

The Stem State: Mesenchymal Plasticity as a Paradigm

Dov Zipori*

Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, 76100, Israel

Abstract: The mesenchyme is a remarkably plastic tissue in the embryo. Recent studies have led to the discovery of mesenchymal cells in the adult organism that can differentiate *in vitro* into unexpected directions, beyond the well-known ability of the mesenchyme to give rise to mesodermal derivatives. These studies highlighted the plastic nature of the mesenchyme, also beyond the embryonic developmental stage. This review discusses the possible functions of the mesenchyme in the adult and the reason for the maintenance of plasticity throughout mammalian life. The properties of the mesenchymal cells clearly exemplify the stem state concept; cells, whether early or late in the differentiation cascade, may assume a stem state that entails high plasticity.

Keywords: Stem cells, stem state, mesenchyme, plasticity.

PLASTICITY OF THE EMBRYONIC MESENCHYME

The development of the mammalian embryo provides the ultimate example of the plastic nature of cellular phenotypes. Cells shift from an epithelial to a mesenchymal phenotype in a sequential manner. This process, called epithelial mesenchymal transition (EMT) also occurs in a reciprocal manner, thus, mesenchymal cells assume an epithelial phenotype, a process designated mesenchymal epithelial transition (MET). These extreme fluctuations in the nature of the cell entail changes in cell-to-cell adhesion, modification of the extracellular matrix and of cell motility, to point out just a few features that underlie these processes, based on marked changes in gene expression (reviewed in [1]). The notion that embryonic mesenchyme has a multipotent nature gained support from the isolation of C3HT101/2 cells. The latter is a stable cell line that originated from embryonic tissues. It is multipotent in that it gives rise to several types of mesodermal derivatives upon induction with agents such as 5-azacytidine [2] and differentiates more specifically upon induction with bone morphogenic protein-2 [3] or transforming growth factor 1 [4]. Therefore, this cell line is regarded as a mesenchymal stem cell (MSC). The C3HT101/2 cell line seems to have physiological counterparts since embryonic stem cells are found in maternal blood [5,6] and thus seem to be present in the embryo and to have migratory properties. A recent report suggests that MSCs are localized during development in the hemopoietic organs and spread from this locality to other sites in the adult [7]. The remarkable plasticity of embryonic mesenchyme was recently demonstrated by the isolation of primary cells from embryonic somatic explants. The cells were termed fetal somatic stem cells (FSSC) and were shown to integrate into embryonic tissues following injection into the blastocyst. The donor cells were found in the tongue, liver and muscle and some of them may have differentiated into lymphoid cells, however, no precise determination of the nature of the cells that arise from FSSC

was reported [8]. Thus, plastic behavior is not foreign to mammalian cells and is well exemplified by embryonic mesenchyme. This plastic property is assumed to be a trait specific to embryonic cells that is lost in the adult, although it may be regained upon tumorigenic transformation and acquisition of metastatic properties (reviewed in [1]). In the review below, I shall discuss some past data as well as recent observations that support the notion of plasticity of the mesenchyme in the adult organism. This plastic behavior is, as I recently argued, the hallmark of the stem state [9,10].

EARLY INDICATIONS FOR THE PLASTIC NATURE OF ADULT MESENCHYME: FRIEDENSTIEN'S BONE MARROW STROMA THAT POSSESSES THE CAPACITY TO RECREATE *IN VIVO* ECTOPIC HEMOPOIETIC MICROENVIRONMENT

Studies performed in the Soviet Union by AJ Friedenstein in the early 1970's have indicated that within the bone marrow reside cells capable of forming fibroblastoid colonies *in vitro* [11,12]. It was assumed, on grounds of histological analysis of the bone marrow that these cells are derived from the mesenchymal stroma that forms the supportive anlagen of blood-forming tissues. By the same token, similar mesenchymal elements were supposed to form the supportive anlagen of any tissue and organ. Are all tissue stromal cells the same or does each organ contain a different type of stroma that creates a specific microenvironment? To examine this issue Friedenstein attempted transplantation of the cultured bone marrow mesenchyme under the kidney capsule. The cultured fibroblasts induced the formation of a bony structure at the site of implantation, and within this bone structure hemopoiesis occurred [13] (Fig. 1). It was shown that the bone structure is of donor origin while hemopoietic cells encapsulated within the bone structure were of host origin, thus, the conclusion drawn by Friedenstein was that the cultured fibroblasts are osteogenic and, in addition, these cells carry the information necessary for the establishment of a hemopoietic inductive microenvironment (reviewed in [14]). Thus, these very early studies have already highlighted the width of activities of the bone marrow mesenchyme:

*Address correspondence to this author at the Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, 76100, Israel; Tel: 972-8-9342484; Fax: 972-8-9344125; E-mail: dov.zipori@weizmann.ac.il

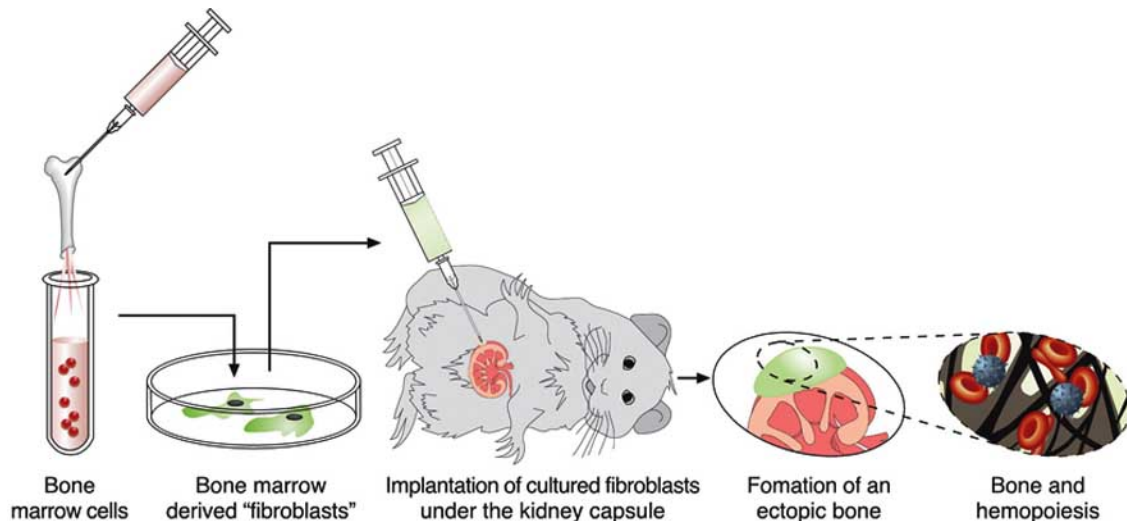


Fig. (1). Friedenstein's ectopic osteogenesis and hemopoiesis model: Bone marrow cells are isolated and seeded *in vitro* in simple medium. This results in elimination of most of the hemopoietic cells and bone marrow derived fibroblasts survive. These cells are implanted under the kidney capsule where they form a bony structure that is colonized by hemopoietic cells from the host.

osteoblastic capacity along with ability to dictate the differentiation of hemopoietic stem cells. As to the original question, whether the bone marrow stroma is specific to this organ; to date this question has remained unanswered since one can derive osteogenic mesenchyme that supports hemopoiesis from other tissues and organs. Whether the organ mesenchymal stroma *in situ* has specific properties that distinguish one organ stroma from the other, should await genomic and proteomic analysis of cells within their natural habitat, rather than the study of cells isolated and propagated *in vitro*.

DEXTER'S LONG-TERM CULTURES AND THE IDENTIFICATION OF VARIOUS PHENOTYPES OF STROMAL MESENCHYME

The *in vivo* observations of Friedenstein obtained strong support from the studies of TM Dexter and colleagues in Manchester during the late 1970's. This research group discovered the means to obtain long-term hemopoiesis *in vitro*, mimicking a bone marrow like situation under culture conditions [15-19]. Hemopoiesis has been studied *in vitro* for many years, and is still being studied extensively, using clonogenic assays in which the stem cells are diluted such that upon induction with a cytokine they proliferate to form an isolated colony. This allowed a powerful means to quantify processes of cell response to cytokines. However, this approach completely ignores the fact that within hemopoietic organs stem cells respond to cytokines, while in intimate contact among themselves, and also with the organ stroma. Dexter *et al*, showed that seeding of dense bone marrow cultures, under specific conditions, including selected horse sera, relatively low temperature and supplementation of hydrocortisone, lead to the formation of an adherent layer of mesenchymal stroma and associated hemopoiesis, that lasted for many months. In these long-term cultures, large numbers of hemopoietic progenitor cells were produced. One important aspect of these long-term cultures was the heterogeneity of stromal cell types observed

