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Stem cells: From embryology to cellular therapy? An appraisal of the present state of art

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Abstract

A series of publications has dealt in the last years with topics as the isolation, properties and applications of animal stem cells (Weissman 2000. Cell 100: 157–168; Weissman 2002. N. Engl. J. Med. 346: 1567–1579; Lovell-Badge 2001. Nature 414: 88–91; Marshak et al. 2001. Stem Cell Biology. Cold Spring Harbor Laboratory Press, New york; Eridani 2002. J. Roy. Soc. Med. 95: 5–8; Borge and Evers 2003. Cytotechnology 41: 59–68; Sgaramella 2003. Cytotechnology 41: 69–73), however, the bonanza of experimental data recently accumulating have raised such an amount of controversial views and discussions that time perhaps has come for a reassessment of the basic facts in this peculiar area of research and an evaluation of possible, not unrealistic, implications.

Definition and properties

We may start from an operational definition of stem cells: Laitha's formulation (1979) is still generally accepted: 'cells which have the capacity to divide symmetrically to expand their number and asymmetrically in order to self-renew and give rise to a differentiated progeny'. An additional point may be made about the essential property of stem cells to be capable of self-renewal *in vivo* throughout the entire life span of an organism: in this regard, not all stem cells of the germ line behave that way, as oocytes are classically known to be produced in a finite number, shortly after birth and never more; only male spermatogonial cells are apparently produced in mammals over the entire life and can therefore be considered true stem cells of the germ line (van der Kooy and Weiss 2000). However in a very recent report it is claimed that juvenile and adult mouse ovaries contain mitotically active germ cells, which may sustain oocyte and follicle production in the postnatal mammalian ovary as well (Johnson et al. 2004).

Another type of embryo stem cells have been considered in a category of their own, namely, those found in the genital ridges of developing foetuses and are commonly termed embryonic germ (EG) cells. EG cells, grown *in vitro*, differentiate spontaneously, forming aggregates containing derivatives of all three germ layers, maintaining their normal karyotype. It is also noteworthy that, at variance from ES cell lines, they do not generate teratomas, when injected into immune-deficient mice (Shamblott et al. 2001). It is not clear whether EG cells can be found in adults.

Embryonic stem cells (ES) from the inner mass of cells in the blastocyst (Figures 1 and 2) remain anyway the best candidates to form every cell type

of the body, while the remaining part of the blastocyst will form extra-embryonic tissue, like the placenta (Borge and Evers 2003). ES cell lines have been developed from mice, monkeys and humans (Evans and Kaufmann 1981; Thompson et al. 1998). Such cell lines can be maintained in media supplemented with growth factors: no change in their karyotype and the conservation of telomerase activity, assuring telomere regeneration, are essential requirements (Lebkowski et al. 2001; Odorico et al. 2001;).

In a recent report it is furthermore claimed that a cell line has been derived from a cloned human blastocyst, with cells displaying typical ES morphology and surface markers, as well as keeping the same genetic identity (Hwang et al. 2004); such cells can differentiate to embryoid bodies *in vitro* (the next step from the blastocyst) and in mice can form teratomas containing all three germ layers. The possible connection between stem cells and cancer will be discussed later.

As far as the genetic features of stem cells, it is generally recognized that they express, at least in murine and human types, a higher number of genes than differentiated cells and also a variety of gene products, defining regulatory pathways (Ramalho Santos et al. 2002). A total of 216 genes are enriched in many types of stem cells (embryonic, neural and haemopoietic) and most of them are clustered. Only 60 have been mapped to a chromosomal location, and 12 of them on chromosome 17, which appears therefore to contain more stem cell-enriched genes than dictated by its size. It has been suggested that such array of gene and gene products may represent a kind of 'signature' of stem cells (Ivanova et al. 2002).

Another attempt of genetic analysis has focused recently on the transcriptome characterization of human ES cells: over 32,000 transcripts, expressed in such cells, have been identified, allowing to unveil changes in the transciptional network which occur during ES cell differentiation. It is felt that these data may help to obtain clinically useful cell types from human ES cells (Brandenberger et al. 2004).

A special mention should be made of a very peculiar way to obtain ES cells, namely by *parthenogenesis*. This is a process by which an unfertilized oocyte may develop into an embryo without participation of sperm. It has been obtained in non-human primates (*Macaca fascicularis*) treating

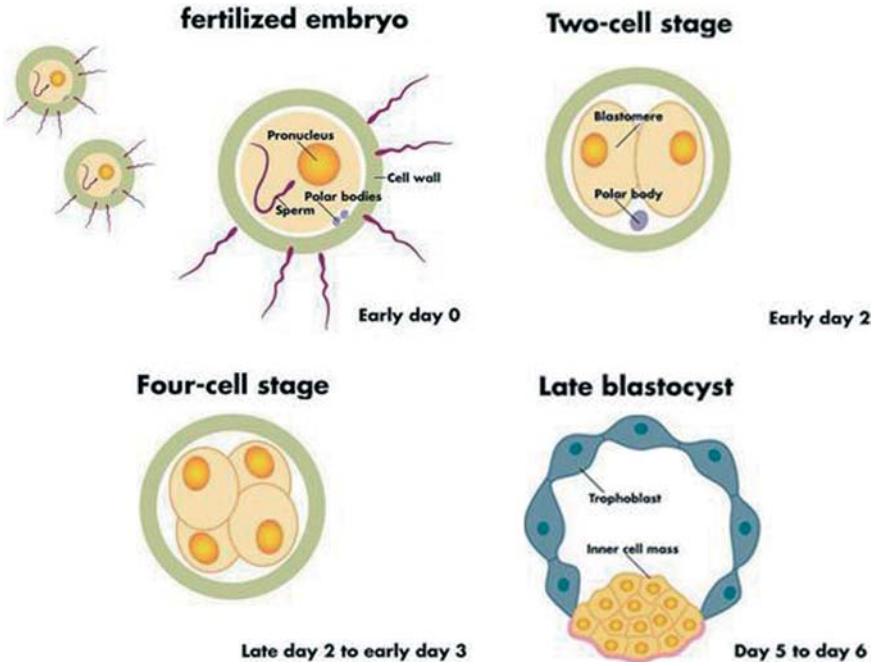

Figure 1. Human blastocysts are formed about 5 days after fertilization (scheme). Each blastocyst consists of about 140 cells of two types: inner cell mass (ICM) and trophoblasts. ICM cells eventually develop into a complete individual while trophoblasts will provide the supporting tissue of the embryo.

