

Embryonic Stem Cells, Telomeres and Aging

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ABSTRACT

Embryonic Stem Cells (ESCs) constitute a small elite group of immortal cells with specific biological mission and mechanisms of rejuvenation, self-renewal and maintenance. Successes in nuclear reprogramming, induced pluripotency and other related protocols make these naturally pluripotent cells “gold standard” for comparison, understanding the basic mechanisms of pluripotency and for development of new biotechnologies. Here, we briefly addressed three aspects of ESCs biology that could be directly linked to anti-aging and longevity-promoting interventions: (i) how ESCs maintain the telomere length and why it is so important for pluripotency; (ii) paradoxical combination of superior protection of ESCs with hypersensitivity to apoptosis; and (iii) the role of hypercapnic/hypoxic conditions in ensuring and keeping the ESC features.

Keywords: Embryonic stem cells, Pluripotency, Telomeres, Telomerase, Hypoxia, Hypercapnia, Aging

Abbreviations: ALT: Alternative Lengthening of Telomeres; DSBs: Double Strand Breaks; ESCs: Embryonic Stem Cells; HSCs: Hematopoietic Stem Cells; iPSCs: Induced Pluripotent Stem Cells; LUCA: Last Universal Common Ancestor; MSCs: Mesenchymal Stem Cells; ROS: Reactive Oxygen Species; TERRA RNA: Telomeric Repeat-Containing RNA; Terc (TERC): Telomere RNA Component; Tert (TERT): Telomerase Reverse Transcriptase

INTRODUCTION

Embryonic Stem Cells (ESCs) are a gold standard of cellular pluripotency and immortality. They can generate any cell of over 200 cell types in our body, known thus far and preserve stable karyotype, pluripotency, proliferative capacity and telomere length after hundreds of population doublings during months and years of continuous maintenance in culture [1-3]. The ever-growing interest to this elite group of cells in gerontology is primarily supported by the expectations of new efficient strategies in tissue regeneration and anti-aging. To promote the study on ESC biology, the NIA Mouse ESC Bank, for example, has generated and held 185 ESC lines which can be differentiated into various cell types by specific transcription factors within 48 h [4].

According to intriguing data by Ratajczak et al. [5-8] adult tissues contain ESC-like pluripotent stem cells as a backup for the tissue-committed stem cells. Most likely, these quiescent cells are remnants of ESCs, can be mobilized at stressful conditions to support tissue repair, and presumably have a role in determination of longevity. Comparison of several murine strains differing in their lifespan showed that

the longer-lived strains have a more abundant pool of ESC-like cells [6].

Another important aspect of putative anti-aging effects of ESCs and probably other pluripotent (e.g. iPSCs) and multipotent (e.g. MSCs) stem cells is that they may also act in a paracrine manner [9-11]. This gained support from the observation that ESC-conditioned medium suppressed

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cellular senescence, maintained proliferative capacity and accelerated the wound healing [12] – all of which undergo age-related changes and contribute to the aging process [13-17].

Before deployment of the ambitious perspectives in cellular rejuvenation and tissue regeneration based on artificial pluripotent cells, it seems relevant studying as much as possible about their natural counterparts. ESCs could be a perfect choice to fulfill the task. Unfortunately, the expectations for application of pluripotent cells in anti-aging, especially in *in vivo* models [18] remain insufficiently explored, supporting the contentions of gearing up their study. Here, we briefly addressed three aspects of ESC biology that could be directly linked to anti-aging and longevity-promoting interventions: (i) how ESCs maintain the telomere length and why it is so important for pluripotency; (ii) paradoxical combination of superior protection of ESCs with hypersensitivity to apoptosis; and (iii) the role of hypercapnic/hypoxic conditions in ensuring and keeping the ESC features.

