

Modulation of mitochondrial biogenesis and bioenergetic metabolism upon *in vitro* and *in vivo* differentiation of human ES and iPS cells

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ABSTRACT Reprogramming somatic cells to induced pluripotent stem (iPS) cells transforms differentiated cells to an embryonic stem (ES) cell-like state characterized by the acquisition of pluripotency and self-renewal capabilities. We recently demonstrated that human ES and iPS cells share similar mitochondrial properties and bioenergetic metabolism, which are distinct from those of fibroblasts. In the present study, we have applied a global transcriptome profiling approach to compare the mitochondrial-related transcriptional signature upon the loss of self renewal and pluripotency in human ES and iPS cells. This was achieved by inducing *in vitro* and *in vivo* spontaneous differentiation. ES and iPS cells showed a similar degree of correlation both in the undifferentiated state and in all the stages of differentiation analyzed, suggesting that their transcriptional similarities are retained upon differentiation. Moreover, comparable induction of transcripts involved in epithelial to mesenchymal transition was observed in both cell types. Analysis of mitochondrial-related nuclear transcripts revealed consensual regulation of genes involved in mitochondrial biogenesis and bioenergetic metabolism upon *in vitro* differentiation of human ES and iPS cells, while specific differences were identified within *in vivo* differentiated cells. Significant changes were not detected for antioxidant-related genes. Finally, we formulate a "metabolic state hypothesis" linking mitochondrial state and cellular metabolism to the stage of differentiation. Overall, our data unveil differences and similarities between human ES and iPS cells during spontaneous differentiation and suggest that the study of mitochondrial and metabolic remodeling may reveal key mechanisms underlying the acquisition, maintenance and exit of a self-renewing pluripotent state.

KEY WORDS: *iPS cells, reprogramming, differentiation, mitochondria, metabolism*

Introduction

Somatic cells have been found able to acquire a self-renewing pluripotent state upon the ectopic expression of a set of genes commonly present in embryonic stem (ES) cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Park *et al.*, 2008; Singh *et al.*, 2009). The somatic cell-derived induced pluripotent stem (iPS) cells share multiple features with ES cells, including cellular morphology and cell cycle structure, transcriptional and epigenetic signatures, unlimited propagation without senescence and most importantly developmental potential. In addition, we recently demonstrated that human ES and iPS cells are characterized by similar bioenergetic

metabolic profiles, redox status and mitochondrial function (Prigione *et al.* 2010).

Mitochondria are double-membrane organelles known to play a fundamental role in controlling ATP production through the electron transport chain (ETC), calcium homeostasis, apoptosis, and cell signaling (Dyall *et al.*, 2004). Mitochondria are also the

Abbreviations used in this paper: EB, embryoid body; EMT, epithelial-mesenchymal transition; ES, embryonic stem; ETC, electron transport chain; iPS, induced pluripotent stem; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species.

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major source of endogenous reactive oxygen species (ROS), common by-products of oxidative phosphorylation (OXPHOS), which can in turn generate oxidative damage to DNA, proteins, and lipids (Balaban *et al.*, 2005). Moreover, mitochondria possess their own DNA, which encodes 13 ETC peptides. Due to the presence of ROS and the lack of efficient repairing mechanisms, mutations within the mitochondrial DNA (mtDNA) can accumulate over time (Cortopassi *et al.*, 1992; Wallace, 1994) causing multiple cellular dysfunctions (Alemi *et al.*, 2007; Prigione *et al.*, 2007).

Approximately 1500 mitochondrial-related genes reside within the nuclear genome, including key regulators of mitochondrial replication (Kelly *et al.*, 2004; Scarpulla, 2008). Transcription of mtDNA requires a small number of nucleus-encoded proteins comprising the mitochondrial-specific DNA polymerase gamma, which is composed of one catalytic subunit (*POLG*) and two accessory subunits (*POLG2*), and the mitochondrial transcription factor A (*TFAM*) (Clayton, 1998). Additional regulating elements include the transcription factor *NRF-1* and members of the *PGC-1* family (*PGC1-a* and *PGC1-b*). In particular, *PGC1a* appears as

a master regulator of mitochondrial biogenesis due to its ability to activate of *TFAM* through direct interaction with *NRF1* (Goffart *et al.*, 2003).

Under physiological normoxic conditions, human somatic cells are characterized by active mitochondria and aerobic ETC-based metabolism. On the other hand, solid tumor cells shift to glycolysis-based metabolism as a result of inefficient vascularization, an event known since the early 1930s under the name of "Warburg effect" (Warburg, 1956). Glycolysis might be advantageous in comparison to mitochondrial respiration. Indeed, it can provide quick energy supplies and avoids toxic ROS generation. In addition, by promoting the flux through the pentose phosphate pathway, it can support antioxidant defenses by inducing the production of NADPH, which is required for the generation of reduced glutathione (GSH), a key antioxidant enzyme (Pfeiffer *et al.*, 2001; Hsu *et al.*, 2008). Early-stage mammalian embryos and *in vivo* stem cell niches are also exposed to extremely low oxygen conditions (Fischer *et al.*, 1993). Thus, hypoxic environment and anaerobic metabolism appear to favorably support self-renewal and pluripotency.

Accordingly, hypoxic culture and mitochondrial inhibition have been associated with reduced ES cell differentiation and more recently with improved generation of iPS cells (Ezashi *et al.*, 2005; Varum *et al.*, 2009; Yoshida *et al.*, 2009).

For these reasons, mitochondrial structure and function have been suggested as indicators of stem cell competence (Chen *et al.*; Lonergan *et al.*, 2006; Siggins *et al.*, 2008; Parker *et al.*, 2009). In particular, relative under-developed mitochondrial network and low mitochondrial activity emerge as common features of 'stemness', as reported in primordial germ cells, inner cell mass, early embryos, and murine and human ES cells (Lonergan *et al.*, 2007; Shoubridge *et al.*, 2007; Facucho-Oliveira *et al.*, 2009; Ramalho-Santos *et al.*, 2009; Van Blerkom, 2009). Recent data suggested that the self-renewal state of human iPS cells may also be supported by glycolysis metabolism (Prigione *et al.* 2010) and by mitochondrial properties similar to those of ES cells, including low mtDNA copy number, immature organelle shape with under-developed cristae, and low levels of oxidative stress (Armstrong *et al.* 2010; Prigione *et al.* 2010). Hence, it appears that somatic cells undergo a metabolic shift in order to acquire ES cell-like features. On the other hand, functionally active mitochondria are necessary for successful differentiation of ES cells, which requires an opposite

