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Minireview

A molecular view on pluripotent stem cells

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Abstract Pluripotent stem cells are undifferentiated cells that are capable of differentiating to all three embryonic germ layers and their differentiated derivatives. They are transiently found during embryogenesis, in preimplantation embryos and fetal gonads, or as established cell lines. These unique cell types are distinguished by their wide developmental potential and by their ability to be propagated in culture indefinitely, without loosing their undifferentiated phenotype. This short review intends to give a general overview on the pluripotent nature of embryo-derived stem cells with a focus on human embryonic stem cells. **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Embryonic stem cell; Germ cell; Teratoma; Embryoid body; Pluripotency; Self-renewal

1. Introduction

Pluripotent stem cell lines, embryonic stem (ES), germ (EG) and carcinoma (EC) cells, are undifferentiated cell lines that are capable of forming virtually any cell type in the body. Their wide developmental potential and unlimited life span in culture make them extremely interesting and important for basic and applied research. They can undergo differentiation in vivo and in vitro, allowing them to be used for the study of basic processes in developmental biology. Moreover, they can be genetically manipulated and could potentially be used as a renewable cell source for restoring tissue function by cell engineering and transplantation. It is possible to characterize them by a distinct repertoire of cell-specific markers, including the presence of certain antigens, enzymes and the expression of a number of developmentally regulated genes. However, neither of these markers is completely restricted to this type of cells. The following review intends to summarize the accumulated information on the pluripotent nature of embryo-derived stem cell lines, and discuss possible mechanisms and functional molecules by which pluripotency is acquired and maintained in these unique cell types.

2. The origin and properties of pluripotent cells

Pluripotent stem cells are transiently present during embryonic development, in preimplantation embryos (zygote to morula and inner cell mass (ICM) of blastocyst) and in fetal gonads (primordial germ cells (PGC)) [1]. They can also be maintained as established cell lines, derived either from the inner cell mass of blastocysts (embryonic stem (ES) cells) [2,3], from primordial germ cells (embryonic germ (EG) cells) [4,5], or from tumorigenic derivatives of germinal tissues (embryonic carcinoma (EC) cells) [6] (Fig. 1). The embryo-derived stem cell lines are considered to be pluripotent according to several criteria (Fig. 1, box). They can develop into a wide range of cell types in vitro and in vivo, and are immortal, can be propagated indefinitely in culture while maintaining their undifferentiated phenotype.

EC, ES and EG have been well characterized in mouse, and are also available in human [7–10]. They are characterized by a unique repertoire of cell surface molecules, including stage-specific embryonic antigens (SSEA), and the activity of specific enzymes, such as alkaline phosphatase and telomerase (summarized in [11,12]). Although neither of these markers is completely cell specific, their presence as a group is associated with the undifferentiated state of the cells. In addition, a short list of molecular markers, which are rapidly down-regulated upon differentiation is available for mouse ES cells. It includes several transcription factors like Rex1 [13], Genesis [14], GBX2 [15], Oct4 [16], UTF2 [17], Pem [18] and L17 [19], which are members of well-known gene families and that are also expressed by the ICM of the blastocyst. Unfortunately, none of them is exclusively expressed by pluripotent cells and can be found in other cell types in the soma.

3. Developmental potential of pluripotent cells

Comparison of the differentiation potential of murine ES, EG and EC cells has shown that although these cells have similar properties, their different origins are often reflected in various developmental potentials. EC cells, for example, have a more restricted potential; they are unlikely to be transmitted through the germ line of chimeric animals and often have karyotype abnormalities [20]. EG cells easily undergo spontaneous differentiation, but may be unable to support normal development due to epigenetic modifications which have occurred during the formation of PGCs [21]. Thus, ES cells were shown to have the greatest developmental potential, differentiating into the widest range of cell types (reviewed in [22]). This chapter will focus on the recent findings that support the wide developmental potential of human ES cells. The pluripotent nature of human ES cells can be demonstrated in vitro and in vivo. In vitro, differentiation may be triggered if the cells aggregate by growing in suspension culture [23].