ESCs GET TO THE TOP IN MAINTAINING THE TELOMERE LENGTH

Telomeres and telomerase

Each end of a chromosome of eukaryotic organisms contains a special region of repetitive nucleotide sequences called a telomere. In vertebrates, telomeric DNA consists of multiple tandem hexomere repeats – GGGATT for the telomere DNA heavy strand and complementary CCCTAA for the light strand. In complex with six proteins known as shelterin, non-translating (TERRA) RNA and proteins associated with the heavy strand overhang, telomeres create a unique telomeric chromatin for protecting the chromosome ends [19,20]. Telomeres are apparently the most vulnerable elements of the genome and are prone to attrition (a loss of small fragments of DNA at each round of replication), because of the DNA incomplete end-replication problem. Yet, occasional damage to telomeric DNA and especially breaks of the single-stranded overhangs could be a main cause of telomere shortening [21]. Overly short or otherwise impaired telomeres could cause chromosome end-to-end fusion or recombination, thus leading to genome instability, with far-reaching consequences including tumorigenesis [22,23].

Telomere length is recovered by a specialized ribozyme – telomerase or by a homologous recombination known as alternative lengthening of telomeres (ALT). Telomerase consists of two functionally diverse subunits – reverse transcriptase (Tert) and telomere RNA template (Terc) [20]. Robust expression of these subunits and especially Tert, is an important attribute of all unlimitedly proliferating pluripotent stem cells (except of early embryo cleavage stages), as they maintain telomere length mostly through telomerase activity. ALT has primarily been found in cells with no or very low telomerase activity – in early embryo

cleavage stages (when the number of cells increases without increasing their total mass) [24] and in somatic cells [25].

Telomeres and telomerase activity in ESCs

Efficient telomere homeostasis is critical for proper functionality of both embryonic and resident stem cells and therefore essential for tissue homeostasis throughout the life of an organism [26]. Telomere elongation is crucial for the self-renewal of ESCs [27], while telomere shortening leads to replicative senescence of their differentiated progeny and was suggested as one of the hallmarks of aging [13]. The rate of telomere shortening seems to be in some proportion to the species-specific longevity. For example, the mouse telomeres shorten 100 times faster than human telomeres [28].

As could be expected, ESCs have one of the longest telomeres as compared to other cell types. In humans, telomere size first decreases in early embryo cleavage stage cells but then reaches its maximum length in the blastocysts (8.4 kb and 12.2 kb, respectively) [29]. Notably, the elongation of telomeres in the blastocysts is accompanied by a gradual exacerbation of the intracellular hypoxia/hypercapnia, especially in ESCs of the inner cell mass of the blastocyst.

The most stringent tests for pluripotency – generation of complete pups and germline-competent chimeras – showed that only ESCs with long telomeres possessed authentic developmental pluripotency, whereas ESCs with short telomeres failed the tests [27]. Additionally, ESCs with short telomeres exhibited a lower proliferative rate and germ cell differentiation, as well as a capacity to modify the expression of genes related to embryogenesis and epigenetics. iPSCs with longer telomeres also were superior in generating chimeras over the cells with short telomeres, thus suggesting that telomere length could be a valuable marker of cell pluripotency [27].

TERT overexpression enhanced telomerase activity, proliferative rate and colony-forming capacity of human ESCs [30]. Differentiated progeny of TERT-overexpressing ESCs also showed an enhanced telomerase activity and resistance to oxidative stress, whereas downregulation of TERT decreased the proliferative rate and resulted in loss of pluripotency [30]. The very ends of eukaryotic linear chromosomes are additionally protected by the telomeric overhangs [19]. As telomeres, they are shorter in differentiated progeny of ESCs [31]. The Tert overexpression in mouse ESCs not only increased their telomerase activity but also extended the length of overhangs, simultaneously elevating ESC proliferative capacity and resistance to apoptosis [31].

