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Telomerase and Oxidative Stress in Embryonic Stem Cells

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1. Introduction

Embryonic stem (ES) cells are derivatives of the inner cell mass from pre-implantation blastocysts of early mammalian embryos. They are uncommitted and pluripotent cells with a characteristic high self renewal potential. If propagated under appropriate tissue culture conditions they can proliferate indefinitely while maintaining a high genomic stability and are in fact immortal. An important contributor to these properties is a high amount of telomerase activity. This occurs mainly via the telomere maintenance function of telomerase. However, recently it has also been shown that telomerase can localise to mitochondria and decrease intracellular oxidative stress.

The final goal for the use of human embryonic stem cells (ESC) is to generate differentiated cells and products for cell replacement therapies. For this purpose, embryonic stem cells can be induced to differentiate into cells from all three germ layers: mesoderm, entoderm and ectoderm and eventually give rise to all somatic cell type of the human body. *In vitro*, this requires specialised growth conditions and factors which are often defined empirically rather than due to scientific evidence. For example, it emerges recently that growing ES cells under low, physiological oxygen conditions rather than atmospheric oxygen (21%) are greatly beneficial for the properties and application of these cells.

This chapter will summarise our current knowledge on telomerase expression and oxygen conditions and their involvement in the maintenance of self-renewal, pluripotency and stem cell differentiation.

2. Telomerase expression in ES cells

There exist several factors which are known to contribute to ESCs pluripotency. Among them there are the transcription factors Oct4, Sox2, NANOG as well as a constitutively high level of telomerase expression. All these factors are down regulated during differentiation of ESCs into various cells and tissues. The only exception to that are germ line cells which continue to express high amounts of telomerase. The mechanisms of maintaining pluripotency differ in mouse and human ES cells. While the murine system requires both LIF and BMP4 for self-renewal human ESC culture needs to be supplemented with basic fibroblast factors and the TGFβ/Activin/Nodal pathway has to be activated.

The ribonucleoprotein telomerase is a unique reverse transcriptase that consists of two main parts: TERT, the catalytic subunit and TR or TERC, the RNA component that contains the

template region for the synthesis of telomeric repeats. The main and best studied function of telomerase is the extention of telomeres. Telomeres are the end of chromosomes and protect them from genomic instabilities. Telomeres in cells without or with rather low levels of telomerase shorten continuously (Harley et al., 1990) due to loss of sequences caused by inability of conventional polymerases to fill the gap of the last RNA primer at the lagging strand synthesis as well as to oxidative stress (von Zglinicki et al., 1995). There is an important difference between mouse and humans regarding the expression of telomerase in somatic cells and tissues. While telomerase expression persists in many adult tissues in mice in humans telomerase levels are high during early embryonic development, but then down regulated during development. Therefore, only some cell and tissue types in the adult human body retain telomerase activity such as endothelial cells and lymphocytes while adult stem cells are able to activate telomerase upon activation. However, a stable maintenance of telomeres is necessary for ongoing cellular proliferation and immortality. Embryonic stem cells share a high level of telomerase expression with cancer cells but they are characterised by a high genomic stability. The physiological regulation and fine-tuning of telomerase expression occurs at multiple levels including transcriptional, post-transcriptional, post-translational and different sub-cellular localisations.

In addition to telomere maintenance that is of particular significance in vigorously proliferating cells, non-telomeric functions of telomerase have been described recently (for review see Saretzki, 2009). These include interference of the catalytic subunit TERT with important signalling pathways of stem cells and early embryonic development such as WNT and myc (Choi et al., 2008). These pathways are also important for embryonic stem cells. Recently it has been demonstrated that TERT can function as a transcriptional co-factor that modulates chromatin structure and activates genes downstream of the WNT signalling pathway (Park et al., 2009). No RNA component or catalytic function are necessary for this non-canonical function of telomerase. Although these studies have been performed in adult stem cells one can speculate that telomerase/TERT might have additional, non-telomere roles in embryonic stem cells as well. However, so far no studies have been performed to address this issue.

It is also known that the presence of telomerase correlates to cellular survival under conditions of DNA damage and stress. While over- expression of telomerase protects various cell types including embryonic stem cells (Fu et al., 2000, Zhu et al., 2000, Zhang et al., 2003, Armstrong et al., 2005, Ahmed et al., 2008, Yang et al., 2008) telomerase inhibition can lead to increased apoptosis and increased sensitivity against cellular stress including treatment with chemotherapeutic drugs (Kondo et al., 1998, Saretzki et al., 2001, Ludwig et al., 2001, Yang et al.2008).

In addition, it has been demonstrated that telomerase can be excluded from the nucleus under oxidative stress and localise to the mitochondria (Santos et al., 2004, Ahmed et al., 2008, Haendeler et al., 2009). This can improve mitochondrial function, decrease cellular oxidative stress and protect cells from apoptosis and DNA damage (Ahmed et al., 2008, Haendeler et al., 2009). However, no detailed studies on the relationship between telomerase expression and mitochondrial properties, including generation of reactive oxygen species (ROS) have been performed on embryonic stem cells although we have preliminary data that show a mitochondrial localisation of TERT protein within mitochondria in human embryonic stem cells (unpublished data). It is likely that other factors than oxidative stress can promote mitochondrial localisation of telomerase in order to provide a pro-survival function. Since telomerase is able to shuttle from the nucleus to the mitochondria it can carry

out nuclear functions such as telomere maintenance or interference with gene expression and transcription in parallel with any potential function at other cellular locations including mitochondria. However, this fact also makes it difficult to separate the various functions of telomerase within a cell. Over-expression of telomerase in mouse and human embryonic stem cells improved many stem cell parameters including proliferation and colony forming abilities (Armstrong et al., 2005, Yang et al., 2008). However, the differentiated progeny from TERT over-expressing stem cells maintained high telomerase levels and showed less intracellular oxidative stress as well as higher apoptosis resistance (Armstrong et al., 2005, Lee et al., 2005, Yang et al., 2008).
In contrast, down regulation of TERT expression in ES cells correlated either with immediate spontaneous differentiation or decreased proliferation rates demonstrating the importance of a high telomerase for pluripotency in human embryonic stem cells (Yang et al., 2008).