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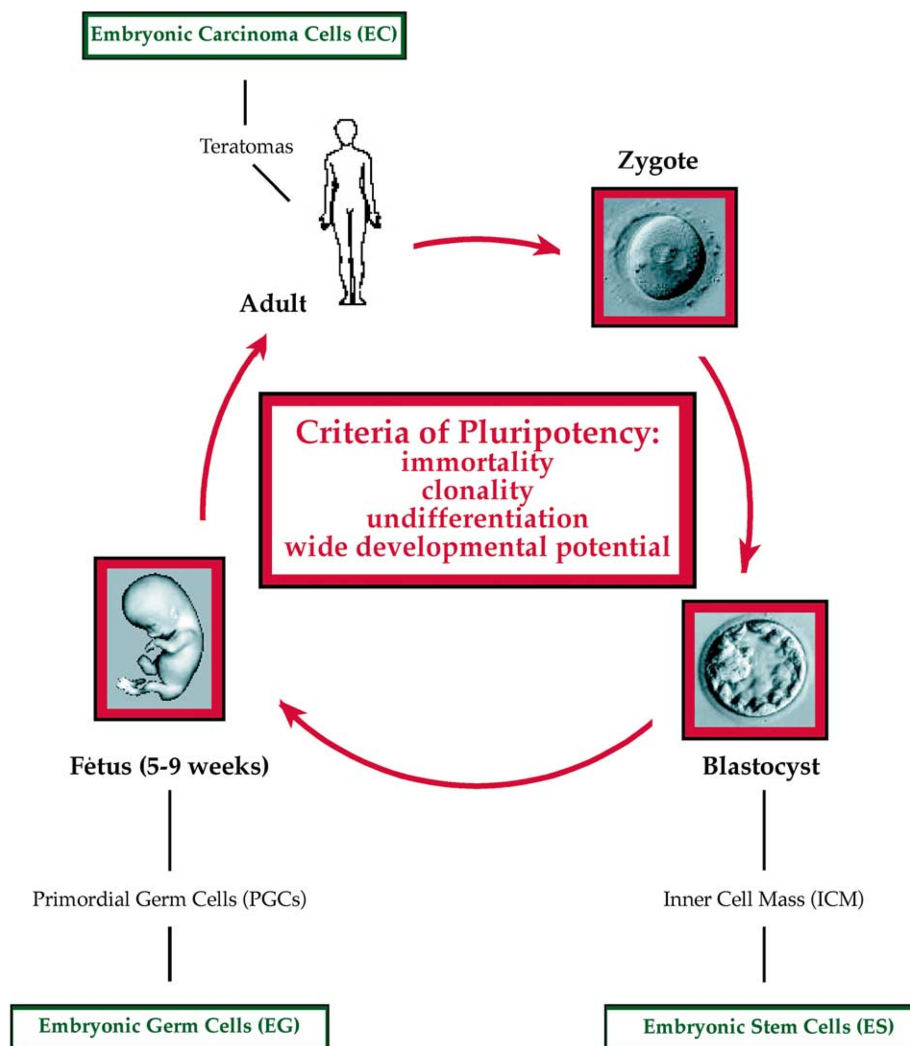

Fig. 1. Origin and derivation of human pluripotent stem cell lines. Pluripotent stem cells are derived either from the ICM, generating embryonic stem (ES) cells, from PGCs, generating embryonic germ (EG) cells, or from tumorigenic germinal tumors called teratomas, generating embryonic carcinoma (EC) cells.

These cell aggregates are called embryoid bodies, and mimic to some extent the process of early embryonic development. By day 20 in suspension culture different types of differentiated cells can be obtained spontaneously, shown either at the level of RNA or protein (see Fig. 2 and [23–33]). Moreover, it is possible to somewhat direct the differentiation of the cells into specific cell types by treatment with growth factors [24,33]. So far, human ES cells were shown to differentiate in vitro into about dozen of different cell types. Alternatively, differentiation may be induced by the expression of transcription factors, which play a major role in early commitment of cells into specific lineages, as has been previously demonstrated for mouse ES cells [34]. In vivo, by injecting the undifferentiated cells into nude mice, non-malignant tumors (teratomas) are formed [8,10,35]. These tumors include differentiated derivatives of mesoderm, endoderm and ectoderm. Clearly, the most definitive proof of pluripotency is the ability of the cells to integrate, proliferate and differentiate to all cell lineages (including germ cells) by introducing them into host blastocysts. This has been well practiced in the mouse, where ES cells are genetically manipulated and delivered to host blastocysts for introducing germ line transmitted genetic modifications [36]. However, such experiments are unacceptable in human for obvious reasons. The ability to efficiently direct the differentiation of human ES cells and allow the mass production of a specific cell type in vitro, illustrates their enormous potential as an unlimited cell source for transplantation and their possible use as vectors in cell-based therapies.

