STEM CELLS

CÉLULAS-TRONCO

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ABSTRACT

Stem cells can be classified as embryonic stem (ES) cells or adult stem cells considering their origin. If plasticity is considered, stem cells can be classified as totipotent, when stem cells retain the ability to give rise to an entire new organism. When stem cells lose this capacity, cells are named pluripotent stem cells, which can give rise to almost all mature cell types that compound an organism. Totipotent and pluripotent stem cells can be obtained from developing early-stage embryos. Multipotent is the group of adult stem cells with restricted plasticity. These cells can differentiate into a defined cell type related with a specific organ or tissue. ES cells can be propagated *in vitro* under undifferentiated system or with a series of protocols to induce cell differentiation. On the other hand, multipotent adult stem cells cannot be maintained *in vitro* in an undifferentiated form, except for a special class of adherent adult stem cells named mesenchimal stem cells, which can be expanded *in vitro* conserving their undifferentiated characteristics. Considering the ability to generate teratomas, ES cells were not used in experimental *in vivo* cell transplant. On the other hand, several experimental adult stem cells transplants have been performed with controversial results.

Keywords: Stem cell, embryonic stem cell, adult stem cell, cell differentiation, cell therapy.

RESUMO

Considerando a origem de obtenção, as células-tronco podem ser classificadas como células-tronco embrionárias (ES) ou como células-tronco adultas. Mas, se a plasticidade for considerada, as células-tronco podem ser classificadas como células totipotentes, quando as células-tronco preservam a capacidade de dar origem a um novo indivíduo completo. Quando as células-tronco perdem esta capacidade, passam a ser classificadas como células-tronco pluripotentes, que podem dar origem a praticamente todos os tipos celulares maduros que compõem um organismo. Células-tronco totipotentes e pluripotentes podem ser obtidas de estágios embrionários iniciais.

O grupo de células-tronco que apresenta plasticidade restrita é denominado de multipotente. Estas células podem se diferenciar em determinado tipo celular comprometido com um órgão ou tecido específico. Células ES podem ser expandidas *in vitro*, mantendo sua forma indiferenciada, ou podem ser submetidas a uma série de protocolos, que irão induzir diferenciação *in vitro*. Por outro lado, as células-tronco adultas multipotentes não podem ser mantidas *in vitro* na forma indiferenciada, exceto uma subpopulação de células-tronco adultas aderentes, denominadas células-tronco mesenquimais, que podem ser mantidas *in vitro* na forma indiferenciada. Considerando a capacidade de gerar teratomas, as células ES não foram utilizadas para transplante celular experimental *in vivo*. Além disso, várias cirurgias de transplantes experimentais com células-tronco adultas têm sido realizadas, porém apresentando resultados controversos.

Unitermos: Célula-tronco, célula-tronco embriônica, célula-tronco adulta, diferenciação celular, terapia celular.

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STEM CELLS CLASSIFICATION

As mentioned by Boheler et al. (1) or reviewed by Wobus & Boehler (2), stem cells are those cells that self-renew and retain the capacity for either embryonic development or tissue regeneration. Stem cells can be classified using different characteristics. If *origin* is considered, stem cells are classified as embryonic or adult stem cells. Embryonic stem (ES) cells can be isolated by early-stage embryo. Stem cells obtained from adult tissues are named adult stem cells.

On the other hand, if *plasticity* is considered, stem cells can be classified as *totipotent*, *pluripotent* or *multipotent* stem cells. Undifferentiated cells with the capacity to generate an entire organism can be encountered exclusively on early stages of fertilized eggs or considering the developing embryo from zygote to 8-cell stage embryos (Figure 1). These undifferentiated cells can give rise to all required embryonic and extraembryonic tissues to generate a new organism and this is the reason to be classified as *totipotent*. Cells obtained from the inner cell mass (ICM) of pre-implantation blastocyst are no longer totipotent. Loss of totipotency is therefore related to the developmental fate of the organism. ES cells obtained from the ICM of blastocyst lose the ability to generate an entire new organism because they lose the capacity to generate extraembryonic tissues. However, these *pluripotent* cells retain the ability, in defined conditions, to generate all cell types derived from the three germ layers: ectoderm (epidermal tissues and nerves), mesoderm (muscle, bone and blood) and endoderm (liver, pancreas, gastrointestinal tract, lungs), including fetal and adult cells. Although these cells alone do not develop into a viable fetus or adult animal, they retain an undifferentiated form and the capability to originate almost all different cell types which compose an entire organism. Lastly, cells with reduced plasticity, with the ability to generate some differ-

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ent cell types in special conditions are considered *multipotent*.