3. Telomerase activity and differentiation

A constant high telomerase expression is a hallmark of pluripotent human stem cells (Amit et al., 2000). In contrast, it is lower, although strongly inducible in adult human stem cells (Hiyama and Hiyama, 2007). Down regulation of telomerase expression strongly correlates with the *in vitro* differentiation process in both embryonic and adult stem cells resembling the situation *in vivo* very closely (Saretzki et al., 2008, O'Connor et al., 2009).
We have shown previously that down regulation of telomerase as well as other pluripotency factors such as Sox2, Oct4 and NANOG and increase of oxidative stress, DNA damage and mitochondrial biogenesis in embryonic stem cells coincided in time when cells were differentiating (Armstrong et al., 2000, Saretzki et al., 2008). However, it is not known whether there is a causal interplay between these factors during the differentiation process. The differentiated progeny of ESCs down regulated telomerase within a week, shorten telomeres and accumulate DNA damage (Saretzki et al., 2008). The mechanism for this down regulation seem to be epigenetic processes such as methylation and acetylation of the TERT and, perhaps to a lesser extend, the TERC promoters (Lopatina et al., 2003, Atkinson et al., 2005, Saretzki et al., 2008). However, additional mechanisms for down regulation of telomerase such as polyunsaturated fatty acids have been described as well (Eitsuka et al., 2005).
In general, it seems important that endogenous telomerase is down regulated during differentiation of ESCs as well as cancer cells (Bagheri et al., 2006, Li et al., 2005). However, it is not entirely clear whether down regulation of telomerase activity is merely associated or even necessary for differentiation of ESC.
Therefore studies which analyse over-expression or inhibition of telomerase in ESCs are very informative to address this question. Over-expression was achieved by constitutively over-expressing TERT, the catalytic subunit of telomerase in ES cells. Over-expression of TERT further potentiated the pluripotency and proliferation capacities of mouse and human ESCs (Armstrong et al., 2005, Yang et al., 2008). TERT over-expression resulted in a higher proliferation rate due to an increased fraction of cells in S-phase in mouse as well as human ES cells (Armstrong et al., 2005, Yang et al., 2008). However, differentiation capabilities after TERT over-expression varied between mouse and human ESCs and between different studies (Armstrong et al., 2005, Lee et al., 2005, Yang et al., 2008). While endogenous telomerase is normally down regulated during differentiation of ES cells ectopic telomerase remained stably expressed in the differentiated progeny (Armstrong et al., 2005, Yang et al.,

2008). TERT over-expressing mouse ESCs showed an almost 5 fold increased efficiency to generate hematopoietic progenitors (CFU-GEMM) as well as other myeloid lineages (Armstrong et al., 2005). Lee et al., (2005) did not find any changes in proliferation and differentiation in mouse ESC when they over-expressed mTERT. However, they found a protection of ES cells against cell death during differentiation and increased resistance to apoptosis induced by oxidative stress and other genotoxic insults that depends on the catalytic activity of mTERT.

In addition, TERT over-expression resulted in a decrease in spontaneous as well as induced apoptosis rates in ESCs and in embryoid bodies (EBs) at day 6 of differentiation. At the same time also the increase in oxidative stress levels during differentiation into EBs was significantly attenuated (Armstrong et al., 2005). However, TERT over-expression in human ESCs only promoted proliferation and accelerated cell cycle progression, but suppressed differentiation properties (number of hematopoietic colonies formed) of TERT over-expressing cells although they could form all three germ layers *in vivo* (Yang et al., 2008). The reasons for these differences in the differentiation capacity of mouse and human ESC after ectopic TERT expression are not clear but could be explained with general differences between mouse and human telomere and telomerase biology. Conversely, when TERT and telomerase activity levels are severely reduced due to over-expression of a transcriptional inhibitor of TERT (Zap) cell growth as well as differentiation potential are significantly compromised (Armstrong et al., 2004).

Comparison of gene expression between normal and TERT over-expressing mESCs revealed an up regulation of cell cycle genes (CyclinD1, which was also up regulated in the human experiment, Yang et al, 2008), detoxification and differentiation as well as a down regulation of two DNA damage and stress-related downstream genes of p53: p21 and Gadd45a (Armstrong et al., 2005). These results correspond well with a decreased oxidative stress and apoptosis sensitivity as well as with a higher proliferation rate and differentiation capacity towards hematopoietic lineages in TERT over-expressing murine ES cells. Interestingly, Tsai et al (2010) published recently that over expression of TERT in human mesenchymal stem cells increases proliferation and differentiation potential and brings those cells close to human ES cells (hESC) in their gene expression and methylation pattern (Tsai et al., 2010).

Together, these results show that ectopically over-expressed telomerase can mitigate increases in ROS and oxidative stress but not diminish them entirely. The mechanism could be the delayed down regulation of genes involved in antioxidant defences as well as various heat shock factors (Armstrong et al., 2005). This data suggests that stably high levels of telomerase might be responsible for the maintenance of high levels of stress defence capacities in accordance with findings from our and other groups on hTERT over-expressing cells (Fu et al., 200, Zhu et al., 2000, Zhang et al., 2003, Ahmed et al., 2008).

The data on a protective function of telomerase in differentiated progeny of ES cells are in good accordance with telomeric protection of telomerase as well as newly described non-telomeric functions. In addition to telomere maintenance TERT can also directly change gene expression in a telomere-independent fashion by functioning as a chromosomal modulator and transcriptional c-factor of the Wnt pathway (Choi et al., 2008, Park et al., 2009). TERT therefore can actively influence gene expression levels and interact with important signalling pathways in stem cells although this observation has not been extended to embryonic stem cells yet. A connection between Wnt signalling and the activation of mitochondrial biogenesis and OXPHOS gene expression involved in oxidative phosphorylation has been described in immortalised mouse myoblast cells recently (Yoon et al., 2010).

In summary, these results also suggest that down regulation of telomerase is usually associated with differentiation of ES cells, however, it does not seem to be essential for the differentiation process since ES cells stably over- expressing telomerase retain their differentiation capacity.

4. Long term cultivation and karyotypic stability

In order to obtain enough stem cells for any potential application in regenerative medicine and cell replacement therapies a large amount of cells and consequently, an extensive expansion of stem cell cultures would be required. Contradictory data exist about genetic instabilities in embryonic stem cells during long term cultivation. ES cells maintain a constant telomere length due to the presence of high telomerase levels and also show lower rates of genomic mutation than somatic cells even after prolonged *in vitro* culture. Some stem cell lineages that had been grown for a long time in culture retained a normal karyotype (Rosler et al., 2004, Buzzard et al., 2004) as well as their epigenetic stability (Rugg-Gunn et al., 2005) while others described an accumulation of chromosomal abnormalities when ES cells were cultured for a prolonged time (Longo et al., 1997, Maitra et al., 2005, Armstrong et al, 2005). This might depend on the species (mouse or human) as well as the (often sub-optimal) culture conditions.

Various groups had cultivated human embryonic stem cell lines for around 100 passages (Maitra et al., 2005, Park et al., 2008, Xie et al., 2010). Park et al. (2008) and Xie et al. (2010) did not find any gross morphological changes or different expression levels for stem cell markers between early and late passages. Both groups consistently reported an increased proliferation of ESCs at higher passages, no changes in telomerase activity as well as a reduced differentiation ability.