4. Stem cell selection

The potential of human embryonic stem cells to be used as an unlimited cell source for cellular transplantation depends on the availability of large and pure populations of undifferentiated cells, and on the ability to efficiently direct their differentiation into specific cell types in vitro. In order to maintain pluripotency, the cells are grown under conditions which support their undifferentiated growth and include the presence of a feeder layer such as inactivated mouse embryonic fibroblasts (MEF). Murine ES cells were shown to be able to sustain undifferentiated growth without a feeder layer in the presence of LIF (leukemia inhibitory factor) [37,38]. Yet, spontaneous differentiation still occurs even under optimal

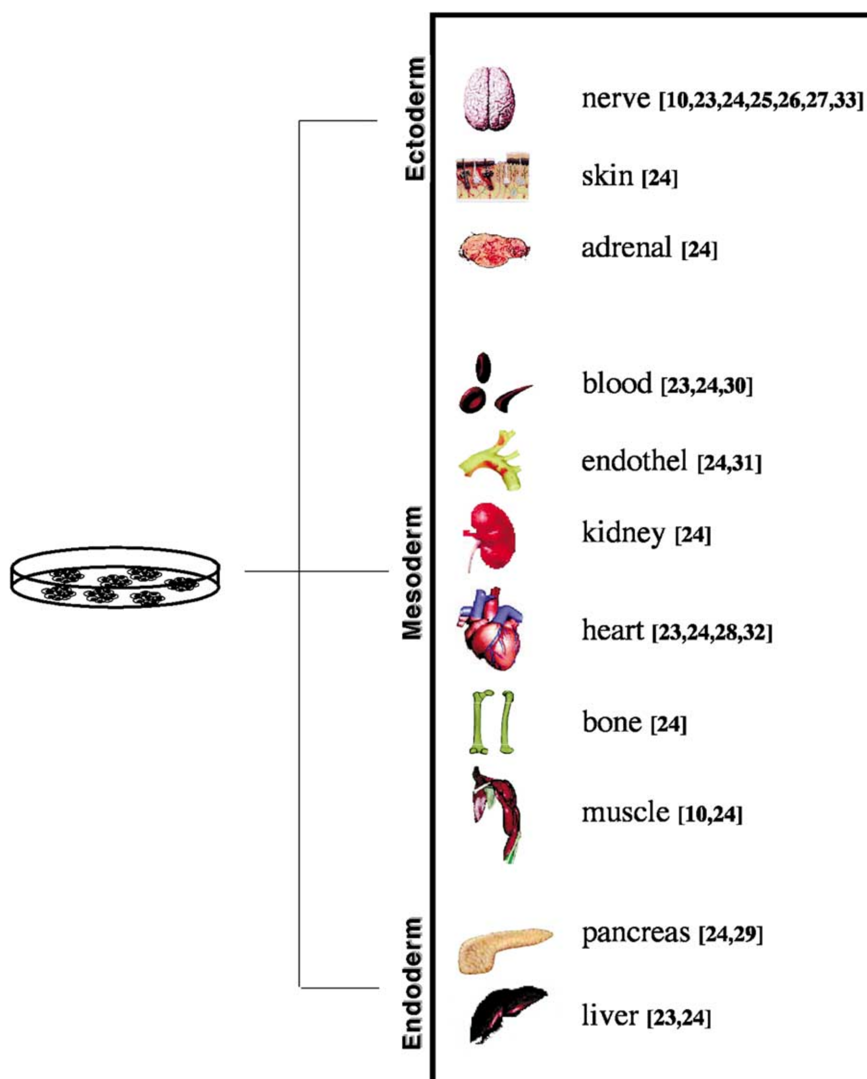

Fig. 2. Developmental potential of human ES cells in vitro. Spontaneous and induced differentiation of human ES cell results in the generation of terminally differentiated cells of ectoderm, mesoderm and endoderm origin in culture. In parentheses are references [10,23–33] to manuscripts demonstrating in vitro differentiation of human ES cells into the different cell types.