These two different systems of stem cell classification can be overlapped and it will be possible to classify stem cells, for example, as pluripotent ES cells obtained from a mouse blastocyst, or pluripotent stem cells obtained from an adult undifferentiated carcinoma, an adult or differentiated tissue. Considering that issue, we will focus our discussion on pluripotent stem cells obtained from blastocysts and multipotent adult stem cells obtained from different adult tissues as described below.

PLURIPOTENT STEM CELLS

In 1975, Martin & Evans (3) obtained the first pluripotent cell line from murine teratocarcinomas. These cells are called embryonic carcinoma cells because they present similar characteristics to cells obtained from early-stage embryos.

Another group of pluripotent adult cells was isolated from spontaneous testis teratocarcinomas. These cells were exhaustively studied by Tam & Zhou (4), who encountered cells with the same characteristics on mouse epiblast embryos. Then this group of pluripotent cells was named as primordial germinative cells.

Later, pluripotent ES cells were isolated from the ICM of blastocyst-stage preimplantation mouse embryos (Figure 1) by Evans & Kaufman in 1981 (5) and independently by Martin (6). The plasticity of pluripotent ES cells should be addressed when teratocarcinomas are formed after the injection of undifferentiated ES cells on recipient animals (7), but not viable embryos. Furthermore, when ES cells are transferred to a developing embryo, they can be reintegrated into ICM and give rise to all mature cell types, including germinative cells (8). It is important to note that this phenomenon can occur also when ES cells are genetically manipulated, for example. In this vein, a series of knockout animal models were established using this methodology. In 1998, Thomson et al. (9) were able to isolate ES cells from *in vitro* fertilized human embryos (blastocysts). Furthermore, Wobus (10) described ES cells as a group of stem cells with an unlimited capability of propagation *in vitro* under undifferentiated forms, besides the ability to differentiate into differentiated somatic cells. In addition, ES cells can also be characterized by high alkaline phosphatase activity; expression of embryonic specific markers as SSEA-1; small cytoplasm; high telomerase activity, self-division modulated by interleucin-6 (IL-6) family (7,11,12).

Several other pluripotent ES cell lines have been established from rodents (13,14), rabbits (15), pigs (16), primates (17) and a considerable number of human cell lines has been established (18-22). These cell lines have been obtained applying almost the same protocol, using blastocysts to isolate ES cells from the ICM. The huge plasticity of pluripotent ES cells was demonstrated *in vivo* and *in vitro*. When injected into blastocysts or under the kidney capsule, these cell lines produce cells from all three germ layers representing endoderm, ectoderm and mesoderm. Otherwise, pluripotent characteristic or plasticity of ES cells can also be addressed *in vitro.* Comparing the three different pluripotent stem cells, ES cells obtained from preimplantation blastocyst present the best way to perform *in vitro* differentiation experiments, considering ES cells ability to propagate and maintain their plasticity in culture systems (7).