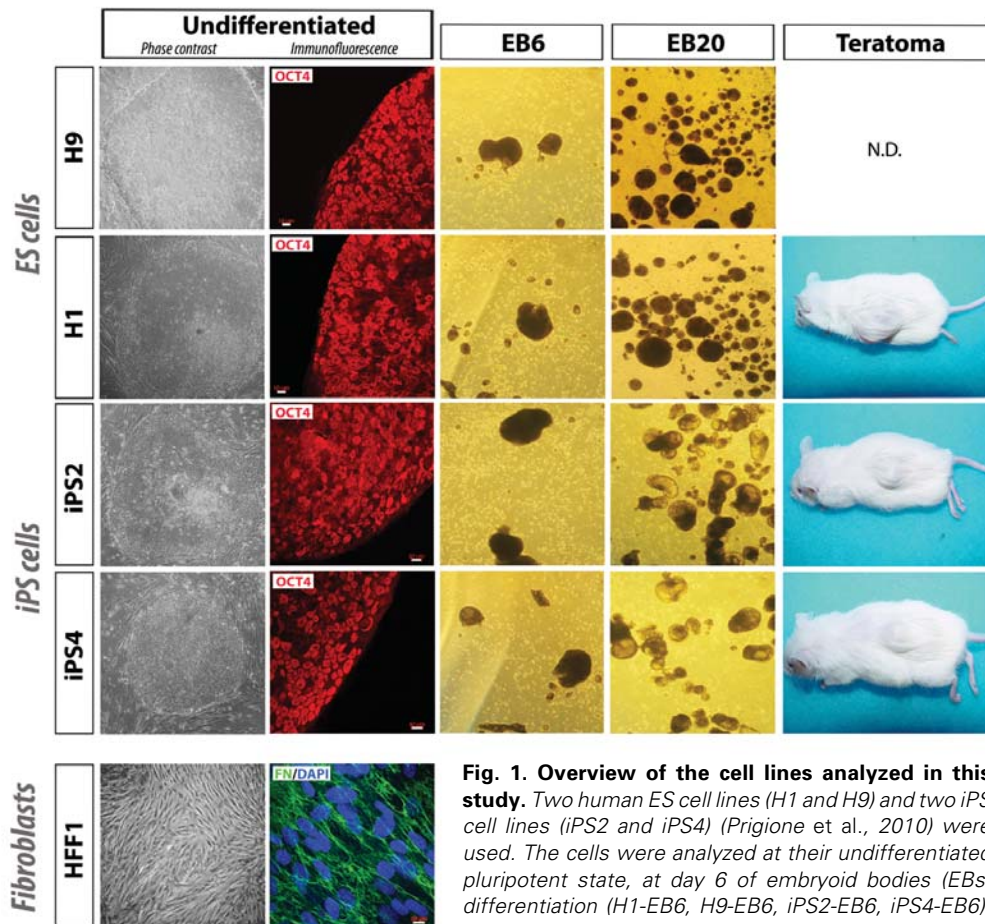


Fig. 1. Overview of the cell lines analyzed in this study. Two human ES cell lines (H1 and H9) and two iPS cell lines (iPS2 and iPS4) (Prigione *et al.*, 2010) were used. The cells were analyzed at their undifferentiated pluripotent state, at day 6 of embryoid bodies (EBs) differentiation (H1-EB6, H9-EB6, iPS2-EB6, iPS4-EB6), at day 20 of EB-differentiation (H1-EB20, H9-EB20, iPS2-

EB20, iPS4-EB20), and after generation of complete teratomas (H1-teratoma, iPS2-teratoma, iPS4-teratoma). H9-teratoma was not analyzed (N.D. = not determined). As an additional differentiated cell type, we included the parental neonatal foreskin fibroblast cell line (HFF1) from which the iPS cells used in this study were obtained. For undifferentiated ES and iPS cells and for HFF1 fibroblasts, Immunofluorescence-based expression analyses of key markers are shown for OCT4 in ES and iPS cells and fibronectin (FN) in HFF1 cells. White bars correspond to 10 μ m. 4X magnification was used for phase-contrast images. All pictures were taken using a digital camera (Canon).

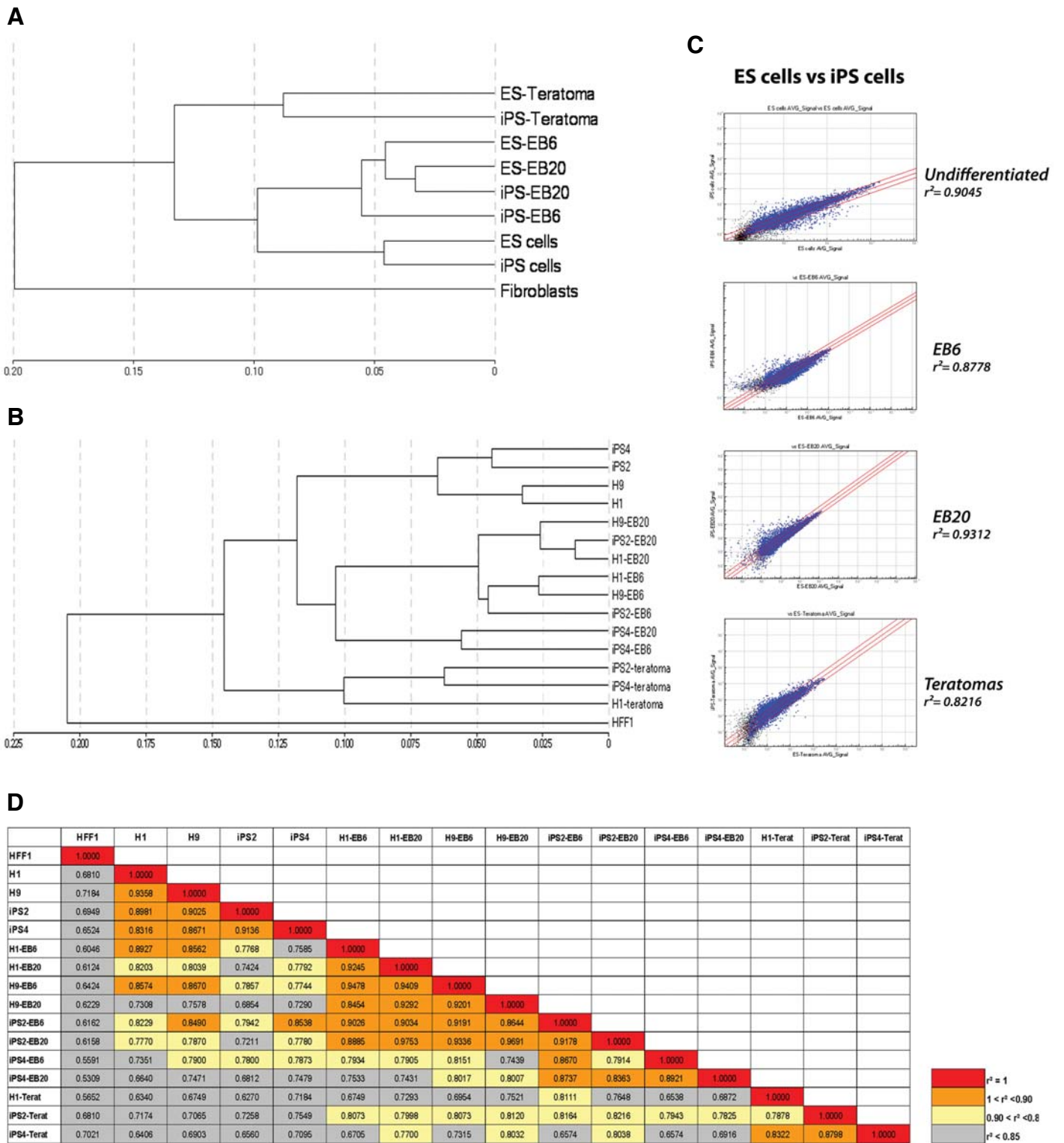


Fig. 2. Transcriptional profiling analysis. (A) Hierarchical clustering of the samples; different cell lines were pooled together to provide an overview of the global correlations between undifferentiated cells and differentiated cells at various stages. (B) Hierarchical clustering of all the samples used in the study. The two ES cell lines and the two iPS cell lines cluster together in all the different cell states (undifferentiation state, EB6, EB20, and teratomas). (C) Scatter plot graphs showing the degree of correlation between ES and iPS cells at distinct stages of differentiation. Blue dots highlight genes whose detection p-value is lower than 0.01. (D) Table showing all the correlation coefficient values between the single samples analyzed. For color coding, four distinct degrees of correlation are represented (red for $r^2 = 1$; orange for $1 < r^2 < 0.9$; yellow for $0.9 < r^2 < 0.85$; and grey for $r^2 < 0.85$).

switch in energy metabolism from anaerobic glycolysis to aerobic OXPHOS (Cho *et al.*, 2006; Facucho-Oliveira *et al.*, 2007).

In the present study, we aimed at comparing mitochondrial-related transcriptional signatures of human ES and iPS cells during *in vitro* and *in vivo* differentiation. Modulation of these pathways may be essential in the regulation of cellular bioenergetic metabolism and might thus reveal important mechanisms for acquiring, maintaining and exiting a self-renewing pluripotent state in human cells.

Results

Transcriptional similarities between human ES and iPS cells are retained upon differentiation

In this study, we used two human ES cell lines (H1 and H9) and two iPS cell lines (iPS2 and iPS4), previously derived by retroviral transduction of OCT4, KLF4, SOX2 and c-MYC (Takahashi *et al.*, 2007) into neonatal foreskin fibroblasts (HFF1) (Prigione *et al.* 2010). The cells were analyzed at their undifferentiated pluripotent

state and at different stages of differentiation. For *in vitro* differentiation, we employed embryoid body (EB)-based differentiation and chose two time-points for mRNA isolation (EB6 at day 6 and EB20 at day 20). The choice of EB stages was based on our previously published work on EB-based differentiation of human ES cells (Fathi *et al.*, 2009). For *in vivo* differentiation, dissected teratomas derived from the two iPS cell lines and from one ES cell line (H1) were employed. In addition, we included in the analysis the parental HFF1 fibroblasts from which the two iPS cell lines (iPS2 and iPS4) were derived. An overview of the cells utilized for the transcriptome profiling is shown in Fig. 1.