conditions, especially in human cells [8,10]. It has been demonstrated that the presence of differentiating cells induces the elimination of the pluripotent stem cells in culture, either by forcing them to further differentiate and/or by inducing apoptosis [39]. Hence, by eliminating the differentiated progeny, it should be possible to facilitate their maintenance and obtain uniform populations of undifferentiated cells. This can be performed by introducing into the genome of ES cells a selectable marker, such as the neomycin, under the regulation of a pluripotent associated promoter sequence [39]. The undifferentiated cells express the neo resistance gene, which is turned off as the cells differentiate, causing their immediate elimination by continuous G418 selection. Alternatively, the undifferentiated cells may be monitored in vitro and in vivo, by expressing the reporter gene green fluorescent protein (GFP), under the control of promoters which are specific to undifferentiated cells (such as the regulatory sequences of Oct4 or Rex1). By tagging the undifferentiated cells with green fluorescence, they can be analyzed and sorted according to the intensity of their fluorescent emission using a fluorescent activating cell sorter (FACS) [40]. This allows obtaining pure populations of undifferentiated cells, which can be re-plated and propagated or be used for further analysis. In addition, this system may be considered for eliminating undifferentiated cells prior to transplantation, avoiding the risk of tumor induction.

5. Functional molecules

Research in mouse ES cells has elucidated two unrelated molecular pathways, the LIF and the Oct4 pathways, that play a role in maintaining pluripotency and supporting undifferentiated growth (Fig. 3). It has been shown that LIF supports undifferentiated proliferation of ES and EG cells by binding to the gp130-LIF receptor heterodimer and activating the STAT3 transcription factor (reviewed in [41]). Yet, in vivo studies of knockout mice show that the maintenance of pluripotency in the embryo does not rely on the gp130 signaling pathway but only under suboptimal conditions of delayed implantation [42]. LIF$^{-/-}$ embryos develop to term and are fertile [43] while LIFR$^{-/-}$ and gp130$^{-/-}$ embryos die late in gestation (12.5–18.5 dpc) or shortly after birth [46–48].

Gene	Mutant mice	Expression during embryogenesis
LIF	develop to term, fertile 25%-30% reduction in body weight essential for embryo implantation [43]	trophectoderm (3.5dpc) and extraembryonic membranes [44,45]
LIF-R	die within 24h of birth [46,47]	ICM (3.5dpc), decidua (5.5dpc), embryonic and extraembryonic tissues (8.5 dpc) [44]
gp130	progressively die from 12.5dpc hypoplastic ventricular myocardium decrease in hematopoietic stem and progenitors [48] absence of pluripotent cells in delayed blastocysts [42]	ICM (3.5dpc), deciduas (5.5dpc), embryonic and extraembryonic tissues (8.5dpc) [44]
STAT3	develop to the egg cylinder stage (7dpc) [49]	many different embryonic tissues [50]
OCT4	develop to the blatocyst stage with no ICM [15]	unfertilized oocytes, zygote-morula, ICM, PGCs, germ cells [15,52,53]