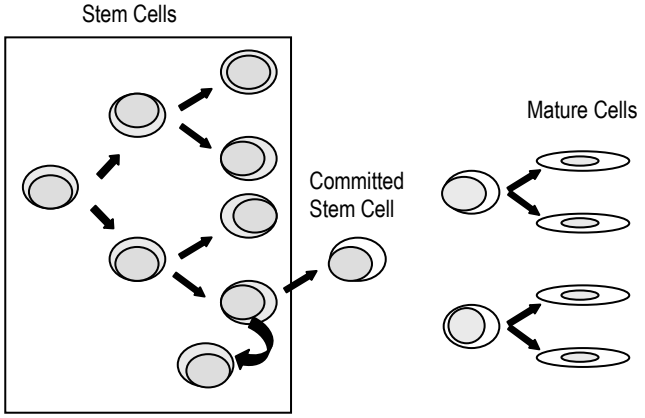

Figure 1 – Early Embryo Development
Spermatozoid fusion with an oocyte produces a unique cell embryo named zygote. With adequate conditions, *in vivo* or *in vitro* zygote divides and produce a 2-cell embryo. In a subsequent stage, both split and give rise to 4-cell embryo. Subsequently, 8-, 16-, 32-cell will be generated by blastomers divisions. Morula is the stage where embryo is formed by a non-measured compact cell mass. The next step will form the pre-implantational blastocyst. Totipotent embryonic stem (ES) cells can be obtained by collecting blastomers from 2- to 8-cell embryos. Totipotent ES cells can give rise to an entire organism. Pluripotent stem cell lines can be established collecting cells from the inner cell mass of a blastocyst. Pluripotent stem cells have no ability to generate extra-embryonary structures, considering that it cannot form a new organism. However, pluripotent stem cells can generate all cell types from the three germ layers: ectoderm, mesoderm, endoderm.

IN VITRO PLURIPOTENT ES CELLS

ES cells can be expanded *in vitro* in two different conditions. Applying specific cell culture conditions it is possible to avoid the natural characteristic of ES cells to divide and differentiate. Co-cultivating ES cells with inactivated embryonic fibroblast cell cultures which secrets inhibitory factors combined with a specific cell culture media supplemented with leukemia inhibitor factor (LIF) (23,24), ES cells can be infinitively propagated in an undifferentiated state. IL-6 and oncostatin have been mentioned as factors that can negatively interfere with ES cell differentiation. Thus, ES cells can be propagated *in vitro* maintaining their undifferentiated state. Alternatively, ES cells could be submitted to different cell culture protocols in order to guide ES cells *in vitro* differentiation. As reviewed by Wobus & Boheler in 2005 (2), three different protocols were described to induce *in vitro* formation of ES cell aggregates named *embryoid bodies* (EB). EB can be cultivated in a series of conditions to produce different mature cell types in culture. In order to obtain EB, Wiles & Keller (25) described a method by cultivating ES cells with methyl cellulose. Or applying the method named *mass culture* ES cells were cultivated under high density and in the presence of specific factors EB can also be obtained (26). Our group and most research groups adopted the *hanging drop technique* established by Wobus et al. (27). With this technique, a number of undifferentiated ES cells were cultivated in a hanging drop to achieve through the formation of cell aggregates the three dimensional structure called EB. EB were maintained under specific conditions to differentiate into different cell types. The kinetic of *in vitro* EB development is similar to *in vivo* embryo development. Therefore, at the beginning of culture it is possible to observe mesoderm and ectoderm precursor cells, while endoderm-derived cells can be detected at least 10 days after EB culture.

It is important to note that today only mixed cell type cultures should be obtained from ES cell cultures. Different protocols have been described in order to enrich a mix cell culture in a specific cell type. However, no protocol can guide ES cells *in vitro* differentiation to a unique specific cell type. Clusters of cardiomyocytes, neurons, smooth muscle cells, skeletal muscle cells and epithelial cells represent the majority of mature cells obtained in ES cell cultures after differentiation induce protocol.

MULTIPOTENT ADULT STEM CELLS

Initially, multipotent adult stem cells were known as stem cells committed with a specific tissue or organ conferring a limited plasticity to them. Later, studies demonstrated that adult stem cells have a wide plasticity, but are not completely understood (28-30).