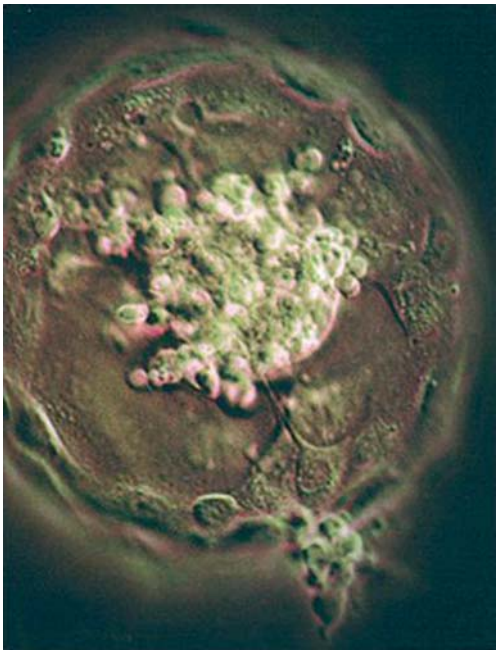

Figure 2. Photomicrograph of a human blastocyst, showing the inner cell mass and the surrounding trophoblast cells.

eggs with chemicals which induce cell division without prior ejection of half the chromosome complement (Cibelli et al. 2002): 4 out of 28 eggs so treated developed into blastocysts, and from one of them a stable cell line could be obtained. Cultures of cells from this line were induced to differentiate into a variety of cells, including neurons and, apparently, cardiomyocytes. It is not known whether such process could be applied to human oocytes, but in this case there would be a remarkable ethical advantage, namely the possibility to obtain pluripotent stem cells from a biological formation, which, as in other primates, could hardly develop into a full-term baby.

Another source of stem cells well known since the seventies is represented by a peculiar tumour, called teratocarcinoma or, briefly, teratoma, found in ovaries and testis: such tumour arises from a stem cell, able to originate many cell types and tissues, which are actually found in teratomas (Pera et al. 1989). Stem cells from such tumour (EC cells) can be recovered and placed in culture, where, after treatment with certain growth factors, can produce a variety of differentiated cells, including neurons, although with a more limited potential for differentiation, compared to ES cells. Moreover, EC cells do not keep a normal karyotype and it has been thought that genetic variations linked to tumorigenesis may be a limiting factor in their capacity of differentiation. It is therefore suggested that the best use for EC cells should be the study of mechanism controlling cell differentiation, with particular emphasis on the neuronal development, which seems to be specially activated in EC cell lines (Andrews 1998).

Identification and characterization of stem cells

An apparent paradox is surfacing in stem cell research: their alleged rarity (one in hundred thousand in circulating blood?) in a fully developed organism is at odds with their reported frequency in the various organs.

This paradox obviously depends first upon a tenaciously elusive definition of 'stemness', if existing at all, as outlined above and then on the accuracy of their characterization (Marshak et al. 2001). In the absence of any morphologically outstanding feature distinguishing stem cells even under the most potent optical microscope, with the use of electron microscopes barred because of the ensuing damages to the samples, researchers have to rely on the presence of surface markers and on their exploitation in purification.

The surface of the cells are coated of molecules, mostly proteins and carbohydrates, which help in performing their integrated functions: they do so by binding to other structures, molecular as well as cellular, hence their collective name of 'cell receptors'. Researchers bind signalling molecules (mostly fluorescent tags) to the receptors: the combination of the physico-chemical properties of the fluorescence and the unique pattern of receptors make stem cells distinguishable not only from other cells but also among themselves, and possibly separable.

Various approaches are available for stem cell separation and enrichment .The most popular method for its ease of use and speed, relative to more elaborate strategies, is *immuno-magnetic separation*, in which targeted cells are labelled with specific antibodies and magnetic beads; such beads are superparamagnetic particles approximately 50 nm in size, not visible with light microscopy, biodegradable and gentle on cells; they can be coupled to highly specific antibodies. like the QBEND 10 monoclonal antibody, directed against

the CD34 antigen: after a passage through a high-gradient magnetic field in order to retain exclusively the labelled cells, the magnetic separator is removed and purified cells can be eluted. This highly enriched cellular fraction can be directly utilized for experiments or grown *in vitro* without any interference on survival/proliferation by the remaining beads. With this method a high degree of purity ($>70\%$) and a good yield of haemopoietic cells can be obtained from sources as the bone marrow (BM), umbilical cord and peripheral blood (De Wynter et al. 1995); in order to improve T-cell depletion (so important for a successful BM transplant) a negative selection step may be added. The clinical applications of such separation technique have been comprehensively reviewed (Farag 2002).

Another method is based on the affinity between *biotin and avidin*, in which mononuclear cells stained with a biotinylated anti-CD34 -antibody are passed through an avidin column, retained and then released by agitation: this system is mainly used for small scale purification but has also been upgraded for clinical application (Brugger et al. 1995). Widely used is also the sorting machine called *Fluorescence-Activated-Cell-Sorter (FACS)*, which is based on the ability of the fluorescent tags to become electrically charged when a laser beam is flashed on them as they pass through it in a tiny drop: this is however a rather time-consuming technique. An extensive comparison between many different ways of haemopoietic stem cell isolation and purification has shown that magnetic activated cell sorting (MACS) and FACS as the best reliable methods for high purity yield and colony forming cells (CFC), while affinity colums gave the same purity values, but a lower amount of CFC's (De Wynter et al. 1995).

A special method to study stem cell morphology uses ultra-thin sections of CD34+stem cells embedded in Araldite and counterstained with uranyl acetate and lead citrate: observation is carried out with a Transmission Electron Microscope (TEM) and the structure of stem cells can be studied (Servida et al. 1996).

As in particular human embryonic stem cells (HESC) are concerned, standards have been set recently by a panel of experts under the aegis of NIH (Kirschstein and Kirsboll 2001). They have listed 13 molecular markers for identifying undifferentiated pluripotent HESC. These markers are expressed or enriched in undifferentiated HESC and are turned off after differentiation. There are two groups: *class I*, cloned markers that are well defined and unambiguous, and *class II*, cell surface antigens detected by antibodies but requiring more extensive characterization.

The problem is reputed of such importance that the NIH have created a Stem Cell Characterization Unit with the mission of providing reliable and standardized data derived from assays performed on HESC lines to be supplied to the research community. One interesting approach being considered involves constructing cDNA libraries from several HESC lines and then sequencing the Expressed Sequence Tags (EST). This should identify genes corresponding to the functions of HESC present in, and intrinsic to, different lines (Brivanlou et al. 2003). An important item on their agenda is also the evaluation of the genetic modifications allowed to the HESC in view of the characterization and subsequent use of millions of *in vitro* cultured human stem cells.

