A metabolic modulator of
embryonic stem cell fate?***

Abstract

Due to the capacity of self-renewal and differentiation in all cell types of an adult individual, embryonic stem cells (ESCs) are a promising field for regenerative medicine and toxicological purposes. Resembling the blastocyst's inner cell mass and many cancer types, ESC rely mostly in a glycolytic over oxidative metabolism to supply their biosynthetic requirements to proliferate. When ESCs undergo differentiation, a metabolic shift occurs from glycolysis to a predominant oxidative flux. As already demonstrated by our group, metabolic manipulation is an important gatekeeper for pluripotency and/or differentiation. Nonetheless, there are no clues concerning epigenetic regulation and its possible influence on the crosstalk between pluripotency and metabolism. In order to address this question, a knockout murine ESC line for Sirtuin 1 was used to unveil possible effects of this epigenetic-related enzyme in the metabolic profile of our cells.

$SIRT1^{-/-}$ mESC, although pluripotent, showed elevated OCT4 levels. Moreover, this could explain the observed impaired differentiation potential into a neuronal cell lineage. Additionally, our results show that the absence of Sirtuin 1 leads to alterations in the metabolic profile of ESCs, given that its absence induces a more pronounced glycolytic metabolism paralleled by a decrease in oxidative metabolism. This metabolic modulation could be, in part, due to modulation of STAT3 mRNA expression levels.

Overall our results suggest metabolism as a powerful tool to induce alterations in pluripotency/cellular fate in ESCs. Although more comprehensive studies need to be done, our results involving Sirtuin 1 suggests that transcription control in ESCs could be an important mechanism on how metabolism could be regulated.

Keywords

Embryonic stem cells, sirtuin 1, mitochondria, metabolism, STAT3

Introduction

Embryonic stem cells (ESC) are pluripotent stem cells isolated from the inner cell mass of the blastocyst and have the capacity for indefinite self-renewal and to differentiate into all cell types that are present in a new individual (Martello and Smith, 2014; Pereira et al., 2014). Although somehow bivalent in their mitochondrial – glycolytic metabolism, it is believed that they have a reduced mitochondrial metabolism, favoring the aerobic glycolytic metabolism (Varum et al., 2011), similarly to the well-described “Warburg effect” in many cancer cells (Ito and Suda, 2014; Warburg, 1956). Nowadays, it is well accepted that a metabolic shift from glycolysis towards an oxidative phosphorylation occurs during differentiation of these cells (Chung et al., 2007). Metabolic perturbations in pluripotency conditions (Rodrigues et al., 2015a; Rodrigues et al., 2015b) can drive cells into spontaneous differentiation. Moreover, metabolic perturbations during differentiation of ESCs can inhibit (Kim et al., 2015; Moussaieff et al., 2015; Pereira et al., 2013) or bias this process for specific lineage cell types (Rodrigues et al., 2015b).

The mammalian silent information regulator 2 homolog 1 (SIRT1) is one member of the NAD⁺-dependent sirtuin deacetylases (Frye, 1999). There are seven sirtuins (SIRT1-7), with SIRT1 the closest homolog of yeast *Saccharomyces cerevisiae* Sir2. Although Sir2 were first described in yeast acting as a regulator of transcription with an important role in DNA compaction and histone deacetylase activity, soon mammalian Sirtuins were found to also regulate gene expression, but many other processes by deacetylating many other targets than histones (Haigis and Sinclair, 2010). SIRT1, a

predominant nuclear sirtuin, is a player on several intracellular processes, such as, for example, gene regulation, metabolism, autophagy and oxidative stress response, among many others. To do so, SIRT1 deacetylates several substrates that directly or indirectly act on those functions such as histones, p53, PGC1 α , FOXO1 or NF- κ B (Houtkooper et al., 2012).

SIRT1 is highly expressed in embryos, as well as in embryonic stem cells and its expression decreases with differentiation (McBurney et al., 2003b; Sakamoto et al., 2004). In ESCs, SIRT1 can regulate pluripotency (Han et al., 2008) and autophagy/apoptosis in response to oxidative stress conditions (Chae and Broxmeyer, 2011; Ou et al., 2014). It can act through tight signaling together with the pluripotent transcription factors NANOG (Han et al., 2008) or OCT4 (Zhang et al., 2014b), suggesting that SIRT1 may indeed be necessary for the pluripotency phenotype of ESCs. Moreover, some studies showed that SIRT1 also has a role in differentiation programs. Development defects usually occur in the newborns knockout animals that can survive during development process (Cheng et al., 2003; McBurney et al., 2003b). *In vitro* studies in embryonic stem cells showed that SIRT1 binds to promoter regions of some differentiation related genes (Calvanese et al., 2010) and SIRT1 KO mESC seem to be more prone for differentiation (Tang et al., 2014). Moreover, mESC differentiation impacts were already shown when SIRT1 expression or activity was manipulated, namely neuronal (Hayakawa et al., 2013; Hu et al., 2014), osteoblastic (Srivastava et al., 2012), hematopoietic (Ou et al., 2011) and smooth muscle cell differentiations (Yu et al., 2015). Sirtuin 1 was also implicated in the regulation of metabolism (Bernier et al., 2011; Nemoto et al., 2005), although the exact mechanisms underlying that modulation are still to be completely understood.

In our study, we aim to understand the possible role for Sirtuin 1 in pluripotency and differentiation potential for mESC. Moreover, we aim to determine if SIRT1 could have a metabolic effect on mESCs.

Materials and Methods

mESC culture conditions

R1 and *Sirt1*^{-/-} R1 mouse embryonic stem cell lines were kindly provided by Dr. Michael McBurney (University of Ottawa and Ottawa Hospital Research Institute, Ottawa, Canada) (McBurney et al., 2003a). Cells were maintained and propagated on a feeder cell layer composed by mitotically inactivated (mitomycin-C) CF-1 mouse embryonic fibroblasts (MEF; GlobalStem) with an estimated density of 18,000-20,000 MEFs per square centimeter. Cells were kept in **ES-DMEM** medium composed of DMEM, 15% embryonic stem cell-qualified fetal bovine serum (ES-FBS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate (Gibco, Life Technologies), 1% non-essential amino acids, 0.1 mM mercaptoethanol (Sigma-Aldrich) and 1,000 U/mL of Leukemia inhibitory factor (LIF) - (Chemicon – Millipore) at 37°C and 5% CO₂ conditions. Medium from both mESC lines were changed daily and cells were passaged every 2/3 days.

For mitochondrial metabolism modulation, cells were plated and kept in galactose supplemented DMEM (**galacDMEM**) medium after passaging. GalacDMEM medium is composed by DMEM (Sigma-Aldrich D5030) supplemented with 15% ES-FBS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate (Gibco, Life Technologies), 1.8g/L galactose, 3.7g/L sodium bicarbonate, 1% non-essential amino acids, 0.1 mM mercaptoethanol (Sigma-Aldrich) and 1,000 U/mL LIF (Chemicon – Millipore). For proper results comparison, cells were also kept in normal glucose DMEM (**gluDMEM**) composed by DMEM (Sigma-Aldrich D5030) supplemented with 15% ES-FBS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate (Gibco, Life Technologies),

4.5g/L glucose, 3.7g/L sodium bicarbonate, 1% non-essential amino acids, 0.1 mM mercaptoethanol (Sigma-Aldrich) and 1,000 U/mL LIF (Chemicon – Millipore).

mESC passage

Before experiments, cells were detached with StemPro Accutase Cell Dissociation Reagent (Gibco, Life Technologies) and MEFs were removed by differential adhesion process, i.e. cells in suspension were plated into gelatin-coated dishes per 30 minutes allowing MEFs to adhere, while mESC keep in suspension. Non-adherent cells were collected, centrifuged at $\sim 280 \times g$, supernatant was discarded and pelleted cells resuspended in fresh medium. A small fraction of resuspended cells (20 μ L) was collected and stained with 20 μ L Trypan Blue solution (Sigma) and counted in a hemocytometer. 5,000 cells were plated in gelatin-coated dishes 12h before start the experiments.

Monolayer neuronal differentiation

For monolayer neuronal-directed differentiations, undifferentiated R1 and *Sirt1*^{-/-} R1 mESC were plated at a 3,000 cells per square centimeter in a gelatin-coated dish with complete ES-FBS medium. 12 hours after, medium was changed for N2B27 medium, composed by MEM (1:4), F-12 nutrient mix (1:2) and neurobasal medium (1:2) supplemented with 7,5 mM Hepes, 1 mM L-glutamine (Gibco, Life Technologies), 1.5 mg/mL Glucose (Sigma), 1.5 mg/mL Albumax I and 1X N2 and B27 supplements (Gibco, Life Technologies). Medium was changed every other day until approximately day 10 and thereafter every day, because at this time point medium is already consumed after 1 day incubation by the higher amount of cells.

Immunocytochemistry (ICC)

For ICC purposes cells were grown in 24-well plates with thermanox plastic cell culture coverslips (Nunc, ThermoScientific). After each experiment, cells were rinsed with 1x PBS and fixed with 4% paraformaldehyde (PFA) for 15 minutes, followed by 3 washes with ice-cold 1x PBS. Cells not immediately used for ICC procedures were kept in 0,1%Azide in PBS at 4°C. Briefly, cells were blocked and permeabilized with 5% bovine-serum albumine (BSA), 0,25% Triton X-100 in PBS at room temperature for no less than 1 hour. Samples were then incubated with the primary antibody – Oct4 1:500 (Cell signaling); Sirt1 1:1000 (Sigma-Aldrich); TOM-20 1:200 (Santa Cruz) diluted in 5% bovine-serum albumine (BSA), 0,25% Triton X-100 in PBS overnight at 4°C. After that, respective secondary antibodies (Life-Technologies) were incubated for 1 hour at RT. Finally, nuclei were counterstained with Hoechst 33342 (Molecular Probes) and coverslips were mounted in slides with Vectashield Mounting Medium (Vector).

Total RNA isolation, DNA cleanup, cDNA synthesis and qPCR

RNA Isolation

RNA was isolated using Trizol Reagent (Ambion, Life Technologies) accordingly to manufacturer. Briefly, after cell detachment and centrifugation (280 xg), 1mL of Tryzol reagent was added and vortexed for ~10 seconds, followed by the addition of chloroform and new vortex for ~20 seconds. The suspension was then centrifuged at ~3200 xg (4000 rpm) for 5 minutes at room temperature. Aqueous phase was then removed to a clean tube, molecular biology grade isopropanol was added and samples were stored overnight at -20°C.

The next day, the tubes were centrifuged at 16,000 xg for 30 minutes at 4°C. Supernatant was discarded and pellet resuspended in 75% molecular biology grade ethanol solution. Samples were

then centrifuged at 16,000 $\times g$ for 10 minutes at 4°C. Supernatant was then discarded and pellets were allowed to dry at room temperature. Finally, dry pellets were resuspended in 20-30 μL of nuclease-free water, depending on the size of the pellet before dry.

DNA clean-up

Possible DNA contamination was removed using the DNA-free DNA Removal Kit (Ambion, Life Technologies). Briefly, 0.1v/v of 10x DNase I buffer and 2 μL of rDNase I were added to the samples and incubated for 30 minutes at 37°C. At the end of incubation time, 0.1v/v of DNase inactivation reagent was added and incubated for 2 minutes with occasional mix. Samples were then centrifuged at 10,000 $\times g$ for 2 minutes and supernatant was collected to a fresh tube.

Concentration and quality of the collected RNA was determined using NanoDrop 2000 (Thermo Scientific) and samples with 260/280 ratio under 1.8 were discarded.

cDNA synthesis

1 μg cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) accordingly to manufacturer's instructions (table III). Briefly, in 0.2 mL tube, the table I reaction was prepared. Two controls were also performed: (1) RT- control where all reagents were added except RNA for proper accessing of possible DNA contamination of reverse transcription reagents and (2) -RT control where all reagents including RNA were added, except iScript reverse transcriptase for accessing of residual DNA contamination of our RNA samples in the subsequent steps.

Table I – First cDNA strand synthesis reaction mix using iScript cDNA Synthesis Kit

cDNA synthesis reaction	Volume (μL)
5x iScript reaction mix	4
iScript reverse transcriptase	1
Nuclease free water	x (1 μ g)
1ug of sample RNA	15 - x
Final Volume	20uL

Next, reaction mixtures were incubated for 5 minutes at 25°C, followed by 30 minutes at 42°C and 5 final minutes at 85°C incubation times in a thermal cycler (S1000 Thermal Cycler; BioRad).

Quantitative real-time PCR (qPCR)

qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad), accordingly to manufacturer's instructions (table IV). Primers sequences used for the reaction PCR described in table V were obtained from PrimerBank (Spandidos et al., 2010) database (<http://pga.mgh.harvard.edu/primerbank/>) and ordered from Integrated DNA Technologies (IDT).

Table II – qPCR reaction mix using SsoFast EvaGreen Supermix

qRT-PCR reaction mix	Volume (μL)
Nuclease free water	7
Primer Forward (100 μ M)	1
Primer Reverse (100 μ M)	1
SsoFast™ EvaGreen® Supermix	10
Final Volume	19 μL
Template cDNA	1

Duplicates were performed for each gene expression and appropriate negative controls were used: the reaction for each primer with RT– and –RT reaction products from cDNA synthesis, besides qPCR reaction without cDNA template addition. 1 µl of template cDNA of each sample was loaded to the 96-well reaction plate (Bio-Rad) and afterwards 19µl of the supermix was added to each well. Reaction and quantification was performed in CFX96 Touch Real-Time PCR Detection System (BioRad) and mRNA fold change was calculated using the $-\Delta\Delta C_t$ method.

Table III – List of primers nucleotide sequences for qPCR.

Target gene	Primer	5' – 3' Sequence	PrimerBank ID
Oct4	Forward	CGGAAGAGAAAGCGAACTAGC	356995852c3
	Reverse	ATTGGCGATGTGAGTGATCTG	
Nanog	Forward	TCTTCCTGGTCCCCACAGTTT	31338864a1
	Reverse	GCAAGAATAGTTCTCGGGATGAA	
Pax6	Forward	TACCAGTGTCTACCAGCCAAT	1405745a1
	Reverse	TGCACGAGTATGAGGAGGTCT	
Nestin	Forward	CCCTGAAGTCGAGGAGCTG	15011851a1
	Reverse	CTGCTGCACCTCTAAGCGA	
βIII-tubulin	Forward	TAGACCCCAGCGGCAACTAT	12963615a1
	Reverse	GTTCCAGGTTCCAAGTCCACC	
β-actin	Forward	GGCTGTATCCCCTCCATCG	6671509a1
	Reverse	CCAGTTGGTAACAATGCCATGT	

Oxygen consumption and extracellular acidification rates analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined using the Seahorse XF24 analyzer (Seahorse).