Global hierarchical clustering of the samples revealed that ES and iPS cell lines share similar transcriptomes, both in the undifferentiated state and in all stages of differentiation analysed (Fig. 2 A,B). ES and iPS cells showed a similar degree of correlation in the undifferentiated state ($r \leq 0.9045$) and in different stages of differentiated cell states (EB6 $r \leq 0.8778$, EB20 $r \leq 0.9312$, and teratomas $r \leq 0.8216$) (Fig. 2C). On the other hand, undifferentiated ES and iPS cells poorly correlated with somatic HFF1 fibroblasts

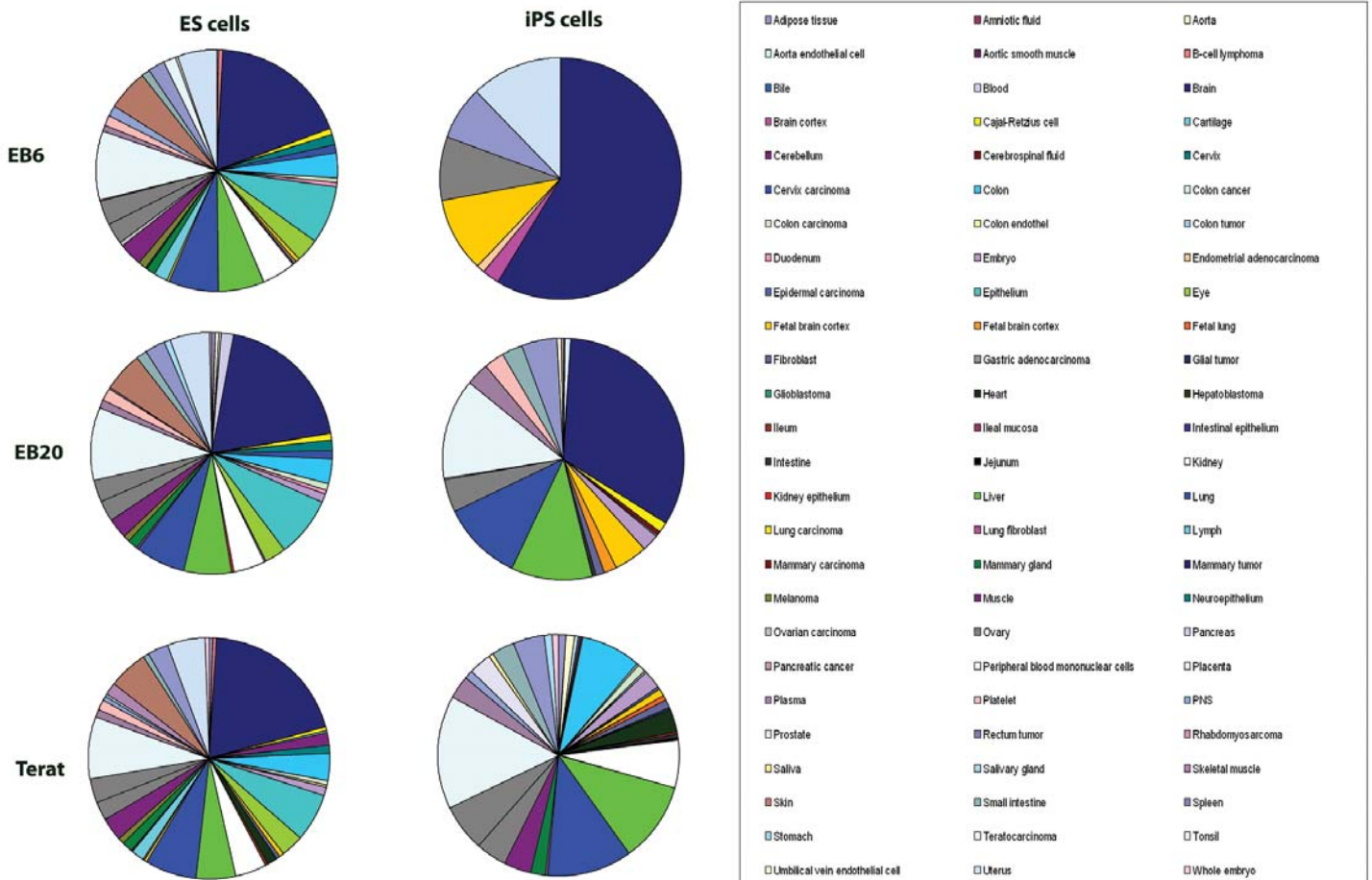


Fig. 3. Increased complexity of tissue-association patterns upon differentiation of ES and iPS cells. For each differentiation stage of ES and iPS cells, a gene list was generated by comparing significantly up-regulated genes (fold change > 1.5, detection p value ≤ 0.01 , and differential p value ≤ 0.01) in comparison to undifferentiated ES and iPS cells, respectively. The lists were then imported as input for the tissue expression annotation tool. In both ES and iPS cells, the degree of complexity of tissue commitment increased over time upon differentiation. Several tissue-related transcripts showed a similar pattern of induction in both pluripotent stem cells, such as colon (light blue), kidney (white), liver (green), and muscle (purple). However, brain-related transcripts (blue) were enriched upon differentiation of ES cells, but appeared to decrease over time in spontaneously differentiated iPS cells.