However, while Park et al. (2008) found no karyotypic changes Xie et al (2010) described some karyotypic abnormalities (translocation) in one of the two hES cell lines analysed while Maitra and co-workers (2005) and found karyotypic anomalies in 8 out of 9 analysed human ESC lines at high passage numbers. These included copy number changes (amplifications and deletions of chromosomes, 45%) and gene promoter methylation (90%). Interestingly, many of those changes are commonly known to occur in cancer. Both Maitra and co-authors (2005) as well as Xie et al., (2010) observed in addition to genomic instabilities also changes in mitochondrial sequences and function. Maitra et al. (2005) found changes in mitochondrial DNA sequence six heteroplasmic sequence alterations in two of nine (22%) late-passage hESC lines, most of them in protein coding regions such as in the ATPase6 gene which most likely probably affected the protein function. Xie et al., (2010) described a compromised mitochondrial function as well as an increased level of ROS production and a higher membrane potential and mitochondrial mass. These two studies emphasise that in addition to nuclear changes there seem to occur changes in mitochondrial sequence and function as well. Decline in mitochondrial function is a hallmark of cellular senescence as well as ageing (Passos et al., 2006, Passos et al., 2010). In general, little research has been conducted on changes in mitochondrial properties of ES cells during prolonged culture.

Since it has been shown recently that telomerase can influence mitochondrial function (Santos et al., 2004, Ahmed et al., 2008, Haendeler et al, 2009) it is possible that in addition to telomere-maintenance by telomerase its intracellular localisation could also be important for proper mitochondrial function in ES cells.

In summary, although contradictory results exist regarding the frequency of various genomic and epigenetic changes that might occur during long-term cultivation of human ES cells a careful karyotypic analysis has to be performed for cells that are intended to be used for regenerative purposes. More data on genome instability will be discussed in connection with various oxygen conditions.

5. Mitochondria, oxidative stress and pluripotency in ES cells: lessons for optimal culture conditions

Mitochondria are important organelles that produce the energy of a cell. In addition, they play an essential role for many additional cellular processes including cellular survival and differentiation. Therefore, a tight regulation of mitochondrial function and integrity is important for all cells, including embryonic stem cells. In contrast, compromising of mitochondrial function results in cellular senescence, ageing and age-related degenerative diseases (Passos et al., 2006, Passos et al., 2010, Wallace, 2005). New connections and mechanisms between mitochondrial properties and stem cells emerge recently and will be summarised here.

Mammalian blastocysts and embryonic stem cells of pre-implantation embryos exist under highly hypoxic conditions. While early stage embryos develop low oxygen conditions (1.5% in rhesus monkeys, slightly higher in rabbits: 3.5% and hamster: 5.3%) oxygen content increases upon implantation of the embryo into the uterus (Fisher and Bavister, 1993). Thus, they have a limited oxidative capacity and immature mitochondria. They generate most of their ATP via aerobic respiration and glycolysis. Only when these cells differentiate do their mitochondria mature, increase in number and switch to oxidative phosphorylation for energy production (Cho et al., 2006). Mitochondrial content increases dramatically when oocytes mature (Shoubridge and Wai, 2007). Decreased mitochondrial content in oocytes correlates to poor fertilisation efficencies (Santos et al., 2006). During early embryogenesis the initial amount of mitochondria (around 150 000-300 000 per human oocyte) gets distributed to the dividing cells and is therefore decreased in number per cell. Consequently, the mitochondrial number is also low in undifferentiated embryonic stem cells that derived from the blastocyst stage embryos. At later stages of pre-implantation development mitochondrial DNA replication and transcription are activated. These processes coincide with mitochondrial maturation into morphologically typical and metabolically active organelles that use oxidative phoshorylation and aerobic respiration for ATP generation (Trimarchi et al., 2000). However, at the blastocyst stage *in vivo* cells of the trophoectoderm start mitochondrial maturation and differentiation while the pluripotent cells of the inner cell mass have still immature mitochondria and mainly rely on glycolysis for energy production (Houghton 2006). Mammalian mitochondrial DNA is around 16kb in size and encodes 13 mitochondrial proteins as well as various RNAs (tRNAs and rRNAs). All other proteins and factors are encoded in the nucleus and then imported into mitochondria. The mitochondrial genome is double stranded and contains no introns. Mitochondrial genomes form complexes-nucleoids that reside in the matrix close to the inner mitochondrial membrane.

Undifferentiated embryonic stem cells from both mouse and humans have very small cytoplasm (Thomson et al, 1998, Varum et al., 2009) and seem to conserve characteristics of small, undeveloped mitochondria which is also reflected in their low mitochondrial mass and low mitochondrial copy number (Saretzki et al., 2004, Saretzki et al., 2008, Facucho-

Oliveira et al., 2007). In addition, these cells have low levels of ATP (Cho et al., 2006) as well as low levels of oxygen consumption (Kondoh et al., 2007).

Facucho-Oliveira et al., (2007) suggested that the maturation of the mitochondrial network and pluripotency are mutually exclusive. The authors showed that activation of aerobic respiration during differentiation of ESCs coincided with down regulation of pluripotency markers. The authors claim that in mouse ES cells the expression of pluripotency markers decreased prior to the expansion and maturation of mitochondria during spontaneous differentiation. However, they used different pluripotency markers from the classical (Oct4, Sox2, NANOG). Surprisingly, they even detected an increased expression of their markers (Dppa5, Pramel 7 and NDP52L1) during early differentiation days (up to day3), only after that the expression of those markers decreased while changes in mitochondrial copy number as well as expression of mitochondrial replication and transcription related factors started to increase at or after day 6 respectively. In addition, no direct experimental prove was given that the initial decrease of pluripotency markers was indeed causal for mitochondrial maturation or just a co-incidence. The same authors claim that a steady-state expression of PolG, the mitochondrial DNA polymerase is essential for the maintenance of a pluripotent state in ES cells (Facucho-Oliveira et al., 2007). It has been demonstrated by various groups that once ES cells differentiated their mitochondrial number increased (Saretzki et al., 2008, St. John et al., 2005). This process coincided with the loss of pluripotency during differentiation of ESC. St. John et al. (2005) suggest that this is in accordance with a concept that each stage of cellular development adjusts its metabolic and energetic demands to it oxidative capacity. However, it is not clear whether the correlation between pluripotency of the inner cell mass and immature mitochondria is purely associative or mutually dependent on each other.

In accordance with the hypoxic nature in the pre-implantation embryo there is accumulating evidence that favours low oxygen for embryonic development *in vitro* and *in vivo*.

Rinaudo et al. (2006) found that mouse embryos cultivated under 5% oxygen showed a gene expression pattern which was much more similar to that of *in vivo* embryos when compared with those cultivated under 20% oxygen. This data corresponds to observations from Harvey et al. (2004) who found a significant increase in the proportion of ICM cells compared with trophectoderm cells when embryos were cultured under 2% oxygen instead of 21% oxygen. These results are in agreement with findings that pluripotent cells display an increased proliferation under reduced oxygen concentrations (see below). There is evidence accumulating that suggests that reducing the oxygen concentration towards physiological levels *in vivo* is beneficial for the *in vitro* maintenance and pluripotency of hES cells. This includes a decrease of spontaneous differentiation while promoting self-renewal and maintaining a stable karyotype (Ezashi et al. 2005, Westfall et al. 2008, Forsyth et al. 2006). Westfall and co-workers compared gene expression patterns in stem cells under low and high oxygen conditions of cultivating stem cells (Westfall et al. 2008). The authors identified various genes whose expression was sensitive to oxygen concentrations, many of them under the control of hypoxia-inducing factors and genes encoding enzymes involved in carbohydrate catabolism and cellular redox state. Although genes associated with pluripotency, including OCT4, SOX2, and NANOG were not directly affected, some downstream genes such as LEFTY2 were decreased under 20% oxygen (Westfall et al. 2008). Zachar and co-workers (2010) analysed human ESC up to 42 month under 20% and 5% oxygen and found that while under 20% many cells spontaneously differentiated the cultures under hypoxia (5%) better maintained their pluripotency: they continued to express