A final step along this line should address the problem of studying the features typically involved in DNA differentiation, de-differentiation and transdifferentiation, such as methylation of DNA bases and of chromatin proteins. The project falls within a broader program aiming at spotting and evaluating intraorganismal genomic discontinuities as indicative if not causative features of gene expression and silencing during the various phases of development: as such they are charged with enormous expectations in therapeutic protocols generally resting on the *in vivo* injection of cells.

Stem cells and cancer

It is of interest to consider the possible relationship between stem cell and cancer. It has been for instance demonstrated that the same signals in individual tissues can trigger the proliferation of both normal and cancer cells: such is the case for Wnt signaling, which includes a large family of highly conserved proteins (Nusse 1999). It appears that the Wnt gene does activate a receptor on the cell membrane, which in turn sets off a series of events leading to cell division and differentiation. This process is called the 'Wnt cascade', with a Tcf-4 protein at the end of such pathway: stem cell renewal in both the intestinal epithelium and the

haemopoietic tissue is prevented in mice lacking this protein and the animals die shortly after birth (Polakis 2000).

Furthermore, it has been found that the Wnt gene exerts an important role in colo-rectal cancer, upregulating 120 genes (including c-Myc) and down-regulating 115 other genes. The same signal from WNT exerts however an opposite effect on normal intestinal cells: this apparently generates mutations, which tend to accumulate in an irrepressible process, eventually producing overt cancer (van de Wetering et al. 2002). Another gene product, called 'nucleostemin', acting within the stem cell nucleus, seems also able to interfere with the proliferation of both normal and some types of cancer cells (Tsay and Mc Kay 2002).

Stem cells capable of giving rise to neoplastic forms have been described in other conditions, like acute leukemia, where it is debated whether the haemopoietic stem cell is potentially leukemic or the leukemic process may originate from a more differentiated stem cell (Marks 2003), Cancer stem cells have also been identified in brain tumours of different phenotypes: such cells show a high capacity for self-renewal, proliferation, and differentiation to the original neoplastic phenotype (Singh et al. 2003).

Evidence is therefore increasing about the possible connection between normal and neoplastic evolution of stem cells, so that a better knowledge of potential cancer stem cells is advocated, in order to understand and counteract the disease (Reya et al. 2002).

Skin stem cells

The possibility of skin transplants has been known and also practised for a long time, for instance as reported by the Italian Baronio in 1804 for animal skin transplants and by Winston Churchill, who described how, during the Sudanese war in 1898, he was asked to donate a portion of his skin to a wounded friend: the transplants was successful and the illustrious donor wrote that he 'kept the scar as a souvenir…' (Converse and Casson 1968).

In the epidermis a population of stem cells has been recognized as responsible for maintaining the potential to differentiate to a variety of epithelial cell types as well as the capacity to self-renew through the course of life (Fuchs and Segre 2000).

The outermost layer of the skin, the epidermis, is indeed a rapidly renewing tissue and relies on the regenerative capacity of keratinocytes. The majority of proliferating epidermal cells, also known as transit-amplifying cells, at the innermost layer of the skin have a finite life span and undergo rapid terminal differentiation. Therefore it is well accepted that the extensive regenerative capacity of the skin is most likely due to the activity of epidermal stem cells.

The structure and function of the epidermal regenerative compartment have been accurately analyzed (Watt 2001): within the epidermis proliferation takes place in the basal layer of keratinocytes that are attached to an underlying basement membrane. Cells that leave the basal layer undergo terminal differentiation as they move towards the tissue surface. The basal layer contains two types of proliferative keratinocytes: stem cells, which have unlimited self-renewal capacity, and transit-amplifying cells, those daughters of stem cells that are destined to withdraw from the cell cycle and terminally differentiate after a few rounds of division. Stem cells express higher levels of the β 1-integrin family of extracellular matrix receptors than transit-amplifying cells and this can be used to isolate each subpopulation of keratinocyte and to determine its location within the epidermis. Constitutive expression of the transcription factor c-Myc promotes terminal differentiation by driving keratinocytes from the stem cell compartment into the transit-amplifying compartment.

To determine the cells responsible for rapid epidermal regeneration, Li et al. (2004) separated epidermal stem cells from their progeny and assayed the ability of both cell types to regenerate epidermal tissue in both *in vitro* and *in vivo* settings. As expected, keratinocyte stem cells displayed robust regenerative capabilities, but unexpectedly, transit-amplifying cells and early differentiating cells, which are more committed progenitor cells, could also form a fully stratified epidermis under appropriate microenvironmental conditions and up to 10 weeks in culture. This work allows new considerations for therapies that require large numbers of epidermal cells (such as those designed for the treatment of extensive burns), removing the need for difficult and limited stem cell selection.

The skin is an interesting source of adult stem cells, not only important for the repair of skin

lesions, but also able to give rise to other cell types and tissues if the debated theory of cell plasticity finds sustained support: as far as skin cells are concerned, it was shown by Toma et al. (2001) that cells derived from mouse dermis, which proliferated clonally in culture in the presence of epidermal growth factor (EGF), could generate both neural and mesodermal progeny. Working on this assumption it may be possible to exploit the skin as a very accessible source of adult stem cells, which might be used for transplantation protocols (Slack 2001); isolation and purification of epidermal stem cells have thus been advocated for use in tissue engineering (Dunwald et al. 2001).

Cornea stem cells

Among the stem cells of epithelial origin, peculiar attention can be devoted for their practical implications to those responsible for the regular maintenance of the corneal epithelium, which is actually undergoing a constant process of renewal and repair. The population of such stem cells resides in the limbus, which is part of the ocular surface and which (beside anomalies due to genetic factors) is subject to severe alterations: chemicals, trauma, auto-immune disease can injure stem cells beyond repair and blindness may follow. It is therefore mandatory to replace the damaged cornea, but transplant of cornea *in toto* has many drawbacks and, above all, does not replace the limbus. It has been therefore tried for some years to transplant limbal stem cells, with an increasing success rate (Holland and Schwartz 1999). The transplant can be done according to three different procedures: (1) by autograft in cases where the injury has destroyed the stem cell population in one eye only, (2) by allograft, obtaining the limbal tissue from a cadaver or a living relative, and (3) by tissue culture, harvesting limbal stem cells from cadaver, growing them in culture and transferring them on a collagen shield, which serves as a carrier to deposit the epithelial cells on the surface of the eye. Another carrier can be the *membrana propria* of the conjunctiva. It appears that the success rate is similar for all these procedures. The immunosuppressant cyclosporine is generally used, both locally and in a systemic way, to prevent rejection (Dua and Azuro-Blanco 2000).

