Plenary session
Pluripotent stem cells
Cellules souches pluripotentes

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Abstract

The isolation of human embryonic stem cells (ESC) in 1998 has created the hope that stem cells will one day be used to regenerate tissues and organs, even though it is obvious that a number of hurdles will need to be overcome for such therapies to become reality. The cloning of “Dolly” in 1997, more than 40 years after the first frogs were cloned, combined with the very fast progress made in our understanding of the molecular processes that govern the pluripotency of ESC has led to the ability of scientists to recreate a pluripotent state in fibroblasts and other cells from mouse, rat and man, named induced pluripotent stem cells (iPSC). This feat makes it theoretically possible to create patient specific pluripotent stem cells whose differentiated progeny could be used in an autologous manner obviating the need for immunosuppression that would be needed to use allogeneic ESC-derived differentiated cells. In addition, the ability to generate custom made pluripotent stem cells will no doubt lead to the development of protein or small molecule drugs that can induce differentiation not only of iPSC or ESC to mature tissue cells, but also endogenous tissue stem cells. Moreover, it allows scientists to create models of human diseases and may aid the pharmaceutical industry in testing more rigorously toxicity of drugs for human differentiated cells. Thus, there is little doubt that progress in stem cell biology will change many aspects of medicine as we know it in the next one to two decades.

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Keywords: Embryonic stem cells; Induced pluripotent stem cells; Cell differentiation

Résumé

L'isolement de cellules souches embryonnaires (CSE) humaines en 1998 a fait naître l'espoir qu'un jour ces cellules seront utilisées pour régénérer des tissus et des organes, bien qu'à l'évidence de nombreux obstacles devront être surmontés avant que ces thérapies ne deviennent réalité. Le clonage de « Dolly » en 1997, plus de 40 ans après que les premières grenouilles aient été clonées, conjugué aux progrès très rapides dans la compréhension des mécanismes moléculaires qui gouvernent la pluripotence des CSE a ouvert la voie permettant aux scientifiques de recréer des états pluripotents dans les fibroblastes et d'autres types cellulaires chez la souris, le rat et l'homme, appelés cellules souches pluripotentes inductibles (SCPi). Cet exploit rend théoriquement possible la création chez un malade donné de cellules souches pluripotentes spécifiques dont la différenciation devrait conduire à des cellules utilisables en conditions autologues, évitant ainsi la nécessité d'une immunosuppression inhérente à l'utilisation de cellules allogéniques dérivées de CSE différenciées. De plus, la capacité de générer des cellules souches pluripotentes sur mesure conduira certainement au développement de médicaments protéiques ou de petites molécules capables d'induire non seulement la différenciation de SCPi ou de CSE en cellules tissulaires matures, mais aussi de cellules souches tissulaires d'origine endogène. Ces travaux devraient aussi permettre de créer des modèles de maladies humaines et s'avérer utiles dans l'industrie pharmaceutique pour tester de manière plus rigoureuse la toxicité des médicaments vis-à-vis de cellules différenciées d'origine humaine. Il ne fait pas de doute que les progrès de la biologie cellulaire des cellules souches changeront de nombreux aspects de la médecine actuelle dans les une ou deux prochaines décennies.

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Mots clés : Cellules souches embryonnaires ; Cellules souches pluripotentes inductibles ; Différenciation cellulaire

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1. Stem cells

Stem cells are defined based on three characteristics. First they can undergo self-renewing cell divisions in which at least one of

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the daughter cells is again a stem cell. To increase the stem cell pool, stem cells must undergo symmetrical cell divisions wherein both daughter cells continue to have stem cell characteristics. This occurs obviously early during development but rarely postnatally, except for instance following a major insult to a stem cell pool or following transplantation of a small number of stem cells that then needs to repopulate the whole stem cell population. Stem cells have as second characteristics that they can generate differentiated progeny. Most stem cells give rise to multiple types of different cell types with as notable exception for instance spermatogonial stem cells that produce only one type of mature cells, spermatogonia. Finally, stem cells must be capable of repopulating a tissue *in vivo*, some would say even without extensive damage of the target tissue. This notion is well established for hematopoietic stem cells (HSC), while repopulation by stem cells of solid organs has only recently been shown and not yet for all stem cells.

All mammals are derived from a single stem cell, the totipotent stem cell. During embryology, stem cells undergo specification giving rise first either extraembryonic endoderm or to cells of the inner cell mass (ICM), which are pluripotent and no longer pluripotent as they can no longer give rise to extraembryonic cell types. Cells in the ICM then specify sequentially to epiblast cells, primitive ectoderm, followed by creation of definitive ectoderm, definitive endoderm and mesoderm and finally to tissue specific stem cells. With each additional step of specification, cells become more and more limited in their differentiation ability, such that the stem cells present in tissues of the three germ layers can only generate cells of that tissue but no longer cells of other tissues within the same germ layer or the other germ layers. These cells are therefore termed multipotent.