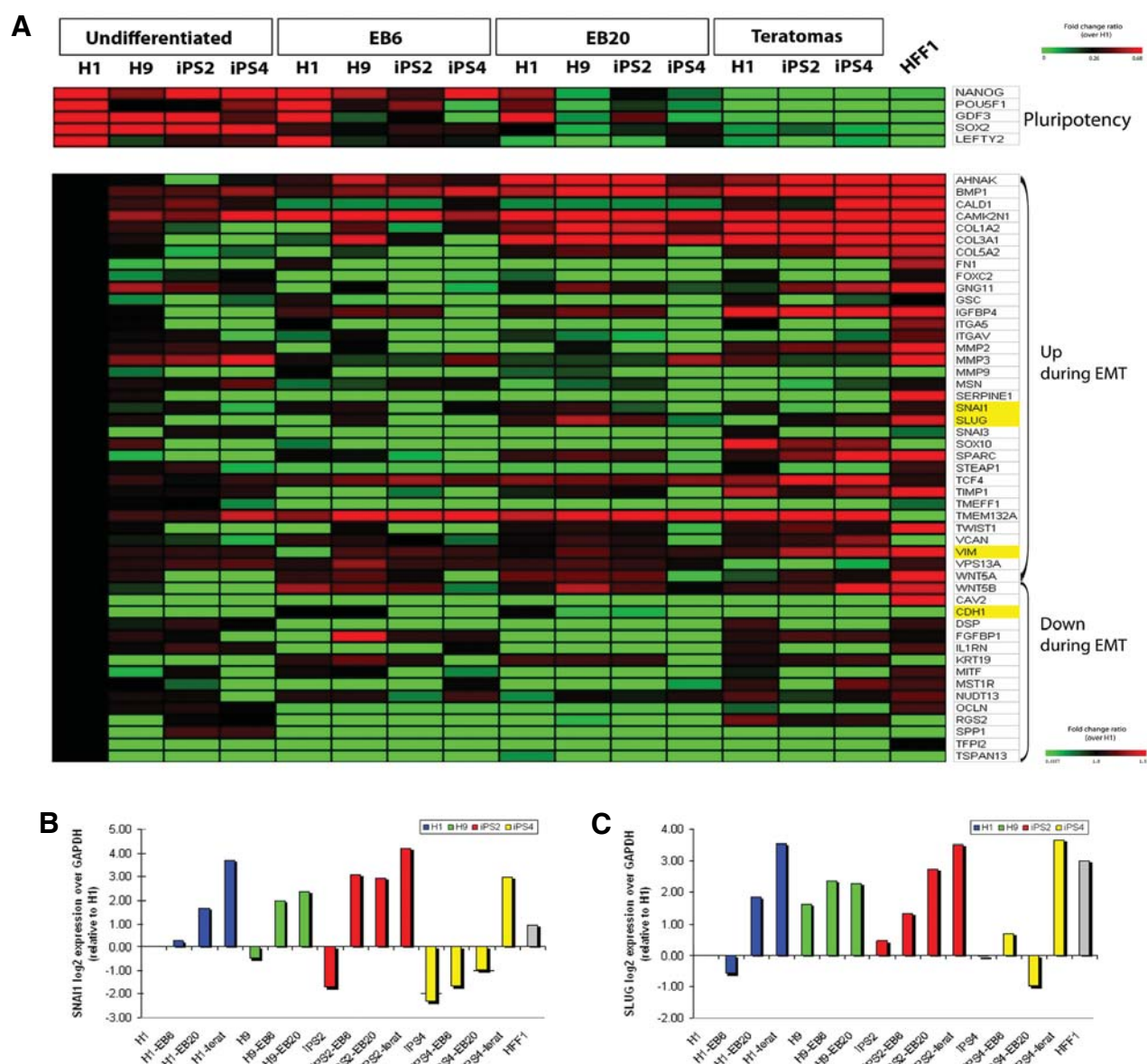


Fig. 4. Activation of epithelial-mesenchymal transition (EMT)-related transcripts upon differentiation. (A) Upper panel, heatmap depicting the regulation of expression of genes related to pluripotency upon differentiation. Lower panel, modulation of Epithelial to Mesenchymal Transition (EMT)-related genes during spontaneous differentiation. EMT transcripts were divided into two categories "Up in EMT" and "Down in EMT", according to the literature (Nakaya et al., 2008) and to the SA bioscience PRC array list (see material and methods for details). Values represent the ratio of the array average signal for the given gene divided by the average signal of H1 ES cell line (fold change 1.5, detection p value ≤ 0.01 , and differential p value ≤ 0.01). Down-regulated genes are shown in green, up-regulated genes in red. Highlighted in yellow are four key EMT genes: SNAI1, SLUG, VIM, and CDH1. The first three were induced in differentiated cells in comparison to undifferentiated cells, while the opposite was found for the epithelial marker CDH1. (B,C) Q-PCR analysis of SNAI1 (SNAI1) and SNAI 2 (SLUG), two driver transcription factors of the EMT response, performed in all analyzed samples confirming the array data.

(correlation coefficient $r \leq$ around 6-7) as we and others have previously shown (Takahashi et al., 2007; Prigione et al. 2010) (Fig. 2D). Overall, the results suggested that human ES and iPS cells retain their transcriptional similarities even upon differentiation analyzed *in vivo* and *in vitro*.

Increased degree of complexity of tissue expression commitment upon differentiation of human ES and iPS cells

The ability of human ES cells to mimic the embryonic process of

germ layer formation *in vitro* can be utilized as a tool to understand the mechanisms underlying human embryogenesis at the molecular and cellular level (Nishikawa et al., 2007). Indeed, previous transcriptome-based analysis employed *in vitro* differentiated human ES cells to dissect the complex molecular signature of early embryogenesis (Ivanova et al., 2002; Bhattacharya et al., 2005; Fathi et al., 2009).

ES and iPS cells displayed a consensual up-regulation of key markers of germ layer commitment during spontaneous differen-

tiation (Supp. Fig. 1). Moreover, by assigning tissue-specific annotations to genes up-regulated at different differentiation stages in comparison to undifferentiated cells, we observed that the complexity of tissue generation increased over time in differentiated ES and iPS cells (Fig. 3). The number of tissue-related transcripts rose significantly upon differentiation in a similar

fashion in ES and iPS cells, including genes associated with adipose tissue, colon, fibroblasts, kidney, liver, lung, muscle, pancreas, plasma, platelets, small intestine, and spleen. The tissue categories called embryo and whole embryo, which comprise genes related to gastrulation and development (such as *AHI1*, *EPHB3*, *FHL3*, *FRZB*, *LAMC3*, *MYST1*, *NDN*, *NELF*, *PITX1*,

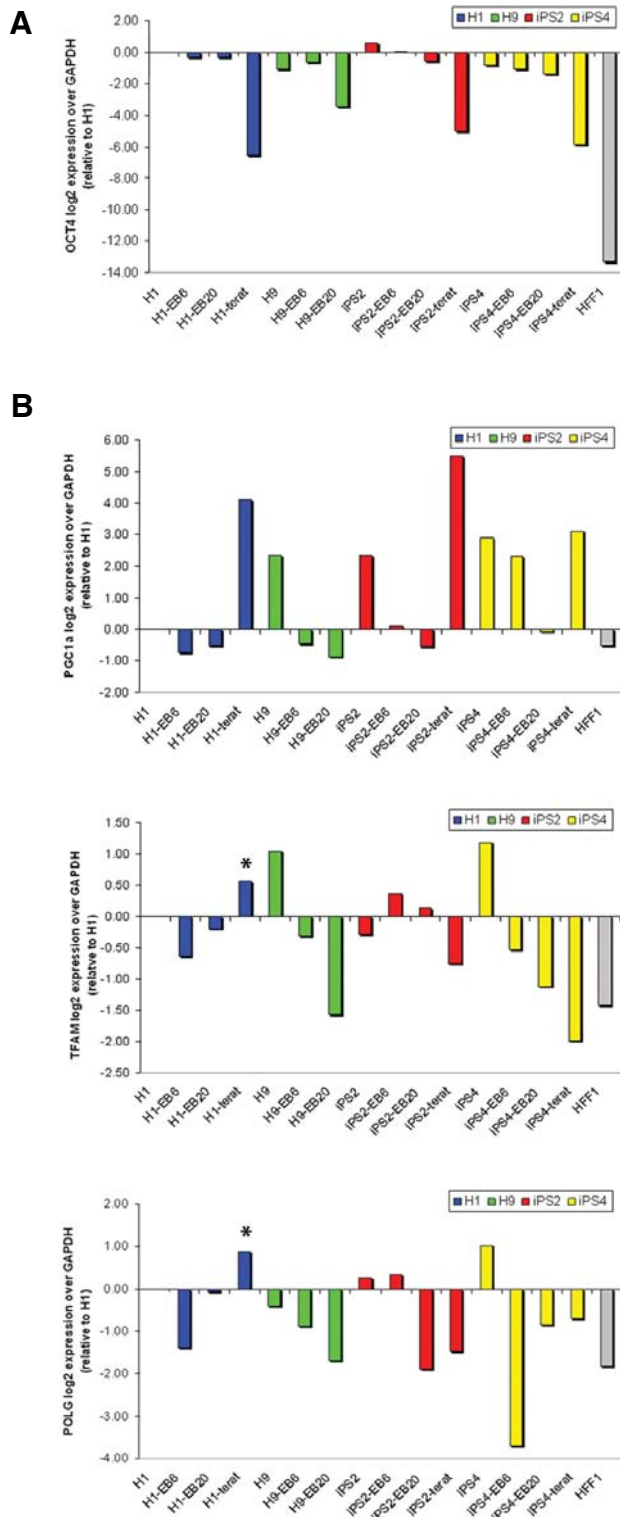


Fig. 5. Mitochondrial biogenesis response during *in vitro* and *in vivo* differentiation. (A) Q-PCR analysis, conducted on the same original mRNA samples used for the hybridization studies, confirmed the significant down-regulation of the *OCT4* transcript upon differentiation. (B) Q-PCR results showing the nuclear response related to mitochondrial biogenesis upon differentiation of human ES and iPS cells. The genes analyzed, which represent the main nuclear components involved in the mitochondrial biogenesis process, included *PGC1a*, *PGC1b*, *TFAM*, *NRF1*, *POLG*, and *POLG2*. Two key genes involved in mitochondrial biogenesis, *TFAM* and *PGC1a*, exhibited a different expression pattern in ES and iPS cell-derived teratoma samples. When compared to undifferentiated cells, these genes were induced in H1-derived teratoma (asterisks) and mainly reduced in both iPS cells-derived teratomas.