Chondro- and osteogenic stem cells

Bone formation *in vivo* is achieved in two ways, either by replacement of a cartilagineous template, as in most skeletal bones, or by direct mesenchymal derivation, as it happens in the flat bones of the skull (Erlebacher et al. 1995). In the first case, chondrogenic cells undergo a process of maturation, which involves the expression of specific proteins, like collagen II A and B, and later collagen X. At some stage chondrocytes become so called hypertrophic cells, which may develop into osteocytes or undergo apoptosis to be replaced by osteoblasts. Specific proteins are in turn produced by osteoblasts like osteonectin, osteopontin, bone sialoproptein and osteocalcin (Cancedda et al. 2000). Research *in vitro* has shown that a similar process takes place in chondrocytes derived from mouse embryonic stem cells, including formation of osteogenic proteins: it has been said therefore that cellular differentiation events in culture tend to recapitulate the process of embryonic differentiation in the living organism, making it a model system for developmental biology (Kramer et al. 2003).

The possible use of stem cells for bone repair and regeneration in a variety of traumatic events and bone diseases is presently a fascinating challenge for tissue engineering: following a trial with rabbit osteochondral progenitors, which showed bone formation in artificially created ulnar defects (Perka et al. 2000), muscle-derived stem cells genetically engineered to express specific growth factors like vascular endothelial factor (VGEF) and bone morphogenetic protein 4 were able to enhance cartilage formation in the early stages of endochondral bone formation (Peng et al. 2002). It has also been shown that BM stromal cells implanted into immunodeficient mice in combination with hydroxyapatite bioceramics were able to form primary bone tissue with haemopoiesis-supporting stroma; however a limiting factor was the loss of differentiation potential with time in culture and it is therefore suggested to transfect the cells to be transplanted with the telomerase gene to prevent ageing (Derubeis and Cancedda 2004).

Neural stem cells

In mammals neural stem cells (NSCs) can be derived both from developing and mature Central

Nervous System (CNS) and are characterized by plasticity, multipotency and capability to give rise to the three principal fully differentiated cell types (astrocytes, neurons and oligodendrocytes) (Bottai et al. 2003). *In vivo* NSCs were thought to derive from two specific regions of the adult brain: the subventricular zone (SVZ) surrounding the lateral ventricles and the subgranular layer of the hippocampal dentate gyrus (DG). As a matter of fact, recent microdissection data suggest that adult DG contains only lineage-restricted progenitor cells with a reduced capacity for self-renewal (thus limiting multipotential stem cell niche into the SVZ region). In addition, current evidence indicates that there may be multiple NSC sub-populations in the adult brain, which may share certain properties but, at the same time, are distinguishable on the basis of other characteristics such as their quiescent or proliferative state (Pevny and Rao 2003).

Unlike other stem cell types, NSCs can be isolated and grown in cultures as clonogenic clusters in suspension (called 'neurospheres', a mixture of stem and progenitor cells), following the appropriate stimulation, such as exposure to mitogens like basic Fibroblast Growth Factor (bFGF) and EGF. In spite of several efforts, until now it was not possible to specifically separate NSCs from their differentiated progeny: all the identified and used markers (see Table 1) allowed only enrichment in the stem cell compartment, but did not allow selective NSCs purification. In order to clarify this issue a wide transcriptome analysis of several mouse tissue-specific stem cells and early embryos has been very recently published, with the aim to define cellular stem potency in terms of molecular biology and gene profiling (Sharov et al. 2004).

In this paper correlation between genes and cell developmental potential has been enlightened, with the selection of 88 genes, suggested as a set of markers for characterizing the cell potential.

Several papers have already clarified that genetic clues are not the only elements acting on stem cell conditions and destiny. It has been recognized that NSCs proliferation or differentiation are regulated and influenced at the progenitor level, both *in vivo* and *in vitro*, by a number of factors: among the most relevant are the availability of appropriate cytokines and growth factors, while a certain role is also played by the receptor types and their density: the metabolic state and the degree of apoptosis have also to be taken into account (Sommer and Rao 2002).

A recent paper has underlined how ageing influences neurogenesis specifically through an age-related reduction in the NSC population (Maslov et al. 2004) probably due to a reduced availability of neurotrophic factors, condition which is exacerbated in neurodegenerative diseases such Parkinson's and Alzheimer's. As a matter of fact, for example, genetically modified grafted NSCs able to secrete neurotrophin 3, could provide locally significant amounts of trophic molecules thus determining a three-fold increase in axonal growth when transplanted into the lesioned murine spinal cord (Lu et al. 2003).

Environmental influences and the fundamental importance of cell-to-cell contact have been recently proved in an Amyotrophic Lateral Sclerosis (ALS) animal model carrying the human mutated Cu-Zn superoxide dismutase gene (SOD1-G93A transgenic mice). In chimeric mice, surrounding wild-type non-neural cells extend survival of SOD1 mutant motor neurons and delay degeneration (Clement et al. 2003). Similarly

Table 1. Specific markers proposed for murine and human NSC compartment enrichment

Markers	Species	References
Nestin	*Mouse-human*	Lendhal et al. (1990)
EGFR	*Mouse-human*	Reynolds and Weiss (1992), Doetsch et al. (2002)
Dlx2	*Mouse*	Porteus et al. (1994), Doetsch et al. (2002)
PNA^{low}/HSA^{low}	*Mouse*	Rietze et al. (2001)
LeX/ssea-1	*Mouse*	Capela and Temple (2002)
AC133	*Mouse-human*	Yin et al. (1997), Uchida et al. (2000)
Musashi1-Musashi2	*Mouse*	Sakakibara et al. (2002)

None of these markers alone allows however an unequivocal selection of neural stem cells

NSCs transplantation in different pathologies, such as multiple sclerosis and a chemically induced Parkinson's like form, induced recovery not only by simple NSC-derived replacement of lost cells, but also by promoting the spontaneous proliferation and reorganization of endogenous stem cells and tissue (Ourednik et al. 2002). On the other hand amplification of spatial complex interactions between donor stem cells, seeded on a reabsorbable polymer scaffold, and host environment facilitated neuronal regeneration, guided repair and mitigated glial scar formation after hypoxic-ischemic injury. Very recently (Silva et al. 2004) *in vitro* encapsulation of NSCs within a three-dimensional network of nanofibres induced selective differentiation of neural progenitor cells into neurons with a marked reduction of astroglial fraction.

Therefore all these recent findings and data indicate that the new approaches in stem cell therapy require transplantation of NSC's, not only to replace the lost/damaged population, but also to induce undifferentiated progenitors or glia to promote endogenous stem cells proliferation and optimal recovery.

At the same time, bio-matrix scaffolds could influence endo-autologous NSCs destiny and promote spatial interaction aimed at reconstituting damaged tissue (see Figure 3). In addition, genetic modification of transplanted cells integrated with pharmacological treatment (i.e. growth factors infusion and/or antiapoptotic drugs) is necessary to synergistically overcome restriction imposed by the degenerated environment. In summary, cell replacement and circuit reconstitution require complex strategies and technical devices in order to translate experimental approaches into clinic and treat the complexity of CNS disorders.