Epigenetic factors, such as histone modifications and DNA methylation, play an important role in establishing and maintaining pluripotency of ESCs [32,33]. Not surprisingly, the telomere homeostasis is also under epigenetic control

[34,35]. Moreover, this control could be species-specific [36]. As typical for pluripotent stem cells, ESCs have more relaxed chromatin conformation, including telomeric regions [27,37,38]. Accordingly, ESCs have a higher number of open chromatin markers, acetylated and methylated lysine 27 of histone 3, in combination with trimethylated histone 3 lysine in non-expressing genes [39]. Modification of the di/tri methylated lysine ratio at regulatory regions of ESCs was sufficient for acquisition or repression of cell lineage transcriptional program and phenotypes [39]. Methylation of cytosine (5mC) is another powerful tool of epigenetics. Oxidation of 5mC by the Tet protein family results in formation of oxidized derivatives of 5mC. They are selectively recognized and excised by thymine DNA glycosylase, thus leading to DNA demethylation. Tet knockout ESCs exhibit elongated telomeres and elevated telomere-sister chromatid exchange, indicating the direct impact of DNA demethylation on telomere homeostasis [40].

In cell cultures, ESCs exist as a mixture of metastable cells sporadically entering into the 2-cell embryo-like state. The genes *Zscan4*, *Testv1* and *Testv3* were shown to be involved in the formation of this state; they were also responsible for telomere maintenance and genomic stability [41,42]. Ectopic overexpression *Testv1* or *Testv3* genes resulted in telomere elongation, whereas their knockdown shortened telomeres of ESC [42].

Telomerase in cellular senescence, aging and rejuvenation

Aging is often referred as to a progressive decline in tissue homeostasis and repair caused by malfunction of somatic stem cells, accompanied by accumulation of senescent cells [17,43,44]. However, there is a growing consensus that cellular senescence and aging are reversible [45-48]. Somatic cells can be rejuvenated back to the ESC-like state by various procedures of induced pluripotency, nuclear transferring, fusing with ESCs, tetraploid embryo complementation or exposure of somatic cells to ESC extracts. The essential point is that the viable pups could be derived from cell clones rejuvenated by all these procedures [27,41,49,50]. For example, treatment of mouse fibroblasts with ESC protein extracts was sufficient for reprogramming the adult fibroblasts into ESC-like cells [41]. Moreover, these cells were functionally and biologically indistinguishable from ESCs and exhibited complete developmental potency, giving rise to fetal animals. The ESC extract-induced alterations in the global gene expression, DNA methylation and histone modifications were typical for the conversion of somatic cells into pluripotent stem cells. In another study [12], the mouse ESC-conditioned medium supplemented to cultured human dermal fibroblasts was shown to suppress cellular senescence and maintain their proliferative capacity,

presumably, through up-regulation of fibroblast growth factor 2 and down-regulation of CS-associated p53.

The microarray analysis showed that the enhanced self-renewal and extended lifespan of cells were associated with activation of a variety of genes. Among them, telomerase was suggested as a “survival enzyme” in ESCs and their differentiated progeny [31]. Cellular rejuvenation is ubiquitously associated with telomerase activation and telomere elongation, supporting the idea of “cause-and-effect” relationships between aging and telomere integrity [51]. Moreover, there is evidence that telomere size established by ESCs could be deterministic for mammalian longevity [52,53].

IT IS BETTER TO DIE THAN TO BE WRONG

This “Samurai Law of Biology” [54] is especially relevant to ESCs. In contrast to the mostly local effects of somatic cell failure, DNA damage and mutations in ESCs could have catastrophic consequences for most tissues and the whole organism and may also be passed to the germline progenies. This assumes that ESCs should evolutionarily be rendered superior defense and selection systems. Indeed, ESCs are characterized by a robust DNA repair and low levels of mutations [55-57]. For example, spontaneous or induced mutation frequency of the reporter genes was several orders of magnitude lower in mouse ESCs than in the embryo fibroblasts. Yet, DNA breaks are rather frequent in ESCs, especially during DNA replication [58]. Homologous recombination is recognized as the main pathway of DNA double strand breaks (DSBs) repair and Rad51 is a key regulator of this process [59]. In mouse ESCs, Rad51 showed a 2-fold increase in mRNA and 15-fold increase in protein expression, compared with the embryo fibroblasts. Moreover, only a small portion of Rad51 protein was recruited to repair DSBs or stalled replication forks in normal conditions, thus indicating substantial reserves of Rad51-dependent DNA repair in stressful conditions [60].