(Fig. 2). Myelopoiesis was associated with "giant fat cells" and macrophages were observed underneath flat "blanket cells", to mention just two examples. Thus, along with the heterogeneity of hemopoietic cell types, a multitude of mesenchymal "types" was observed. Although the studies of long-term bone marrow cultures did not discover the means to segregate between mesenchymal cell types, they did highlight the possibility that such heterogeneity that may have functional significance, does exist.

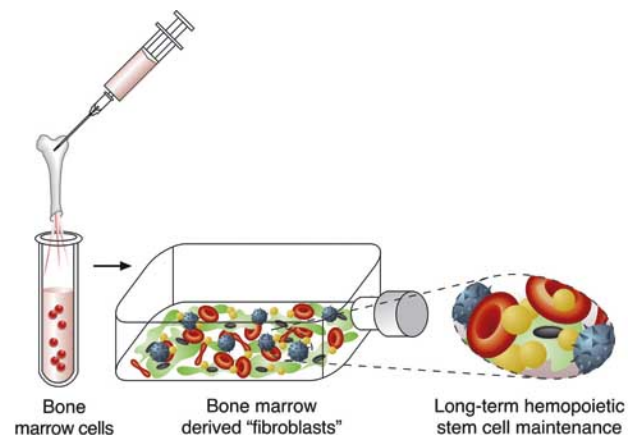


Fig. (2). Dexter's long-term bone marrow cultures: Bone marrow cells cultured at high densities form an *in vitro* simulation of a hemopoietic tissue in which stem cells are maintained and differentiate for months. This process is highly dependent on the development of an adherent layer of supportive stroma made of a variety of mesenchymal phenotypes.

STROMAL CELL LINES AND FURTHER ANALYSIS OF MESENCHYMAL PHENOTYPES

During the early 1980's, we were engaged in attempts to derive adherent cell lines from bone marrow cultures with the aim of cloning them, to arrive at a point of better characterization of mesenchymal phenotypes, or cell types.

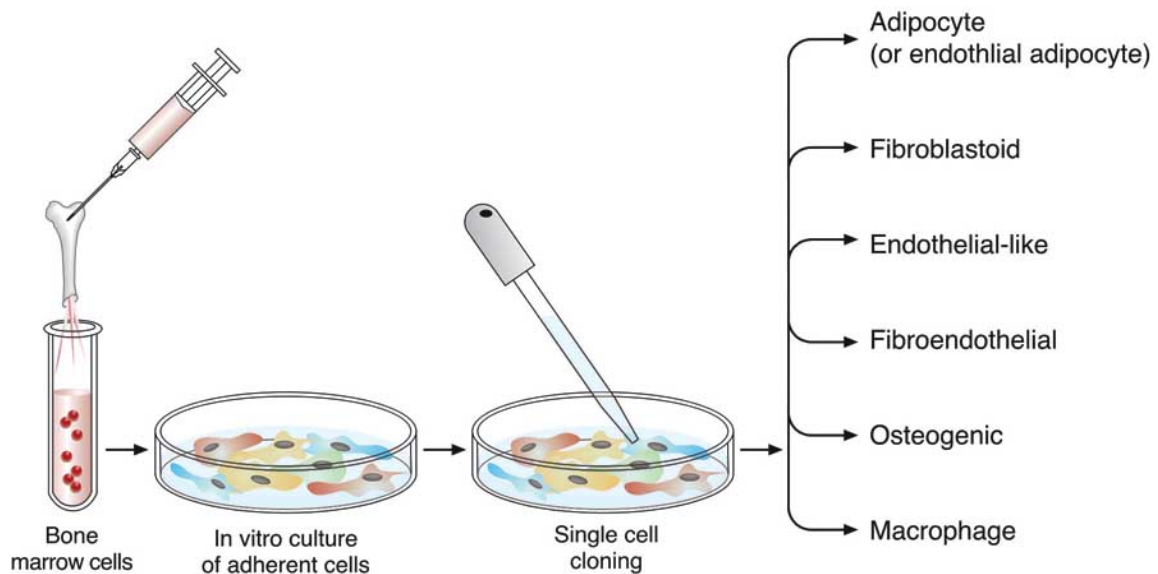


Fig. (3). Adherent cell layers that result from *in vitro* seeding of bone marrow cells are a source for permanent cell lines that can be propagated for years while maintaining a relatively fixed phenotype under constant and strict conditions. However, changing the culture conditions can easily shift their phenotype.