Nowadays, adult stem cells can be classified as multipotent, considering their reduced plasticity. Adult stem cells are cells obtained from adult or differentiated tissues. The main category of adult stem cells is the hematopoietic stem cells (HSC) encountered in the bone marrow (BM). HSC is currently the best characterized multipotent adult or somatic stem cell population and was prospectively isolated from mouse BM about 15 years ago (31). Interestingly, there are approximately 5x 10^{11} cells on BM but only 1% of these cells are progenitor cells involved with hematopoietic system and 0.001% are stem cells (32). On 50th of last century, HSC were known, but could not be isolated considering that no specific markers were described to this cell type neither monoclonal antibodies technology was available. However, in cell culture systems where a cell suspension obtained from BM was cultivated in an *in vitro* system called "limit dilution", it was possible to have evidence of the existence and the potentiality of HSC. In this system, a preparation of BM cell suspension was plated with a density of one cell per well in a 96 well plate. In this way, only one cell per well was cultivated in a defined condition and it develops and gives rise to a colony composed by different blood cell types. This experiment demonstrated that only one cell could give rise to a series of different mature blood cells, such as lymphocyte cell line or granulocyte-macrophage cells, for example. In this vein, it was possible to detect all mature blood cells in other wells. This clear-cut procedure demonstrated that a mix of stem cells reside at the BM. The most undifferentiated one was called HSC and these cells give rise to all mature blood cell lines. In addition, other committed stem cells were detected. For example, it was possible to show the existence of a stem cell committed specifically with granulocyte-macrophage (GM) cell lines and other stem cells committed with lymphocyte cell lines. These data are collected in a "pyramid"-like cell differentiation system proposed to the hematopoietic cells, where the top of the pyramid is occupied by the HSC, which is the most undifferentiated cell from blood cell lineages. Downstream, other stages of more differentiated stem cells from HSC were detected. Later the blood cell-lineage-committed stem cells and the different stages of adult or differentiated blood cell lines were encountered, as pro-B, pre-B and B lymphocytes, for example. At pyramid base resides mature blood cell lines.

Recently, a new cell division mechanism was demonstrated to adult stem cells *in vitro* by Sherley (33). Stem cells apparently divide into a special process named *asymmetric division,* represented in Figure 2. During the cell division process, the mother cell divides and produces two daughter cells. One of the daughter cells is equal to the undifferentiated mother stem cell and the other daughter cell is different from the mother cell. This other cell undergoes a cell differentiation process and will be committed with a group of lineage cells. In this vein, stem cell replication occasionally produces two different daughter cells, an undifferentiated one equal to the mother cell, and a second one different from the mother-cell which starts to differentiate and gradually loses its stem cell potentiality, because it becomes specialized to perform functions related to a specific mature cell type. This hypothesis tries to explain the self-renewal of stem cells in adult tissues or adult or-

ganism, besides the stem cell ability to differentiate and give rise to different cell types.

Another multipotent stem cell type has been isolated from BM, submitting BM cell suspension to an adherent cell culture system. *In vivo* and *in vitro*, most hematopoietic cells encountered on BM cell suspension were not adherent cells, except monocyte-macrophage cells. When BM cell suspension was submitted to adherent cell culture systems, most cells became on the supernatant phase and macrophage, fibroblast, endothelial cells and other adherent cell types became attached to the culture flask. The hematopoietic non-adherent cells were discarded with the cell culture medium some days latter. A mix of adherent cell types was obtained with this system. One to 2 weeks latter, the majority of differentiated cells, such as macrophage, fibroblast, endothelial cells and other mature cell types died. Some undifferentiated fibroblast-like adherent cells persist for several weeks in this system and these are named mesenchimal stem cells (MSC). MSC is a multipotent adherent stem cell obtained from BM aspirates and was firstly obtained from mouse BM (30,34-36). Only recently specific cell markers for the prospective isolation were described (37).

In parallel, other adult stem cell populations have been described in differentiated tissues. Furthermore, BM adult stem cells have been described on umbilical cord, blood, brain, spinal cord, skeletal muscle, epidermal tissues, and pancreas (7). Stem cells encountered on umbilical cord presents similar characteristics to BM stem cells, but with higher plasticity (38,39). These other adult stem cells were known to be committed with tissue-specific repair. Despite the lack or controversial identification of cell markers in this group of adult committed stem cell populations, the specific mechanism of action of these cells is also unclear. Tissue- or organ-specific committed stem cells have been described as tissue-resident stem cells from peripheral nervous system (40,41); and from central nervous system (42-44). Furthermore, there is evidence of the existence of other organ-specific committed stem cells encountered on skeletal muscle (45), or epidermal stem cells located on the boundary of hair follicle (46); on gut, where committed stem cells reside near the bottom of the intestinal crypts (47,48). Yet, muscle-committed stem cells have been described (49-52) and brain-resident stem cells too (53,54). Moreover, other groups demonstrated evidence of the presence of liver stem cells (55,56) and pancreas-resident stem cells were described by Bonner-Weir & Sharma (57) and by Murtaugh & Melton in 2003 (58). In fact, the controversial existence and whether tissue regeneration is mediated by adult stem cells or not, must be elucidated. Forbes et al. (55) proposed that different tissue regenerative populations may function in response to tissue injury as opposed to tissue homeostasis. As reviewed by Wagers & Weissman (59), adult stem cell populations that appear to normally give rise to tissues of multiple germ layers have recently been found by culture methods from the skin (60), BM (61,62), muscle (62-64) or brain (62). Complementary experiments will be necessary to understand tissue regeneration in parallel with adult stem cell plasticity.