Further supporting superior protection of ESC vs. differentiated cells are lower rates of free radical generation in combination with a higher antioxidant defense [61]. Measurements of 8-OH-G (8-hydroxyguanine), a well-known marker of oxidative stress damage in DNA, showed that ESCs cultured with 300 μ M hydrogen peroxide had lower levels of 8-OH-G than more differentiated cells. The better protection of ESC DNA against free radicals was further supported by an enhanced expression of 8-OH-G repair-associated genes [62]. It appears that ESCs and their genome are more resistant to oxidative stress. Degradation of misfolded, aggregated or otherwise damaged proteins via autophagy or proteasomes was also superior in ESCs, thus underpinning their generally ameliorated intracellular environment [63,64].

Apart from a robust DNA repair and a low mutational level, hypersensitivity of ESCs to apoptosis is another important

tool to protect their genome integrity. ESCs with unrepaired DNA damage readily undergo apoptosis or differentiation, thus removing the damaged cells from the pluripotent pool [65,66]. In fact, ESCs and their differentiated progeny adhere to different stress response strategies [67]. For example, sub lethal heating activated apoptosis in human ESCs while induced premature senescence in the ESC-derived fibroblast-like cells [68]. Another example includes hypersensitivity of ESCs to camptothecin, a topoisomerase I inhibitor which induces DNA DSBs and an intensive p53-mediated apoptosis in human ESCs, but to a much lesser degree in differentiated ESCs [69]. The importance of genome integrity in ESCs is further exemplified by mice deficient in Cdk12 (cyclin-dependent kinase 12), a multifunctional protein involved in maintaining the genomic stability and pluripotency of ESCs [58]. The Cdk12^{-/-} embryos displayed a reduced expression of DNA damage response genes and insufficient DNA repair, with subsequent activation of apoptosis. This resulted in abrogated accumulation of the inner cell mass in blastocysts and lethality of the embryos shortly after the implantation [58]. Of note, an increased DNA repair and sensitivity to apoptosis have been observed in several models of lifespan extension [44].

ESCs RESIDE IN HYPOXIC/HYPERCAPNIC MICROENVIRONMENT AND RELY ON GLYCOLYSIS

Increased sensitivity to apoptosis and completely de-differentiated status of ESCs apparently require a low metabolic rate associated with less production of ROS. This could be supported by hypoxic/hypercapnic microenvironment which is optimal not only for ESCs but for other stem cells as well [70-72]. ESCs are usually obtained from the inner cell mass of pre-implantation embryos (blastocysts). While moving down the oviduct towards the uterus, embryos continue dividing and reach a considerable size at the pre-implantation blastocysts. At this stage, there are usually several hundreds of the inner mass cells and comparable number of cells of the outer layer of the blastocyst-trophoblast [73]. Lack of vascularization or other mechanisms of O₂/CO₂ active transport, aggravated by the assembly of substantial number of ESCs, means that ESC environment in the blastocyst cavity (blastocoele) is essentially hypoxic/hypercapnic. We hypothesized that the severe hypoxia/hypercapnia in blastocysts is required for suppression of ESC differentiation, thus allowing a highly efficient DNA repair — a crucial event before ESCs start to differentiate. Besides, this temporary suppression allows synchronizing a consequent differentiation of ESCs.