Among the multipotent stem cells, HSC are the best characterized. Murine HSC have been purified to homogeneity, as a single CD34⁺/cKit⁺/Lin[−]/Sca1⁺ (CD34-KLS) cell can repopulate the hematopoietic system of a lethally irradiated mouse [1], even though this has not yet been achieved for human HSC. Over the last decade, epidermal and gastrointestinal epithelial stem cells have also been isolated to homogeneity [2,3]. In common between HSC, gastrointestinal and epidermal stem cells is that they are responsible for maintaining a very rapidly turning over differentiated cell pool. However, also in tissues where cell turnover is considerably less, such as the brain, stem cells persist throughout adult life [4]. Whether stem cells are present in all tissues is still a matter of debate. For instance, it remains unresolved whether true stem cells reside in adult heart muscle in postnatal life, or in the pancreas, among other tissues. Although multipotent stem cells have considerable selfrenewing capacity, they appear to age. This has again been best studied in the hematopoietic system. For both murine and human HSC, telomere shortening has been found following multiple rounds of replication after transplantation [5,6]. This aging of HSC is associated with skewed differentiation towards the myeloid lineage [7]. In dyskeratosis congenita and Fanconi anemia, two disorders with telomerase dysfunction, premature aging of stem cells leads to exhaustion of the HSC pool [8].

2. Pluripotent stem cells: embryonic stem cells

As indicated above, in contrast to multipotent stem cells found in tissues and organs, cells in the ICM of the blastocyst are pluripotent, as was shown by Gardner et al. in 1968, when he created chimeric animals by transferring cells from the ICM of one blastocyst into another blastocyst [9]. In 1981, two groups demonstrated that when such ICM cells were plated onto mouse embryonic fibroblasts (MEF), continuous growing cell lines could be generated that continued to have the pluripotent characteristics of cells in the ICM, termed embryonic stem cells (ESC) [10,11]. That ESC are pluripotent can be demonstrated *in vitro* by allowing the cells to self-assemble in cell clusters or embryoid bodies (EB), without support of the MEF. ESC then undergo spontaneous differentiation to many cell types of the three germ layers. *In vivo*, pluripotent stem cells will generate teratomas, wherein small organoid structures are found representing the three germ layers, and for mouse ESC, final proof of pluripotency is the generation of chimeric mice, wherein somatic as well as germ cells are derived from the ESC. It was not until the late 1990s that ESC were also generated from higher mammalian species, first from blastocysts of non human primates and subsequently from human blastocysts [12,13]. Like murine ESC, human ESC are generated by explantation of cells from the ICM on supportive feeders, creating continuous growing cell lines that differentiate in cells of mesoderm, endoderm and ectoderm when allowed to form EB or as teratomas, when injected *in vivo* in immunodeficient recipient animals. Whether hESC and mESC are identical is not yet clear. Some have suggested that the gene expression profile of hESC may be more similar to that of epiblast committed cells (EpiSC) during embryogenesis rather than ICM cells. EpiSC have more recently also been isolated from slightly later stage mouse and rat blastocysts. They have a gene expression profile more consistent with that of hESC rather than mESC [14,15]. Furthermore, rodent EpiSC do – like hESC – not depend on leukemia inhibitory factor (LIF) for their propagation, but require – like hESC – activin-A and basic fibroblast growth factor (bFGF). Compared with mESC, mEpiSC contribute poorly to chimeras but still generate teratomas and form EB. Irrespective of whether hESC are more alike epiblast cells and mESC more like ICM cells, hESC and mESC share, aside from the much broader differentiation potential than multipotent stem cells, also significantly greater proliferation ability than multipotent stem cells. For instance, there is no telomere shortening even after several 100 population doublings. However, upon differentiation to lineage committed cells, ESC, like differentiating ICM cells *in vivo*, lose the ability to proliferate without ultimate signs of senescence.

Significant progress has been made in our understanding of the molecular mechanisms underlying the unique features of ESC. Pluripotency is maintained by a combination of epigenetic, transcriptional and posttranscriptional mechanisms. Loss of function studies have shown that several transcription factors (TF) are necessary for pluripotency, including *Oct3a/4*, *Nanog* and *Sox2*, as well as other TFs such as *Lef*, *Tcl1*, *Essrb*,