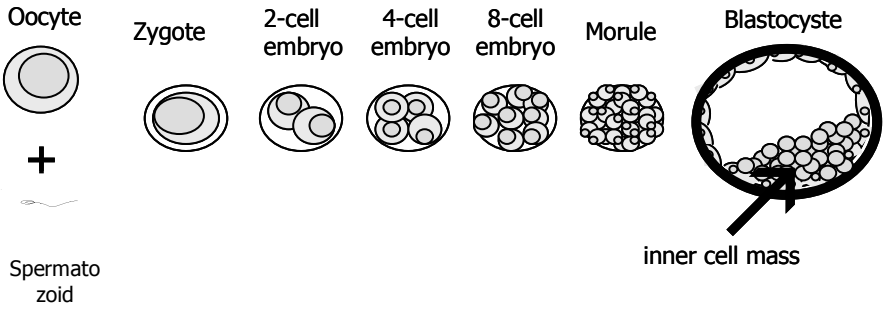

Figure 2 – Asymmetric Division
Adult stem cells present a special self-renewal process named asymmetric division *in vitro* observed. Frequently a single stem cell divides and generates two daughters-cells equal to the undifferentiated mother cell. Occasionally, stem cells perform a special division process, where mother cell produces two distinct cells. One cell will be equal to the undifferentiated mother cell. The second daughter cell will differentiate and compromise with a specific cell type differentiation process. In asymmetric division, stem cells divide and generate new undifferentiated cells for self-renewal. In parallel new differentiated committed stem cells will be generated to give rise to new cells of different tissues and organs.

IN VITRO ADULT STEM CELL

Differently from ES cells, there is no established protocol to expand undifferentiated adult stem cells *in vitro*, except to the MSC. When BM aspirate, for example, is submitted to special cell culture system, stem cells propagate, but give rise to differentiated cells. In fact, adult stem cells (again, except MSC) cannot be cultivated to propagate undifferentiated stem cells. However, it is acceptable that researchers have not understood exactly in

which *in vitro* systems adult stem cells must be cultivated to be propagated as undifferentiated stem cells. It means that nowadays, only the conditions to propagate MSC were described. MSC can be obtained from BM too. To isolate MSC, BM aspirate must be centrifuged in a Ficoll gradient to isolate peripheral blood mononuclear cells (PBMC). PBMC fraction must be cultivated in an adherent system. After 2 days, the non-adherent cells are discarded with the medium change. A mixture of adherent cells becomes attached to the culture flask and it is possible to encounter fibroblasts, endothelial cells, macrophages, nerve cells and MSC. In appropriate conditions, MSC will divide maintaining the undifferentiated state for long periods of culture. In the meantime, mature cells die and detach from culture flask. Then, in subsequently media changing, cultures are enriched in MSC almost to the totality. In this vein, considering adult stem cells, only MSC can be propagated *in vitro* under the undifferentiated state. Consequently, several analyses have been performed with this group of adult stem cells to characterize it. Thus, Wagner & Ho (65) define by flow cytometry a panel of surface markers that must be expressed on MSC membrane and at the same time there is another group of cell markers that could not be present on cell surface of MSC. Considering this panel, MSC must be CD105+, CD73+, CD90+ and CD45-, CD34-and CD11b/c. Despite the mentioned results, the existence of MSC is controversial.