Haemopoietic stem cells

Stem cells from the blood and blood-forming organs have been the first ones to be studied, identified and even employed in transplantation practice (Thomas 1991). The enormous potential of haemopoietic stem cells (HSCs) has been therefore recognized for long time and received a solid experimental support from the demonstration that a single mouse HSC, negative for all lineage markers, could activate a full long-term reconstitution of the lympho-haemopoietic system (Osawa et al. 1996).

Further evidence of the potential of stem cells from the blood-forming organs came from studies on the purification of marrow stem cells, showing that there are single bone-marrow derived stem cells capable not only to regenerate the haemopoietic system, but also to differentiate into other cell types, like liver, lung, gastro-intestinal tract and skin (Krause et al. 2001).

This and similar findings point to a considerable diversity for HSCs and their potential and call for a revised classification. We shall therefore briefly attempt to outline the main features of different HSC's, keeping into account the reservations that such attempts require, as well as the basic objections about a real hierarchy of such cells.

Starting from the intermediate progenitors, we can recognize *committed stem cells*, which can induce the formation of well-characterized colony types, like CFU-GM, giving rise to granulomonocytic colonies, BFU-E and CFU-E's producing erythroid colonies, CFU-MK, giving megakaryocytic colonies and so forth (Eridani and Morali 1993). An exclusive lymphoid progenitor was also identified (Kondo et al. 1997).

Associations between two types of colony-forming cells are also known, as exemplified by the already mentioned case of the granulocytic-monocytic precursor and by the occurrence of spontaneous erythroid colonies in cases of Essential Thrombocythemia (Eridani et al. 1987). Another kind of stem cell seems to act as a common myeloid progenitor, giving rise to all myeloid lineages (Akashi et al. 2000).

It is of interest that from these data the impression emerges that, at a more differentiated level, some kind of hierarchy exists among various types of progenitors. The picture is variable if we, however, consider the most primitive stem cell: it was at one stage postulated that such population is made of quiescent cells in the G0 phase of the cell cycle and is thereby protected from depletion or exhaustion (Rosendaal et al. 1979). In contrast with this view, it has been claimed that such quiescent state is relative, as a good number of stem cells may be continuously cycling and contributing to blood cell formation (Harrison et al. 1987). It has been therefore proposed, on the basis of recent experiments, that instead of a rigid stem

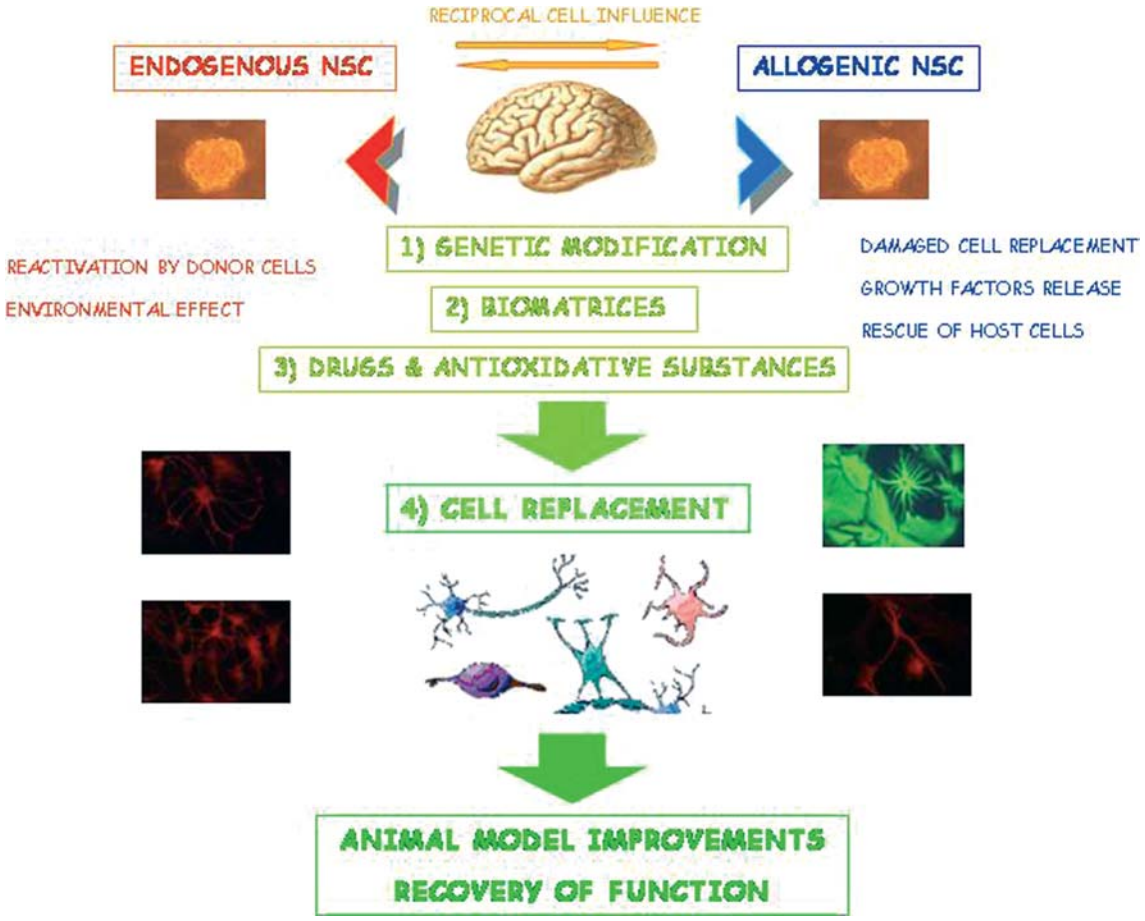

Figure 3. Effective NSC therapy in neurodegenerative diseases requires a complex critical multilevel strategy, which involves several molecular and cellular techniques. NSC, endogenous (on the left) or allogenic (on the right) eventually genetically modified for trophic factors (TF, **1**) release, require interaction with the surrounding cells in order to reconstitute the damaged tissue architecture. Use of synthetic biomatrices (**2**) could improve donor and endogenous cell differentiation and facilitate structural interactions in order to promote neuronal connectivity. A permissive niche (combining utilization of pharmacological drugs, artificial extra-matrix proteins, anti-oxidative substances, (**3**) will increase graft survival and integration into the damaged host tissues. Cell replacement (**4**) could be even indirectly reached through an effect on environment with the reactivation of host cells *in situ* without any functional integration of transplanted cells. Therefore all of these different, and in part unclear, factors and mechanisms could contribute to animal model improvements and recovery of function after transplantation in neurodegenerative diseases.

cell/progenitor hierarchy, there may be a 'fluctuating continuum', which can be altered by changing cell surface phenotype of the stem cell and by environmental stimuli: this would thus provide a flexible system for the regulation of haemopoiesis (Quesenberry et al. 2002).