After cell passaging and MEF adhesion, approximately 100,000 cells were plated into the gelatin-coated 24-well XF24 cell culture plate in the respective culture medium for 12 hours.

For measuring mitochondrial function, cell culture medium was removed and replaced by XF Assay Medium Modified DMEM (Seahorse), adjusted pH=7.4 supplemented with 4,5g/L glucose, 1 mM pyruvate and 2 mM L-glutamine and incubated for 37°C, 1 hour before start the experiment. Oligomycin (1 μ M), FCCP (1.25 μ M) and rotenone + Antimycin A (1 μ M each) were injected after measurements 3, 6 and 9, respectively.

To measure glycolytic function, cell culture medium was removed and replaced by XF Assay Medium Modified DMEM (Seahorse), adjusted pH=7.4 supplemented with L-glutamine (2 mM), but without glucose and pyruvate. Cells were incubated for 1 hour without CO₂ in order to let cells starving before first injections during the experiment. Glucose (10 mM), Oligomycin (1 μ M) and 2-deoxyglucose (100 mM) were injected after measurements 3, 6 and 9, respectively.

After measurements, cells were detached and counted in order to normalize the measurements for cell number per well. Blank gelatin-coated plates incubated with medium without cells were used for blank control measurements.

Flow cytometry analysis: mitochondrial mass, Mitochondrial membrane potential, and Mitochondrial superoxide production

All fluorescent dyes were analyzed by flow cytometry (Becton Dickinson BD FACSCalibur cytometer) and 20,000 gated cells were acquired/analyzed per condition with the Cell Quest Pro Acquisition software (BD Biosciences). Geomean of the curve for each condition was used for quantification and statistical analysis

An indirect measure of cell **mitochondrial mass** was obtained by using MitoTracker® Green FM (Life Technologies), a dye that binds to mitochondria independently of mitochondrial membrane potential. Cells grown in 60 mm dishes were harvested, centrifuged and resuspended at 10⁶ cells/mL

in PBS1x. 50 nM of dye was added and incubated for 30 minutes at 37 °C in the dark. Dye was washed, cells resuspended in PBS and analyzed by flow cytometry using appropriate settings.

MitoSox Red (Life Technologies) is a mitochondria-directed dye that allows for detection of **mitochondrial superoxide** anion. After cell harvesting and centrifugation, cells were resuspended in PBS at 10^6 cells/mL. 3 μ M of MitoSox Red was added to the cells and incubated at 37 °C for 30 minutes in dark. Cells were then washed, resuspended in PBS, kept on ice until being analyzed by flow cytometry using appropriate settings.

Mitochondrial membrane potential was measured with TMRM (Tetramethylrhodamine methyl ester; Life Technologies). Briefly, cells grown in 60 mm dishes were detached with accutase, centrifuged and resuspended at 10^6 cells/mL in PBS. 150 μ M of TMRM was added and incubated for 20 minutes at 37°C in the dark. Dye was washed out, cells resuspended in PBS1x and analyzed by flow cytometry using appropriate settings.

Protein collection and Western Blot

Protein extracts for Western blot were obtained by lysing mESC with RIPA buffer (Sigma-Aldrich) supplemented with 2mM of phenylmethanesulphonyl fluoride-PMSF (Sigma-Aldrich) and 2x Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL). Protein quantification was performed using the PierceTM BCA (Bicinchoninic Acid) Protein Assay Kit, following the manufacturers' protocol. Both samples and calibration curves were determined in duplicate.

For Western blot analysis 30 μ g of protein were diluted in Laemmli sample buffer (Bio-Rad) and water and denatured at 95°C in a dry bath. Thereafter, samples were loaded into a 12% Acrilamide Tris-HCl gel and electrophoresis was performed in a Mini-protean tetra-cell Bio-Rad apparatus. Proteins were then blotted to a PDVF membrane (Bio-Rad). At the end, membranes were washed

and 5% powder milk (Bio-Rad) diluted in Tris-Buffered Saline with Tween (TBST) was used for blocking purposes. Overnight incubation at 4°C was used for the following primary antibodies: rabbit anti-Oct4 (Cell Signaling Technology, 1:1000) and mouse anti-Sirt1 (1:1000) and mouse anti- β -Actin (Sigma-Aldrich, 1:15000). Membranes were washed with TBST and incubated with the respective HRP-conjugated secondary antibody (Cell Signaling Technology, 1:2000) for 1 hour at room temperature. Clarity Western ECL Substrate (Bio-Rad) was used for protein detection using VersaDoc Imaging system (Bio-Rad). Quantity-One (BioRad) software was used for protein quantification. All results were normalized for the respective β -Actin of the same sample.

Data analysis and statistics

Prism 6 (GraphPad) was used to perform statistical analysis. All data is expressed as mean values \pm standard error of mean (SEM). Paired Student's T-test was used for statistic analysis between R1 and Sirt1^{-/-} R1. Multiple comparisons were performed by ANOVA followed by Bonferroni post-hoc test. Statistical analysis was performed on the raw data and, for a comprehensive analysis of the results, data were then normalized to 100% of the control condition. Statistical significance was determined at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Results

SIRT1 absence confirmation in $Sirt1^{-/-}$ R1 mESC

R1 and $Sirt1^{-/-}$ R1 mouse embryonic stem cell lines were first accessed for SIRT1 protein expression. By western blot and immunocytochemistry analyses we confirmed that SIRT1 protein is absent. As shown in figure 2.1A, there is no signal from $Sirt1^{-/-}$ R1 cells, when cultured in pluripotency or non-pluripotency (removal of LIF: -LIF) conditions, for the anti-SIRT1 antibody after Western blot analysis, while WT counterparts clearly show the presence of SIRT1 protein. Similar results were achieved by immunocytochemistry (Fig. 2.1B) where it is possible to observe that $Sirt1^{-/-}$ R1 cells do not present Sirtuin 1, but react to another antibody, in this case TOM-20. As expected, TOM-20 staining is cytoplasmatic, as it is expected, considering that this is a mitochondrial protein.

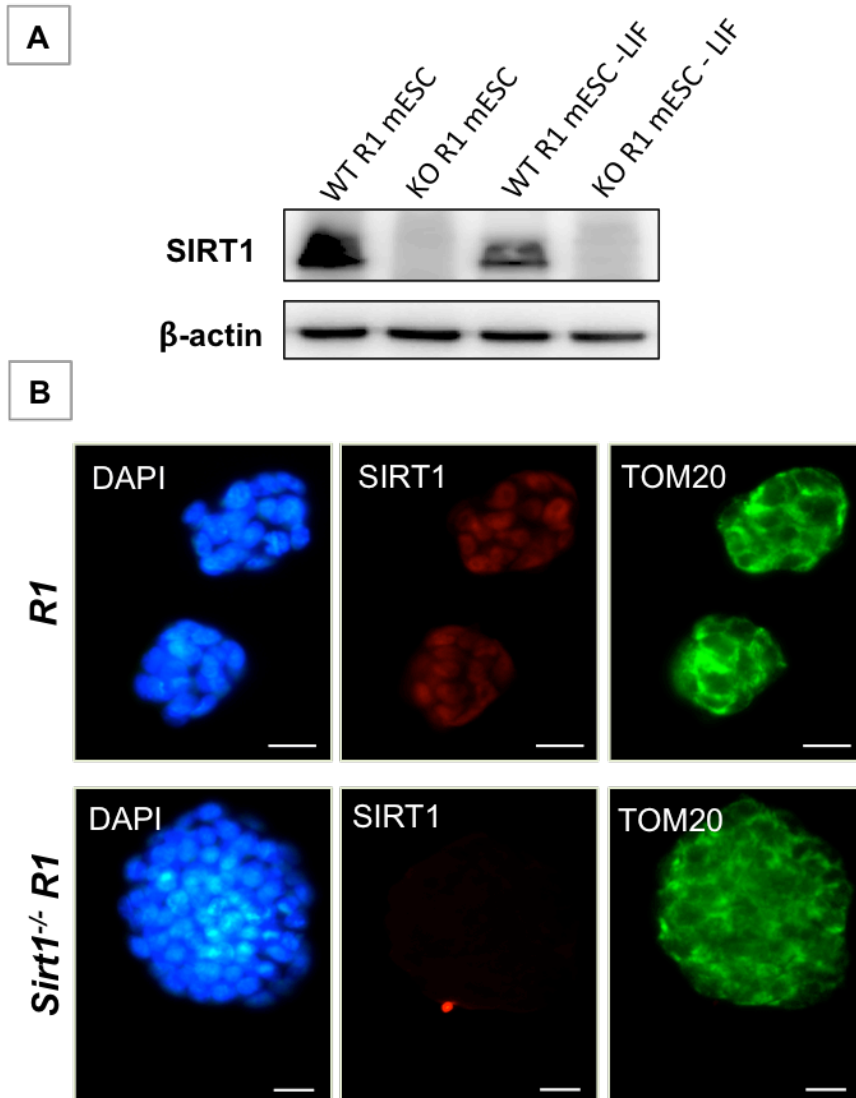


Figure 2.1 –Confirmation of SIRT1 protein absence in *Sirt1*^{-/-} R1 mESC

Total protein extracts of R1 and *Sirt1*^{-/-} R1 in the presence or absence of LIF (-LIF) were analyzed by western blot for SIRT1 (A). R1 and *Sirt1*^{-/-} R1 cultured in ES-DMEM medium were analyzed by ICC for SIRT1 (red) and TOM-20 (green) and counterstained with Hoechst 33342 (blue; nuclei); scale bar corresponds to 25 nm (B).

Checking for pluripotency markers of WT and Sirt1^{-/-} R1 mESC

Next, we checked for the pluripotency status of Sirt1^{-/-} R1 cells. In our culture conditions we were only able to keep these cells morphologically in a pluripotent state when cultured over a feeder cell layer of mitotically inactivated mouse embryonic fibroblasts. When cultured in tissue culture dishes coated with gelatin, Sirt1^{-/-} R1 mESC tend to lose the colony shape, starting to spread a huge amount of cells in the edges and individual cells start to be distinguished in the center of the colonies, while WT R1 mESC grow in perfectly good and in apparent pluripotent conditions.

Therefore, we evaluated the mRNA expression of pluripotency-related genes Nanog and Oct4, but similar levels are observed for both cell lines as can be seen in figures 2.2A and 2.2B, respectively. At the protein level, cells express OCT4, and its expression is nuclear, as observed after OCT4 staining by ICC within colonies of both cell lines (Fig. 2.2C), suggesting that it is acting as a pluripotency transcription factor. However, when we looked into the protein expression levels (Figs. 2.2D and 2.2E) it is noted that OCT4 is more abundant in Sirt1^{-/-} R1 cells. Oct4 expression must be tightly regulated in order to maintain cells pluripotency and for proper differentiation potential (Niwa et al., 2000; Niwa et al., 2005). Allied to the more prone differentiation, it suggests that SIRT1 can be priming cells to differentiate. Nonetheless, just the increase of OCT4 expression *per se* does not allow us to conclude about the pluripotency or differentiation commitment of these cells.

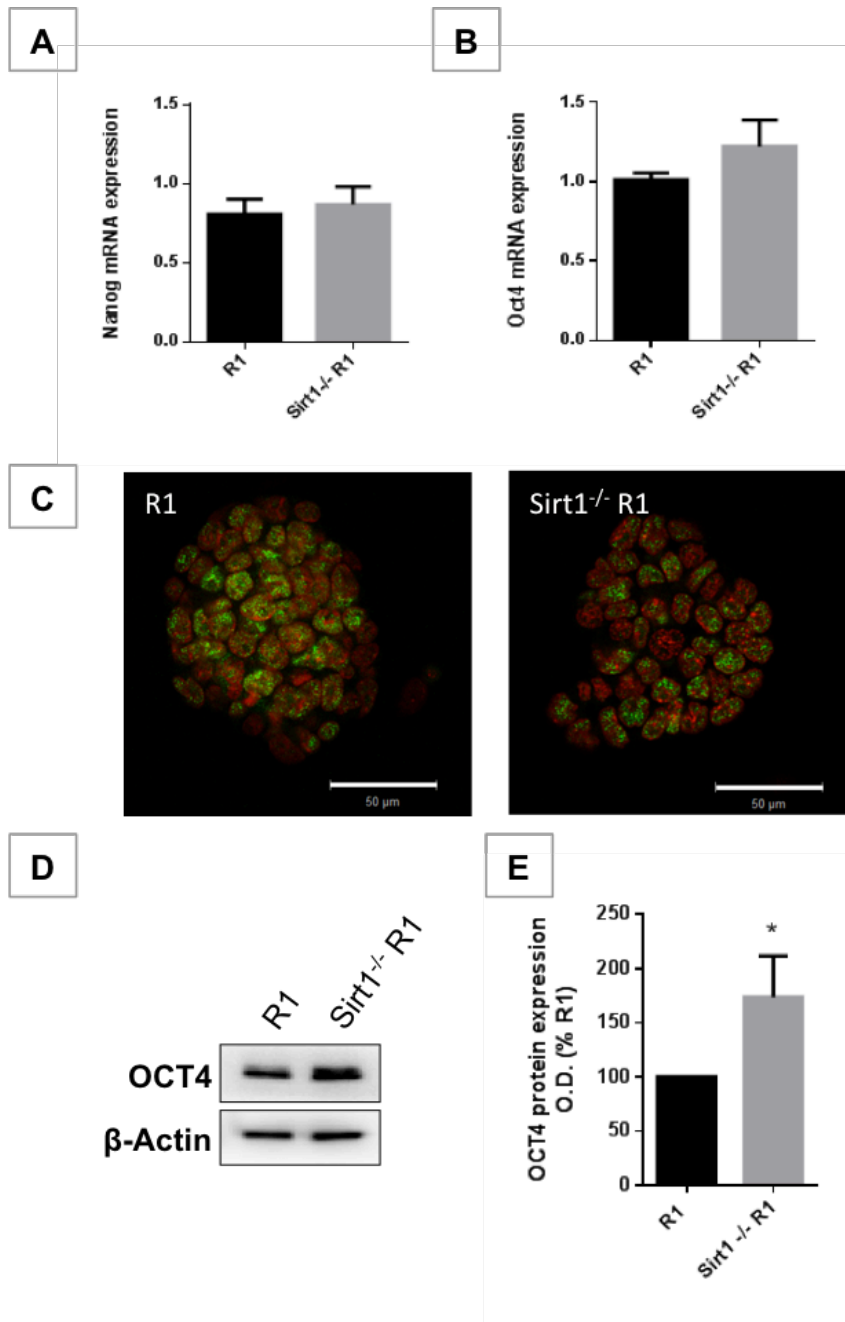


Figure 2.2 - Pluripotency of R1 and Sirt1^{-/-} R1 mESC.

mRNA levels for Nanog (A) and Oct4 (B) were evaluated by qPCR. Graph data is normalized for R1 mRNA levels. R1 and Sirt1^{-/-} R1 cultured in ES-DMEM medium were analyzed by ICC for OCT4 (green) and counterstained with propidium iodide (red; nuclei); scale bar corresponds to 50 nm. Images were acquired in a confocal microscope (C). Total protein extracts of R1 and Sirt1^{-/-} R1 were analyzed by western blot for OCT4 and loading control β-actin protein levels (D). Relative OCT4 expression normalized to R1 levels is represented in (E). The values represent means ± SEM of three independent experiments; * $p < 0.05$.