STEM CELL THERAPY AND PERSPECTIVES

Considering the high plasticity of ES cells that can give rise to all cell types of an adult organism, undifferentiated ES cells cannot be used for cell transplant to an individual, because they will obligatorily induce formation of teratomas. As mentioned before, at the moment, there is no defined protocol to induce *in vitro* ES cell differentiation to obtain a pure cell type culture of cardiomyocytes cells or other. It is possible to enrich ES culture system in a specific cell type, but no guarantees can be offered to produce *in vitro* mature cells for *in vivo* cell transfer without a contamination of undifferentiated ES cells and/or other mature cell types. This technical problem avoids application of ES cell for cell transplant. However, it is important to note that ES cell culture systems is the best *in vitro* system to study cell differentiation, and the development of research projects on *in vitro* ES cells are extremely important to help us understand several cell differentiation processes. In the future, with the acquisition of knowledge to manage ES cell *in vitro* differentiation, this option will certainly be available as a new alternative for cell or organ transplantation.

On the other hand, transplant with adult stem cells has been a real practical alternative for human medicine and others since BM transplant has been performed on 50th of last century. In fact, the reconstitution of hematopoietic system in patients with hematopoietic neoplasias was performed by BM aspirates obtained from healthy donors. Since the beginning, the exact mechanism of hematopoietic recipient system recovery is not well understood. However, with today's technical development, it is possible to imagine that HSC and other committed stem cells encountered on BM donors can help reconstitute the recipient hematopoietic system. Based on this procedure, many experimental therapeutic alternatives have been performed to treat a series of distinct diseases, as reviewed by Krause in 2002 (66) and by Kraitchman et al., 2008 (67), for example. Successful experiments with BM transplant to hematopoietic neoplasia patients, as well as the difficulties related to ES cell transplantation, inspire several groups to look for benefits conferred by unprocessed BM suspension cells or *in vitro* processed BM derived cells to almost all pathologies. The success of this new therapeutic alternative can be observed with the high number of scientific articles reporting results obtained in 2008 using this approach to repair cartilage defects (68), heart failure (69,70), osteoarthritis (71) or neurological diseases (72). Nowadays, many groups around the world are trying to establish this therapeutic alternative to their patients, but little is known concerning the mechanism of action and the exact benefits for tissue injuries with stem cell transplantation. Most projects today are focused on bioengineering, concerning this topic to define the mechanism of action of *in vivo* stem cell and the reconstitution of tissue lesions.

Despite these positive results, many groups detected no influence of stem cell transplant on tissue repair (73). In this vein, there were almost no detectable cells expressing Y chromosome in different organs of woman recipients of male HSC. On the other hand, it was possible to observe completely hepatic recovery in animals submitted to mouse experimental induced hepatic failure followed by HSC transplant. In addition to hepatic regeneration, stem cell donor markers were detected on recipient mature hepatocytes. These results corroborate the idea of *transdifferentiation*, in which a stem cell differentiates to a specific cell type in response to microenvironmental signals and factors produced in the local injury. Later, other experiments demonstrated that specific hepatocytes expressed at the same time recipient and donor cell markers. These results suggested that donor HSC fuses with a recipient cell (such as a macrophage, for example). The same fusion phenomenon was observed with other tissues, such as cardiomyocytes or neurons (74). Nonetheless, other results demonstrated that these cells, which have donor and recipient markers simultaneously, expressed a normal number of chromosome suggesting that no fusion occurred. One more time, there is no consensus concerning the mechanism of interaction between transferred stem cells and recipient tissues. It is important to note that no negative or deleterious results were described. It is a fact that stem cells represent a new therapeutic alternative and it is important to continue investigating to establish an adequate treatment protocol for each pathology. Finally, election of three researchers investigating stem cell technology to receive the Nobel Prize of Medicine and Physiology in 2007 is the best demonstration of the real impact of stem cells around the world.