high levels of Oct4 and NANOG as well as telomerase activity. The cultures also retained their normal karyotype and were able to differentiate into all 3 germ layers.
However, other studies found no apparent advantages of culturing hES cells under reduced oxygen tension when cells are passaged sufficiently frequent (Chen et al. 2009).
At least mouse ES cells seem to be not very sensitive against oxidative stress that is even higher than 21% oxygen levels (Saretzki et al., 2004) while hESCs might be more delicate in their requirements and many studies show an advantage of hypoxic culture conditions. Ezashi et al. (2005) found that cultivating human ESC under low oxygen (5%) compared to 21% of ambient oxygen reduced the amount of spontaneous differentiation dramatically.
Several lines of evidence seem to explain the beneficial effect of hypoxia with the induction of. hypoxia-inducible factors (HIF). For example, Ji et al. (2009) demonstrated the positive effect of transcription factor HIF-1alpha on pluripotency and the maintenance of an undifferentiated phenotype in human ESCs. The authors co-cultured human embryonic cells with human feeder cells that stably expressed HIF-1alpha. Their results demonstrated that HIF-1alpha was critical for preventing differentiation of hES cells in culture (Ji et al., 2009). At the same time hypoxia also upregulated the expression of NANOG and Oct-4 and of some soluble factors including βFGF and SDF-1alpha which are released into the microenvironment to maintain the undifferentiated status of hES cells (Ji et al., 2009).
These results have been confirmed and extended recently by Forrestal et al. (2010). The authors also found a faster proliferation and larger colony size in human ES cells cultivated up to 12 month under hypoxia (5%) compared to normoxia (21%). Consequently, hES cells cultured under 20% oxygen had to be passaged more frequently prior to confluency in order to remove differentiated areas, while hES cells under low oxygen were only passaged when they reached confluency. Importantly, upon exposure to hypoxic conditions, cells displayed a physiological response that ensures availability of sufficient oxygen levels for oxygen-dependent processes. This response is regulated by hypoxia inducible factors (HIFs) which regulate the expression of many downstream genes including various involved in erythropoiesis, apoptosis and proliferation (Semenza, 2000). HIFs are transcription factors consisting of three oxygen-dependent subunits: HIF1α, HIF2α and HIF3α. Although HIF1α in mouse is the main regulator of hypoxic responses in human ES cells, HIF1α is only transiently expressed after hypoxic exposure and predominantly regulates different (mainly glycolytic) target genes than HIF2α. The authors identified HiF2α as the directly responsible one for the maintenance of pluripotency under hypoxic (5%) oxygen (Forrestal et al., 2010) corresponding to earlier findings in mouse ES cells that HIF2α is a direct upstream regulator of OCT4 (Covello et al.2006). This data correlated well with a 60-80% reduction in expression of pluripotency markers under normoxia. Inhibition of HIF2α and HIF3α, but not HIF1α decreased the expression of the pluripotency markers Oct4, Sox2 and NANOG. However, only HIF2A silencing correlated to morphological changes of more differentiation, a proliferation stop and loss of pluripotency markers (Forrestal et al., 2010). Taken together, HIF2α regulated the long-term hypoxic response by controlling hES cell pluripotency and proliferation.
In contrast to other cell types, HIF1α is only responsible for the initial adaptation of cells to hypoxia in embryonic stem cells and it was observed in mouse ES cells that expression of HIF1α under hypoxia suppressed the LIF–STAT signalling pathway resulting in the inhibition of self renewal and the promotion of cell differentiation (Jeong et al. 2007). This data provide a greater understanding of the mechanisms which regulate ES cell function

and the pluripotent state and confirm that environmental oxygen tension has a critical role for maintaining of pluripotency in hES cells.

A direct influence of hypoxia and HIF1α on telomerase expression in mouse ES cells was recently established by Coussens et al. (2010). A role for HIF1α for the activation of telomerase had already been established in cancer cells previously (Nishi et al., 2004, Yatabe et al., 2004). Coussens et al. (2010) used a RNAi screen in order to identify factors that are responsible for maintaining a high TERT expression in murine ES cells. Knocking down HIF1α did not result in any differences in pluripotency, morphology or growth rate in mES cells but it down regulated TERT expression as well as telomerase activity in those cells and consequently, also telomeres shortened over 120 population doublings (PDs). The authors identified a functional HIF1α binding site at position +1 of the TERT promoter. The mechanism for up-regulation of Hif1α levels in cells in response to hypoxia involves a posttranslational stabilization of Hif1α via posttranslational modification of specific prolyl residues in the oxygen-dependent degradation domain (Ulrich, 2007), however the factors that are needed for HIF1α stabilisation are not well understood yet. Coussen et al. (2010) observed that under hypoxic growth conditions both, the targeted and non targeted mES cells induced Hif1α levels as well as TERT levels and telomerase activity. The ongoing telomere shortening in HIF1α targeted mES cells under long term cultivation resulted eventually in a decreased growth rate, increased γH2A.X levels and greatly enhanced p21 levels-indicative for the occurrence of senescence (although it was not directly shown). However, growing Hif1α-targeted cells under hypoxic conditions restored telomerase levels and telomere length. This study is the first to demonstrate a direct connection between hypoxic culture conditions and telomerase expression that is an important factor for pluripotency and unlimited proliferation capacity of undifferentiated stem cells. The authors also speculate that HIF1α might be required to sustain high telomerase levels at the inner cell mass of the blastocyst since early embryos develop under hypoxic conditions.

Another paper that seems at odds with the conclusion that a low oxygen atmosphere is beneficial for the maintenance of pluripotency was published recently by Varum and colleagues (2009). Normal cells decrease the amount of oxidative phosphorylation under hypoxic conditions, therefore the authors hypothesised that a further decrease of mitochondrial function in ES cells might directly promote pluripotency. They inhibited complex III of the mitochondrial respiratory chain pharmacologically and detected a high preservation of stem cell features such as high nuclear/cytoplasmic ratio and a specific increase in NANOG expression while other pluripotency markers (Oct4 and Sox2) or general stemness markers were not changed. In accordance with earlier observations that NANOG over-expression prevented neuroectoderm differentiation (Vallier et al., 2009) the authors found that expression of genes involved in ectoderm differentiation was significantly reduced in their system due to antimycin A. However, antimycin A treatment had a specific interaction with FGFβ signalling in human ES cells. Complex III is directly involved in the electron flow and ATP production of mitochondria, but has also been shown to directly generate ROS (Hamanaka and Chandel, 2009). Treatment of ESCs with antimycin A resulted in impairing of mitochondrial respiration and to a shift from oxidative phosphorylation to glycolysis while ATP levels were maintained at a constant level. In addition, this treatment increased ROS generation while apoptosis and cell proliferation were unchanged (Varum et al., 2010). There seemed to be a direct correlation between ROS generation after antimycin A treatment and NANOG expression since quenching ROS by

using a MnSOD mimetic diminished any increase in NANOG expression. These results suggest that ROS generated at complex III could be at least partially responsive for the effects of antimycin A on NANOG expression levels.