PLXNA3, *TAPT1*, *TSPAN9*, and *VIM*), showed a similar induction in both ES and iPS cells. Interestingly, however, brain-related transcripts exhibited a distinct expression pattern in the two pluripotent stem cells. In ES cells, the number of brain-related genes gradually increased upon differentiation while it appeared reduced over time in iPS cells (Fig. 3). These results may reflect distinct properties of the specific iPS cell lines used in this study and further analysis conducted on several iPS cell lines will be needed to substantiate this observation.

Comparable induction of EMT-related transcripts during spontaneous differentiation of human ES and iPS cells

During the developmental process of gastrulation, a critical role is played by epithelial to mesenchymal transition (EMT) (Nakaya *et al.*, 2008). By analyzing numerous published transcriptome datasets related to human ES and iPS cells, we previously observed that genes involved in the progression of EMT were mainly down-regulated in undifferentiated cells compared to somatic fibroblasts (Wang *et al.*, 2010). Inhibition of EMT and activation of its reciprocal process MET, likely occurring through the modulation of the TGF- β pathway, might thus represent a key aspect for efficient reprogramming to pluripotency of cells of mesenchymal phenotype, such as somatic fibroblasts.

To further validate this observation, we analyzed the transcriptional regulation of EMT-related genes during differentiation of human ES and iPS cells. Upon differentiation, pluripotency-associated genes were significantly down-regulated, while EMT-related genes showed a trend of up-regulation (Fig. 4A). In HFF1 fibroblasts, EMT genes were strongly up-regulated in comparison to undifferentiated ES and iPS cells. Gradual activation of EMT-related genes could be observed in spontaneously differentiated ES and iPS cells. In particular, the induction of EMT genes was detected in the latest *in vitro* differentiation stage (EB20) and even more evidently within the teratoma samples (Fig. 4A). Overall, the expression of key EMT

drivers such as *SNAIL1* (*SNAI1*), *SNAIL 2* (*SLUG*) and *VIMENTIN* (*VIM*) was induced in differentiated cells in comparison to undifferentiated cells, while the epithelial markers *E-CADHERIN* (*CDH1*), *DSP* and *KRT19* were down-regulated. Q-PCR analysis of *SNAI1* and *SLUG* confirmed that two factors, known to repress transcription of the epithelial specifying gene *CDH1* (Ullmann *et al.*, 2007; Thiery *et al.*, 2009), were gradually activated upon differentiation in both human ES and iPS cells (Fig. 4 B,C).

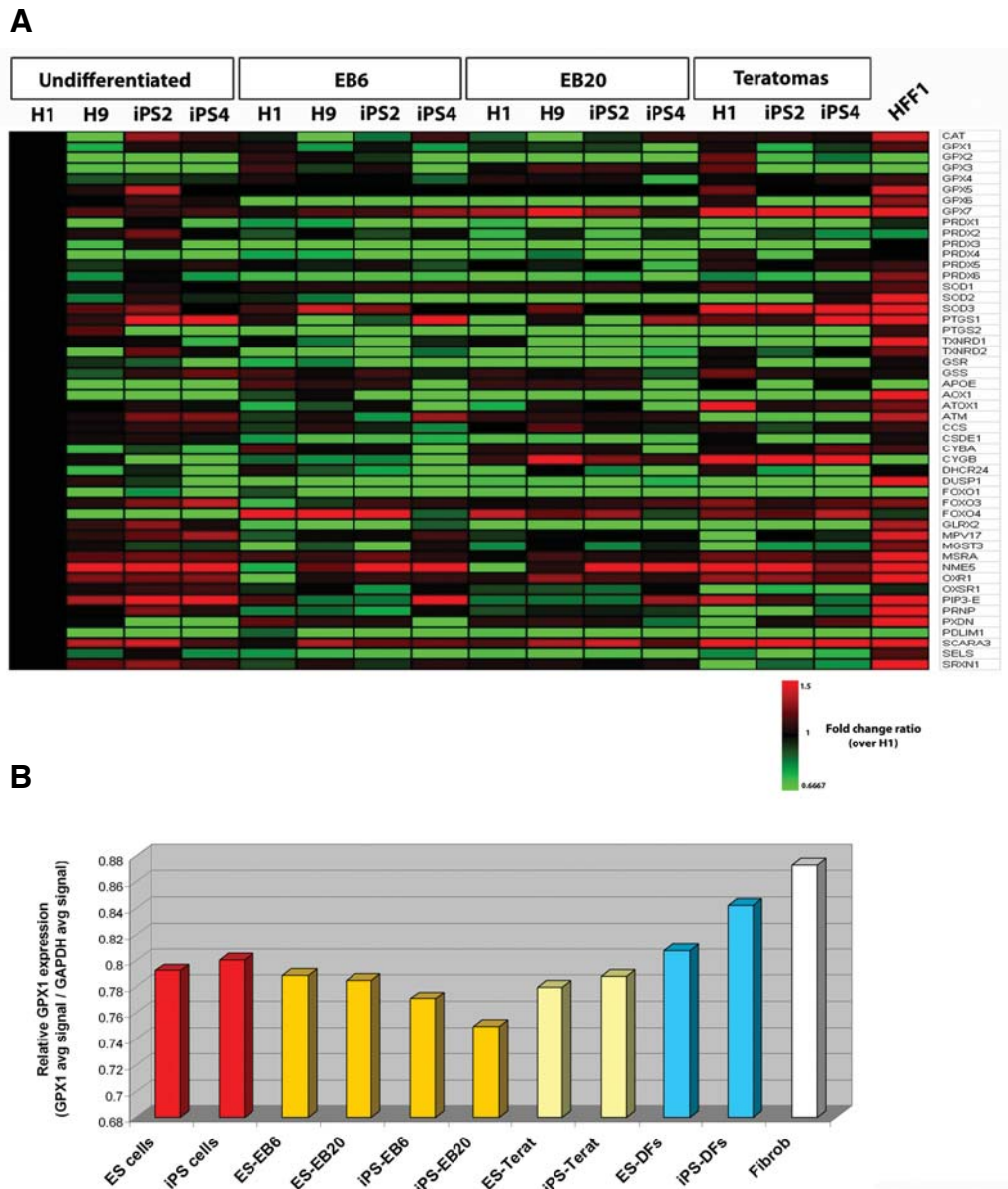


Fig. 6. Expression analysis of genes involved in the response to oxidative stress. (A) Heatmap figure showing a panel of gene transcripts related to oxidative stress and antioxidant defense. Values represent the ratio of the array average signal of the given gene divided by the average signal of H1 ES cell line (fold change 1.5, p value ≤ 0.01 , and differential p value ≤ 0.01). Down-regulated genes are shown in green, up-regulated genes in red. **(B)** GPX1 gene expression measured as the ratio of GPX1 average array signal over GAPDH average array signal. The expression values within the analyzed samples were compared to the values previously detected by microarray in derived fibroblasts (DFs) obtained from ES and iPS cells (Prigione *et al.*, 2010).

Modulation of mitochondrial biogenesis upon spontaneous differentiation of human ES and iPS cells

In order to compare the mitochondrial-related signatures upon differentiation of human ES and iPS cells, we analyzed by Q-PCR the expression of key genes involved in mitochondrial biogenesis (Fig. 5). The Q-PCR data showed consensual down-regulation of OCT4 expression during differentiation of ES and iPS cells, which was in accordance with the array analysis (Fig. 4A and 5A). In agreement with previous reports (Armstrong *et al.* 2010; Prigione *et al.*, 2010), undifferentiated human ES and iPS cells presented similar expression levels for most of the genes involved in mitochondrial biogenesis (*PGC1a*, *PGC1b*, *TFAM*, *NRF1*, *POLG*, and *POLG2*), which appeared up-regulated in both ES and iPS cells in comparison to somatic HFF1 fibroblasts (Fig. 5B).