REFERENCES

1. Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ Res. 2002;91:189-201.
2. Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev. 2005;85:635-78.
3. Martin GR, Evans MJ. Differentiation of clonal lines of teratocarcinoma cells:formation of embryoid bodies *in vitro*. Proc Natl Acad Sci U S A. 1975;72:1441-5.
4. Tam PP, Zhou SX. The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. Dev Biol. 1996;178:124-32.
5. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154-6.
6. Martin, GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981;78:7634-8.
7. Prelle K, Vassiliev IM, Vassilieva SG, Wolf E, Wobus AM. Establishment of pluripotent cell lines from vertebrate species-present status and future prospects. Cell Tissues Organ. 1999;165:220-36.
8. Bradley, A, Evans M, Kaufman MH, Robertson E. Formation of germ line chimeras from embryo-derived teratocarcinoma cell lines. Nature. 1984;309:255-6.
9. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from humas blastocysts. Science. 1998;282:1145-7.
10. Wobus AM. Potential of embryonic stem cells. Mol Aspects Med. 2001;22:149-64.
11. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. 1998;12:2048-60.
12. Vassilieva S, Guan K, Pich U, Wobus AM. Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth. Exp Cell Res. 2000;258:361-73.
13. Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol. 1985;87:27-45.
14. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early passage embryonic stem cells. Proc Natl Acad Sci U S A. 1993;90:8424-8.
15. Graves KH, Moreadith RW. Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. Mol Reprod Dev. 1993;36:424-33.
16. Ropeter-Scharfenstein M, Neubert N, Prelle K, Holtz W. Identification, isolation, and culture of pluripotent cells from the porcine inner cell mass. J Anim Breed Genet. 1996;113:427-36.
17. Thomson JA, Kalishman J, Golos TG, et al. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A. 1995;92:7844-8.
18. Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol. 2000;227:27127-8.
19. Reubinoff BE, Pera MF, Fong CY, Trounson, Bongso A. Embryonic stem cell lines from human blastocysts:somatic differentiation *in vitro*. Nat Biotechnol. 2000;18:399-404.
20. Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol. 2002;20:933-6.
21. Hovatta O, Mikkola M, Gertow K, et al. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod. 2003;18:1404-9.
22. Mitalipova M, Calhoun J, Shin S, et al. Human embryonic stem cell lines derived from discarded embryos. Stem Cells. 2003;21:521-6.
23. Williams RL, Hilton DJ, Pease S, et al. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature. 1988;336:684-7.
24. Smith AG, Heath JK, Donaldson DD, et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature. 1988;336:688-90.
25. Wiles MV, Keller G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development. 1991;111:259-67.
26. Keller GM. In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol. 1995;7:862-9.
27. Wobus AM, Wallukat G, Hescheler J. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca2+ channel blockers. Differentiation. 1991;48:173-82.
28. Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? Cell. 2001;105:829-41.
29. Morrison SJ, White PM, Zock C, Anderson DJ. Prospective identification, isolation by flow cytometry and in vivo self-renewal of multipotent mammalian neural crest stem cells. Cell. 1999;96:737-49.
30. Prockop DJ, Gregory CA, Spees JL. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proc Natl Acad Sci U S A. 2003;100:11917-23.
31. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241:58-62.
32. Gage FH. Cell therapy. Nature. 1998;392:18-24.
33. Sherley JL. Asymmetric cell kinetics genes: the key to expansion of adult stem cells in culture. Stem Cell. 2002;20:561-72.

34. Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol. 1976;4:267-74.

35. Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet. 1987;20:263-72.

36. Pereira RF, Halford KW, O'Hara MD, et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage and lung in irradiated mice. Proc Natl Acad Sci U S A. 1995;92:4857-61.

37. Gronthos S, Zannettino AC, Hay SJ, et al. Molecular and cellular characterization of highly purified stromal stem cells derived from human bone marrow. J Cell Sci. 2003;116:1827-35.

38. Kogler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med. 2004;200:123-35.

39. Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cell. 2005;23:220-9.

40. Stemple DL, Anderson DJ. Lineage diversification of the neural crest: in vitro investigations. Dev Biol. 1993;159:12-23.

41. Kruger GM, Mosher JT, Bixby S, Joseph N, Iwashita T, Morrison SJ. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential and factor responsiveness. Neuron. 2002;35:657-69.