Neuronal differentiation potential of R1 and Sirt1^{-/-} R1 mESC

OCT4 protein expression seems to be different between cell lines. Since OCT4 levels must be tightly regulated by ESC in order to maintain their pluripotency and/or properly differentiate, our next goal was to understand if SIRT1 absence could impact proper mESC differentiation into neuronal lineages using an established protocol for directed differentiation through a monolayer differentiation protocol with N2B27 medium (Pereira et al., 2013). After 7-8 days in differentiation conditions, neuronal projections from differentiating R1 cells were already visible, clearly evident at day 14 of differentiation (Fig. 2.3A). However, neuronal projections were not found in differentiating Sirt1^{-/-} R1 cells, even at day 14 of differentiation (Fig. 2.3A).

Thus, we next evaluated by the expression of neuroectodermal lineage markers (Pax6 and Nestin) and neuronal cell marker (beta-III tubulin) by qPCR. Apparently, Sirt1^{-/-} R1 cells express lower mRNA levels for the analyzed neuroectoderm markers, suggesting that Sirt1^{-/-} R1 cells have a lower differentiation potential into this lineage than their WT cells. Thus, we speculate that SIRT1 can be involved in the neuronal differentiation potential of mESC.

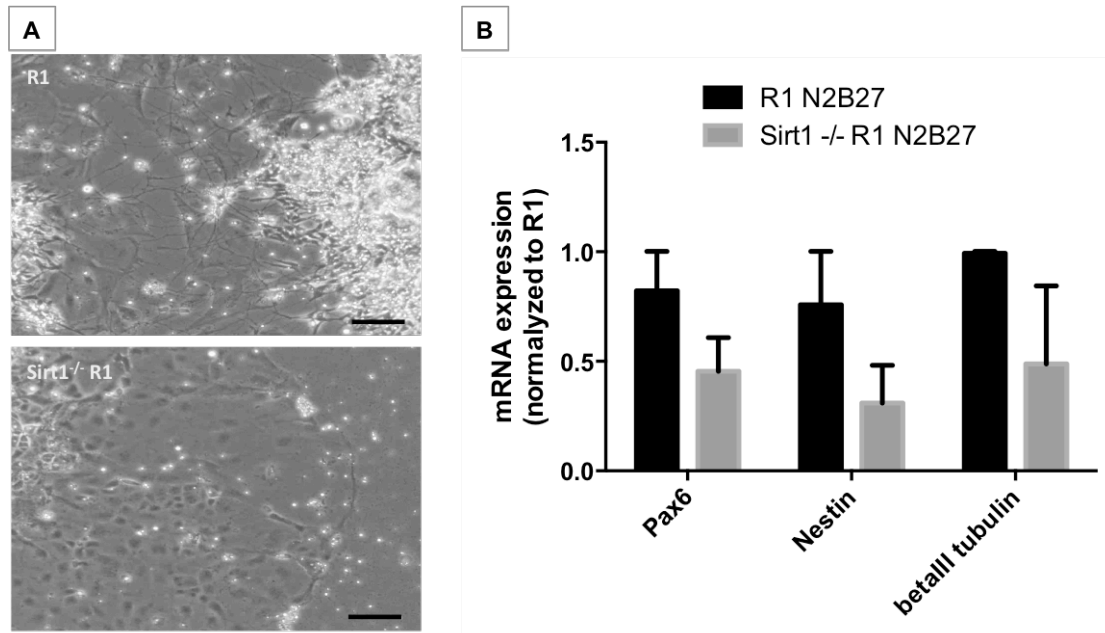


Figure 2.3 – Monolayer directed-neuroectodermal differentiation of R1 and Sirt1^{-/-} mESC.

N2B27 neuronal directed differentiation technique was used to differentiate R1 and Sirt1^{-/-} R1. The represented pictures are representative of the cultures at day 14 of differentiation. Scale bar represents 100 μ m (A). mRNA levels for Pax6, Nestin and β III-tubulin were evaluated by qPCR. Graph data is normalized for R1 mRNA levels (B). The values represent means \pm SEM of two independent experiments

Mitochondrial implications in R1 and Sirt1^{-/-} mESC

Our group recently showed that cell metabolic status during directed neuronal differentiation is important for the proper differentiation capacity of mESC E14Tg2.a and R1 cell lines (Pereira et al., 2013). Indeed, OXPHOS inhibition via the specific inhibitor Antimycin A was able to significantly decrease the differentiation efficiency of these mESC into dopaminergic neurons (Pereira et al., 2013).

Given that we observed a decreased differentiation potential of *Sirt1*^{-/-} R1 cells, we therefore checked the metabolic and mitochondrial status of R1 and *Sirt1*^{-/-} R1 mESC to infer if SIRT1 can act through metabolic modulation. Indirect measure of mitochondrial mass using the Mitotracker green fluorescent dye (Fig. 2.4A) and mitochondrial superoxide anion reactive oxygen species evaluation using the Mitosox Red dye (Fig. 2.4B) showed no differences between both cell lines for mitochondrial mass and production of mitochondrial ROS superoxide anion. However, the mitochondrial membrane potential sensitive dye TMRM (Fig. 2.4C) indicated that *Sirt1*^{-/-} R1 stained less than their WT counterparts, as observed by epifluorescent microscopy. In order to quantify TMRM staining intensity, cells were detached and analyzed by flow cytometry. GeoMean analysis of TMRM intensity showed a tendency of decreased TMRM staining in *Sirt1*^{-/-} R1 cells ($p = 0.0645$; Fig. 2.4D).

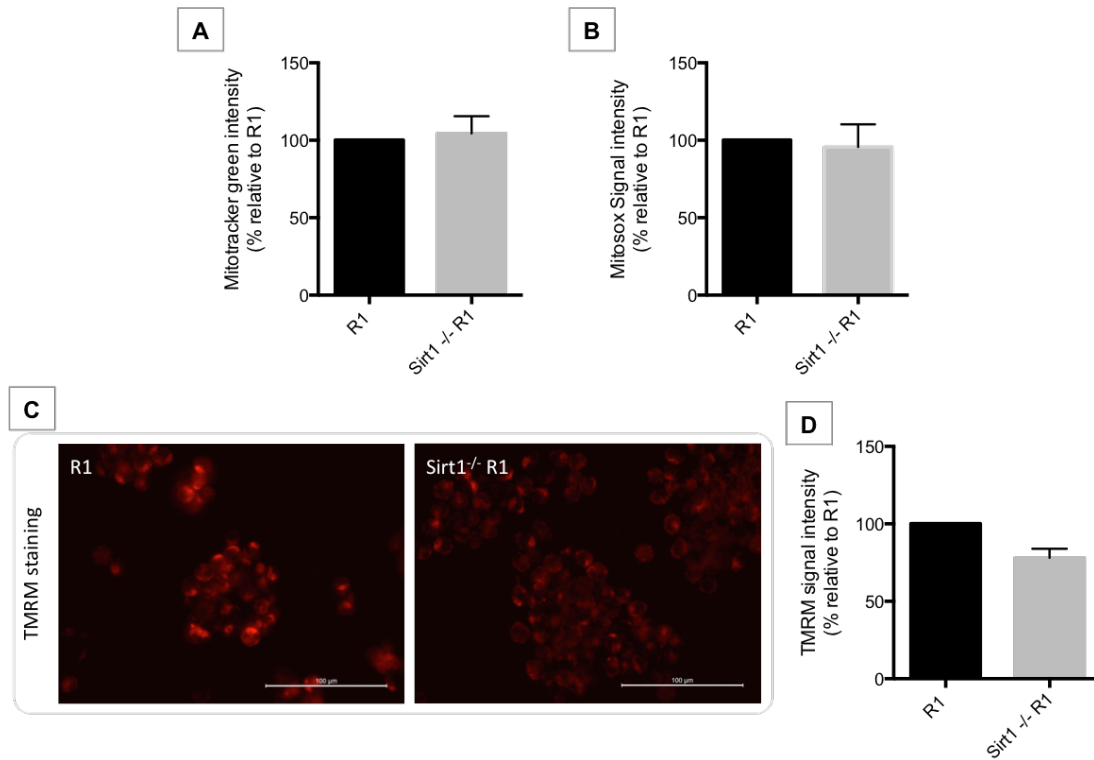


Figure 2.4 – Mitochondrial parameter of R1 and Sirt1^{-/-} R1 mESC.

Mitochondrial mass (A) and mitochondrial superoxide anion (B) levels were evaluated by flow cytometry using Mitotracker green (A) and MitoSox (B) dyes. Mitochondrial membrane potential (MMP) was evaluated by tetramethylrhodamine, methyl ester, perchlorate (TMRM) dye, by fluorescence microscopy (C) and flow cytometry – Geomean quantification (D) were used to evaluate MMP. Scale bar represents 100 μm. GeoMean were used for quantification purposes of flow cytometry data. Graph data is normalized for R1 mRNA levels. The values represent means ± SEM of three independent experiments

Despite these observations, proper mitochondrial function should be addressed by cellular oxygen consumption, as a direct measure for OXPHOS activity. Thus, we took advantage of the Seahorse technology to precisely and quantitatively evaluate oxygen consumption of both cell lines, and consequently infer about their mitochondrial respiration. This instrument allows us to quantify the rates as pmol of O₂ changes with time [oxygen consumption rates (OCR)], as a measure for mitochondrial oxidative metabolism. At the same time the rate of changes in pH of cultured cells is

also measured [extracellular acidification rates (ECAR)], which is an indirect measure for glycolytic activity, due to lactate production.

As can be seen in figure 2.5A, basal OCR (measurements performed in basal conditions before oligomycin injection) were higher in R1 cells when compared to Sirt1^{-/-} R1 cells ($p < 0.05$; Fig. 2.5A and 2.5B). Oligomycin injection, which inhibits ATP synthase, decreases OCR, indicating that mitochondrial respiration is in fact used for ATP production (Brand and Nicholls, 2011). The ATP production related respiration is measured as the difference between respiration rates after oligomycin injection and basal respiration rates. As R1 cells present higher basal OCR, they also use higher mitochondrial respiration for ATP production purposes than Sirt1 knockout R1 cells ($p < 0.05$; Fig. 2.5C). FCCP is a mitochondrial uncoupler that dissipates the mitochondrial proton gradient and consequently the MMP, forcing the mitochondria to reach their maximal electron transport and oxygen consumption (Brown et al., 1990). After FCCP injection SIRT1^{-/-} R1 cells present a significant reduced maximal respiration capacity than R1 cells ($p < 0.05$; Fig. 2.5D). However, the basal OCR for both cells is similar to their maximal respiration capacity, showing that both cells are consuming oxygen near their maximal capacity (Fig. 2.5E), which is a feature of embryonic stem cells (Zhang et al., 2011). Finally, rotenone and antimycin A, complex I and III blockers, respectively, block electron transport across the ETC and inhibit the OCR that is related to mitochondrial respiration (Abe et al., 2010; Dott et al., 2014). Indeed, injection of these two compounds inhibited OCR of both cell lines to residual levels (Fig. 2.5A) demonstrating the specificity of the assay for mitochondrial oxygen consumption in relation to other sources of oxygen consumption within the cells.

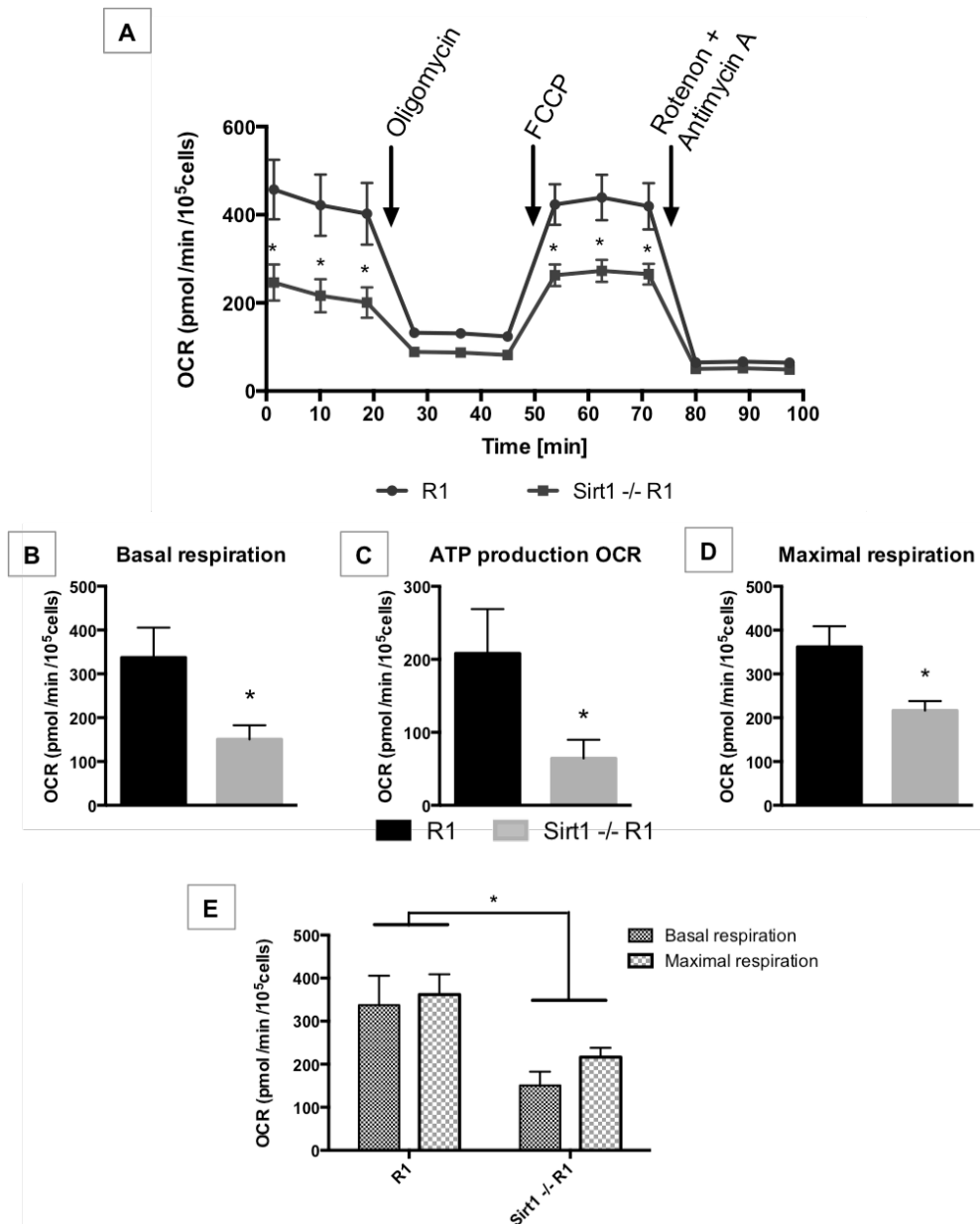


Figure 2.5 – Mitochondrial metabolism of Sirt1^{-/-} R1 is reduced relative to R1 mESC.

mESC were incubated with basal assay medium for 1 hour prior to OCR measurements. Three measures of OCR were performed before and after each drug injection (A). Basal OCR (B), OCR related to ATP production (C) and maximal respiration (D) are represented as the mean values of all three corresponding measurements. Basal OCR and Maximal respiration are represented for each cell line for comparative purposes (E). Used concentration of compounds: oligomycin (1 μ M), FCCP (1,25 μ M), rotenon (1 μ M) and antimycin A (1 μ M). The values represent means \pm SEM of five independent experiments; * $p < 0.05$.