There are different sources of haemopoietic cells: bone marrow (BM), peripheral blood (PB) and umbilical cord blood (CB) cells, which can be isolated and amplified in culture. A special class of bone-marrow-derived elements are the mesenchymal cells (MC), which have raised a lot of interest for their 'plasticity'.

Selection of haemopoietic cells from different sources is now fairly standardized, based as it is on the positivity for CD34 antigen (positive selection) and/or negativity for lineage markers (negative selection). A widely used method is based on anti-CD34 antibodies and paramagnetic beads, as before mentioned: labelled cells are released by switching off the magnetic field, without apparent alteration of cell function. Florescence activated cell sorting is also employed (Clark et al. 2003). An interesting new molecule, expressed on early haemopoietic stem cells, is the CD133 + antigen: purified CD133 + cells show a high engraftment

potential and may also give rise to other populations, like endothelial cells; it is suggested that CD133 + cells may prove useful for reasearch as well as having clinical applications (Handgretinger et al. 2003).

With a good use of such methods a highly purified amount of stem cells can be obtained, but the total yield of course is different, depending on the source used. Comparison of the three most used sources has shown that BM is by far the richest source of HSCs, so that a sufficient dose of haemopoietic progenitors can be obtained for subsequent expansion and clinical use from a relatively low number of bone aspirates (Emerson 1996).

PB HSCs have also been extensively studied and used, with the added attraction of possible mobilization before collection (To et al. 1994). A particular finding was that among PB cells there are endothelial stem cells, which can be isolated and expanded in culture, so that a common progenitor could be postulated (Nishikawa 2001).

The cord blood (CB) source was first discussed by Broxmeyer et al. (1989), as stem cells were found to be present there in considerable amounts. Furthermore it was shown that in culture they could generate a high amount of mature cells without reducing the number of CD34 cells, thus showing a persistence of primitive self-renewing cells (Mayani and Lansdorp 1995). More recent studies have confirmed both the amplification and self-renewal in culture of CB stem cells (Piacibello et al. 1997; Eridani et al. 1998), which are currently used for transplantation. Cord blood banks are now established and progenitor cell assays have now been devised to predict the engraftment speed in cord blood transplants (Migliaccio et al. 2001).

A possible subtype of circulating haemopoietic progenitors has been claimed to be neonatal blood (NB), although in lesser amount than CB: cells from that source, obtained shortly after birth, have shown to be able to repopulate sublethally irradiated immune-deficient mice (Zhang et al. 2002).

Clinical results with HSC transplantation for a variety of disorders have steadily improved since the first attempts a few decades ago. While most transplants were performed utilizing BM as a source of HSCs, later experience with sources like peripheral blood and umbilical cord blood has been quite satisfactory: in particular the practice of mobilizing PB stem cells by the administration of factors like granulocyte colony stimulating factor (G-CSF) allows a collection of a sufficient number of HSC to achieve the engraftment (Bensinger et al. 1995). Success of HSC transplants is of course dependent on many factors, including tissue compatibility between donor and recipient: in this context the introduction of very large registries of potential donors has dramatically enhanced the chance of finding a compatible unrelated BM donor (Appelbaum 1997; Thomas 1999).

CB stem cells, which have a reduced risk of graft-versus-host disease are also currently used for transplantation, mainly in children, but also in adults; results are also encouraging for transplantation from unrelated donors (Wagner et al. 1996).

Many advantages have been found for CB cells, like the high content of lymphocytes with a 'naive' phenotype, the reduction of graft-versus-host reaction and a greater transduction efficiency (De Wynter 2003).

Of special interest are the so called 'directed donations', whereby cord blood collection is recommended for siblings born into a family at risk from a genetic disease which may be cured by HSC transplantation (Hows 2001).

A special alternative to post-natal HSC transplants has been introduced in recent years for the treatment of congenital blood disorders: *in utero transplantation* (IU-HSCT). This has been possible by advances in prenatal diagnosis (10–12 weeks of gestation), so that an increasing interest is devoted to new techniques, which may facilitate such practice. However the limited receptivity of the recipient and, in some cases, a lack of advantage by the donor HSC's may constitute a barrier to engraftment, which needs to be overcome. Among diseases, which are the best candidates for IU-HSCT are therefore those which offer a selective advantage for donor cells: for example the severe combined immune deficiency (SCID) disorders and the Wiscott–Aldrich syndrome (Flake and Zanjani 1999).

Increasing attention is now devoted to an interesting source of bone marrow-derived progenitors, namely *mesenchymal stem cells* (MSC). These adherent cells actually constitute the support for haemopoietic cells and provide them with a functional micro-environment (Caterson et al. 2002). The main point about MSCs is the demonstration that, beside giving rise (only in mouse) to haemopoietic cells, they can generate cells of

such diverse phenotypes as bone, fat, muscle cartilage, and fibroblasts in human subjects (Pittenger et al. 1999). Moreover, mouse MSC have been shown to be able to differentiate into neural cells *in vitro* (Sanchez-Ramos 2002).

These and similar findings have raised the possibility that MSC could represent a promising stem cell source for tissue replacements: for a review see Koc and Lazarus (2001).

Further speculation was prompted by the finding that mesenchymal cells with high-differentiation potential could be isolated from non haemopoietic organs, like human fetal kidney: in culture such metanephric mesenchymal cells developed hepatocyte features and, after transplantation into sheep fetuses produced a multilineage haemopoietic engraftment. This seems to support the claim that MSC may represent a widely distributed multipotent stem cell pool, present in various organs of the body (Almeida-Porada et al. 2002).

The problem of cell 'plasticity'

The above-mentioned findings about multilineage potential of progenitor mesenchymal cells keep alive a question which has been debated for some years now, namely the so called 'transdifferentiation' of primitive stem cells: this was prompted by the observations that BM haemopoietic cells were apparently able to differentiate into neural cells (Eglitis and Mezey 1997) or muscle cells (Ferrari et al. 1998), as well as into the above mentioned pluripotent skin cells: it has been thus optimistically assumed that adult stem cells could turn into almost any other cell type, avoiding thus the necessity to use ES cells. Cautionary views were, however, expressed on this subject (Weissman et al. 2002) and some experimental results have also proposed alternative hypotheses. It has been indeed reported that both brain and BM cells can be fused in culture with ES cells, resulting in hybrid cells endowed with the pluripotent capacity of ES cells (Terada et al. 2002; Ying et al. 2002).