While many other studies had demonstrated an increase of pluripotency under low oxygen levels Varum and co-authors (2010) showed that a certain level of ROS seemed necessary specifically for a high expression of NANOG. From these data it could be speculated that modulation of mitochondrial function has a rather specific effect on skewing the differentiation probability of specific, in this case ectodermic, lineages. It could also mean that specific oxidative conditions favour the differentiation of certain tissues, however, more evidence for that has to be gathered before any meaningful conclusion can be drawn.

A possible explanation for the apparent difference between the papers that showed a benefical effect of hypoxia on pluripotency of ES cells and the one published by Varum et al. (2009) is that it had been shown that ROS generation at mitochondria is responsible for the propagation of a hypoxic signal (Chandel et al., 1998). Complex III produces increased amounts of ROS under hypoxia which is similar to inhibition of complex III with antimycin A (Hamanaka and Chandel, 2009). Hypoxia-induced mitochondrial ROS inhibit HIF-α subunit turnover. Therefore it seems that mitochondrial ROS are required for hypoxic activation of HIFs and that ROS generation at mitochondrial complex III in particular is critical for hypoxia signalling. While complex I inhibitors such as rotenone inhibit the induction of HIF-1α, antimycin A does not (Chandel et al., 1998, Chandel et al., 2000). Guzy et al. (2005) found that a functional complex III of the mitochondrial electron transport chain (ETC) is required for the hypoxic stabilization of HIF-1α and HIF-2α and that an increase in ROS from complex III results in a stabilisation of HIF-α subunits. These results suggest that mitochondria function as cellular oxygen sensors and signal hypoxic HIF-α stabilisation by releasing ROS to the cytosol. Thus, mitochondrial ROS seem to act upstream of prolyl hydroxylases in regulating the stability of both HIF-1α and HIF- 2α in mammalian cells.

However, whether oxidative stress and ROS production is either decreased (Richter and von Zglinicki, 2007) or increased (Hanaka and Chandel,2009) under hypoxia is still controverse and might to a large degree depend on the actual oxygen percentage (1-5%) as well as changes in oxygen concentration during cell handling which might favour re-oxygenation that leads to increased ROS.

Evidence for very specific influences of mitochondria on stem cells has been recently demonstrated by Todd et al. (2010). The authors used loss and gain of function experiments in order to show that the growth factor Gfer plays a very specific role in stem cells, but not in somatic cells. The factor is present in mitochondria as well as in the nucleus and cytoplasm and seems to be highly enriched in various types of stem cells including embryonic. By influencing the mitochondrial fission factor Drp1 Gfer promotes survival and pluripotency in mouse ESCs (Todd et al., 2010). Down regulating Gfer in mouse ESCs decreased pluripotency markers, mitochondrial membrane potential and resulted in mitochondrial fission and fragmentation, a sign of cellular stress. At the same time the damaged mitochondria were eliminated by autophagy and apoptosis. This data suggests that dysfunctional mitochondria are effectively cleared from embryonic stem cells thereby ensuring a high quality of mitochondria remaining within the stem cells. While the level of Drp1 was increased when Gfer levels were decreased a decrease of Drp1 rescued mitochondrial dysfunction, pluripotency and apoptosis in Gfer depleted cells (Todd et al., 2010). Although the authors have not directly characterised glycolysis and energy

generation in those ES cells their data would suggest that embryonic stem cells rely on functional and healthy mitochondria rather than undeveloped and non functioning ones. However, nothing is known about the role of Gfer in human ES cells and more research is needed to clarify and understand this apparent contradiction as well as the direct role of mitochondria for pluripotency.

6. Redox regulation in ES cells

Oxidative stress can be generated endogenously by dysfunctional mitochondria and NADH oxidases or applied externally via cell culture conditions or drugs and agents. It recently has been shown to control self renewal and differentiation of embryonic stem cells. ROS are generated in cells by enzymatic as well as non-enzymatic pathways. NADPH oxidases and xanthine oxidase generate superoxide anions by reducing molecular oxygen. In mitochondria superoxide leaks during electron transport from complexes I and III. However, at the same time there are many different ways in a cell to counteract oxidative stress. A first defence are antioxidant enzymes such as superoxide dismutases that dismutate the superoxide anions to hydrogen peroxide, which then is further scavenged by peroxidases, catalase or other molecular scavengers such as vitamins, carotinoids etc. Therefore, there generally exists a very balanced homeostasis of oxidative stress and redox state within a cell. Disturbing that balance can lead to increased oxidative stress and result in damage of DNA, lipids and proteins. On the other hand, ROS are also an important intracellular signalling factors (Finkel, 2001, Schieke and Finkel, 2006 Starkov 2008).

A better understanding of the influence of culture and environmental conditions should help to optimise growth conditions for undifferentiated and differentiated ES cells. Thus, it will also impinge on the successful application of those cells for the purpose of regenerative medicine.

Evidence is emerging currently that distinct functions of stem cells are under the influence of a redox potential. For example, low oxygen keeps hematopoietic stem cells in a quiescent state while higher ROS promote their differentiation (Hosokawa et al., 2007).

Recently, Yanes and colleagues (2010) uncovered a mechanism how increased oxidative stress not only accompanies differentiation of ES cells as described earlier (Saretzki et al., 2004, Saretzki et al., 2008) but seems to drive it actively. The authors performed an untargeted metabolomics approach in order to compare pluripotent mouse embryonic stem cells and their differentiated progeny. They also used metabolites involved in and produced by oxidative pathways in order to characterise their role in pluripotency and differentiation. They found that embryonic stem cells are characterised by an abundance of metabolites with highly unsaturated structures that decrease during differentiation. Some of the most up-regulated metabolites were secondary lipid messengers as well as inflammatory mediators. These unsaturated metabolites are preferentially susceptible to pro-oxidative events that occur due to intracellular changes in redox status. As evidence the authors monitored the ratio of reduced and oxidised glutathione (GSH/GSSG) during the course of differentiation. Oxidative stress is known to change that ratio due to conversion of GSH into GSSG. While the GSH/GSSG levels decreased by day 4 of differentiation ascorbic acid (an important antioxidant) levels increased during the same time pattern. The authors conclude that cell differentiation is associated with an increase of oxidative stress, an observation corresponding to findings from our laboratory (Saretzki et al., 2004, Saretzki et al., 2008). To prove that activation of an oxidative pathway is indeed necessary for cellular differentiation

they inhibited the eicosanoid signalling pathway. That resulted in a promotion of pluripotency (increase of Oct4 and NANOG expression) and a reduction of differentiation in a dose dependent manner of inhibitors. Accordingly, when treated with eicosanoids, differentiation was promoted. These pathways acted similarly in human ESCs (Yanes et al., 2010). These results correspond well to an increased pluripotency in ESCs grown under physiological (5%) oxygen instead of normoxia (see above). They also correspond to findings of Eitsuka et al. (2005) who found that unsaturated, but not saturated fatty acids decreased telomerase activity and hTERT expression. In particular, polyunsaturated fatty acids (PUFAs) like EPA and DHA, were identified as powerful telomerase inhibitors mediated via protein kinase C (Eitsuka et al. 2005). These results identify an additional mechanism (in addition to epigenetic changes) how telomerase might be down regulated during ES cell differentiation and adds another component to the study (Yanes et al. 2010).