Glycolytic capacity is increased in Sirt1^{-/-} R1 mESC

Given that Sirt1^{-/-} R1 have lower mitochondrial metabolic activity, we aimed to check if this could be, at least in part, compensated with increased glycolysis, as an alternative way for energy production in order to meet cellular energy demands. Thus, we measured the extracellular acidification rates of both cell lines, which is an indirect measure for glycolysis, assuming that the lactate acid produced from glycolysis can act as an acidifying agent (Wu et al., 2007; Xie et al., 2009).

After a starving period of 1 hour without CO₂, 10mM glucose was added to the cells and ECAR measured. Glucose injection induces a fast glycolytic rate, generating lactic acid, which is released and changes extracellular pH (Wu et al., 2007). Glucose injection increased ECAR in both cell lines as expected (Fig. 2.6A). However, this glycolysis measurement was significantly higher in Sirt1^{-/-} R1 cells than R1 cells ($p < 0.05$; Fig. 2.6A and 2.6B) demonstrating that glycolytic activity is higher in the cells that have a lower OCR capacity. Regarding ECAR, oligomycin inhibits ATP synthase forcing cells to use glycolysis as an alternative source for ATP production, allowing the quantification of maximal glycolytic capacity (Brand and Nicholls, 2011). Thus, oligomycin injection increased ECAR for both cells. Although Sirt1^{-/-} R1 cells present a slightly higher glycolytic capacity, the difference between both cells is not statistically significant ($p = 0.1776$; Fig. 2.6C). 2-deoxy-glucose (2DG) is a glucose analog that binds and inhibits Hexokinase, thus inhibiting glycolysis (Brown, 1962). 2DG injection decreased ECAR to residual levels for both cells demonstrating the specificity of the measured results for glycolysis rather than other metabolic processes that could change extracellular pH.

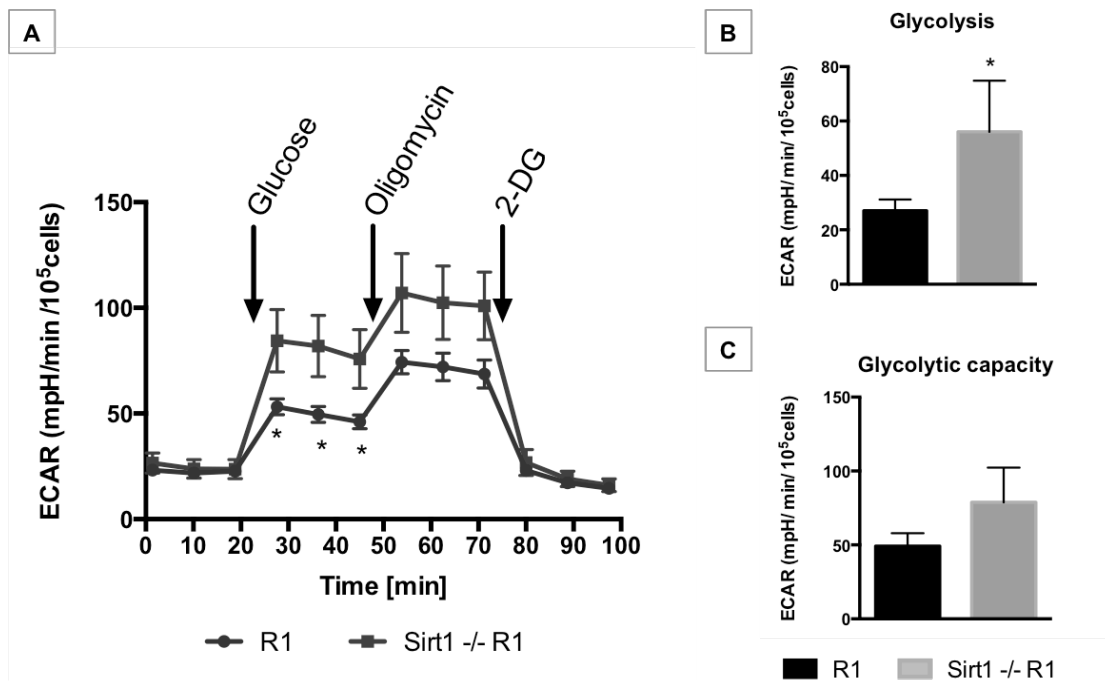


Figure 2.6 – Glycolytic metabolism of R1 and Sirt1^{-/-} R1 mESC.

mESC were incubated with basal assay medium without glucose and pyruvate for 1 hour prior ECAR measurements. Three measures of ECAR were performed before and after each drug injection (A). Glycolysis activity (B) and glycolytic capacity (C) are represented as the mean values of all three corresponding measures. Used concentration of compounds: glucose (10mM), oligomycin (1 μ M) and 2DG (100mM). The values represent means \pm SEM of three independent experiments; * $p < 0.05$.

Could Galactose-mediated OXPHOS reinforcement modulate pluripotency maintenance?

MEF cells are used as feeder-cell layer as an adjuvant of LIF to maintain cells pluripotent in culture. The R1 cell line is commonly kept in culture in gelatin-coated dishes without MEFs (Pereira et al., 2013). Indeed, we plated our R1 cells in these culture conditions and pluripotency-associated morphology was observed for these cells (Fig. 2.7A) for, at least, 3 passages. However, Sirt1^{-/-} R1 cells are difficult to maintain in a pluripotent state under those conditions (Fig. 2.7B), and tend to lose

the typical pluripotent colony shape, individual cells start to be distinguishable within the colonies and periphery cells tend to emit filopodia (Fig. 2.7A), suggesting an initial commitment for differentiation.

Our results showed that R1 cells differentiate more efficiently into neuronal lineages rather than *Sirt1*^{-/-} R1 cells, which can be related to their increased mitochondrial metabolism and decreased glycolysis relatively to their SIRT1 knockout counterparts found under pluripotent conditions. Moreover, under differentiation conditions, WT mESC could select the best substrate for metabolic and cellular requirements under these stringent conditions, while *Sirt1*^{-/-} R1 do not.

Glucose absence and galactose-supplemented culture medium have been used as a strategy to force cells to use oxidative phosphorylation for ATP production (Marroquin et al., 2007; Rossignol et al., 2004; Vega-Naredo et al., 2014). Thus, in a preliminary study, we applied this culture medium strategy for both cells when kept in culture without the feeder cell layer. From a morphological point of view, *Sirt1*^{-/-} R1 cells tend to retain the pluripotent appearance when kept in galactose-supplemented medium than the above-described glucose-supplemented medium (Fig. 2.7A). R1 cells apparently do not show major morphological differences in glucose- or galactose-supplemented medium (Fig. 2.7A).

We also evaluated the mitochondrial respiration in those conditions to check if the hypothesized galactose-related OCR increase occurs in our mESC and to check if major differences are observed for WT *versus* *Sirt1*^{-/-} R1 mESC. Indeed we confirmed an increase in OCR for both cell lines as expected with galactose supplementation (Fig. 2.7B). However, a surprising result was observed. While OCR of R1 in galactose-supplemented medium was highly increased, *Sirt1*^{-/-} R1 galactose supplemented OCR was increased to levels similar to R1 glucose-OCR (Fig. 2.7B). Although interesting, more experiments must be conducted to confirm this data.

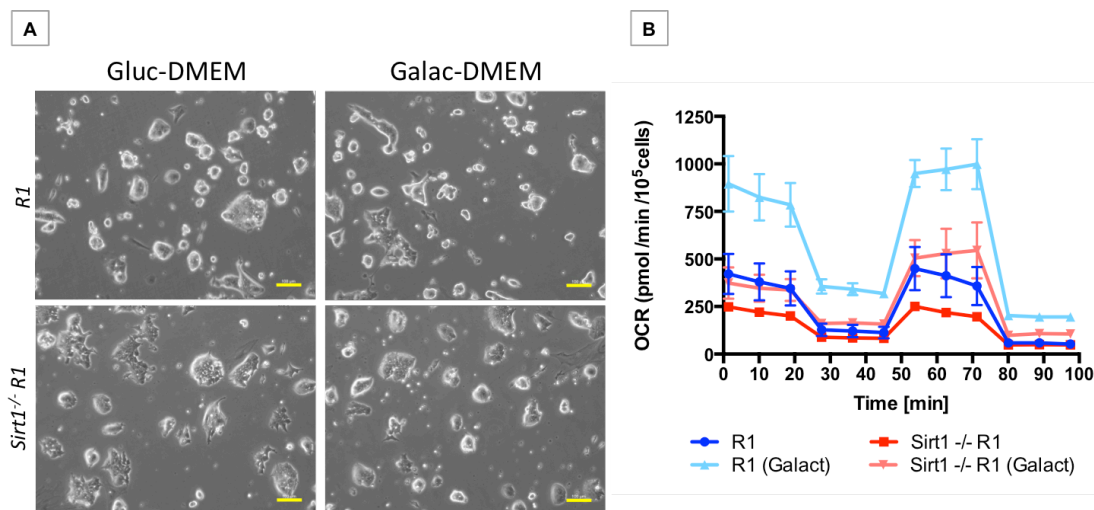


Figure 2.7 – Effects of galactose-supplemented medium on mESC morphology and respiration.

R1 and Sirt1^{-/-} R1 mESC were plated in gelatin-coated dishes and maintained in glucose-supplemented (gluc-DMEM) or galactose-supplemented medium (galac-DMEM). Pictures were acquired after 2 days in culture in the presence of LIF (A). Scale bar represents 100 μm. R1 and Sirt1^{-/-} R1 mESC cultured in gelatin-coated dishes were passaged and plated in 24-well XF24 cell culture plate 12 hours prior the assay. 1 hour before the assay, cells were incubated with basal assay medium for 1 hour. Three measures of OCR were performed before and after each drug injection (B).

STAT3 mRNA levels are downregulated in Sirt1^{-/-} mESC

In order to disclose possible genes involved in glucose metabolism, which expression could be altered, we used the Glucose mRNA array (SABiosciences) custom designed by our group (Rodrigues et al., 2015a), which comprise the evaluation of 12 genes that are related to glucose metabolism. From the twelve genes studied, only Stat3 gene is downregulated in Sirt1^{-/-} R1 mESC.

Interestingly, metabolic modulation mediated by SIRT1 – STAT3 in differentiated cells were reported recently. These metabolic alterations were accompanied by alteration of STAT3 levels, in an NF- κ B deacetylation dependent manner (Bernier et al., 2011).

In order to avoid possible false positives from the array and confirm Stat3 regulation, we evaluated Stat3 mRNA expression by qPCR. Similar results were observed, i.e. Stat3 gene expression is, in fact, downregulated in *Sirt1*^{-/-} R1 mESC compared to WT cells (Fig. 2.8B).

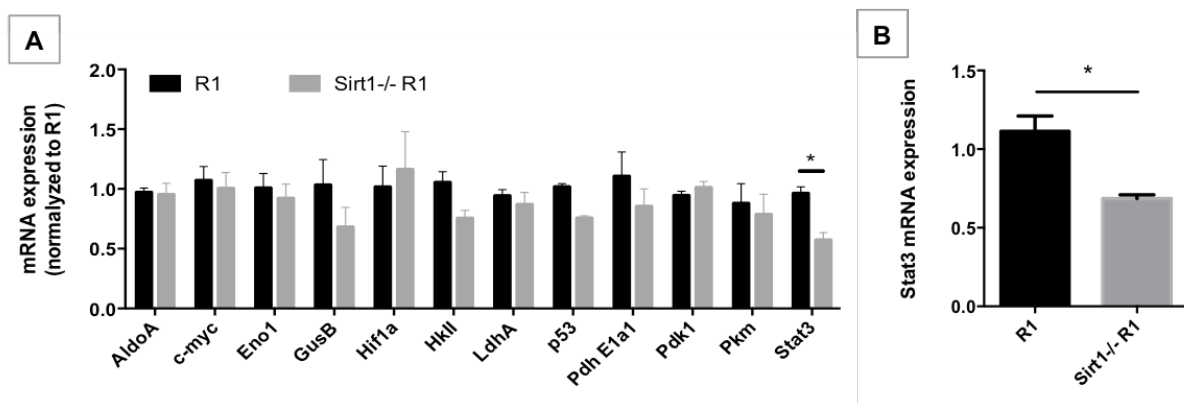


Figure 2.8 – Evaluation of metabolic-related gene expression in *Sirt1*^{-/-} R1 mESC.

Normalized expression for various genes using the $-\Delta\Delta C_t$ method relative to the R1 mESCs (A). mRNA levels for Stat3 were evaluated by qPCR. Graph data is normalized for R1 mRNA levels (B). The values represent means \pm SEM of four independent experiments. * $p < 0.05$

Discussion

Sirtuins are class III histone deacetylases that gained a particular interest in the last few years by their possible role in an increase in longevity and ageing-related mechanisms (Kaeberlein et al., 1999), which can be somehow related with metabolism and autophagy regulation (Houtkooper et al., 2012; Ou et al., 2014). Sirtuin 1, the most studied sirtuin, is mainly located into the nucleus where it is involved in histone deacetylation reactions thus regulating gene expression (Haigis and Sinclair, 2010). Moreover, histones are not the only Sirtuin 1 targets; other targets related to other functions were identified. PGC-1 α and FOXO1 are examples, related with mitochondrial biogenesis and function (Motta et al., 2004; Nemoto et al., 2005).

Sirtuin 1, as a regulator of ageing, is also required for stem cell self-renewal and proper differentiation (Rodriguez et al., 2013). Focusing on embryonic stem cells, Sirtuin 1 was shown to be involved in autophagy (Ou et al., 2014), oxidative stress (Chae and Broxmeyer, 2011; Han et al., 2008) and possible regulation of pluripotency (Han et al., 2008).