This process of cell fusion has been known for a long time and it has been recognized as a spontaneous occurrence in multicellular organisms (Ringertz and Savage 1976). Induced cell fusion was then developed to study the expression of traits of two differentiated states in new hybrids: a typical example is the reactivation of the inactive X chromosome when thymocytes were fused with teratocarcinoma cells (Takagi et al. 1983).

Presently we shall discuss the concept of 'plasticity', namely the capacity of a cell to express genes which are typical of another differentiated state (Lucas and Terada 2003). A case in point is the apparent *muscle regeneration* by haemopoietic stem cells: the question is whether this process involves a true generation of myogenic progenitors or it implies a fusion between HSCs and newly formed myotubes. Recent reports showed that HSCs could be found in muscle tissue long time after transplantation and that integration of marrow cells into myofibres occurs spontaneously and increases with muscle damage, suggesting that HSCs actually have a myogenic potential (Courbel et al. 2003). More evidence was provided to support the theory that in transplanted mice single HSC could produce both haemopoietic and myogenic cells (Camargo et al. 2003), but these authors found that this process is due to circulating inflammatory cells (myeloid cells), which are incorporated by fusion during formation of myotubes: a real myogenic potential by HSC's was therefore ruled out.

It is probably more likely that muscle regeneration is mediated by stem cells resident within the muscle itself, as shown by recent data about a population of specialized C45+ stem cells, which is present in muscle and can give rise to myogenic progenitors in response to Wnt signaling (Polesskaya et al. 2003). It is therefore suggested that systemic marrow transplantation is not at the moment a valid option for the treatment of muscle disorders, while efforts should be made to stimulate proliferation of resident stem cells (Rudnicki 2003).

Research has also been directed towards possible regeneration of *cardio-myocytes*, and here again favourable results obtained in culture (Figure 4) await confirmation by transplantation experiments *in vivo* . It was indeed claimed that BM stem cells injected into damaged hearts became cardiac cells, improving heart function in 40% of treated mice (Orlic et al. 2001). There were even reports of patients treated with their own marrow cells who apparently showed a remarkable improvement. Marrow mesenchymal cells, inserted with a survival gene Akt1, were also given to rats with ischemic hearts, halting the subsequent heart failure (Mangi et al. 2003).

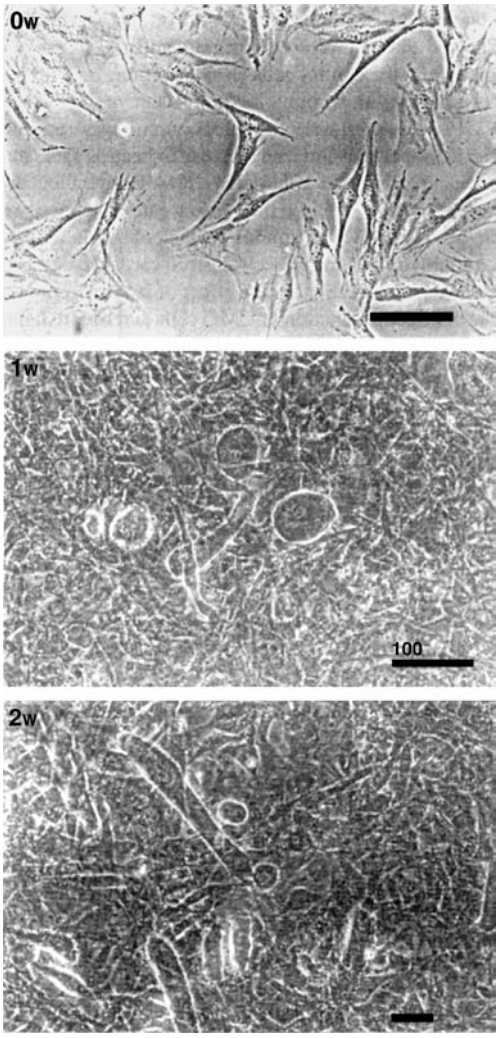

Figure 4. Phase-contrast photograph of fibroblast-like mesenchymal stem cells (at 0 week), then after treatment with 5-azacytidine, evolving at 1 week. Into ball-like or stick-like appearance, and finally, at 2 weeks, developing into pulsating. cardiomyocytes. (by kind permission of Dr K. Fukuda, from Cytotechnology, (2003), 41, 169.).

However two recent reports seem to deny the possibility of transformation of marrow progenitors into cardiac muscle cells, as experiments carried out as the original ones failed to demonstrate a single cardiac cell which was derived from the injected haemopoietic cell (Balsam et al. 2004; Murry et al. 2004). It has also been found that after 6 months from the administration of G-CSF to patients and the injection of purified blood stem cells into their hearts there was, beside some improvement in heart function, a development of abnormal growths around the implanted devices used to keep arteries open (Kang et al., 2004).

The reason for such contrasting observations is not easy to explain: many factors are involved, like different methods for purification of BM cells and different methods to follow the fate of injected cells. It is also still unknown which type of signal may come from a damaged tissue in order to attract stem cells for repair. The whole issue is therefore still debated and more investigations are obviously required.

Another differentiation pathway on which attention has been recently paid is one leading to *pancreatic β-cells:* while this has been accurately investigated as far as the potential of embryonic stem cells is concerned, much less is known for a possible transdifferentiation from adult cells: in the former case, the process of differentiation in embryonic cell cultures can be targeted by transfecting stem cells with expression cassettes for a cell type specific promoter and thus selecting one cell lineage only ('gating technology'). This has been brilliantly achieved to obtain insulin-secreting cells (Roche et al. 2003).

The possibility of transdifferentiation from adult BM cells to pancreatic β-cells is however a subject of intense debate and it has been reported that after BM transplantation in a mouse model of diabetes mellitus regeneration of pancreatic β-cells can occur, perhaps via an intermediate vascular endothelial cell (Hess et al. 2003). However, a very recent study could not support such hypothesis, as mice transplanted with sex-mismatched BM showed a good chimerism in the peripheral blood, and also a contribution of transplanted cells to the interstitial cells of the pancreas, but no significant contribution to pancreatic β-cell renewal (Lechner et al. 2004).

Another interesting process of transdifferentiation, recently described, concerns new bone formation from adipose-derived adult stromal (ADAS) cells: by implantation of these cells in mice with critical-size skeletal defects it has been possible to induce bone formation and bone bridges without any genetic manipulation or addition of specific growth factors (Cowan et al. 2004). These results highlight the remarkable potential of the ADAS cell compartment.