During spontaneous *in vitro* differentiation of ES and iPS cells, we detected a consensual down-regulation of nuclear genes responsible for regulating mitochondrial biogenesis, as earlier demonstrated in human ES cells [27]. At the EB6 stage, the expression of mitochondrial biogenesis genes (with the exception of *NRF1*) showed decreased gradually in all four pluripotent stem cells (Fig. 5B). At the latest stage of *in vitro* differentiation (EB20), both ES and iPS cell-derived EBs showed consistent down-regulation of genes related to mitochondrial biogenesis in comparison to undifferentiated H1, still with the exception of *NRF1* (Fig. 5B).

On the other hand, in fully-differentiated teratoma samples some mitochondrial biogenesis factors appeared up-regulated compared to undifferentiated cells. These included *PGC1a* and *PGC1b*, *TFAM* and *POLG*. Most of the genes were consensually expressed in ES and iPS cell-derived teratomas. When compared to undifferentiated stem cells, *PGC1a* and *PGC1b* were up-regulated, *NRF1* was unchanged, and *POLG2* was down-regulated (Fig. 5B). However, although ES and iPS cell-derived teratomas showed quite similar global transcriptional signatures (Fig. 2), two key genes involved in mitochondrial biogenesis, *TFAM* and *PGC1a*, were differentially expressed in ES and iPS

cell-derived teratoma samples. Their expression level was induced in H1-derived teratoma compared to undifferentiated cells (Fig. 5B, asterisks); while it was mainly decreased in teratomas obtained from the two iPS cell lines.

Transcriptional regulation of bioenergetic metabolism upon spontaneous differentiation of ES and iPS cells

There is a paucity of data available on the regulation of genes involved in bioenergetic metabolism during stem cell differentiation. Employing global transcriptome analysis, we analyzed nuclear-encoded genes involved in mitochondrial energy metabolism (ETC genes) and compared their expression within cells at different stages of differentiation in relation to undifferentiated H1 cells.

ETC genes were mainly similarly expressed in both ES and iPS cells compared to somatic fibroblasts (Supp. Fig. 2), in accordance to our previous results (Prigione *et al.*, 2010). Interestingly however, one iPS cell line (iPS2) showed higher expression of some of these genes than the other undifferentiated cell lines (Supp. Fig. 2), possibly due to different patterns of viral integration within different iPS cell lines.

Within the EB6 and EB20 stages, both ES and iPS cell-derived samples showed down-regulation of ETC genes compared to undifferentiated cells -although less evident in the case of Complex III- suggesting a possible occurrence of a metabolic shift at these differentiation stages (Supp. Fig. 2). The expression of ETC-related genes was not identical within *in vivo* differentiated ES and iPS cells, in a similar fashion to transcripts involved in mitochondrial biogenesis (Fig. 5 and Supp. Fig. 2). ETC transcripts within teratoma samples show a trend of up-regulation compared to undifferentiated cells to a level more similar to somatic fibroblast. However, the up-regulation of transcripts related to Complex I, III, IV, and V was more evident in H1-teratoma rather than in iPS2 or iPS4-teratomas (Supp. Fig. 2). This may imply further differences related to mitochondrial activity between the two pluripotent cell types at this stage of differentiation,

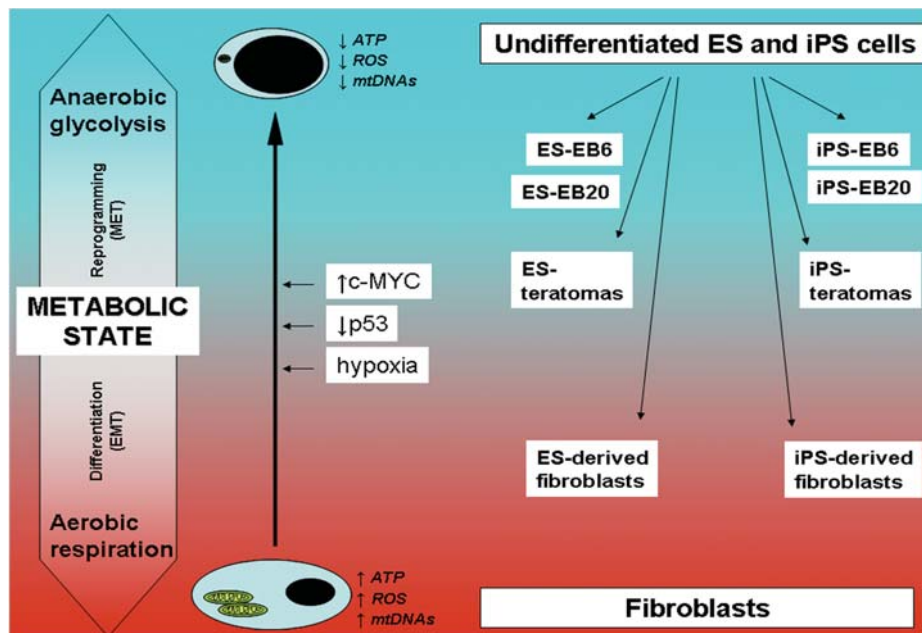


Fig. 7. The "metabolic state hypothesis". Schematic illustration depicting the possible role of the metabolic cellular state during cellular reprogramming and differentiation. We hypothesize that the metabolic cell state might play a role in the two divergent processes. During cellular reprogramming of fibroblasts, cells undergo a MET transition and acquire a metabolic state typical of undifferentiated ES cells. These features include decreased mitochondrial number and activity and induced anaerobic glycolysis. During differentiation, an EMT response is activated in ES and iPS cells. The differentiated cells acquire a metabolic state more dependent on aerobic ETC respiration and mitochondrial activity. However, the metabolic state attained upon spontaneous differentiation may not be as complete as that which is characteristic of the parental somatic cells. On the other hand, specific differentiation protocols may generate cell types with metabolic states more similar to that of somatic cells, such as the case of ES and iPS-derived fibroblast-like cells (see discussion).

despite the detected global transcriptional similarities.

Lack of transcriptional modulation of antioxidant genes within spontaneously differentiated ES and iPS cells

Finally, we aimed to analyze the expression level of genes related to the response to oxidative stress in undifferentiated and spontaneously differentiated stem cells (Fig. 6). We earlier detected a different redox status between undifferentiated and fully-differentiated fibroblast cells (Prigione *et al.*, 2010). In accordance, HFF1 somatic fibroblasts presented a consistent up-regulation of most of antioxidant genes in comparison to undifferentiated stem cells (Fig. 6A). However, these genes did not show transcriptional induction within *in vitro* and *in vivo* differentiated ES and iPS cells. A mild up-regulation could be identified only in teratoma samples in comparison to undifferentiated stem cells; however, the expression level was not as high as in HFF1 fibroblasts (Fig. 6A).

To address whether this lack of transcriptional change of antioxidant genes upon differentiation was specific to spontaneously differentiated EBs and teratomas, we compared the array expression level of *GPX1*, a key antioxidant gene, within cells at all different stages of differentiation and in derived fibroblasts (DFs), fibroblast-like cells previously obtained from ES cells (H1 and H9) and iPS cells (iPS2 and iPS4) (Prigione *et al.*, 2010). *GPX1* expression was significantly increased in somatic fibroblasts and in iPS-DFs compared to undifferentiated cells, in accordance with our previous immunoblot data (Prigione *et al.*, 2010), but mostly unchanged in EBs and teratomas (Fig. 6B). Accordingly, DFs clustered together with somatic HFF1 fibroblasts and far more distant from ES and iPS cells in either undifferentiated or spontaneously differentiated states (Supp. Fig. 3A). The correlation values between undifferentiated cells and DFs ($r \leq 0.7216$ for ES cells and $r \leq 0.6646$ for iPS cells) were similar to those between undifferentiated cells and somatic fibroblasts ($r \leq 0.6979$ for ES cells and $r \leq 0.6782$ for iPS cells) (Fig. 2D and Supp. Fig. 3B).

Overall, the results could imply that differentiation towards a specific lineage may generate transcriptional changes in the response to oxidative stress in a fashion more similar to the representative somatic tissue. On the other hand, the EB and teratoma-based spontaneous differentiation models might be masking the specific changes occurring within cells due to the simultaneous generation of distinct cell types representing the three germ layers. Future studies would have to confirm these findings in a larger number of samples and determine whether spontaneously differentiated cells might exhibit a different redox status compared to cells committed to a lineage-specific differentiated state.