These attempts yielded a series of cell lines; the mouse bone marrow adherent (MBA) cell line series [20-22]. Analysis of clonal populations revealed great resemblance between the different clones, along with clear differences. According to morphology, intracellular enzymes and extracellular matrix components, the cell lines were designated as endothelial like, adipogenic, fibroblastoid, fibro-endothelial, osteogenic and macrophages [23] (Fig. 3). With the exception of the latter that are from hemopoietic descent, all the rest are mesenchymal in nature although they clearly show properties of other lineages. The best example is the MBA-2.1 cell that is derived from bone marrow mesenchyme but shares properties with endothelium. Most of the cell lines shared osteogenic properties, but in some, such as the MBA-15, these were more pronounced compared to the others [24-26]. The capacity to support hemopoiesis was by far more restricted. Only the adipogenic cell lines exhibited this trait [27-29]. Many investigators have reported the derivation of stromal cells line using different methodologies and several of these cell lines support hemopoietic cell generation [30-36]. The derivation of cell lines with diverse properties, such as those exhibited by the MBA series, could suggest that the bone marrow mesenchyme is divided into different cell types. Indeed, it has been suggested that all these different phenotypes are derived from a single MSC. Yet, our study of these cell lines indicated that the cells tend to change phenotypes even when obtained as clonal populations. Enzyme expression varied extensively among individual cells of the same clone, adipogenesis was an inducible and reversible trait in all of the clones, and the capacity to support hemopoiesis could be induced or lost according to the *in vitro* conditions used to grow the cells. Thus, the bottom line conclusion of the study of bone marrow derived mesenchymal cell lines is that their phenotype is very flexible [37]. Contrary to hemopoietic cell lines that upon *in vitro* derivation maintain a relatively fixed phenotype, with the exception of phenomena of lineage promiscuity, the

study of cultured mesenchymal cell lines lead to the conclusion that these cells continue to change fate upon culture, and are thus plastic.

ACCUMULATION OF EVIDENCE FOR MESENCHYMAL PLASTICITY

The study of bone marrow derived mesenchymal cell lines thus confirmed that highly variable phenotypes of adherent cells arise in bone marrow cultures. These experiments could be interpreted in two ways: the heterogeneity observed could result from the presence of many types of cells having different origins, or alternatively, these different phenotypes could be derived from a common stem cell. This latter conclusion gained support from cloning experiments. Pittenger *et al.* propagated single cells from human bone marrow that were expanded to form colonies. Cells from these clones could differentiate into adipocytes, chondrogenic and osteocytic cells, thus confirming the multipotency of the mesenchymal colony forming cell [38]. This rather limited differentiation potential was dramatically widened following the discovery of Jiang *et al.*; a cell population from the adult bone marrow, which was also found in the brain and muscle, was termed multipotential adult progenitor cells (MAPC). These cells co-purify with MSC (and thus maybe a subtype of this population) were characterized by unexpected pluripotency; by analysis at the single cell level and genetic labeling of the cells the investigators confirmed that MAPC differentiate *in vitro* into mesodermal derivatives including endothelium, neuroectoderm and endoderm cells. Moreover, upon inoculation into mouse blastocyst, these cells contributed to the formation of a multitude of somatic cells [39-42]. Such cells were characterized in the mouse, rat and man [43]. These studies were followed by a series of publications that support the pluripotent nature of mesenchymal cells. Marrow isolated adult multilineage inducible (MIAMI) cells reside in