Here, we observed that Sirtuin 1 could affect mESC differentiation in neuronal cells, which could in part be due to the regulation of mitochondrial and glycolytic metabolism.

For this purpose we took advantage of an established Sirtuin 1 knockout mouse embryonic stem cell line R1 (McBurney et al., 2003a). After confirmation of SIRT1 absence, both cells similarly express the pluripotency markers Nanog and Oct4 at mRNA level, but OCT4 was increased at protein level in Sirt1^{-/-} R1 cells relative to R1. Moreover, when cells were submitted to a differentiation protocol

directing for neuronal enriched populations, cells lacking SIRT1 showed a lower differentiation potential. OCT4 levels must be tightly regulated for proper differentiation (Niwa et al., 2000), as increased or decreased OCT4 can bias differentiation into mesoderm or ectoderm, respectively, which is related with the inverse expression of SOX2 (Masui et al., 2007). Interestingly, SOX2 was already shown as being deacetylated and thereby stabilized by SIRT1 in bone marrow-derived mesenchymal stem cells (Yoon et al., 2014). Although we did not check SOX2 expression, decreased neuronal differentiation together with elevated OCT4 levels, suggests that SIRT1 can modulate differentiation potential of mESC by modulation of pluripotency factors. More studies must be conducted to confirm if SOX2 may also be a linker between Sirtuin 1 and differentiation bias.

Our group recently showed that an active mitochondrial metabolism is required for an efficient neuronal differentiation (Pereira et al., 2013). Direct influence of SIRT1 in the metabolism of embryonic stem cells however has not yet been reported. Thus, we checked for a possible relation between Sirtuin 1 and mESC metabolism. Although we did not see any difference in terms of mitochondrial mass and mitochondrial ROS production, a tendency for decreased MMP was observed for *Sirt1*^{-/-} R1 mESC. Furthermore, we observed that lack of SIRT1 decreased relative respiration and increases the glycolytic capacities of mESC when compared to the WT cells, thus pointing towards a “more pluripotent” metabolic phenotype following *Sirt1* deletion. A difference in mitochondrial mass would be expected once SIRT1 can modulate the action of some transcription factors related to mitochondrial biogenesis (PGC-1 α and FOXO1) (Motta et al., 2004; Nemoto et al., 2005). Nonetheless, cellular metabolic function is altered in these cells, suggesting that in mESC SIRT1 may be modulating proteins more related with metabolic function rather than mitochondrial biogenesis. To note, ESC present few and undeveloped mitochondria (Folmes et al., 2011; Varum et al., 2011), thus SIRT1 modulation of transcriptions factors related with mitochondrial biogenesis could be ineffective.

In an attempt to force mitochondrial metabolism, we used glucose-free, galactose-supplemented medium to culture R1 and *Sirt1*^{-/-} R1 mESC without the feeder-cell layer. Although preliminary, our data suggest that forced mitochondrial metabolism with galactose-supplemented (glucose-free) medium can in fact modulate SIRT1 effects on maintenance of mESC in pluripotency culture conditions. More work is being done to confirm this data and understand its implications for mESC. If these first clues were proven to be true, then forced mitochondrial metabolism through galactose-supplemented medium could be applied into neuronal differentiations on WT and KO mESC, allowing to truly unveiling if metabolic modulation by SIRT1 is indeed the one master regulator controlling the neuronal differentiation potential.

Nonetheless, we took advantage of a glycolytic mRNA array (SAbiosciences) custom designed by our group (Rodrigues et al., 2015a) to dissect possible genes that could be on basis for the reported metabolic changes. Interestingly, only STAT3 gene was significantly downregulated, which was confirmed by qPCR. SIRT1 implication in mitochondrial metabolism of MEFs modulated by NF- κ B and STAT3 was already reported (Bernier et al., 2011). STAT3 is the downstream regulator of metabolism, which seems not to be related to its canonical function as nuclear transcription factor, but rather to its role in mitochondria (Wegrzyn et al., 2009). *Per se*, different STAT3 post-translational modifications were already shown to be necessary for the regulation of pluripotency (Huang et al., 2014). Nevertheless, our results show the opposite of what was obtained by others in terms of SIRT1 effects on mitochondrial respiration (Bernier et al., 2011). This difference could be explained by the opposite “potency” of those cells relative to the ones used by us. While their study was conducted in well-differentiated cells, our study focus in the pluripotency of R1 cells. Different pathways and machinery may be used for metabolism regulation, and thus, lack of SIRT1 can differently affect those pathways in these different “potency” conditions.

Moreover, more studies may be conducted to disclose the role of SIRT1 in metabolic regulation. Above all, rescue experiments are currently being performed to confirm the importance of SIRT1 and

exclude other factors that can be modulating metabolism. Additionally, the in-between pieces of the puzzle from SIRT1 till metabolism should be characterized. STAT3 seem to be a candidate target, but others may also be involved in this process.

Conclusion

In our study, we intended to unveil possible role of Sirtuin 1 in embryonic stem cell pluripotency and differentiation potential.

Overall, our data suggest that deletion of Sirt1 decreases neuronal differentiation efficiency of mESC, which seems to be correlated with exacerbated metabolic characteristics of pluripotent cells, including a decrease in mitochondrial OXPHOS function and an increase in glycolytic metabolism that may hamper differentiation to more mitochondrial active cells, such as neurons. Although more studies must be conducted to understand the basis for such metabolic perturbations, STAT3 appeared as a potential target for such modulation, once its expression is downregulated in cells lacking Sirt1.

***Distinct concentrations of kaempferol
distinctly modulate murine embryonic
stem cell function***

Abstract

Kaempferol (3,4',5,7-tetrahydroxyflavone) is a natural flavonoid with several beneficial and protective effects. It has been demonstrated that kaempferol has anticancer properties, particularly due to its effects on proliferation, apoptosis and the cell cycle but possible effects on pluripotent embryonic stem cell function was not yet addressed. Embryonic stem cells have the ability to self-renew and to differentiate into all three germ layers with potential applications in regenerative medicine and *in vitro* toxicology. We show that exposure of murine embryonic stem cells (mESC) to high concentrations of kaempferol (200 μ M) leads to decreased cell numbers, although the resulting smaller cell colonies remain pluripotent. However, lower concentrations of this compound (20 μ M) increase mESC pluripotency markers expression. Mitochondrial membrane potential and mitochondrial mass are not affected, but a dose-dependent apoptosis increase takes place, probably due to an increase in mitochondrial reactive oxygen species (ROS). Moreover, mESC differentiation is impaired by kaempferol, which was not related to apoptosis induction. Altogether our results show that low concentrations of kaempferol can be beneficial for pluripotency, but inhibit proper differentiation of mESCs. Additionally, high concentrations induce apoptosis, which could be related to increased mitochondrial ROS.

Keywords

Kaempferol; stem cell; natural flavonoids; mitochondria; ROS; apoptosis.

Introduction

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are characterized by their unlimited self-renewal potential and pluripotency. ESCs are derived from the inner cell mass (ICM) of the pre-implantation blastocyst (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Mouse embryonic stem cells (mESCs) are derived from the inner cell mass of blastocysts and commonly maintained in cell culture under normoxia conditions with medium supplemented with leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988) and serum containing bone morphogenic proteins (BMPs) (Ying et al., 2003), required to support and maintain cell culture pluripotency. These signaling pathways are able to induce gene expression of the core pluripotency genes *Nanog*, *Oct4* and *Sox2*, and also contribute for the maintenance of self-renewal (Chen et al., 2008). Another interesting feature of ESCs is their resemblance to some types of cancer cells considering growth capacity, membrane surface markers, activity of some signaling pathways (e.g. *Jak/STAT3*, *PI3K/Akt*, *WNT/GSK3* (Pereira et al., 2014)) and their metabolism (Folmes et al., 2011; Pereira et al., 2014; Varum et al., 2011). When comparing metabolic characteristics, it is interesting to note that both cells rely mostly on glycolysis rather than oxidative phosphorylation for ATP generation, and this is related to the production of building blocks required for self-renewal (Pereira et al., 2014; Varum et al., 2011). More recently, iPSCs were generated by reprogramming of somatic cells to pluripotency so that they resemble embryonic stem. iPSCs represent a huge promise for

regenerative medicine given that in theory patient-derived cells could be derived, differentiated and used to avoid rejection issues, besides overcoming the major ethical concerns regarding embryonic stem cells (Carvalho and Ramalho-Santos, 2013; Ramalho-Santos, 2011).

Flavonoids are compounds widely present in plants (Barros et al., 2012), which have a wide range of beneficial properties (Calderon-Montano et al., 2011; Toh et al., 2013; Yao et al., 2004), namely due to their antioxidant capacity (Crespo et al., 2008; Zhang et al., 2014a) that can potentiate cardiovascular protection (Toh et al., 2013; Weseler et al., 2011) and be harnessed in an anticancer capacity (Kandaswami et al., 2005; Sak, 2014; Sak and Everaus, 2015), among other potential uses. Kaempferol (3,4',5,7-tetrahydroxyflavone) is a natural flavonoid widely present in numerous edible plants which are present in a regular diet, such as broccoli, onions, green tea, pumpkin, strawberry and others (Calderon-Montano et al., 2011; Miean and Mohamed, 2001). It has been described that kaempferol can have beneficial and/or protective effects against several diseases (Calderon-Montano et al., 2011; Chen and Chen, 2013), such as cancer, due to its ability to induce apoptosis on cancer cells (Kang et al., 2009; Li et al., 2009; Sharma et al., 2007), which can be correlated with its anti-proliferative, cell cycle arrest, caspase activation and pro-oxidative capacity (Chen and Chen, 2013; Huang et al., 2010b; Lee et al., 2014).

Twenty percent of the mitochondrial proteins are believed to have 1 or more possible residues passive of being acetylated (Kim et al., 2006). Although there were not yet discovered none histone acetyl-transferase in mitochondria, sirtuins 3-5 are responsible for deacetylation within that organelle (Huang et al., 2010a; Lombard et al., 2007). Sirtuins 3 is the major mitochondrial deacetylase (Lombard et al., 2007) and has been repeatedly associated with metabolic regulation processes (Cimen et al., 2010; Jing et al., 2013). In cancer cell studies, kaempferol is a potential Sirtuin 3 activator, as a strategy behind its anti-cancer properties (Cimen et al., 2010; Marfe et al., 2009)

The effects of kaempferol in cancer cells have been studied, but there is no information regarding embryonic stem cells. Taking into account the similarities described between cancer and embryonic stem cells (Folmes et al., 2011; Pereira et al., 2014), we aim to evaluate the possible effects of kaempferol on the pluripotency of embryonic stem cells and in their ability to differentiate. In our study we observed that kaempferol potentiates the pluripotent associated markers expression of mESC at low concentrations. However, high concentrations of kaempferol induce apoptosis accompanied with an increase in ROS production. Additionally, sub-lethal concentrations of kaempferol impaired proper differentiation of mESCs.

Material and methods

mESC culture conditions

Mouse embryonic stem cell line E14Tg2a was kindly provided by Dr. Miguel Ramalho-Santos (University of California, San Francisco, USA). Cells were maintained and propagated in complete medium – KODMEM medium composed of KnockOut-DMEM, 15% KnockOut serum replacement, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (Life Technologies), 1% non-essential amino acids, 0.1 mM mercaptoethanol (Sigma-Aldrich) and 1.000 U/mL of Leukemia inhibitory factor (LIF) - (Chemicon – Millipore) at 37°C and 5% CO₂ conditions. All plates were coated with 0.1% gelatin (Sigma) before cell plating.

mESC passage

Before experiments, cells were detached with StemPro Accutase Cell Dissociation Reagent (Gibco, Life Technologies), centrifuged at ~280 x *g*, supernatant was discarded and pelleted cells resuspended in fresh medium. A small fraction of resuspended cells (20 µL) was collected and stained with 20 µL Trypan Blue solution (Sigma) and counted in a hemocytometer. 5,000 cells were plated in gelatin-coated dishes and experiments were then performed. E14Tg2.a mESCs were allowed to grow

for 72 hours in the complete medium with kaempferol supplementation and LIF. Medium and drug supplementation was renewed every day (Fig. 3.1A).

Embryoid body differentiation

mESC differentiation was performed by the embryoid bodies (EBs) suspension protocol according to the Life Technologies protocol, with minor modifications. Briefly, 10^6 mESCs were plated in a 60 mm non-adherent petri dish and maintained using KODMEM medium (without LIF supplementation). Cells were cultured at 37°C in a 5% CO₂ incubator and remained in suspension for three days. Medium was changed every day using the EB sedimentation technique. Briefly, medium-containing EBs was added into a canonical 15mL tube, supernatant medium without cells was removed and fresh medium was add to the cells. After three days, EBs were allowed to adhere in a 100mm tissue-culture petri dishes in KODMEM medium supplemented with 10% fetal bovine serum (FBS – Life Technologies). EBs were incubated overnight in a 37°C, 5% CO₂ incubator. From that day on (day4), EBs were grown with KODMEM medium with no LIF supplementation and kaempferol was added to the medium. Medium was changed every day, with fresh drug supplementation. Pictures were taken from EB culture at days 6 and 12 of differentiation and total protein was extracted at day 14.

Sulforhodamine B

Cell viability was measured by inferring protein content in a 24 well with Sulforhodamine B (Sigma-Aldrich), an indirect measure of cell proliferation according to the manufacturers instructions. Briefly, after 72h in culture cells were washed and fixed for 15 minutes with paraformaldehyde 4% and allowed to dry overnight. A Sulforhodamine B 0.5% solution prepared in 1% acetic acid was added to each well and incubated for 30 minutes. Unincorporated dye was removed by several washes with

1% acetic acid and samples allowed to dry. Incorporated dye was solubilized in 10 mM Tris and stirring in a gyratory shaker for 10 minutes. Absorbance was measured at 565 nm. Blank wells without plated cells were used for background correction.

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay (MTT)

Viability was also measured using the MTT assay. Briefly, after 72h in culture medium was renewed without drug supplementation. MTT (Sigma-Aldrich) was added with a final concentration of 0.5mg/mL and incubated at 37 °C for 4 hours. Dye crystals were then solubilized with acidic isopropanol and absorbance was measured at 570 nm. Wells with medium and without cells were used for background correction.

Flow cytometry analysis for Apoptosis/necrosis, Mitochondrial mass, Mitochondrial membrane potential, Mitochondrial superoxide production and Cell Cycle analysis.