Discussion

Human iPS cells have enormous potential for regenerative medicine, both as an isogenic source for cellular replacement therapy and as an *in vitro* tool for studying complex human disorders and discovering new drug approaches (Saha *et al.*, 2009; Deng 2010; Kiskinis *et al.* 2010). The capability to achieve these invaluable goals is linked to the ability of iPS cells to efficiently differentiate into relevant functional cells. Nonetheless, recent findings suggest that differentiated cells derived from

human iPS cells might show premature senescence and reduced efficiency and increased variability in comparison to those obtained from ES cells (Feng *et al.* 2010; Hu *et al.* 2010). This raised concerns over the real potential of the somatic-derived reprogrammed cells. Thus, in depth comparisons of the differentiation competence of human ES and iPS cells are needed to help determine the extent of these possibly relevant differences.

By analyzing the global transcriptional changes accompanying the spontaneous differentiation of human ES and iPS cells, we observed that the similarities between the two types of pluripotent cells are retained upon differentiation suggesting that ES and iPS cells might share analogous molecular mechanisms both for the maintenance of the self-renewal state and for the exiting from pluripotency due to spontaneous differentiation. Interestingly, the number of tissue-associated genes increased over time in both spontaneously differentiated ES and iPS cells but attained a different degree of complexity in the two pluripotent stem cells. These results might imply a distinct potential in embryonic and somatic cell-derived stem cells. Future studies conducted on several iPS cell lines are warranted to investigate whether this is a trait common to all iPS cells or restricted to specific iPS cell lines obtained from a definite tissue.

Spontaneous stem cell differentiation can mimic gastrulation and have potential applications for the study of early embryogenesis at the molecular level (Ivanova *et al.*, 2002; Bhattacharya *et al.*, 2005; Fathi *et al.*, 2009). In particular, a critical component of these developmental processes is considered to be the epithelial to mesenchymal transition (EMT) (Nakaya *et al.*, 2008). In accordance, differentiated cells derived from both human ES and iPS cells showed transcriptional changes consensual with an EMT response. These data could also be of specific interest in the context of cellular reprogramming, as a reciprocal process (mesenchymal to epithelial transition, MET) has been detected upon full reprogramming of human fibroblasts to iPS cells, whereas partially reprogrammed cells were characterized by incomplete MET (Wang *et al.*, 2010). Thus, manipulation of the EMT-MET responses may prove to be useful for enhancing or opposing the two inverse processes of reprogramming to pluripotency (especially for mesenchymal-derived cells, such as fibroblasts) and differentiation. Interestingly, during the course of revision of this manuscript, new findings demonstrated that MET is indeed playing a critical role in initiating cellular reprogramming events in fibroblast cells (Li *et al.* 2010; Samavarchi-Tehrani *et al.* 2010).

Applying the "metabolic state hypothesis" to reprogramming, self-renewal and differentiation

Metabolic alterations are a known common feature of cancer cells, in which anaerobic glycolysis rather than mitochondrial respiration became the preferred route for energy production (Hsu *et al.*, 2008; Vousden *et al.*, 2009). A similar metabolic remodeling may take place during reprogramming of somatic cells to a pluripotent state (Prigione *et al.*, 2010). Glycolysis, although less efficient in ATP generation compared to mitochondrial respiration (respectively 2 and 36 ATP molecules are produced with glycolysis and OXPHOS), can produce ATP at a higher rate (Pfeiffer *et al.*, 2001), without the undesirable presence of toxic free radicals. Thus, undifferentiated ES and iPS cells may adopt a metabolic program similar to cancer cells favoring fast rather than efficient energy production which will require a

high rate of glucose uptake. On the other hand, an opposite switch from anaerobic to aerobic metabolism can occur when undifferentiated cells exit self-renewal and start differentiating (Chen *et al.* 2010).

In order to better understand how this latter transition may be regulated at the molecular level, we focused on the nuclear response of genes involved in mitochondrial biogenesis, bioenergetic metabolism and oxidative stress in both human ES and iPS cells. Based on our previously published findings (Prigione *et al.*, 2010) and the data reported here, we have formulated a possible hypothesis linking the cellular metabolic state to the degree of differentiation. The hypothesis, named 'metabolic state hypothesis', is schematically represented in Fig. 7. At the two extremes of the metabolic arrows are undifferentiated ES cells/ iPS cells and somatic fibroblasts, respectively.

Nuclear-encoded genes involved in mitochondrial biogenesis were mainly up-regulated in undifferentiated ES and iPS cells compared to somatic fibroblasts. This may represent a nuclear response to decreased content of mitochondrial DNA (mtDNA) (Lloyd *et al.*, 2006), which was found reduced in undifferentiated human ES and iPS cells (Armstrong *et al.* 2010; Prigione *et al.*, 2010). Indeed, cells depleted of mtDNA have been shown to induce the expression of mitochondrial biogenesis factors (Holmuhamedov *et al.*, 2003) and *POLG* expression was up-regulated in cells harboring large deletions of mtDNA (Alemi *et al.*, 2007). Although one iPS cell line showed mild up-regulation of some ETC genes compared to somatic fibroblasts, most of the nuclear encoded ETC components were similarly expressed in both undifferentiated pluripotent cells and somatic fibroblasts, suggesting that the metabolic shift occurring upon reprogramming may not be strictly dependent on transcriptional regulation of ETC.

A similar effect has been described for cancer cells, in which the reduced dependence on mitochondrial respiration is not generally due to defects in OXPHOS or altered expression of ETC components, but rather to upstream mechanisms involving factors associated with oncogenesis able to promote glycolysis such as c-MYC, the hypoxia-related factor HIF1 α , and p53 (Vousden *et al.*, 2009). Changes in bioenergetics may then induce modifications of mtDNA copy number and modulate the expression of genes involved in mitochondrial biogenesis (Leary *et al.*, 1998; Lebedeva *et al.*, 2009).

Some of these factors and associated mechanisms may also be in place during cellular reprogramming. c-MYC, whose up-regulation has been associated with elevated lactate dehydrogenase-a expression and glycolytic activity (Shim *et al.*, 1997) and with induction of gene expression programs favoring mitochondrial biogenesis (Li *et al.*, 2005), is one of the four factors comprising the Yamanaka reprogramming cocktail (Takahashi *et al.*, 2007). Hypoxia-related pathways, which have been found to change metabolic homeostasis by inducing glycolysis (Carmeliet *et al.*, 1998; Silvan *et al.*, 2009), may also play a role during cellular reprogramming, as hypoxic conditions have been recently demonstrated to enhance iPS cell generation (Yoshida *et al.*, 2009). Finally, p53 can modulate cellular metabolism by reducing the flux through the glycolytic pathway and inducing OXPHOS (Hu *et al.*; Bensaad *et al.*, 2006; Matoba *et al.*, 2006). Interestingly, p53 activation leads to rapid ES cell differentiation (Maimets *et al.*, 2008), while its abrogation can improve the derivation of human

and mouse iPS cells (Hong *et al.*, 2009; Marion *et al.*, 2009; Utikal *et al.*, 2009). Moreover, reduced mtDNA levels, which have been detected within undifferentiated human ES and iPS cells (Armstrong *et al.* 2010; Prigione *et al.* 2010), have been linked to loss of p53 (Lebedeva *et al.*, 2009). Hence, p53 might also represent a barrier for cellular reprogramming due to its metabolic-related function of opposing the "Warburg effect".

Overall, although the ectopic introduction of c-MYC, hypoxia and p53 inhibition might not be essential for reprogramming, as functional iPS cells can be derived even in their absence, they all appear to play a critical role in enhancing the process (Nakagawa *et al.*, 2008; Hong *et al.*, 2009; Marion *et al.*, 2009; Utikal *et al.*, 2009; Yoshida *et al.*, 2009). Thus, it is tempting to speculate that the same mechanisms may be already in place during cellular reprogramming and modulation of the common downstream metabolic pathways might represent an important step for efficient generation of iPS cells (Fig. 7).