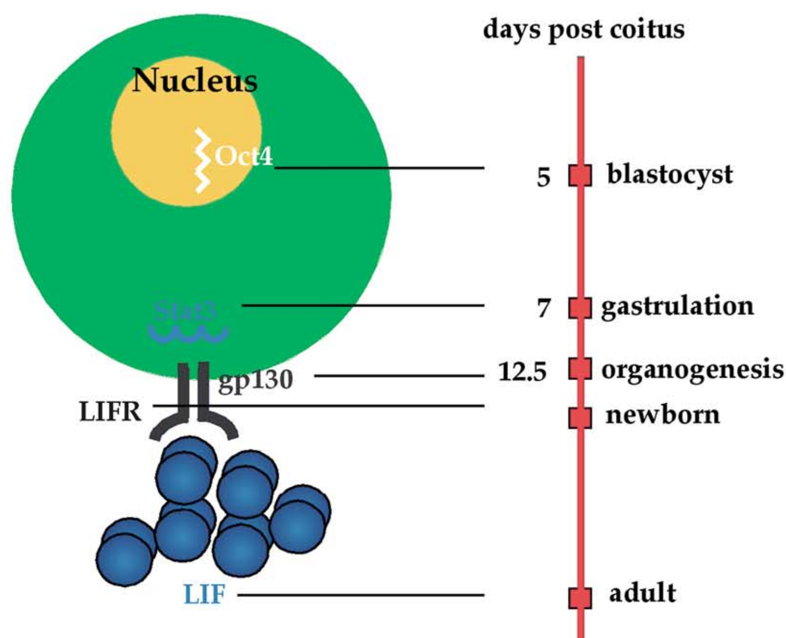

Fig. 3. Functional molecules and related pathways associated with pluripotency. Two unrelated molecular pathways, LIF and Oct4, are associated with the maintenance of pluripotency and self-renewal of ES cells in mice. A table and a schematic illustration summarize the phenotypic effects of knockout mice, that have been produced for each molecule that is known to be directly involved in either the Oct4 or the LIF pathway [15,42–50,52,53].

$STAT3^{-/-}$ embryos, however, develop until the egg cylinder stage (7 dpc) and rapidly degenerate with no obvious mesoderm [49]. In addition, it seems that LIF is not active in supporting undifferentiated growth of human ES cells in culture [8,10]. These findings support the existence of alternative pathways, which operate in sustaining self-renewal.

Oct4, which is a POU-V-related DNA binding transcription factor, has been shown to be associated with the phenotype of totipotent/pluripotent cells in mice (reviewed in [51]). It is expressed by all pluripotent cells during embryogenesis and is also abundantly expressed by ES, EG and EC cell lines [52,53]. Knockout mice deficient in Oct4 develop only to the blastocyst stage [16]. They are unable to produce any differentiated derivatives other than trophoblast cells and as a consequence, are absorbed shortly after implantation. These results demonstrate that Oct4 is required for preventing somatic differentiation of the ICM and is crucial for maintaining the undifferentiated state during embryonic development. Furthermore, in vitro manipulation of Oct4 expression in ES cells shows that it is the relative amount of the protein, as com-

pared to other transcription factors, that ultimately determines cell fate [54]. Therefore, even though both LIF and Oct4 pathways may be involved in controlling pluripotency, the molecular mechanisms and the genes that govern them remain, as yet, largely unknown.

6. Search for pluripotent associated genes

There is only little information regarding the genes which are directly associated with pluripotency. In fact, Oct4 is the only gene that has been shown to be involved in vivo in the maintenance of the undifferentiated state of cells. The search for additional genes which are specific to all pluripotent cells will allow to further investigate their unique developmental potential and study the mechanisms by which pluripotency is maintained. The search for genes, which are exclusively expressed by pluripotent cells, could be performed by comparing closely related populations of undifferentiated and differentiated cells, i.e. the ICM and trophectoderm of early stage blastocysts. Identification of differentially expressed genes in preimplantation embryos is technically difficult due to the limited amount of biological material, but has been previously performed by two-dimensional protein analysis [55] and PCR-based enriched methods [56,57], resulting in the identification of only a limited number of genes. Recently, a large-scale cDNA sequencing analysis, based on the collection of over 25 000 ESTs from preimplantation embryos, was reported [58]. Stage-specific cDNA libraries from unfertilized oocytes, zygotes, 2-cell, 4-cell, 8-cell, morula and early blastocysts were constructed and compared to available database. Based on sequence similarity searches, over 9700 unique genes were identified. Half of them were found to be novel, supporting the impression that many of the genes which are expressed by preimplantation embryos have not yet been characterized. Many (about 17%) were suggested to be stage specific and only about 0.1% show constitutive expression throughout preimplantation development. An alternative approach would be to compare ES to their immediate differentiated derivatives, simple EBs, bypassing the problem of limited amount of cells available for analysis. This has been practiced in an effort to identify downstream genes of Oct4 through the use of a suppression-subtractive hybridization method, resulting in the identification of new genes which may have a role in the establishment or maintenance of pluripotency in mouse cells [59]. Furthermore, it should be possible to perform large-scale cDNA comparisons by the use of microarrays, allowing genome-wide expression profiling of these unique cells.