All fluorescent dyes were analyzed by flow cytometry (Becton Dickinson BD FACSCalibur cytometer) and 20 000 gated cells were acquired/analyzed per condition with the Cell Quest Pro Acquisition software (BD Biosciences). Unless for the case of apoptosis and necrosis where percentage of gated cells *per* region were evaluated, geomean of the curve for each condition was used for quantification and statistical analysis

Apoptosis and necrosis were evaluated using annexin V (Immunostep) and propidium iodide (PI; Life Technologies). Cells were harvested, centrifuged and resuspended in Annexin V binding buffer (10mM HEPES/NaOH (pH 7.4) 140mM NaCl, 2,5mM CaCl₂) at 10⁶cells/ml. Cells were incubated with propidium iodide (PI; Life Technologies) and Annexin V (5 mL/mL) in the dark for 15 minutes. Cells

were then washed and resuspended in AnnexinV Binding buffer and analyzed by flow cytometry using appropriate settings.

An indirect measure of cell **mitochondrial mass** was obtained by using MitoTracker® Green FM (Life Technologies), a dye that binds to mitochondria independently of mitochondrial membrane potential. Cells grown in 60 mm dishes were harvested, centrifuged and resuspended at 10^6 cells/mL in PBS1x. 50 nM of dye was added and incubated for 30 minutes at 37 °C in the dark. Dye was washed, cells resuspended in PBS and analyzed by flow cytometry using appropriate settings.

Mitochondrial membrane potential was measured with TMRM (Tetramethylrhodamine methyl ester; Life Technologies). Briefly, cells grown in 60 mm dishes were detached with accutase, centrifuged and resuspended at 10^6 cells/mL in PBS. 20 μ M of TMRM was added and incubated for 20 minutes at 37°C in the dark. Dye was washed out, cells resuspended in PBS1x and analyzed by flow cytometry using appropriate settings.

MitoSox Red (Life Technologies) is a mitochondria-directed dye that allows for detection of **mitochondrial superoxide** anion. After cell harvesting and centrifugation, cells were resuspended in PBS at 10^6 cells/mL. 3 μ M of MitoSox Red was added to the cells and incubated at 37 °C for 30 minutes in dark. Cells were then washed, resuspended in PBS, kept on ice until being analyzed by flow cytometry using appropriate settings.

For **cell cycle analysis**, cells were detached, a wash step was performed and cells were fixed in ice-cold 70% ethanol and kept at -20°C overnight. The next day, after a washing step, fixed cells were treated with 0.1 mg/ml RNase A (Life Technologies) for 15 minutes, and then incubated with 5 μ g/ml PI at room temperature for additional 15 minutes. Dye was washed out, fixed cells resuspended in PBS1x and analyzed by flow cytometry using appropriate settings.

Protein collection and Western Blot

Protein extracts for Western blot were obtained by lysing mESC with RIPA buffer (Sigma-Aldrich) supplemented with 2mM of phenylmethanesulphonyl fluoride-PMSF (Sigma-Aldrich) and 2x Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL). Protein quantification was performed using the Pierce™ BCA (Bicinchoninic Acid) Protein Assay Kit, following the manufacturers' protocol. Both samples and calibration curves were determined in duplicate.

For Western blot analysis 30 µg of protein were diluted in Laemmli sample buffer (Bio-Rad) and water and denatured at 95°C in a dry bath. Thereafter, samples were loaded into a 12% Acrilamide Tris-HCl gel and electrophoresis was performed in a Mini-protean tetra-cell Bio-Rad apparatus. Proteins were then blotted to a PDVF membrane (Bio-Rad). At the end, membranes were washed and 5% powder milk (Bio-Rad) diluted in Tris-Buffered Saline with Tween (TBST) was used for blocking purposes. Overnight incubation at 4°C was used for the following primary antibodies: rabbit anti-SirT3, rabbit anti-Oct4, mouse anti-SSEA1, rabbit anti-caspase 3 (Cell Signaling Technology, 1:500), rabbit anti-acetylated-Lysine, and rabbit anti-cleaved Caspase-3 (Cell Signaling Technology, 1:1000), rabbit anti-Nanog (Abcam 1:500), and mouse anti-β-Actin (Sigma-Aldrich, 1:15000). Membranes were washed with TBST and incubated with the respective HRP-conjugated secondary antibody (Cell Signaling Technology, 1:2000) for 1 hour at room temperature. Clarity Western ECL Substrate (Bio-Rad) was used for protein detection using VersaDoc Imaging system (Bio-Rad). Quantity-One (BioRad) software was used for protein quantification. All results were normalized for the respective β-Actin of the same sample.

Data analysis and statistics

Prism 6 (GraphPad) was used to perform statistical analysis. All data is expressed as mean values \pm standard error of mean (SEM) reflecting at least three separate experiments. ANOVA followed by the Bonferroni post-hoc test was used for multiple comparisons between different cell treatments. Paired-sample statistic was done since all experiments (with different concentrations) were performed and collected at the same time. Statistical analysis was performed on the raw data and, for a comprehensive analysis of the results, data were then normalized to 100% of the control condition. Statistical significance was determined at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Results

mESC growth is affected by kaempferol

Kaempferol is a natural flavonoid that is present in several types of foodstuffs, and that has been suggested as a potential anticancer drug due to its capacity to induce death in cancer cells. When considering that these cells share many metabolic and signaling similarities with embryonic stem cells (ESC), we attempted to address the possible impact of kaempferol on pluripotent embryonic stem cells.

Initially, the kaempferol impact on mESC colony growth was explored. To address this question, E14Tg2.a mESCs were grown for 72 hours in the presence of Kaempferol, added immediately after cell plating. Medium with kaempferol was renewed every 24 hours (Fig. 3.1A). Experimental conditions included the addition of leukemia inhibitory factor (LIF), which is required for maintenance of mouse embryonic stem cells in a pluripotent state. After a 72h incubation time it is possible to observe that mESC grew with spherical 3d colony shape in all conditions, however they tend to be smaller as kaempferol concentrations increased (Fig. 3.1B). Specifically, mESC incubated with 200 μ M presented smaller colonies, resembling control mESC colonies 24h after plating in normal pluripotent growth conditions. However, these cells maintain the pluripotent morphology (dome shaped 3D colonies). Cells were then harvested and counted and, as shown in figure 3.1C, kaempferol 200 μ M leads to a statistically significant reduction in the number of mESC per dish. Similar results were observed by monitoring cellular protein content per dish using sulforhodamine B (SRB). These levels were significantly affected in the presence of 100 and 200 μ M kaempferol

($p < 0.01$ and $p < 0.001$ respectively; Fig. 3.1D). Moreover, we performed a MTT assay and observed that only 200 μM kaempferol significantly reduces mESC viability ($p < 0.05$; Fig. 3.1E), similarly to what was seen for SRB assay. These results suggest that kaempferol has deleterious effects on the growth of mouse embryonic stem cells with rising drug concentrations, leading to decreased cell viability.

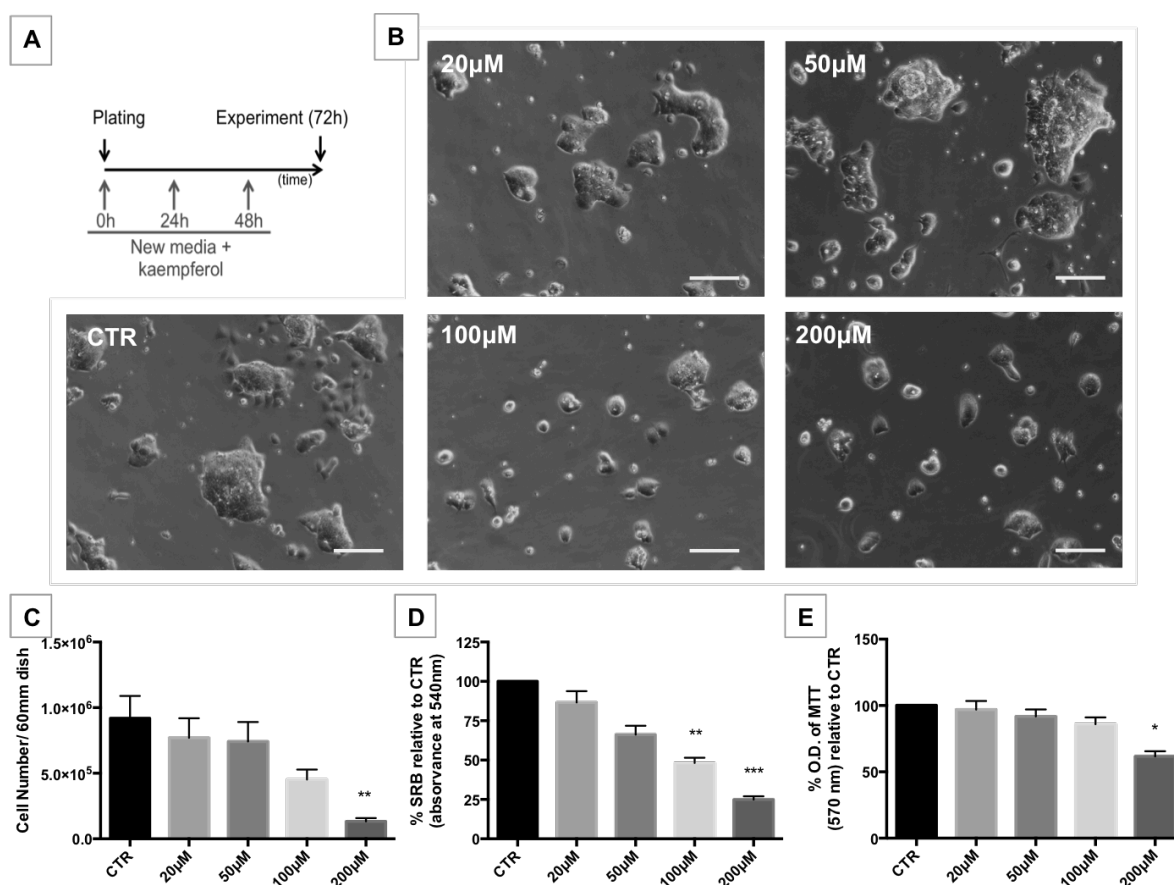


Figure 3.1 - Kaempferol causes a reduction in cell numbers and mESC colony size.

mESC were plated at a density of 5000 cells/cm² and kept in culture for 72h, with medium and kaempferol changed every 24 hours (A). Pictures were acquired from randomly selected fields using phase contrast microscopy at the end of the experimental procedure (100x magnification) (B). Total number of cells per dish at 72h from at least three independent experiments (C). Protein content per dish from three independent experiments was measured by sulforhodamine B and normalized to 100% CTR (D). MTT assay from 4 independent experiments was carried out as described and the percentage of formazan crystals relative to 100% CTR are represented (E). Mean \pm SEM are represented in histograms representations. Scale-bar corresponds to 100 μm . One-way ANOVA with pos-hoc Bonferroni's multiple comparisons statistical analysis was performed. Statistical significance considered when: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Kaempferol does not increase SIRT3 expression nor affect acetylation levels

Our primary goal using kaempferol was trying to modulate SIRT3 expression and activity as was reported previously elsewhere (Cimen et al., 2010; Marfe et al., 2009). Thus, we evaluated SIRT3 expression by WB analysis (Fig. 3.2A). At least in our cells we were apparently not able to induce any alteration in terms of SIRT3 expression. Despite not affecting SIRT3 expression, kaempferol could affect its activity independent of its expression levels. Thus, we evaluated the total acetylated levels of lysine acetylation in cells (Fig. 3.2B), but again no apparent decrease of cellular lysine acetylated levels were observed as an indirect measure for possible increase of SIRT3 activity.

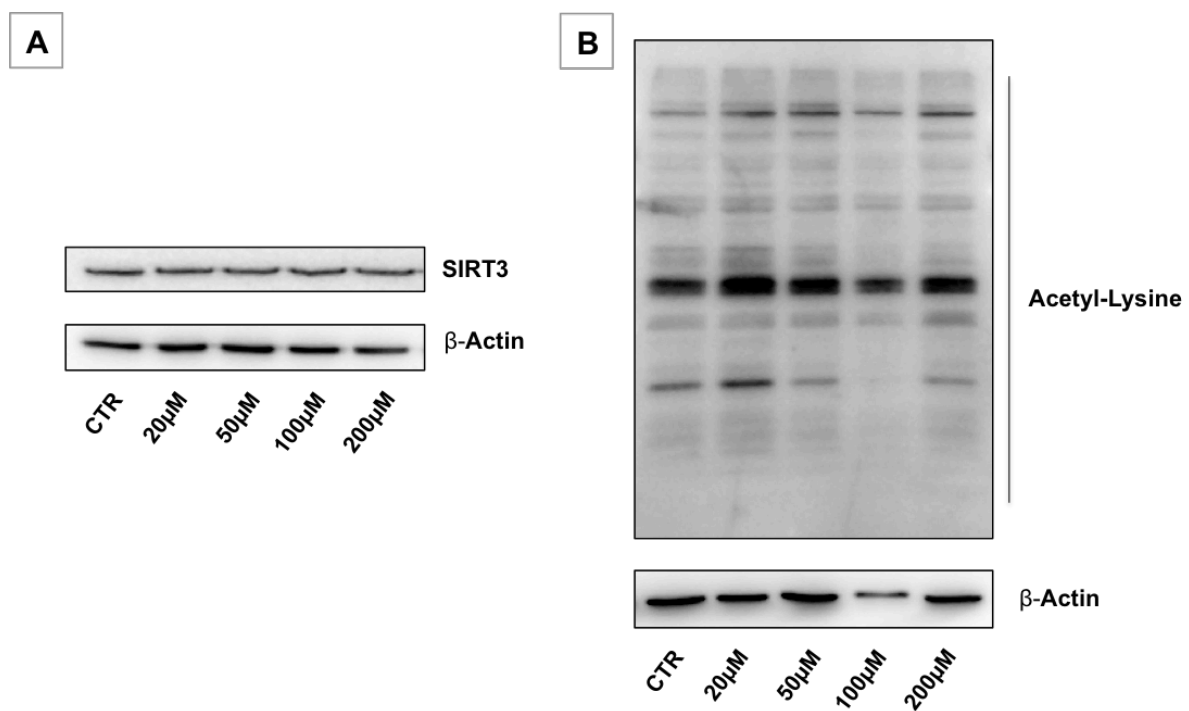


Figure 3.2 - Kaempferol causes a reduction in cell numbers and mESC colony size.

Western blot analysis for SIRT3 (A) and total acetylated proteins (pan-Acetyl-Lysine) (B) and respective loading Actin controls.