Such hypoxic/hypercapnic microenvironment will decrease the rate of oxidative phosphorylation (and accordingly, ROS generation) and activate glycolysis, in part due to the hypoxia inducible factor 1 alpha (Hif1alpha) which concomitantly promotes telomerase expression and enhance

self-renewal of stem cells [74]. Anaerobic metabolism could also be necessary to supply substrates for the anabolic processes, which is typical for intensively dividing cells (Warburg effect) [75,76]. In fact, activated glycolysis and associated higher concentrations of pyruvate and lactate are metabolic hallmarks of apparently all actively dividing cells, ESCs included [77-79]. In the *in vitro* cultured early mouse embryos, glucose consumption was undetectable until the blastocyst stage but became the main source of energy generation in the later stages (embryo's day 6.5 and 7.5), in contrast to the opposite dynamics of pyruvate utilization [80]. Of note, almost the same pattern of metabolic alterations was observed in various models of induced pluripotency which usually started by inhibition of oxidative phosphorylation and activation of glycolysis [81,82]. Remarkably, even at high levels of O₂, ESCs utilized primarily the anaerobic metabolism [83]. This apparently allowed them to survive hyperoxia by reducing the ROS generation. Another important finding is that the main transcription factors of pluripotency, Oct4 and Nanog, can directly induce expression of the key glycolytic enzymes hexokinase 2 and pyruvate kinase M2, thus delaying differentiation and preserving pluripotency of ESCs [84]. In turn, the genes involved in the control of glucose uptake (GLUT3) and metabolism (PKM2) are also involved in regulation of Oct4 expression [85]. It should be emphasized that even though the pluripotent stem cells rely on glycolysis, mitochondria also play an important role in the maintenance of pluripotency [79]. In particular, several mitochondrial genes (e.g. POLG, Gfer, Drp1) were shown to regulate the expression of pluripotent factors.

CONCLUDING REMARKS

ESCs belong to the enigmatic group of immortal, pluripotent cells. Successes in nuclear reprogramming, induced pluripotency and other related protocols make these naturally pluripotent cells “gold standard” for comparison, understanding of the basic mechanisms of pluripotency and for the development of new biotechnologies. The huge potential of ESC application in anti-aging remains insufficiently explored, warranting further research in the field.

Pluripotency of ESCs is supported by complex networks of the growth and transcription factors, primarily by Oct4, Sox2 and Nanog, which expression is under a strict epigenetic control. Survival and proper functionality of ESCs and their differentiated progeny strongly depend on the telomere homeostasis [26], which apparently has wider assignments than just canonical protection of the chromosomes ends. Whatever the case, telomeres and telomerase are key regulators of stem cell proliferation, simultaneously restraining cancer and delaying aging [86].

ESCs have superior mechanisms for DNA repair and genome maintenance. Yet, being potentially immortal in cultures, ESCs are predisposed to apoptosis instead of trying

to repair the damage — an additional line of protection of the ESC progeny. Despite the efficient DNA repair, generally well-maintained telomere homeostasis and sensitivity to apoptosis, long-term manipulations with ESCs *in vitro* often lead to genomic and epigenomic abnormalities, with a subsequent decrease in cell viability or acquiring tumorigenicity [32,87,88]. These aspects of ESC and iPSC biology have not yet been fully addressed and definitely require more attention.

An important point is that ESCs live in hypoxic/hypercapnic microenvironment, as apparently once did the last universal common ancestor (LUCA) of all animal species in primordial Earth atmospheres. On the one hand, such microenvironment ensures minimal generation of ROS due to glycolysis-based metabolism. On the other hand, it promotes conditions for efficient DNA repair. This is especially true for ESCs of the pre-implanted blastocyst, when hypoxia/hypercapnia reaches its peak. This short period of ESC life provides a unique opportunity for the “last-minute” correction of the DNA damage, before ESCs start to differentiate. The very fact that the life still exists, and the accumulated damage does not pass on from generation to generation is, to a great extent, guaranteed by that “last-minute” correction of ESCs.

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