7. Cell reprogramming

The recent advancements in the derivation of human pluripotent stem cell lines and the success in mammalian embryo cloning by nuclear transfer (NT), provide an attractive possibility for restoring tissue function by cellular transplantation through therapeutic cloning. In this method a nucleus from a somatic cell of a patient is introduced into an enucleated oocyte and allowed to develop in vitro to the blastocyst stage. Such NT-derived blastocysts may be used for the establishment of perfectly matched ES cell lines that can be induced to differentiate in vitro and provide the patient with an autologous graft. Although sophisticated, therapeutic cloning is not unreasonable as it has been previously demonstrated to be feasible in mice [60,61]. Yet, before this procedure can be considered for clinical application it should be determined how well can a somatic nuclear be reprogrammed without being transmitted through the germ line.

Although embryo cloning by somatic cell NT has been successfully achieved in several mammalian species, efficiencies are still low due to embryonic lethality and increased incidence of malformations among first generation viable offspring. It has been speculated that nuclei from adult and advanced stage embryos could not be fully reprogrammed by the ooplasm. Even though X inactivation was shown to occur properly in somatic cell-derived cloned mice [62], it remains to be determined how complete is this process by examining additional markers that are liable to epigenetic modifications during embryonic development. Indeed, subtle abnormalities in expression and methylation of several imprinted genes were shown to occur in NT-derived cloned mice [63]. Yet, viable offspring survives to adulthood despite the widespread dysregulation in expression of these genes. It has been suggested that the somatic clones fail to re-activate key embryonic genes, such as Oct4 for example, during preimplantation and early postimplantation development and therefore are incapable of forming embryonic lineages [64]. However, since cell transplantation therapy by embryo cloning does not require full-term embryonic development but rather the formation of blastocysts and the differentiation of only specific cell types, it may be sufficient. An alternative approach suggested for obtaining autologous ES cell lines is to induce de-differentiation through the insertion of a somatic nucleus into the cytoplasm of a pluripotent stem cell, bypassing the problematic step of embryo cloning. In order to understand whether pluripotent cell lines can reverse gene re-programming and restore pluripotency, hybrids between undifferentiated EC, EG or ES cells and fully differentiated cells were examined. The hybrid cells show a loss of stage- and tissue-specific markers of the somatic cell parent (gene extinction) and de novo activation of traits and genes which are characteristic to pluripotent cells [21,65,66]. X inactivation, for example, is a process that is tightly linked with differentiation and occurs in the soma of females. By demonstrating the re-activation of X following cell fusion and it's random inactivation during differentiation, it was possible to prove that pluripotent stem cells can reset the inactivation mark correctly. Furthermore, when induced to differentiate, either in vitro or in vivo, a wide spectrum of differentiated cells could be obtained. The hybrid cells can also be examined for their developmental potential by injecting them into host blastocysts. By generating viable chimeras, it was possible to show cell hybrids in different tissues, indicating their ability to contribute to all cell lineages. It remains to be further investigated how pluripotency is maintained, lost and restored during cell commitment and differentiation, tumorigenesis, gametogenesis and embryo cloning.

8. Conclusions

Pluripotent stem cells are undifferentiated cells that have the potential to develop into all cell types in the body. They are transiently present in the embryo and are available as embryo-derived established cell lines. Their pluripotent character, as determined by their ability to form all embryonic germ layers and their differentiated derivatives in vitro and

in vivo, has made them an extremely interesting and important cell source for basic and applied research, especially for cell-based therapy and the study of early embryonic development. They can be manipulated in vitro, by controlling their growth conditions or by introducing genetic modifications into them, allowing to direct their differentiation into specific cell types or to monitor and select for them during growth in culture. However, since the mechanisms by which these cells acquire such a wide developmental potential and remain undifferentiated are yet largely unknown, it remains to be discovered what are the functional molecules and how do they operate in sustaining self-renewal and maintain pluripotency.

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