Powers et al (2008) analysed mouse ES cells under various oxygen levels and found that these cells adapted well to all oxygen conditions while maintaining pluripotency markers and an undifferentiated state. The authors found that the cells adapted their metabolism accordingly and generated constant ATP levels, although to a different degree by aerobic respiration in dependence on the oxygen conditions (Powers et al., 2008). However, once differentiated oxygen conditions had a substantial effect on the function and properties of those cells. Only extremely low oxygen concentrations lower or equal 1% induced differentiation of mouse ES cells. That could mean that a certain amount of oxygen is needed to maintain a pluripotent and undifferentiated state in mouse ES cells confirming the results obtained by Varum et al., (2009). The results from Powers et al (2008) are in accordance with our findings that mouse ES cells proliferate undistinguishable under normoxia (21%, ambient oxygen) and under hyperoxia (40% oxygen) (Saretzki et al., 2004). Powers et al (2008) did not find any difference in DNA damage due to different oxygen concentration, but could correlate the damage levels with increasing amounts of hydrogen peroxide. However, these results seem to be at odds with the findings of Li and Marban, (2010) who found a four fold higher DNA damage levels under 20% oxygen than under 5%. However, while Powers et al (2008) used mouse ES cells, Li and Marban (2010) used human stem cells which might explain the differences in DNA damage sensitivities.

In addition to growing human ES cells under physiological oxygen (5%) Li and Marban (2010) as well supplemented the culture media with various antioxidants. The latter were much more powerful in reducing intracellular ROS levels than hypoxia. Surprisingly, there was a biphasic dosage-dependent response to the treatment with antioxidants: while at low concentrations they reduced DNA damage, high concentrations promoted DNA damage. Importantly, the authors found that high doses of antioxidants decreased the level of proteins involved in DNA damage response such as DNA damage mediating kinases ATM and ATR and other downstream DNA repair factors such as Rad50, Rad 51, Chk1 and Chk2 (Li and Marban, 2010). The authors discussed this observation as a concept of "oxidative optimum" and concluded that a certain level of ROS are necessary in order to activate the DNA repair pathway and maintain chromosomal stability and genomic integrity, at least in stem cells. This data suggests that ROS not also induce DNA damage but at the same time are also able to promote the DNA damage response that in turn induces DNA repair.

Thus, instead of a low oxygen content (hypoxia) also antioxidants could be supplemented to the culture medium, however their concentrations have to be carefully optimised in order to avoid any detrimental effect as demonstrated by Li and Marban (2010).

To read more about the current knowledge of cultivating ES cells under low oxygen see Millman et al, (2009) for review.
Napier et al. (2010) showed recently that 20% oxygen concentration down regulated the levels of the RNA component hTR of the telomerase complex in endothelial cells thus hampering telomere maintenance by telomerase. These findings correspond well with the up regulation of TERT expression due to HIF1α under hypoxia (Coussens et al., 2010).

7. P53 and DNA damage response in ES cells

Cells are constantly under environmental and metabolic conditions that may cause genetic damage. Various environmental factors can lead to DNA damage that, if not repaired lead to either apoptosis and senescence or to genomic instabilities.
It is imperative for ES cells which give rise to all cells of the body and, most importantly, any germ cells, to guarantee a low mutation load and minimise any influence of genomic instability and DNA damage. ES cells have extremely stringent mechanisms in place that ensure a high degree of genomic integrity. Spontaneous mutation rates are around 100 fold lower in ES cells than in differentiated somatic cells (Cervantes et al, 2002). One of the most remarkable properties of ES cells is their high stress resistance (Saretzki et al., 2004, Saretzki et al., 2008, Guo et al, 2010). Mouse ES cells are very resistant against an increase in external oxidative stress and proliferated with the same rate in 20% and 40% oxygen (Saretzki et al., 2004). In addition, when compared to other immortal mouse cells such as 3T3 which both express high and comparable telomerase levels, mES cells accumulated less DNA damage when gamma-irradiated and had a better or faster DNA repair capacity (Saretzki et al., 2004). This seems to reflect a better DNA repair capacity as well as a high amount of antioxidant enzymes as shown at the gene expression level for mouse and human ES cells alike (Saretzki et al., 2004, Saretzki et al., 2008). In general, there seem to be two main pathways how ES cells cope with DNA damage: apoptosis and differentiation.
The tumour suppressor molecule p53 is one of the most important response mechanisms that operate in most somatic cells and play an important role in maintaining genomic integrity. Without any endogenous or exogenous stress p53 is expressed at a low level and remains inactive due to interaction with MDM2 and rapid ubiquitinylation and degradation of the protein. Only after stress occurs it is quickly stabilised and activated. The pathway ensures that cells that underwent DNA damage can halt their cell cycle in order to repair the damage or alternatively, if the damage is too high and cannot be repaired, drive cells into apoptosis or senescence. However, the p53 signalling pathway seems very differently regulated in ES cells compared to somatic cells. p21 is considered to be an important component in the senescence pathway and thought to correlate with ROS generation (Macip et al., 2002, Passos et al., 2010). In somatic cells the DNA damage response pathway activates p53 which in turn induces the expression of its downstream target genes such as p21. Consequently, high levels of p21 following extensive DNA damage had been implicated as a hallmark for DNA damage-induced premature senescence (Waldman et al., 1995).
Aladjem et al. (1998) have challenged mouse ES cells with an antimetabolite that depleted ribonucleotides and DNA damaging agents. They found that mouse ES cells have a high level of p53 protein but it was mainly localised in the cytoplasm and did not localise to the nucleus as in differentiated cells. P53 was therefore not able to act as a transcription factor for downstream genes such as p21 in the nucleus. Consequently, it did not activate a cell cycle arrest after rNTP depletion or DNA damage. Instead, cells went into apoptosis that

was independent of p53 (Aladjem et al., 1998). The authors showed that the p53 checkpoint became operational again once the ES cells were differentiated (Aladjem et al., 1998). On the basis of this and other papers (Hong and Stambrook, 2004) it was thought that mouse ES cells lack a G1 dependent cell cycle arrest and have a deficient p53 DNA damage response. Miura et al. (2004) suggested that p53 is not required for ES cell self renewal and early embryogenesis and that also the RB/E2F pathway is inactive in ES cells thereby uncoupling mitogenic signalling from cell cycle progression.