among others [16,17]. The combined action of *Oct3a/4*, *Nanog* and *Sox2* reinforces the expression of one-another in a positive feedback loop. At the same time, they serve as suppressors of lineage specific gene expression while reinforcing the expression of a complement of genes involved in histone modification, chromatin remodeling and DNA methylation that maintain the epigenetic state of the ESC. In contrast to lineage committed cells, ESC contain very little heterochromatin, making many regions of the chromosomal DNA readily accessible to TF to initiate lineage specification. Consistent with this, transcriptome studies have clearly demonstrated that genome-wide low-level transcription of lineage-specific genes occurs in ESC [18]. ESC also appear to have large domains of the genome that use a specific histone code, not found in more mature cells, which allows very fast inactivation or activation of gene expression [19]. Therefore, differentiation is more a matter of silencing of large chromatin domains and formation of heterochromatin, which prevents low level gene expression seen in ESC but not allowed in lineage specified cells and enhanced expression of a small number of genes to allow lineage specification. Obviously, much still needs to be learned regarding the epigenetic, transcriptional, and posttranscriptional mechanisms responsible for pluripotency and the mechanisms underlying the exit from the pluripotent state to a lineage specific state.

3. Reacquisition of pluripotency: somatic cell nuclear transfer (SCNT)

Well before any insights were available on regulators of pluripotency, or proof that such characteristics were present in ICM cells in the blastocyst, Gurdon al. demonstrated between 1952 and 1962 that a pluripotent state could be reacquired [20,21]. In their seminal work, they proved that transfer of the nucleus of an albino frog in an enucleated oocyte of a brown frog reprogrammed the somatic cell nucleus to a pluripotent state creating a cell capable of developing into albino frogs. It lasted until the end of the 1990s before this was confirmed for other species, even though this has not yet been accomplished with human cells [22]. The amazing feat is that reprogramming via SCNT occurs within 24–48 h following introduction of the nucleus in the enucleated oocyte. Obviously, extensive epigenetic and transcriptional changes must occur to undo the differentiated state of the cell and reinstall the pluripotent state. However, the mechanisms and molecules responsible for this phenomenon remain to date still largely not understood.

4. Reacquisition of pluripotency: cell fusion

In 2002, two groups demonstrated that pluripotency could be reimposed on somatic cells by fusing the somatic cell with ESC. Within the ensuing tetraploid cell, the molecular program of the somatic cell becomes erased and a molecular program akin to that of an ESC reappears [23,24]. As in SCNT technology, the reacquisition of the pluripotency features occurs within a few days. Here again, it is not understood how this is accomplished, although Pereira et al. recently demon-

strated that Polycomb genes, expressed in ESC, are required [25] and Silva et al. demonstrated that the process is much more efficient when a key TF of ESC, *Nanog*, is force expressed [26].

5. Reacquisition of pluripotency: induced pluripotent stem cells

As the studies described above demonstrated that factors present in ESC are sufficient to reinduce a pluripotent state in somatic cells, Yamanaka et al. went on a quest to identify some of the potential factors. And in silico study identified 20 genes that are relatively uniquely expressed in ESC [27]. His lab then developed a strategy wherein these 20 genes as well as a few others were introduced in somatic cells, questioning whether this would lead to cells with pluripotent features. When this appeared to be possible, they determined the minimal complement of genes needed to do so. In 2006, an initial study was published by his group, demonstrating that transduction of murine fibroblasts with only four TFs, *Oct4*, *Sox2*, *cMyc* and *Klf4*, could dedifferentiate the fibroblasts to cells with almost all features of murine ESC [28]. Subsequently, the same group optimized the method creating cells from murine fibroblasts that – like ESC – can generate EB, teratomas and chimeric mice with all somatic and germline cells derived from the reprogrammed fibroblasts [29]. Such cells have been named induced pluripotent stem cells (iPSC). iPSC have now been generated from multiple terminally differentiated somatic cells from mouse, as well as from human cells [29–31]. The key TF appear needed for the reprogramming to be *Sox2* and *Oct4*, whereas the other TF used allow for increasing the efficiency of reprogramming likely by opening up the closed chromatin typical of somatic cells. Initially, reprogramming was accomplished using integrating retroviral or lentiviral vectors which could cause insertional mutagenesis; however, a series of very recent studies demonstrates that this can also be achieved using short term overexpression of the TF using plasmids, non-integrating adenoviral vectors, or using transposons that are subsequently removed by a Cre-Lox system [32–35]. In contrast to reprogramming by SCNT or ESC fusion, reprogramming via defined TF requires 3 to 4 weeks, suggesting that many more factors may take part in the very quick reprogramming seen by SCNT or fusion.