human bone marrow and upon *in vitro* isolation differentiate into osteoblasts, chondrocytes, adipocytes, neural cells and cells expressing genes associated with pancreatic islet phenotype [44]. In a separate study, small airway epithelial cells were heat shocked to cause injury. The cell damage was corrected by the addition of human MSCs that differentiated into epithelial-like cells as indicated by expression of epithelial genes. Along with such direct differentiation, cell fusion was observed and accounted for part of the contribution of the MSC to epithelial repair [45]. Like other phenomena of cell plasticity, this phenomenon suggests a possible use of MSC in cell therapy, and in this case, in lung diseases such as cystic fibrosis [46]. Additional reports focused on neuronal differentiation of bone marrow stromal cells. Such cells were shown to readily differentiate into neurons [47]. An independent study indicated that it is possible to derive neural cells from human bone marrow stroma with high efficiency; MSC were isolated, grown in neurosphere-like structures and were shown to differentiate into astroglia, oligodendroglia and neurons [48]. Transplantation of rat marrow stromal cells under xenogenic conditions, into chick embryos, resulted in expansion of the transplanted population and integration into the heart and other tissues. Some of the donor cells in the heart expressed heart muscle markers [49]. It has been shown that MSC are capable of migrating into the heart upon systemic administration [50]. Kawada *et al.* have isolated a clonal population of cardiomyogenic cells from MSC. They have compared the capacity of isolated hemopoietic stem cells to contribute to ischemic myocardium following myocardial infarction. The study indicated that hemopoietic stem cell (HSC) do not contribute much, whereas the cardiomyogenic cell line generated actinin positive heart cells [51]. Whether this property is indeed shared by primary MSC remains to be examined. A recent publication by Yokoo *et al.* showed that human bone marrow derived MSCs participate in the

kidney organogenesis upon injection into rodent embryos [52]. These cells seem, therefore, to undergo a transdifferentiation process to form entire nephrons. Thus, similar to the MET that occurs during normal development in kidney organogenesis, bone marrow mesenchyme from an adult source maintains its plastic properties that are exhibited upon introduction into a permissive embryonic environment (Fig. 4) for additional review of mesenchymal stem cells see [53-55].

WHY ARE PLURIPOTENT MESENCHYMAL STEM CELLS SO ABUNDANT IN HEMOPOIETIC ORGANS?

Mesenchymal stem cells have been isolated from the embryo as well as from the adult. In adult organism MSC have usually been isolated from hemopoietic organs, particularly from the bone marrow. However, such cells are also found in other organs and the type called MAPC was also isolated from muscle and brain [40]. One possibility that still needs clarification is whether MSC are direct descendants, or actual embryonic mesenchyme, that distributes to various organs and tissue during ontogeny and remain there throughout the mammalian life span. Indeed, MSC are found also in the spleen and thymus and we have derived mesenchymal cell lines from thymic origin that were very similar in their properties to bone marrow derived stroma [56, 57]. MSC were found in fatty tissues and are probably found in additional sites. However, they seem to be found in the bone marrow in a relatively higher proportion. The bone marrow space may be therefore used as a depository and a possible source for stem cells that upon injury could be recruited to other sites in the body. Many studies in the mouse and in humans suggest that stromal mesenchyme may indeed be transplantable [58-64] although