More evidence seems to be available for a possible derivation of *hepatic cells* from haemopoietic progenitors: this is suggested by the presence of

haemopoietic markers like Thy-1 and CD34 in hepatic oval cells after transplantation, as well as by the demonstration of the Y chromosome in gender mismatched chimeric organs (Petersen et al. 1999; Lagasse et al. 2000). However doubts have been raised recently when BM stem cells were injected into mice with damaged livers: a substantial improvement of liver morphology was obtained, but the analysis of restored livers revealed the presence in hepatocytes of both donor and recipient genes, with double or even triple DNA content: such results obviously pointed to a process of cell fusion, and not to the production of new liver cell production (Grompe et al. 2003). Research is anyway presently carried out to understand which signalling pathway is involved in the regulation of hepatic engraftment from marrow cells: it seems that more than one mechanism may be operating, including a c-kit/SCF pathway (Theise 2003).

More types of stem cells from various tissues are currently under study to clarify the modality of self-renewal, differentiation and possible reprogramming. The issue of adult cell regression to undifferentiated state and its implications are discussed in the following section.

Induction of de- and trans-differentiation

A wide-ranging investigation is presently going on to explore the possible induction of transdifferentiation in adult cells, in order to circumvent the technical and ethical problems connected with the creation of purpose-built human embryos. This has been pursued in many ways, but always with the aim to induce adult cells to revert to a more undifferentiated stage and/or acquire some characteristics of primitive (and multipotent) stem cells.

One simple approach has been to fuse mammalian adult cells with ES cells in order to exploit the programming factors present in ES cells and try to apply them to adult cells: this has been actually done fusing thymocytes with ES cells, obtaining in these hybrid cells the reactivation of a Oct-GFP transgene, normally repressed in thymocytes; moreover such hybrid cells showed pluripotency *in vivo* (Tada et al. 2001). The stumbling block here is the problem of getting rid of the nucleus of the adult cell (much harder to remove than the egg's counterpart) in order to avoid a mixture of genetic material from both fused cells.

Another simple approach has been the use of ES cell extracts, which must be injected into the adult cell through a hole in the outer cell membrane, to be subsequently sealed: some ES cell features have actually been observed in treated cells but that does not give any firm indication of a true regression to an undifferentiated state (Hakelien et al. 2002).

Another factor which seems involved in cell reprogramming has been identified, following observations on transplantation in interphase eggs: a protein complex from Xenopus germ cells can disassemble the nucleolus of somatic cells, inducing nuclear restructuring and changes in cell differentiation (Gonda et al. 2003).

A more elaborate approach has been to try to identify genes, which are involved in the process of regeneration of mammalian tissues, for instance muscles. The properties of the Msx gene, involved in the formation of mouse myotubes, have been studied and it was found that muscle cells respond to the switch-off of such gene, by reverting gradually to more primitive stages, breaking off from the myotubes, starting to divide again and, when grown in presence of appropriate growth factors, evolving in other directions, like fat and bone (Odelberg et al. 2000). In this context attempts have been made to isolate from newt myotubes a protein responsible for de-differentiation of mammalian myotubes: it is obvious that the finding of a single compound capable to induce such regression would be much easier to work with than a gene activation or switch-off mechanism.

An important step in this direction seemed the demonstration of inducible myotube de-differentiation by extracts derived from newt regenerating limbs: a reduction of muscle differentiation proteins to very low levels was found as well as an extensive cleavage of treated myotubes, with proliferation of mononuclear cells (Mc Gann et al. 2001).

Following this, a screening of a myriad of compounds was carried on to find how to induce true de-differentiation: eventually a disubstituted purine analogue was identified as capable to induce myogenic committed cells to become pluripotent mesenchymal progenitors, which in turn may proliferate and differentiate into bone, cartilage and fat cells: such molecule has been named 'reversine' (Chen et al. 2004).

Many indications seem therefore to point out an increasing availability of either natural products or

synthetic compounds, which may selectively induce differentiation towards different pathways, following a pattern which was already identified for compounds like 5-azacytidine and *all-trans* retinoic acid: it is foreseen in a recent review that small molecules of the 'reversine' type may eventually contribute to effective treatment for tissue repair and regeneration (Ding and Schultz 2004).

Concluding remarks

Scientific progress has always been accompanied by strong debate, but the discussion about the derivation, properties and possible use of stem cells is at present reaching very high intensity at all levels, including ethical and political issues. Two main fields are the subject of debate: one is the derivation and use of embryonic stem cells, the other is the potential for use and manipulation of somatic cells (we prefer this term to 'adult cells', which is misleading). The difficulty in dealing with embryo stem cells is exemplified by the huge variety of legislation in different countries in the world: thus we have 1) Countries where no human embryo research is permitted (France, Germany, Austria and Poland), 2) Countries which allow research on already-derived stem cells, but not their derivation, 3) Countries where both derivation and use of stem cells from spare embryos are permitted, like Canada, Japan, Spain and Australia, and finally 4) Countries where use of stem cells created for research is permitted, as in United Kingdom, China and private Institutions in the US. Moreover, partial concessions are often accompanied by stringent recommendations, like in the Report by the UK Expert Advisory Group (2001), including a definite ban on the implantation of an embryo created by nuclear replacement into the uterus of a woman ('reproductive cloning').

It should be pointed out, anyway, that for pure research purposes mouse embryonic stem cells (MESC) are much easier to work with than human embryonic stem cells (HESC): in the first place, MESC grow much more rapidly in culture, can be derived from genetically manipulated background and are obviously more suitable for *in vivo* assays, while HESC are difficult to obtain, are slow to grow and the molecular mechanisms involved in their self-renewal are still unknown. For all these problems it has been suggested in the US to create a HESC repository, which would collect all human stem cell lines, test them for incorporation into host tissues, and make them available to academic institutions (Brivanlou et al. 2003).

The second great challenge is represented by the attempts of the so called *de-differentiation*, namely, as above mentioned, the induction of primitive features into already differentiated cells, in order to obtain the plasticity of a pluripotent stem cell. In this direction some results have been obtained, as shown by the reappearance of a stem cell marker, the *Oct-4* gene, in mammalian somatic cells (Byrne et al. 2003). However it has been demonstrated that trans-differentiation occurs rarely, if at all, in many organ systems, while cell fusion appears the reason for some apparent success. Perhaps one avenue worth exploring is the use of foetal cells, which, although somatic in character, retain a considerable potential for development. In this context it is interesting to note that foetal heart cells have been designed as good tools for cell therapy: they graft easily, adopt the features of an adult cardiac cell type without fusion and are electrically coupled (Chen 2004). Promising results have also been obtained with foetal brain stem cells, which are able to differentiate into mature neural cells (Vescovi, unpublished data, 2004).

In summary, the field of stem cell biology is characterized by a steady flux of new information and it is reasonable to predict that such advance in our knowledge will promote a remarkable progress into the fascinating and rewarding area of cell therapy.

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