6. Reacquisition of pluripotency: cell culture?

There is evidence that some the TF (*cMyc* and *Klf4*) provide access of the *Oct4* and *Sox2* to the cell chromatin. Indeed, there is evidence that these can be replaced by chemicals, such as DNA demethylating agents, or agents that modify histone acetylating and methylation [36,37], suggesting that reprogramming may ultimately be possible by chemical mediators alone. That this may be possible follows from another series of studies wherein germ line cells and even somatic cells have been cultured in vitro, via which they appear to acquire greater potency. Between 2004 and 2008, a series of studies was published demonstrating initially in mouse but now also in humans, that culture of spermatogonial stem cells in vitro for a

few weeks to months re-endows these single lineage-fated cells with the characteristics of ESC, including EB formation, teratoma formation and – for mouse cells in the study by Guan et al. – also generation of chimeric mice [38–41]. This reprogramming occurs without addition of TF. In 2002, Jiang et al. have shown that cells, termed multipotent adult progenitor cells (MAPC), can be found in cultures of rodent bone marrow [42]. Such MAPC express high levels of Oct4 and have the ability to generate differentiated progeny of mesodermal, endodermal and ectodermal lineages. Although it was initially not clear whether such cells were present in the donor tissue, there is mounting evidence that this is not the case and that the potency may be acquired in vitro as is likely true for the spermatogonial derived cells with ESC like features. Anjos-Afonso et al. demonstrated that although a population of premenenchymal stem cells could be isolated from fresh bone marrow, that express low levels of *Oct4*, *Nanog* and *Sox2*, which upon culture under MAPC conditions expressed significantly higher levels of these TF [43]. The latter cells had the same potency as MAPC described Jiang et al. These studies suggest that a subpopulation of murine bone marrow cells may be spontaneously reprogrammed under defined culture conditions. In comparison with iPSC, generation of cells with features of ESC from spermatogonial stem cells or somatic cells requires even more time (up to 2–3 months in culture) and the mechanisms underlying such culture mediated dedifferentiation are not at all understood [40,42,43].

7. Implications for medicine in the near and further-off future

What does all the progress in stem cell research, more specifically pluripotent stem cells, mean for medicine in the next years and decades? Obviously, the isolation of human ESC in 1998 has created hope that differentiated progeny of such cells may lead to tissue replacement for diseases for which currently no therapy exists, from Parkinson, to diabetes, cardiac and liver disease, among others. Hurdles to overcome when considering ESC derived therapies include the allogenicity of ESC which would require life long immunosuppressive therapy. Obviously, this complication would not be true if patient-specific ESC-like cells (iPSC for instance) would be used. Although generating patient specific stem cells is, at the current costs to generate good manufacturing processing (GMP) grade cells, not financially tenable, it is not unreasonable to speculate that costs will decrease with time, making personalized stem cell therapy for at least some diseases possible. One drawback of autologous iPSC is that they would harbor the same known or unknown genetic mutations that underlie the disease in the first place. For known genetic defects this can be overcome by in situ repair of the defect using homologous recombination, as has been shown in a mouse model of sickle cell anemia [44]. Another hurdle to overcome is the generation of cell products that do no longer harbor undifferentiated ESC or iPSC, as these will lead to teratoma formation. Finally, although a number of differentiation protocols induce lineage specification in vitro, engraftment

of the pluripotent stem cell-derived differentiated progeny in vivo in animal models has been disappointing. For instance, HSC generated from mouse ESC can only engraft when they are modified to express high levels of HoxB4 [45,46], while human ESC derived HSC only lead to minimal levels of hematopoietic reconstitution in immunodeficient mice [47]. Likewise, transplantation of ESC derived cardiomyocyte- or hepatocyte-like cells still does not lead to robust repopulation of the target organ [48–50]. The reasons for the inability of pluripotent cell-derived progeny to engraft in a postnatal organ remain an enigma, but this problem will need to be solved before ESC/iPSC-based tissue replacement will be possible.

The availability of pluripotent stem cells will however in the near future influence medicine in many other ways than tissue replacement. For instance, availability of cells that can generate many differentiated cell types, may lead to the development of protein or small molecule drugs that influence differentiation not only from pluripotent stem cells but also of multipotent stem cells residing in different tissues and this not only in vitro but also in vivo, much like the ability to culture HSC led to the development of erythropoietin and granulocyte colony stimulating factor used in hematological disorders and beyond. The ability to generate cells with pluripotent characteristics that per definition differentiate in most cell types will also make it possible to generate in vitro models of human disease, heretofore not imaginable [51–53]. Availability of human ESC that differentiate to neuron-, cardiomyocyte- and hepatocyte-like cells was already of great interest for the pharmaceutical industry for toxicity and metabolism studies. The possibility to create pluripotent cells from fibroblasts of any donor should now make it possible to create cell lines and differentiated progeny from human donors with different abilities to detoxify and metabolize drugs, suitable for use in the pharmaceutical industry. Finally, tumor formation is at least in some instances due to the dedifferentiation of a committed cell that reacquires more primitive characteristics, including extensive proliferation potential. The models developed for reinducing pluripotency will hence no doubt aid in shedding light on some aspects of malignancy development.

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