42. Uchida N, Buck DW, He D, et al. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A. 2000;97:14720-5.

43. Rietze RL, Valcanis H, Brooker GF, Thomas T, Voss AK, Bartlett PF. Purification of a pluripotent neural stem cell from the adult mouse brain. Nature. 2001;412:736-9.

44. Capela A, Temple S. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron. 2002;35:865-75.

45. Mauro A. Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol. 1961;9:493-5.

46. Alonso L, Fuchs E. Stem cells of the skin epithelium. Proc Natl Acad Sci U S A. 2003;100:11830-5.

47. Bjerknes M, Cheng H. Clonal analysis of mouse intestinal epithelial progenitors. Gastroenterology. 1999;116:7-14.

48. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. Nature 2001;414:98-104.

49. Bel A, Messas E, Agbulut O, et al. Transplantation of autologous fresh bone marrow into infarcted myocardium: a word of caution. Circulation. 2003;108:11247-52.

50. Jankowski RJ, Deasy BM, Huard J. Muscle-derived stem cells. Gene Ther. 2002;9:642-7.

51. Parker MH, Seale P, Rudnicki MA. Looking back to the embryo: defining transcriptional networks in adult myogenesis. Nat Rev Genet. 2003;4:497-507.

52. Polesskaya A, Seale P, Rudnicki MA. Wnt signaling induces the myogenesis specification of resident CD45+ adult stem cells during muscle regeneration. Cell. 2003;113:841-52.

53. Chiasson BJ, Tropepe V, Morshead CM, van der Kooy D. Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cells characteristics. J Neurosci. 1999;19:4462-71.

54. Pevny L, Rao MS. The stem-cell menagerie. Trends Neurosci. 2003;26:351-9.

55. Forbes S, Vig P, Poulsom R, Thomas H, Alison M. Hepatic stem cells. J Pathol. 2002;197:510-8.

56. Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. The origin and liver repopulation capacity of murine oval cells. Proc Natl Acad Sci U S A. 2003;100:11881-8.

57. Bonner-Weir S, Sharma A. Pancreatic stem cells. J Path. 2002;197:519-26.

58. Murtaugh LC, Melton DA. Genes, signals and lineages in pancreas development. Annu Rev Cell Dev Biol. 2003;19:71-89.

59. Wagers AJ, Weissman IL. Plasticity of adult stem cells. Cell. 2004;116:639-48.

60. Toma JG, Akhavan M, Fernandes KJ, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol. 2001;3:778-84.

61. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature, 2002;418:41-9.

62. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle and brain. Exp Hematol. 2002;30:896-904.

63. Cao B, Zheng B, Jankowski RJ, et al. Muscle stem cells differentiate into hematopoietic lineages but retain myogenic potential. Nat Cell Biol. 2003;5:640-6.

64. Qu-Petersen Z, Deasy B, Jankowski R, et al. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol. 2002;157:851-64.

65. Wagner W, Ho AD. Mesenchymal stem cell preparations: comparing apples and oranges. Stem Cell Rev. 2007;3:239-48.

66. Krause DS. BM-derived stem cells for the treatment of non-hematopoietic diseases. Cytotherapy. 2002;4:503-6.

67. Kraitchman DL, Gilson WD, Lorenz CH. Stem cell therapy: MRI guidance and monitoring. J Magn Reson Imaging. 2008;27:299-310.

68. Saris DB, Vanlauwe J, Victor J, et al. Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. Am J Sports Med. 2008;36:235-46.

69. Mishra PK. Bone marrow-derived mesenchymal stem cells for treatment of heart failure: is it all paracrine actions and

immunomodulation? J Cardiovasc Med (Hagerstown). 2008;9:122-8.

70. Strauer BE, Brehm M, Schannwell CM. The therapeutic potential of stem cells in heart disease. Cell Prolif. 2008;41:126-45.

71. Black LL, Gaynor J, Gahring D, et al. Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. Vet Ther. 2008;8:272-84.

72. Hess DC, Borlongan CV. Stem cells and neurological diseases. Cell Prolif. 2008;41:94-114.

73. Camargo FD, Chambers SM, Goodell MA. Stem cell plasticity: from transdifferentiation to macrophage fusion. Cell Prolif. 2004;37:55-65.

74. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature. 2003;425:968-73.