Higher kaempferol concentrations induce apoptosis in mESC

Given the reduction in cell number when cells were incubated with higher concentrations of kaempferol, we wondered if this effect could be due to the described effects of the compound in other cell models (Jeong et al., 2009; Lee et al., 2005). In order to determine if cells were undergoing apoptosis and/or necrosis upon drug treatment live mESCs were labeled with Annexin V and PI, allowing for the detection of apoptosis (by externalization of phosphatidylserine) and cell death, respectively. Increased cell death and apoptosis is clearly detected with increasing concentration of kaempferol, namely with an increase in necrotic (PI⁺/AnnV⁻ cells) and apoptotic cells (PI⁻/AnnV⁺ cells) (Fig. 3.3A). It is important to highlight the significant increase in apoptosis (PI⁻/AnnV⁺ cells; $p < 0.05$) for 200 μ M kaempferol (Fig. 3.3B). Comparing results for cell number and viability we wondered of a possible effect of kaempferol in the cell cycle, already shown in other cells (Bestwick et al., 2007; Bestwick et al., 2005). In fact the increase of apoptosis caused by 200 μ M kaempferol is also visible by an increase of the sub-G1 events (which can be used as an indirect indication for apoptosis) (Fig. 3.3C). Moreover, the cell cycle histograms also demonstrate that mESCs treated with 200 μ M kaempferol present higher number of cells in the G2/M phase, which may indicate a cell cycle arrest at this checkpoint (Fig. 3.3C).

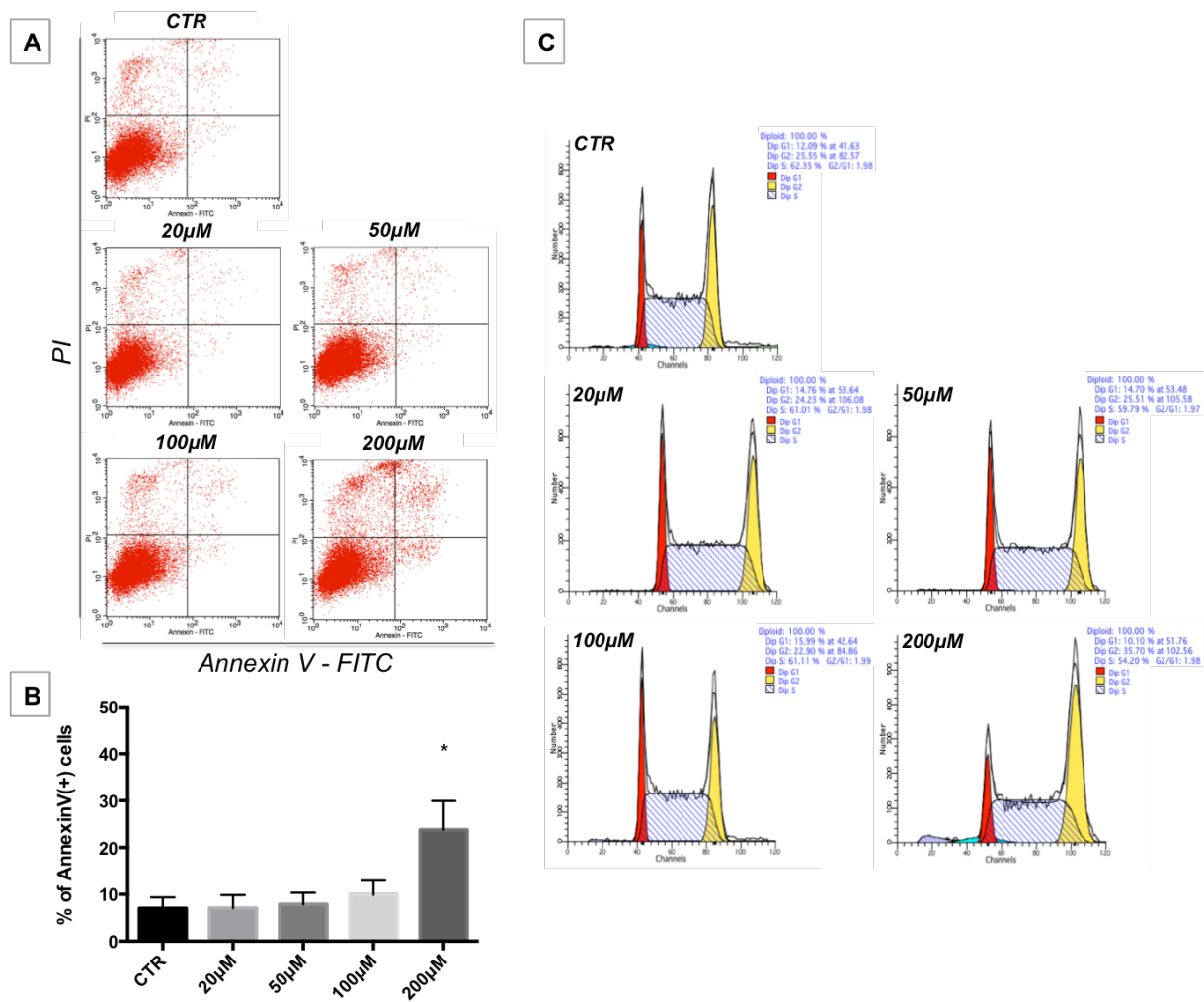


Figure 3.3 - Kaempferol negatively affects mESC viability

Flow cytometry dot plot from necrosis/apoptosis detection via Annexin V and propidium iodide staining after 72h of culture (A). Percentage of Annexin V positive (apoptotic) cells (B). Flow cytometry analysis of the cell cycle using PI staining of fixed cells after each experiment (C). Mean \pm SEM are represented in histograms representations. Statistical significance was considered when * $p < 0.05$.

Mitochondrial activity is not affected, but mitochondrial ROS production increases

Given previous data, we also evaluated changes on the mitochondrial content of mESCs. Although there is an apparent tendency for an increase of mitochondrial mass in response to kaempferol, as observed both by Western Blot for TOM-20 (an outer mitochondrial membrane translocase; Fig. 3.4A and 3.4B) and by flow cytometry using MitoTracker green (Fig. 3.4C), these differences are not statistically significant. In addition mitochondrial membrane potential (MMP) monitored using TMRM was not altered as a consequence of kaempferol treatment (Fig. 3.4D).

We then addressed possible effects of kaempferol on reactive oxygen species levels, particularly superoxide anion, as it was previously reported that kaempferol and other flavonoids could have antitumoral effects at least in part due to an increase in reactive oxygen species production (Sharma et al., 2007). Therefore, mitochondrial production of superoxide was measured by flow cytometry using the MitoSox Red dye. As seen in figures 3.4E and 3.4F, only 200 μ M kaempferol significantly increases the amount of mitochondrial superoxide ($p < 0.05$), which could therefore be one of the mechanisms mediating the higher apoptosis detected under this very same treatment condition.

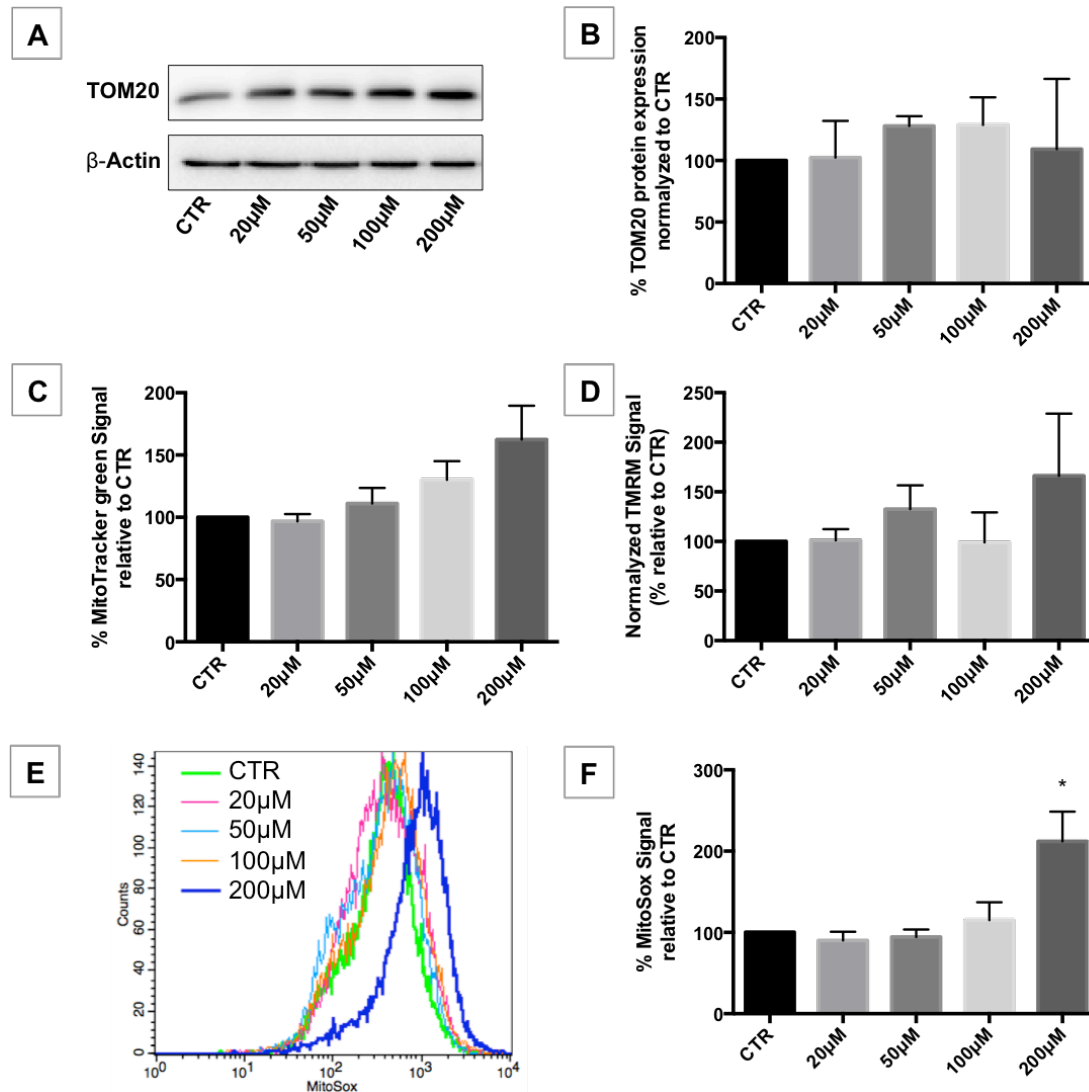


Figure 3.4 - Kaempferol induces mitochondrial ROS production

Western blot analysis was used to indirectly monitor mitochondrial mass by measuring TOM-20 expression and loading control Actin (A). Normalized data to the control is represented in (B). Mitochondrial content and mitochondrial membrane potential were evaluated by flow cytometry of live cells stained with Mitotracker green (C) and TMRM (D), respectively. Mitochondrial superoxide anion production was measured by flow cytometry using MitoSox Red dye in live cells (E). Superoxide anion ROS staining quantification relative to the control is also shown (F). Three independent experiments were performed. Mean \pm SEM are represented in histograms. Statistical significance considered when * $p < 0.05$.

Kaempferol positively affects mESC pluripotency markers at low concentration

Although kaempferol negatively affects mESC colony size and this effect is due to induced apoptosis after 72 hours with higher kaempferol concentrations, low kaempferol concentrations do not affect mESC viability, and may affect ESC pluripotency. Gross morphological daily analysis of all E14Tg2.a mES cells treated with kaempferol show doom-shape colonies (Fig. 3.1B), commonly accepted as a pluripotent feature. This indirect assessment could mean that cells are not differentiating when compared to control. In order to properly assess putative effects on pluripotency, western blot (Fig. 3.5A) analysis was performed for the pluripotency transcription factors OCT4 and NANOG and also for the membrane surface marker SSEA1. As shown in figure 3.5, although there is a tendency for increase of OCT4 and NANOG expression following incubation in 20, 50 and 100 μ M concentrations of kaempferol, only NANOG expression was significantly increased for 20 μ M (Fig. 3.5B).

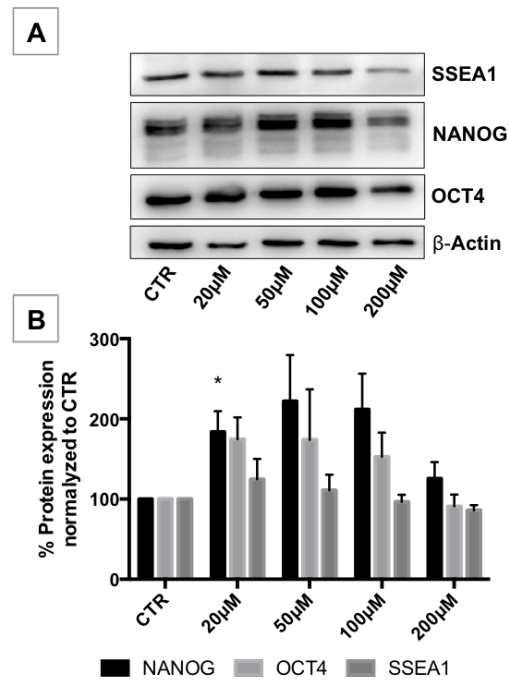


Figure 3.5 - Reduced concentrations of kaempferol positively affect ESC pluripotency.

Western blot analysis for the pluripotency factors OCT4, NANOG, SSEA1 and loading control ACTIN (A) was performed. Data normalized to the loading control is represented in B. Mean \pm SEM are represented in histogram. Statistical significance was considered when * $p < 0.05$.

Kaempferol has deleterious effects on mESC differentiation

Although high concentrations of kaempferol induce apoptosis in mouse pluripotent stem cells and to some extent impact the cell cycle when kept under pluripotency conditions, we aimed to determine if sub-lethal concentrations could affect mESC when cell differentiation is induced. It was shown above that 20 μ M kaempferol increased pluripotency associated markers expression of embryonic stem cells when kept under pluripotent conditions, while 200 μ M caused significant cell death. Thus, we aimed to check if the in-between concentrations 50 and 100 μ M of kaempferol could affect differentiation of mESC using the embryoid body assay. As observed in Fig. 3.6A, embryoid bodies

treated with kaempferol are smaller and have less cells spreading out of the colony compared to control differentiation conditions. This effect starts to be observed at the first days of differentiation, but is clearly evident at latter stages (day 12; Fig 3.6A). Additionally, we checked for the differentiation into the three germ layers (Fig. 3.6B and 3.6C) and both concentrations of kaempferol decreased the expression of a marker of each of the three germ cell layers, namely the expression of the markers α FP (alpha FetoProtein), α SMA (alpha Smooth Muscle Actin) and β III-tubulin (Beta III tubulin), markers for endo, meso and ectoderm, respectively (Fig. 3.6B and 3.6C).

These detrimental effects on differentiation could be related with prolonged exposure to kaempferol. To unveil if apoptosis was induced in EBs differentiation incubated with kaempferol, we checked by WB the expression of the effector caspase 3 and its cleaved form (Fig. 3.6D). No apparent differences were observed (Fig. 3.6E) suggesting that differentiation is being affected by other mechanisms rather than apoptosis.