It is well accepted that mouse and human ESC have a unique cell cycle structure different from somatic cells. They show only a very short and rather transient G1 and G2 cell cycle phases while most cells are in S-phase (Becker et al., 2006). Becker et al. (2006) described the shortened G1 cell cycle phase of human embryonic stem cells as necessary for their self renewal properties. Due to their very short G1 phase it was believed for some time that ES cells lack a G1 dependent growth arrest upon DNA damage.

However, recently a transient cell cycle arrest has been described in mouse and human ES cells. Barta and colleagues (2010) exposed human ES cells to UVC irradiation in G1 phase and found a cell cycle arrest just before DNA synthesis in S-phase. They found a down regulation of the cyclin-dependent kinase 2 activity depending on the checkpoint kinases Chk1 and Chk2 (Barta et al., 2010). The authors conclude that human ES cells are able to prevent entry into S-phase upon DNA damage by activating a G1/S arrest that is independent of p53 and p21 activation.

Lin et al. (2005) have described a role for p53 in the maintenance of a high genomic stability of ES cells by driving damaged ES cells into differentiation and thereby eliminating them from the ES cell pool. The mechanism for that seemed to be a direct down regulation of NANOG expression by p53 in mouse ES cells after DNA damage. NANOG is an important pluripotency associated transcription factor and essential for embryonic survival. The authors found a marked increase in p53 expression 4 hours after the treatment when apoptosis was not detectable yet. The authors also showed the activation of downstream genes such as Noxa and Puma proving that p53 was transcriptionally active. The decrease was specific for NANOG and not due to differentiation since other pluripotency markers such as Oct3/4 were stably highly expressed. P53 directly bound the NANOG promoter with higher efficiency than the p21 promoter. They also demonstrated that p21 was increased during differentiation of mouse ES cells due to active p53 (Ser 315 phosphorylation) although the overall p53 protein level decreased (Lin et al., 2005). Most importantly, down regulation of NANOG as well as successful differentiation induced by retinoid acid was dependent on Ser 315 phosphorylation of p53. Apoptosis induction was dependent on the presence and activity of a functional p53 (Lin et al., 2005) contradicting findings from others that a p53 dependent cell cycle arrest and apoptosis induction might not be operating in ES cells after various DNA damage inducers (Aladjem et al.,1998, Guo et al., 2010).

Mora-Castilla et al (2010) recently confirmed the role of p53 Ser315 phosphorylation for NANOG down regulation during differentiation of mouse and human ES cells following the exposure to NO-inducing agents. They showed that both p53 Ser315 and trimethylated histone H3 bound and repressed the NANOG promoter. Epigenetic modification of Oct4 and NANOG had been previously demonstrated during ESC differentiation (Wang et al., 2009).

Qin and colleagues (2006) demonstrated a direct link between p53 and apoptosis induction in human ES cells. They confirmed that treatment with DNA damaging agents accumulated

p53 but did not activate its transcriptional targets such as p21. The authors demonstrated that this could be due to differential modification/phosphorylation of p53 in ES cells although p53 still binds to the promoters of p21 and Noxa after DNA damage. p53 was still able to bind to the promoters and down regulate the pluripotency genes Oct4 and NANOG. However, instead of transcriptional regulation p53 induced apoptosis in ES cells via a transcription-independent mitochondrial pathway. Like others the authors also observed that p53 is involved in spontaneous differentiation. This study proves the important role of p53 for the survival and differentiation of ES cells. P53 seemed also directly responsible for down regulating Oct4 and NANOG after DNA damage treatment via p53 binding to the promoters of both genes, while decreasing p53 levels correlated with an amelioration of this down regulation. Over-expressing Mdm2 could inhibit p53 accumulation and reduced spontaneous apoptosis levels. The authors also suggested that a decrease of p53 levels due to lowering stress during cultivation such as free oxygen radicals could be beneficial for growth and expansion of ES cells in culture. In summary, the study of Qin et al (2006) suggests that there is a very specific mechanism of p53 regulation that is active in ES cells but not in differentiated and somatic cells.

In general, it seems that p53 is a suppressor of pluripotency in ESC because it suppresses self-renewal of ESC after DNA damage. Thus, p53 seems to play an important role for the maintenance of genomic stability of ES cells by influencing their DNA damage response and self-renewal properties (Zhao and Xu, 2010).

Both mouse and human embryonic stem cells (ESCs) are unique among all stem cells by replicating continuously and being immortal in the absence of replicative senescence (Zheng and Rao, 2007, Guo et al., 2010).

8. Telomerase and mitochondria during the induction of pluripotency from somatic cells

Induced pluripotency cells (iPSCs) are a stem cell population that is experimentally derived from somatic cells by forced expression of various transcription factors that are normally expressed in embryonic stem cells. These cells acquire many properties of normal ES cells, including a high self-renewal potential, gene expression, high telomerase activity and differentiation potential as well as low mitochondrial number (Armstrong et al., 2010).

The generation of iPS cells is very promising in order to analyse and model detailed disease mechanisms *in vitro*. In addition, this procedure opens even greater regenerative potential than normal ES cells since in theory a somatic cell can be taken from the same donor who then receives the transplant from differentiated iPS cells. This type of custom tailored cell generation has enormous potential, but several processes have not yet been understood sufficiently and have to be solved in order for any potential cell replacement to be safe.

Induced pluripotency cells (iPSCs) were for the first time generated from mouse somatic cells by Takahashi and Yamanaka in 2006 (Takahashi Yamanaka, 2006). The authors used a cocktail of 4 essential transcription factors: Oct4, Sox2, KLF4 and c-myc. The latter two are thought to induce telomerase (Wong et al., 2010) however myc seems not really necessary for iPS induction. Meanwhile many groups repeated and refined this method in the mouse and the human system (Marion et al., 2009, Armstrong et al., 2010). In principle the 4 transcription factors re-programme the epigenetic signature of a somatic cell into that of an undifferentiated embryonic stem cell, including telomeres (Marion et al., 2009).

These properties are also reflected during the generation of induced pluripotency cells (iPSCs). One striking example is the activation of telomerase during the induction of pluripotency. It occurs in human, but is much less robust in mouse ES cells (Mathew et al., 2010). iPS cells, as normal stem cells are characterised by a high level of telomerase expression. Apparently, a high telomerase expression level in necessary in human iPS cells in order to re-elongate telomeres to the length of embryonic stem cells. A stable long telomere length is an essential prerequisite for self-renewal capacities of embryonic as well as iPS cells. Maria Blascos' group has provided a direct evidence for the importance of telomere length reset in iPS cells. Efficiency of iPS generation was dramatically reduced when cells from late generation telomerase (TERC) knockout mice were used that do not have telomerase activity and have very short telomeres (Marion et al., 2009). However, they also noticed, that some iPS clones from G2 and G3 generation TERC knock-out mice could have activated telomerase-independent telomere elongation mechanisms. The importance of telomere length and telomerase activity is evidenced by the finding that re-introduction of telomerase into telomerase knock-out mice is able to re-adjust telomere length and promote efficient iPS cell derivation. However, more important than the average telomere length for iPS cell derivation is probably the absence of very short individual telomeres. The authors also found that during the normal iPS cell derivation process telomeres acquire embryonic stem cell properties including telomere length and epigenetic changes (Marion et al., 2009).