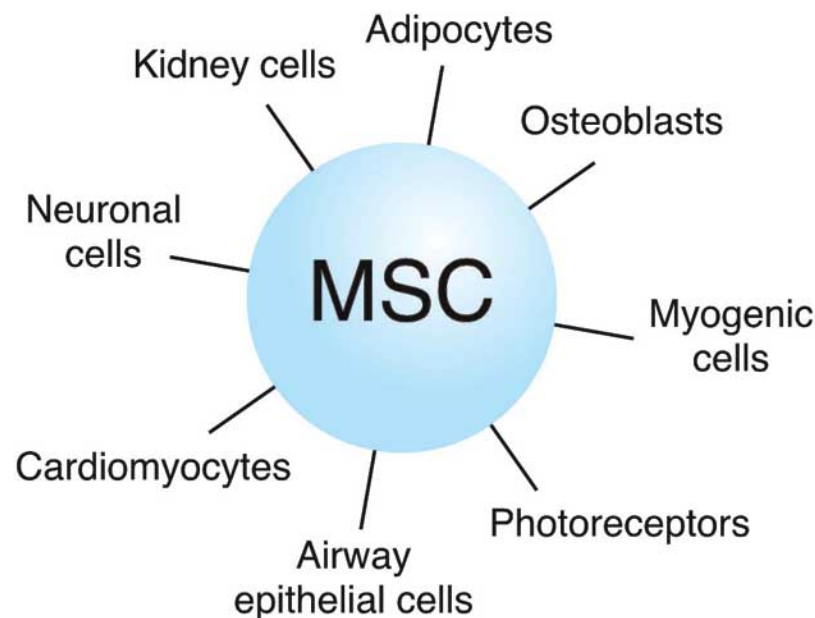


Fig. (4). Bone marrow derived stromal mesenchyme from mouse, or MSC of rat and human origin have plastic nature as revealed by their capacity to differentiate into a multitude of cell types traditionally classified as belonging to the three embryonic germ layers. The figure depicts directions of differentiation reported by different laboratories, as discussed in the text.

there is an ongoing controversy regarding this issue. Are those cells also migrating in a physiological manner? In fact, there are several indications that this is the case. First, the bone marrow was reported to be the source for endothelial precursors that gave rise to capillaries in solid tumors [65]. Furthermore, gastric ulcers induced by *Helicobacter pylori*, attract, by an unknown mechanism, bone marrow derived cells, probably MSC, which travel to the ulcer, transdifferentiate there to gastric epithelium and eventually transform into gastric adenocarcinoma [66]. The investigators proposed that tumor formation, in general, may be mediated by such migration of cells from the bone marrow. Several additional reports support the notion that mesenchymal cells are migratory and that this migration is mediated by the chemokine receptor CXCR4 [67,68]. Obviously, assisting tumor formation could not be the physiological role of bone marrow mesenchymal cells. Alternatively, they may be normally recruited to injury sites to mend damages. If the process is unsuccessful or not properly restrained, tumors may arise. In addition, I would like to propose that under milder conditions, when injury is restricted to the loss of a few cells within a tissue, correction of the damage might be done by resident MSC without the need to recruit cells from the bone marrow. The selection of the bone marrow as a depository site for stem cells that occurred during evolution is probably due to the fact that it is in the crossroad of the vascular system and constantly seeds the circulation and the peripheral organs with derivatives of the HSC. The bone marrow structure is thus ideal for responding to demand, due to tissue damage, by

fast release of cells capable of traveling through the vasculature and of contributing to the correction of damages. Therefore, the adult mesenchyme is not different in principle from embryonic mesenchyme and the existing differences are primarily quantitative (Fig. 5). In the embryo tissue and organ constructions are dramatic and robust processes, they entail very extensive plastic cell behavior. By contrast, the adult organism requires relatively little changes, mostly related to tissue maintenance in replacement of damaged or aged cells. These processes occur at a low incidence and are much more modest, compared to those in the embryo. Therefore, the plastic nature of adult MSC is suppressed and revealed only upon their *in vitro* culture in the absence of tissue constraints.

THE STEM STATE AND MESENCHYMAL PLASTICITY

I recently suggested that stemness is a state that, theoretically, any cell may enter (Fig. 6). Thus a stem cell, as a stable cellular entity, does not exist. Rather, stemness is an unstable state characterized by promiscuous gene expression that puts the cell in a standby state, ready to commit to a variety of different directions. Analysis of all the different traits ascribed to stem cells, i.e. the capacity to self-renew, or to self-renew indefinitely, the capacity to proliferate extensively, etc., suggests that these are options rather than true prerequisites of stemness. I recently suggested that the stem state entails pluripotency and