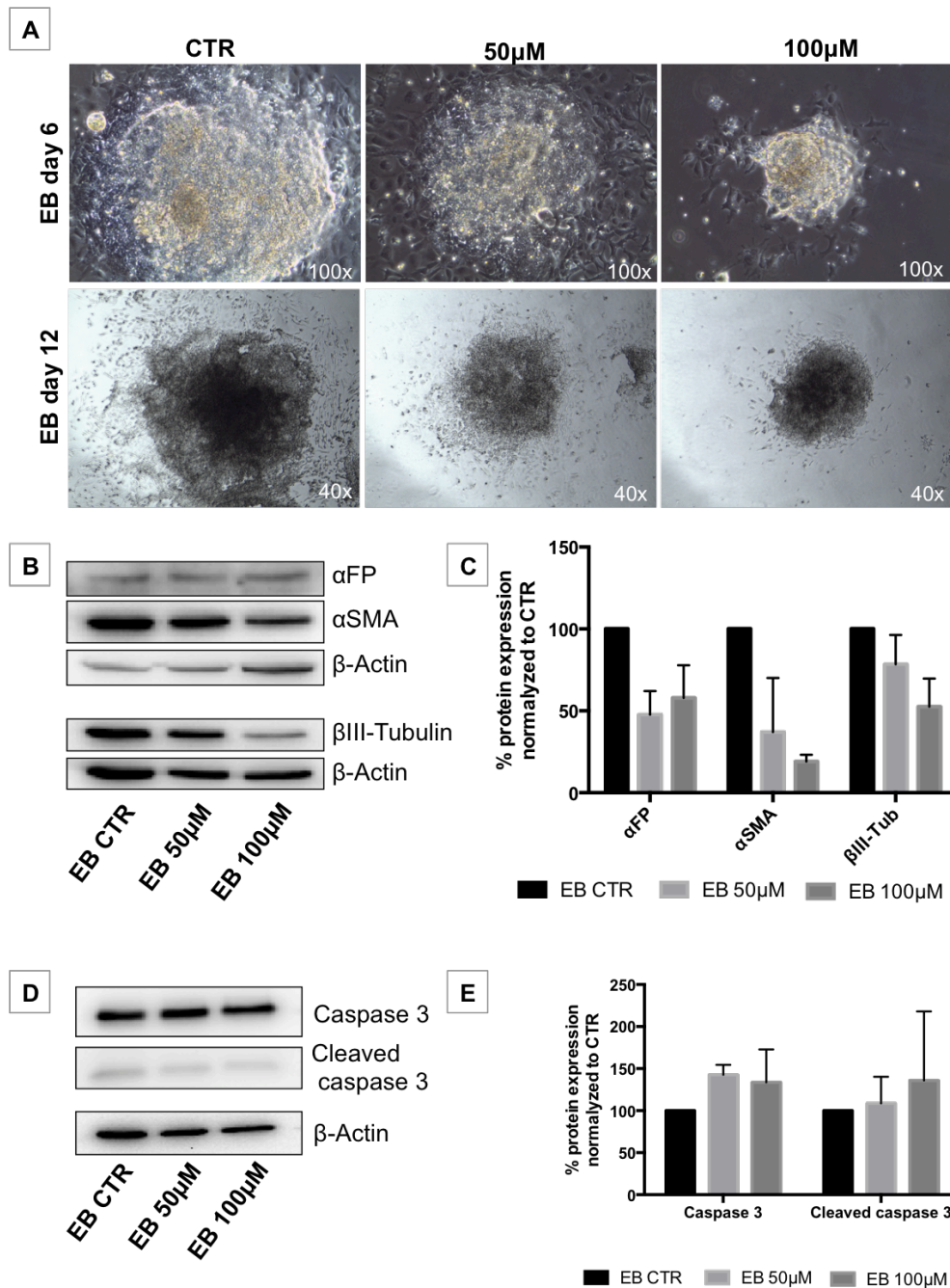


Figure 3.6 - Kaempferol inhibits proper mESC differentiation.

Embryoid bodies (EBs) were generated from mESCs and after adhesion to petri dishes at day 4 of differentiation were incubated in the presence of 50 or 100 μ M kaempferol (A). Western blot analysis (B) was performed for alpha-fetoprotein (α FP), alpha Smooth Muscle Actin (α SMA), beta III Tubulin (β III-Tub) and corresponding loading control beta-actin (β -Actin). Expression of Caspase 3 and its cleaved form were also evaluated by WB (D) and quantified (E). Mean \pm SEM are represented in histogram. Normalized data to 100% of control differentiation condition are represented in C. Magnification is indicated in each image.

Discussion

Kaempferol (3,4',5,7-tetrahydroxyflavone) is a natural flavonoid present in many edible plants. Although many studies focused on its effects on cancer cells, the same approach was not applied to pluripotent stem cells, which share similarities with cancer cells [6, 8, 35]. Additionally, kaempferol was shown to induce activity and expression of Sirtuins3 (Marfe et al., 2009), the major mitochondrial deacetylase (Lombard et al., 2007). However, our results do not corroborate those data, at least in our model of mESC. Therefore the present work aimed to disclose the possible effects of kaempferol in embryonic stem cells.

We show that high concentrations of kaempferol negatively affect mESC viability and growth as monitored both by simple microscope visualization, cell count and SRB protein content. Interestingly, it has already been shown that cancer cells behave similarly in the presence of kaempferol (Bestwick et al., 2007; Dang et al., 2014; Nguyen et al., 2003).

The inhibition of cell growth cannot be associated with changes in pluripotency status due to kaempferol, since no significant differences were observed in expression of the pluripotency factors at higher compound concentrations. However, it was clearly evident that 200 μ M kaempferol reduces cell viability, as observed by several different methods: 1) reduction of formazan optical density suggesting decrease of cell viability through decreased reductive capacity of MTT, 2) increase of necrotic cells and/or apoptotic cells in culture and 3) increase of cells at G2/M phase suggesting a possible cell cycle arrest effect.

Flavonoids, and in particular kaempferol, were already shown to induce changes in mitochondrial physiology (Choi, 2011; Marfe et al., 2009; Yans et al., 2015). Therefore, we aimed to determine mitochondrial changes induced by kaempferol in mESCs. There were no differences in mitochondrial membrane potential as evaluated by TMRM, the same being true for mitochondrial content as measured by MitoTracker green staining and TOM-20 expression, markers that are commonly used for that purpose (Vega-Naredo et al., 2014). However, kaempferol positively affects the accumulation of mitochondria-produced superoxide. Indeed some studies show that kaempferol can have pro-oxidative effects on cancer cells (Jeong et al., 2009; Sharma et al., 2007). Although this pro-oxidative activity can be somewhat ambivalent to the common anti-oxidative effects that are characteristic of flavonoids, it is possible that in cells with high growth rates, as the case of cancer cells and ESCs, the mechanisms of action of Kaempferol could differ from those present in normal, non-dividing, terminally differentiated cells. It is interesting to note that high concentrations of kaempferol [50-200 μ M] are required where pro-oxidative effects in other cell models were observed (Bestwick et al., 2005; Jeong et al., 2009; Sharma et al., 2007).

Although apoptosis is induced by kaempferol at high concentration, it was also evident that lower concentrations seem to have beneficial effects on pluripotency given that the expression of pluripotent marker NANOG significantly increases under these conditions. These results seem to suggest that 20 μ M kaempferol can be used as a supplement for routine maintenance of pluripotent stem cells in *in vitro* culture, at least for E14Tg2.a mES cell line.

Nonetheless, we further analyzed if the intermediate concentrations (50 and 100 μ M) could have an impact on the differentiation of mESCs. Given that 200 μ M induced cell death and that 20 μ M seems to be beneficial for pluripotency, these concentrations were excluded. It was evident that both concentrations used impaired proper mESC differentiation using embryoid bodies, with a concomitant decrease in the expression of one marker of each germ cell layer. Yet, the differentiation still occurs since all markers, even with lower expression, are present, suggesting that

differentiation is delayed or less differentiated cells arise from the embryoid body. It has already been described that *in vivo* administration of kaempferol to rats leads to increase of the population of multipotent osteoprogenitor cells with a concomitant decrease of their differentiated adipocyte counterparts (Trivedi et al., 2008). Further studies should be done to understand the mechanism how kaempferol impacts differentiation.

Conclusion

Kaempferol, a natural occurring flavonoid that is present in regular diets is deleterious for mouse embryonic stem cells at high concentrations, inducing apoptosis which is at least in part due to increase of mitochondrial reactive oxygen species. However, when present in lower concentration it can act as a pluripotency markers expression enhancer in those very same cells. Further studies must be conducted in order to better understand the impact of kaempferol on mESC differentiation.

Therefore, other natural molecules could be screened in order to determine if they can modulate cell fate by controlling stemness or differentiation, thus avoiding the use of non-natural and non-physiological chemical engineered factors in ESC culture conditions. This could be used to improve cell culture formulations and procedures for embryonic stem cells.

***General conclusions and
Future perspectives***

General conclusions

The work that composes this thesis was conducted aiming to understand the possible role of sirtuins in embryonic stem cells and to investigate a possible contribution to metabolic modulation.

On the first part of the work we studied the role of sirtuin 1 in a pluripotency and differentiation context and its influence in metabolism (glycolysis *versus* oxidative phosphorylation) of embryonic stem cells.

On the second part, we studied the effects of kaempferol in pluripotency, apoptosis and differentiation of embryonic stem cells. Unfortunately, we showed that in our model kaempferol was not sufficient to modulate SIRT3 activity and expression and consequently, there was no possibility to disclose a potential role for SIRT3 in this context.

We firstly focused our attention on Sirtuin 1 and to do that we used a mouse embryonic cell line where Sirtuin 1 is not expressed ($Sirt1^{-/-}$ R1) and comparatively evaluate that cell line against WT cells. We demonstrated that $Sirt1^{-/-}$ R1 cells are apparently more prone for differentiation by two reasons: these cells required a feeder-cell layer to keep pluripotency, tending to differentiate when cultured in gelatin-coated dishes, while WT cells easily grow pluripotent within this conditions; and OCT4 protein levels are increased in comparison to normal R1 mESC. Our assumption that $Sirt1^{-/-}$ R1 cells have a predisposition for differentiation is similar to a recent report with similar mESCs (Tang et al., 2014). In line with this, $Sirt1^{-/-}$ R1 apparently are less prone for differentiation into neuronal lineages under a directed differentiation protocol, which could be explained by a possible biased

differentiation potential into another lineages that not ectoderm, suggestion supported by the increased Oct4 levels seen in undirected differentiation (Niwa et al., 2000). Focusing on metabolism, we identified a potential new role for Sirt1 control in embryonic stem cells. Lack of sirt1 encourages a more pronounced glycolytic flux that is accompanied by a decrease in mitochondrial oxidative metabolism. If this metabolic switch is the source or the consequence for the altered differentiation potential in a sirtuin 1 mediated way remains unclear. However, STAT3 seems to be a player in this equation. Its mRNA downregulation in Sirt1^{-/-} R1, accompanied by its well known role in pluripotency signaling pathways (Niwa et al., 1998), but also in metabolism (Wegrzyn et al., 2009) suggests that in fact STAT3 downregulation could be on the basis for the glycolytic shift in these cells. Nonetheless, the impact of STAT3 downregulation on its post-translational modifications, namely phosphorylation in tyrosine 705 and serine 727, responsible for an ultimately control for the role of STAT3 as a transcriptional regulator or mitochondrial modulator, even in embryonic stem cells should be addressed (Bernier et al., 2011; Huang et al., 2014). Interestingly, our preliminary results, suggested that forced oxidative metabolism through galactose containing medium could sustain Sirt1^{-/-} R1 cells pluripotent, even in the absence of a feeder-cell layer. Thus, it seems that sirtuin 1 indeed play a role in metabolism modulation of mESC, contributing for regulation of their cell fate. One report on differentiated cells also implicates sirtuin 1 in metabolism changes via STAT3 (Bernier et al., 2011).

On the second part of this thesis, we evaluated the effect of kaempferol in embryonic stem cells and their differentiation. Indeed, the reason behind the use of this natural flavonoid was the fact that it was described as a positive modulator of Sirtuin 3 expression and activity in cancer cell lines (Marfe et al., 2009). Conversely, we did not observed a difference in terms of Sirtuin 3 expression or the acetylation levels of cellular proteins. Although not making part of this thesis or my own work, our lab already started to implement a new strategy for sirtuins 3 modulation, by attempting to downregulate its expression via short-hairpin RNA interference. In order to do that, plasmid

expansion and isolation and cell transfection is still being optimized as a new powerful technique for new studies in embryonic stem cells, cells that are normally difficult to transfect.

Although not attributable to sirtuin 3 modulation, kaempferol supplementation differently impacts mESC. In pluripotency, elevated concentrations of kaempferol have a negative impact on viability, mainly through apoptosis induction and increased levels of reactive oxygen species, similarly to what has been proposed in cancer cells kaempferol (Bestwick et al., 2007; Dang et al., 2014; Nguyen et al., 2003). Although no effects were observed in terms of pluripotency markers expression at elevated concentrations, reduced concentrations can be beneficial for Nanog expression (20 μM), suggesting that kaempferol have the potential to be used as a supplement in routine culture medium for mESC. Nonetheless, this must be well considered, because intermediate concentrations (50 and 100 μM) could have an impact on the mESC differentiation in all germ cell layers when subjected to a random differentiation protocol in embryoid bodies, as suggested by reduced levels of at least one marker for each lineage. Thereby, we suggest that kaempferol can potentiate a stem cell phenotype, as was also advanced by *in vitro* studies for the population of multipotent osteoprogenitor cells (Trivedi et al., 2008).

Thereby, the understanding of the mechanism behind kaempferol impacts on pluripotency differentiation may be required to safely use it in culture cell medium.

As an overview of the presented studies, we revealed new insights under embryonic stem cell biology that may contribute for a better understanding of some molecular mechanisms that may be explored to further enhance and achieve a more efficient use and application of stem cells.

Ongoing work and Future perspectives

Similarly to every other studies, on-going work is being performed to address old and new raised questions.

Sirtuin regulation of metabolism and cell fate still need to be clarified. At the moment, Sirtuin 1 protein rescue on Sirt1^{-/-} R1 mESc is already being conducted in order to further confirm specific roles of this protein rather than cellular adaptations to their KO state. In line with this, we aim to understand how changes in metabolic requirements across differentiation protocol affects cellular fate and how oxidative stress can also be implicated. Metabolic modulation induction, similar to the one reached by galactose enriched medium, in WT, KO and rescued cells could help to disclose the true importance of specific metabolism to the differentiation machineries of each one of these cells. Additionally, a better understanding on STAT3 role must be addressed by down or overexpression methods.

Regarding sirtuin 3, the potential role this protein might have on mES is still elusive. Current work on silencing procedures in mESC will definitively unveil the potential of sirtuin 3, probably also through metabolic modulation. Nevertheless, the absence of a kaempferol-induced effect on Sirtuin 3 could and should be addressed. Do kaempferol modulate sirtuin 3 in normal differentiated cells? Or is it effect only possible in disease conditions as described for cancer cells? These are some of the answers that still need to be revealed.

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