During spontaneous *in vitro* differentiation of ES and iPS cells, we detected a consensual down-regulation of nuclear genes responsible for regulating mitochondrial biogenesis and mitochondrial bioenergetic metabolism (ETC genes). Although relative little data exist on the regulation of genes involved in bioenergetic metabolism during stem cell differentiation, earlier works on mitochondrial biogenesis in human ES cells, showed a similar down-regulation of these genes during differentiation (St John *et al.*, 2005; Cho *et al.*, 2006; Armstrong *et al.* 2010; Prigione *et al.*, 2010). However, the same genes have been found up-regulated upon differentiation of human mesenchymal stem cells (Chen *et al.*, 2008) and mouse ES cells (Facucho-Oliveira *et al.*, 2007). The most likely explanation of these conflicting results may be that the nuclear response is depending on the mtDNA content (Mercy *et al.*, 2005; Lloyd *et al.*, 2006) which may vary within different cell types or different time points. Interestingly, within teratoma samples, some genes involved in both mitochondrial biogenesis and bioenergetic metabolism were up-regulated compared to undifferentiated cells, suggesting a different mitochondrial response during *in vitro* and *in vivo* spontaneous differentiation of human pluripotent cells, possibly due to the tumorigenic nature of teratomas. Moreover, specific differences could be detected between teratoma samples derived from ES and iPS cells, despite a high level of global transcriptional similarities. These results might imply a potential distinct mode of mitochondrial regulation between *in vivo* differentiated human ES and iPS cells and may possibly reflect random viral integration effects occurring within viral-derived iPS cells. Alternatively, they could reflect specific properties of the original somatic cell lines.

Finally, genes involved in the response to oxidative stress, which were over-expressed in somatic fibroblasts and ES and iPS-derived fibroblast-like cells (Prigione *et al.*, 2010), did not appear up-regulated in spontaneously differentiated cells compared to undifferentiated ES and iPS cells. Thus, spontaneously differentiated cells may undergo a metabolic shift but the extent of this might be linked to the particular differentiated cell type generated. Indeed, ES and iPS cell-derived fibroblasts showed similar metabolic features to somatic fibroblasts (Prigione *et al.*, 2010), while *in vitro* and *in vivo* spontaneously differentiated ES and iPS cells may not attain a similar state, probably due to the fact that they are composed of several cell types and thus cell-specific changes may be masked. For these reasons, future work

addressing the mitochondrial-related changes upon differentiation should focus on cell-type specific differentiation protocols in order to better dissect the modulation of mitochondrial function in the context of a selected cell type.

Summary

We have reported the mitochondrial-related transcriptional signatures of spontaneously differentiated human ES and iPS cells and suggested that mitochondrial and metabolic remodeling may play a key role during the acquisition of pluripotency upon cellular reprogramming and loss of pluripotency due to differentiation.

Interestingly, malignant transformation and cellular reprogramming might share a similar metabolic shift. Since proposed anti-cancer agents include glycolytic inhibitors (Pelicano *et al.*, 2006), future studies are warranted to distinguish the mitochondrial and metabolic profiles of stem cells to that of transformed cells (Chen *et al.* 2010; Yanes *et al.* 2010) in order to prevent a possible elimination of stem cells by treatments aimed against tumorigenic cells.

In conclusion, understanding the mechanisms underlying the modulation of mitochondrial and bioenergetic metabolism may eventually shed some light on the processes of acquisition, maintenance and exit of a self-renewal state in pluripotent human cells.

Materials and Methods

Cell lines and culture conditions

Human ES cell lines (H1 and H9) were obtained from the WiCell Institute (Madison, WI, USA). iPS cell lines iPS2 and iPS4 were generated by retroviral transduction of the Yamanka cocktail (OCT4, KLF4, SOX2 and c-MYC) from neonatal foreskin fibroblasts HFF1 (ATCC, #1041, Manassas, VA, USA) and fully characterized (Prigione *et al.*, 2010). ES and iPS cells were cultured in human ES media containing knockout DMEM supplemented with 20% knockout serum replacement (SR), nonessential amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate, 0.1 mM β -mercaptoethanol (all from Invitrogen, Carlsbad, CA), and 8 ng/ml bFGF (Preprotech, Rocky Hill, NJ). Cultures were maintained on mitomycin-inactivated mouse embryonic fibroblast (MEFs) and manually passaged using a cut-and-paste technique (Babaie *et al.*, 2007). Prior to RNA isolation, cells were grown under feeder-free conditions on Matrigel (BD Bioscience, San Diego) - coated dishes as previously described (Greber *et al.*, 2007).

In vitro and in vivo differentiation

For *in vitro* differentiation, embryoid bodies (EBs) were generated as previously described (Prigione *et al.*, 2010). Briefly, ES and iPS cells were seeded onto low-attachment dishes in differentiating medium (human ES medium without bFGF supplementation). Two time-points (day 6 and day 20) were chosen on the basis of previous results (Fathi *et al.*, 2009) and EBs were harvested for RNA isolation. *In vivo* differentiation experiments were performed by EPO-Berlin GmbH using NOD scid gamma mice as previously described (Prigione *et al.*, 2010). Teratomas obtained from ES and iPS collected 50-70 days after injection, dissected and fast-frozen for RNA isolation.

Microarray-based global gene expression analysis

Total messenger RNA (mRNA) was isolated using the RNeasy mini kit (Qiagen, USA) and quality-checked by Nanodrop analysis (Nanodrop Technologies, Wilmington, DE, USA). 400ng of mRNA was used as input

for generating biotin-labelled cRNA (Ambion, Austin, TX, United States). cRNA samples were then hybridized onto Illumina human-8 BeadChips version 3. Hybridizations, washing, Cy3-streptavidin staining and scanning were performed on the Illumina BeadStation 500 platform (Illumina, San Diego, CA, USA), according to the manufacturer's instruction. The following samples were hybridized in duplicate: HFF1, H1, H9, iPS2, iPS4, H1-EB6, H1-EB20, H9-EB6, H9-EB20, iPS2-EB6, iPS2-EB20, iPS4-EB6, iPS4-EB20, H1-teratoma, iPS2-teratoma, iPS4-teratoma. Expression data analysis was carried out using the BeadStudio software 3.0 (Illumina, San Diego, CA, USA). Raw data were background-subtracted, normalized using the "rank invariant" algorithm, and filtered for significant expression on the basis of negative control beads. Genes were considered significantly expressed with detection p values ≤ 0.01 . Differential expression analysis was performed using the illumina custom method; the following parameters were set to identify statistical significance: differential p values ≤ 0.01 , fold change ratio > 1.5 . Pathway analysis and tissue expression annotation were performed using DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov>). Heatmaps were generated using Microarray Software Suite TM4 (TMEV.bat). Selected gene lists were derived from SA Biosciences PCR arrays: EMT PCR array, Mitochondrial Energy Metabolism PCR Array, and Oxidative stress and Antioxidant defense PCR array (www.sabiosciences.com).

Quantitative real-time polymerase chain reaction (Q-PCR)

Real-Time PCR was performed in 384 Well Optical Reaction Plates (Applied Biosystems, Foster City, CA, United States) using SYBR@Green PCR Master Mix (Applied Biosystems). Reactions were carried out on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using the following program: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 1 min, 95°C for 15 s, 60°C for 15 s and 95°C for 15 s for a total of 40 cycles. Triplicate amplifications were carried out for each target gene with three wells serving as negative controls. Quantification was performed using the comparative Ct method (ABI instruction manual), normalized with the housekeeping gene *GAPDH* and presented as a percentage of the expression of ES cell line H1. All primer sequences are provided in Supplementary Table 1.

Immunofluorescence and microscopy

For immunocytochemistry, cells were fixed with 4% paraformaldehyde for 20min at RT, washed two times with PBS and blocked with 10% chicken serum (Vector Laboratories) and 0.1% Triton X-100 (Sigma). Primary antibodies included OCT4 (1:100 Santa Cruz #sc-5279) and Fibronectin (1:100, Sigma #F3648). Secondary antibodies used were conjugated with either Alexa 488 or Alexa 594 (Invitrogen). Nuclei were counter-stained with DAPI (200ng/ml, Molecular Probes, # D-1306). Coverslips were mounted using Dako fluorescent mounting medium (Dako #S3023) and visualized using a confocal microscope LSM 510 (Zeiss). Phase-contrast pictures were taken using a digital camera (Canon).

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