Mathew et al. (2010) demonstrated in detail that telomerase activation and TERT promoter activation occurred at discrete steps during reprogramming of somatic cells. Comparing mouse and human cells they found a very modest threefold increase of the mTERT promoter activity during reprogramming while the hTERT promoter was induced between 10 fold (partial reprogramming) to hundred fold (complete reprogramming) in different colonies. While many clones had telomerase activity up regulated only those with the highest levels were able to maintain their telomeres (Mathew et al., 2010). Since mice have much longer telomeres in their somatic cells than humans it seems highly likely that human cells have to activate hTERT and telomerase to a much larger extent than it is necessary in mouse cells.

Many promoters such as the one from NANOG were hypomethylated in ES cells and demethylated during iPS cell generation (Mathew et al., 2010). However, the hTERT promoter was not highly methylated in somatic cells and demethylation events did not play an essential role in its re-activation while the histon modification of the promoter changed substantially during re-programming (Mathew et al., 2010). A general similarity but not identity had been shown for gene expression patterns of ES cells and iPS cells (Prigione et al., 2010). Similarities were described for most pluripotency and self-renewal genes while differences could be due to a conservation of some donor cell characteristics since both cell types did not derive from the same individual.

Progione et al. (2010) recently demonstrated that iPS cells derived from human skin also shared similarities with ES cells regarding their reduced p53 activity and mitochondrial activity including low ROS levels. A decrease in mitochondrial copy number and mass were also confirmed by others (Armstrong et al., 2010).

Since all mitochondria within iPS cells derive from a somatic cell the authors were interested in the analysis of how mitochondria from somatic cells are able to acquire stem cell like

properties. They showed that somatic mitochondria re-acquire an immature phenotype which resembled that of ES cells. This included an ESC like state of morphology, biogenesis factors, mitochondrial copy number and a decreased level of ATP as well as oxidative stress generation (Prigione et al., 2010). iPS cells like ES cells relied on glycolysis rather than oxidative phosphorylation for their energy generation.

Progione and co-authors also showed a lower amount of oxidatively modified DNA lesions (oxodG) in undifferentiated ES cells and iPS cells and confirmed also a higher resistance to oxidative stress and DNA damage shown by our group previously (Saretzki et al., 2004, Saretzki et al., 2008, Armstrong et al., 2010). However, while Prigione et al. (2010) found a decrease in the expression of antioxidant enzymes such as catalase, GPX1 and all three SODs in undifferentiated iPS and ES cells that increased during differentiation our group detected the opposite: high antioxidant gene expression in undifferentiated ES cells, but a decrease during differentiation (Saretzki et al., 2004, Saretzki et al., 2008). Further analysis, such as comparing the expression at the protein or activity levels seem necessary to resolve this discrepancy.

Prigione et al. (2010) concluded that reprogramming restored a cellular state characterised by immature mitochondria and low oxidative stress. It is an intriguing finding that it seems to be possible to rejuvenate mitochondria so that they escape cellular senescence. Mitochondria and mitochondrially generated oxidative stress are thought to be important components in the induction of cellular senescence and human ageing alike (Wallace, 2005, Passos et al., 2010). In contrast, long telomeres, low p53 and p21 activity as well as low levels of oxidative stress and immature mitochondria are hallmarks of embryonic stem cells (Saretzki et al., 2004, Saretzki et al., 2008, Facuchi-Olivera et al., 2007). Furthermore, there is evidence to suggest that telomerase that resides inside the mitochondria actively decreases oxidative stress although the mechanism remains elusive yet (Ahmed et al., 2008, Haendeler et al., 2009).

Recapitulation of immature mitochondria might also suggest that this state is important for the generation of iPS cells and possibly causally involved in the establishment of pluripotency. However, further research is necessary to establish a direct connection between mitochondrial state and pluripotency.

However, some mechanisms that enhance the efficiency of iPS cell derivation at the same time also raise concern about the possible risks of application of cells derived from iPS cells generated after inhibition of the p53 pathway. A decrease of p53 has been shown to increase the efficiency of iPS cell derivation (Hong et al., 2009). However, it remains elusive whether the p53 induced senescence or apoptosis are involved in this block of reprogramming. Since many cancer types inactivate this important tumour suppressor pathway it remains controversial whether this could increase the cancer risk for patients treated with iPS cells that have been derived by compromising the p53 checkpoint (Zhao and Xu, 2010).

9. Conclusion

High telomerase expression as well as a special mitochondrial state are both characteristic properties of embryonic stem cells in addition to pluripotency. Our group was the first to analyse oxidative stress and stress resistance in normal and TERT over-expressing ES cells (Saretzki et al., 2004, Armstrong et al., 2005, Saretzki et al., 2008, Yang et al., 2008).

Recently, evidence emerges that favours hypoxic condtitions for the improvement of proliferation, pluripotency and differentiation capacities in ES cells. A direct link between HIF1α and telomerase function provides some rational for that (Coussens et al., 2010).

More data and possible mechanisms emerged on the role of reactive oxygen species (ROS) and the differentiation and pluripotency of ES cells. In particular, the role of mitochondria for ES cell properties gains more and more interest. Specific mitochondrial regulatory pathways that operate only in embryonic stem cells have been demonstrated recently (Todd et al., 2010) and ROS have been identified as possible differentiation inducer (Yanes et al., 2010).

Other new results emerge recently to explain why the tumour suppressor checkpoint p53 operates via a mitochondrial pathway that promotes apoptosis rather than cell cycle arrest or premature senescence as in somatic cells rather than via transcriptional control (Qin et al., 2007). Others have shown that p53 promotes differentiation of ES cells via downregulation of NANOG (Lin et al., 2005).

Induced pluripotency derived cells recapitulate mitochondrial numbers and properties known from early embryonic development under low oxygen conditions. However, no direct causal relationship between mitochondria and pluripotency has been investigated to date.

My group was the first to show a direct correlation between telomerase that shuttles to mitochondria and improved mitochondrial properties and a decreased oxidative stress (Ahmed et al., 2008). Other non-canonical functions of telomerase include its involvement in gene expression and Wnt signalling (Choi et al., 2008, Park et al, 2009). Interestingly, Yoon et al (2010) uncovered a new link between Wnt signalling and mitochondrial biogenesis and gene expression involved in oxidative phosphorylation. Bringing the different components involved in regulation of stem cell properties including mitochondria and telomerase together will certainly result in a better understanding of ES cell properties.

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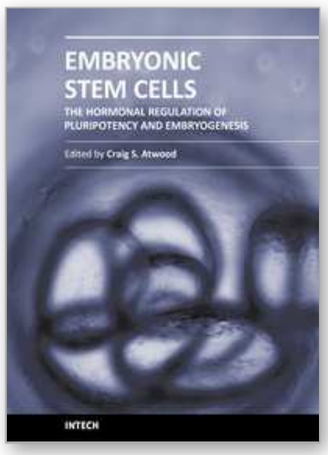
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