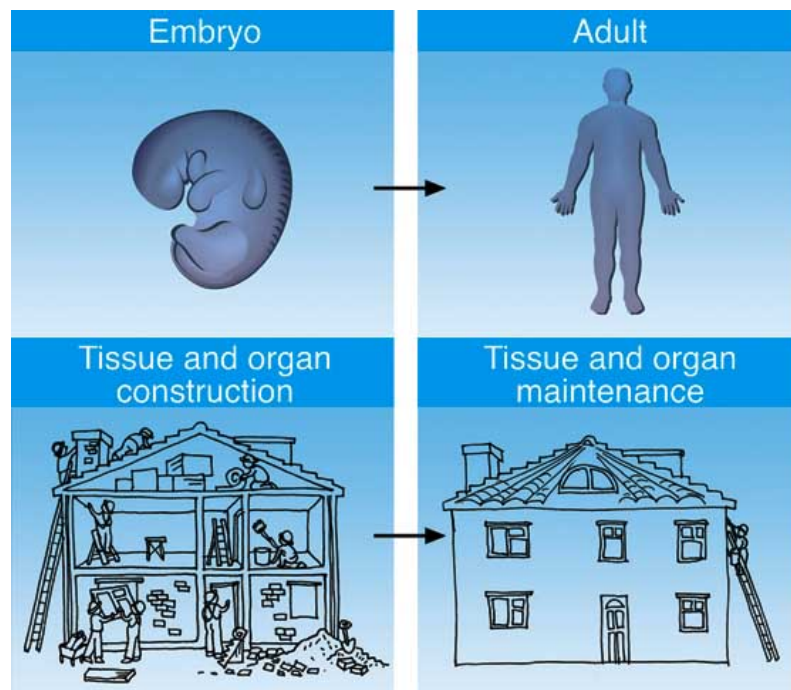


Fig. (5). Mesenchymal functions in the embryo and the adult differ quantitatively only:

Whereas in the embryo the mesenchyme is involved in robust processes of tissue and organ construction, the task of these cells in the adult may be to mend small tissue and organ damages. House illustrations in the lower panels: During constructions many builders are employed and use a series of tools and methods that are operated extensively and robustly to reach the final goal, a complete house in a limited time frame. This finished facility should, however, be further maintained throughout the years. Naturally, only a few workers would be needed. The tools and methods used for house maintenance are identical or similar to those used for construction yet they are employed on a small scale and with much caution, so as not to cause damage to the already functional home.

plasticity, rather than the other properties commonly ascribed to stem cells [9,10]. The MSC demonstrates this notion very well: mesenchymal cells shift fates, and as discussed above, may differentiate into one cell type while maintaining the ability to go back into the original “stem cell” phenotype. Further, mesenchymal cells express a variety of genes that are supposed to characterize their differentiated progeny. Gene expression analysis shows in fact that stem cells express the majority of their genome [69]. Most importantly, mesenchymal cells are pluripotent and highly plastic, in that they may give rise to cells of all three embryonic germ layers.

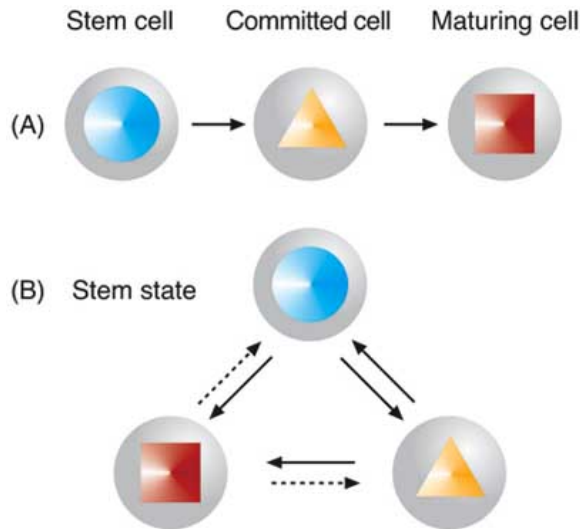


Fig. (6). The traditional stem cell model, assuming the stem cell to be an origin of an irreversible hierarchy of descending potency for renewal (A) as opposed to the stem state notion in which cells may assume a stem state even when already in a differentiating stage (B).

Thus far it has been generally accepted that renewing tissues and organs are designed such that a stem cells is positioned at the top of a hierarchy of descending renewal ability and increasing differentiation. The mature state is viewed as a stable and irreproducible situation. The stem state notion challenges this current view. It suggests that in mammalian tissues de-differentiation does occur, albeit at a low rate. Such de-differentiation phenomena may lead to the creation of stem cells derived from maturing or even mature cells. That de-differentiation occurs, in plants and amphibians, is indisputable. However, several reports suggest the existence of de-differentiation phenomena also in mammals [70-72]. Thus the linear and irreversible differentiation model (Fig. 6A) is suggested to be replaced by a reversible scheme in which the stem state is an option that may be reached at any point in the cell's existence (Fig. 6B). The frequency of entering the stem state is suggested to be dependent on the environment (niche) in which the cell resides.

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