Review Article

Mesenchymal Stem Cells: *In Vitro* Differentiation among Bone and Cartilage Cell Lineages

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Abstract

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The capacity of mesenchymal stem cells (MSCs) to differentiate among skeletal cell lineages and to undergo extensive proliferation *in vitro* renders them an appropriate source for cell-replacement therapy to heal defects of bone and cartilage tissue.It is argued that in bone and cartilage defects, MSCs would better be transplanted as fully-differentiated cells otherwise they may produce non-specific cells in defect sites. This notion may emphasize the importance of the studies considering *in vitro* bone and cartilage differentiation of MSCs. Indeed, the capacity of producing osteoblastic and chondrocytic cell lineage is among the earliest differentiation potentials of MSCs, being reported at first isolation of the cells. Recent studies, however indicate that MSCs could differentiate into more cell lineages than expected. The present study provides evidence for, *in vitro* potential of MSCs to differentiate into bone and cartilage cell lineages. As an introduction to the differentiation, the characteristics of MSCs have been described and MSCs differentiation reviewed. The culture condition for bone and cartilage differentiation, molecular regulation of the differentiation, signaling pathway involved during the differentiation, and the genes up-regulated upon bone and cartilage differentiation have also been described.

Keywords: Mesenchymal Stem Cells, Bone and Cartilage Differentiation, Signaling Pathway, Culture Condition

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Introduction

Stem cell criteria

Stem cells are defined as undifferentiated somatic cells having the ability to produce varieties of fully-differentiated progenies in response to the appropriate environment either *in vitro* or *in vivo* (1). Nowadays, at least four criteria are considered for cells to be known as stem cells. Stem cells should have the ability to undergo extensive proliferation for a long time. This property is called as self-renewal potential. Stem cells should also have the capacity to undergo multilineage differentiation in the presence of appropriate culture condition, for example, mesenchymal stem cells represent the potential to generate fibroblast, osteoblast, chondrocye and adipocyte. In addition, stem cells need to have the potential to regenerate a tissue defects upon transplantation. The last but not the least, stem cells should have the potential to produce fully-differentiated progenies even in the absence of any tissue damages *in vivo* (2).

MSCs characteristics

MSCs, having first been isolated and described from bone marrow aspirates, are a multipotent cell population resided in many tissues of adult body. These cells were originally recognized by their ability to produce three distinct phenotypes of osteoblastic, chondroblastic and adipocytic cell lineages *in vitro* and *in vivo* (3, 4).

The first definitive discovery of mesenchyamal stem cells was reported by Friedenstein (1974) who isolated the cells by their plastic adherent property and described them as a non-hematopoietic, clonogenic and fibroblastic cells having the capacity of producing osteoblastic, chodrocytic and adipocytic cell lineages (3, 4). The following studies indicate that MSCs occur in low quantity in bone marrow

aspirate, they constitute approximately 0.01%-0.001 of the whole bone marrow cells (5). In spite of their limited number, MSCs can easily be isolated owing to their plastic adherent property. The expansion of the cells is strongly dependent on bovine serum content of culture media. The cells assume spindly-shape morphology upon cultivation.

Mesenchymal stem cells (MSCs) have been successfully isolated from human, cat, dog, rabbit, rat, chicken, sheep, goat and pig bone marrows, thanks to their plastic adherence property (6-13). The isolation of murine mesenchymal stem cells is far more difficult than that of other species due to the unwanted growth of non-mesenchymal cells in both primary and passaged cultures (14, 15). To date, several protocols have been developed to purify the fibroblastic cells of murine bone marrow. The purified isolation of these cells would be important because they are suitable models for human disease (16-20). Recently; we have shown that the purified murine mesenchymal stem cells could be isolated by low-density primary culture system.

In the past, bone marrow derived-fibroblastic mesenchymal stem cells have been referred to by different terminology as cloney forming unit fibroblasts (CFU-F), marrow stromal fibroblasts (MSF), mesenchymal stem cells (MSC) and mesenchymal progenitor cells (MPC) (2, 3, 21-23). Nowadays, most denominations have been abandoned in favor of MSCs that have mostly been cited in various articles.

One of the defining characteristics of mesenchymal stem cells is their self-renewal potential, the ability to generate identical copies of themselves through mitotic division over extended periods. The absolute self-renewal potential of MSCs, however, remains an open question, due in large part to the different methods employed to derive populations of MSCs and the varying approaches used to evaluate their self-renewal capacity as a population (24).

In contrast to embryonic stem cells that undergo indefinite proliferation, mesenchymal stem cells reach to senescence after a several cell doubling *in vitro*. It has been shown that *ex vivo* expansion leads to progressive decrease of MSCs proliferation and loss of their multilineage differentiation potential. In this regard, Bruder et al reported that MSCs isolated from fresh bone marrow aspirates extensively expand *in vitro,* undergo senescence and a change in shape and morphology after about 38 population doublings. During differentiation, the adipogenic potential is the first to be lost while osteogenic potential is maintained even up to 38 population doublings (24-26). MSCs, as most somatic cells, do not express telomerase and as a result, their successive subcultures are characterized by progressive decrease of the telomere length and consequent cell aging.

Several studies provide evidence that the addition of FGF2 to culture of MSCs can delay the culture induced senescence in MSCs population by selecting a subpopulation of earlier progenitor among the total bone marrow stromal cells (27, 28). For this very reason, it is assumed that a stem cell compartment does reside in bone marrow within the MSCs population, but culture conditions or the environment so far have not been permissive to support its isolation and expansion.

The issue regarding MSCs for further investigation is the genetic signature. The phenotypes of MSCs are different among various laboratory studies and at present, there is a lack of unifying definition as well as information on specific markers that defines the cell types characterized as MSCs (29). In all studies concerning MSCs isolation, the only way to show the mesenchymal nature of the cells in question is examining the differentiation potential of the cells among the mesenchymal lineages by providing appropriate environment in culture. Recently, we indicated that Thy 1.2 surface antigen increases significantly during murine mesenchymal stem cells culture period and thus, could be used as enriching antigen for these cells (30).

MSCs differentiation potential

Heterogeneity of MSCs cultures

One of the specific characteristics of

MSCs is their colonogenic ability, even in primary culture. Individual colonies derived from single MSCs precursor have been reported to be heterogeneous in terms of their multilineage differentiation potentials. Reports indicated that only one-third of initial adherent bone marrow-derived MSC colons is pluripotent and capable of differentiating into osteoblastic, chondrocytic, and adipocytic cell lineages, while the remainder display a bi-lineage (osteo/chondro) or uni-lineage (osteo) potential (31, 32). In this regard, the interesting point is that all colons represent the bone differentiation potential. Baksh et al. proposes a model that may explain the heterogeneity of MSCs *in vitro*. According to their model, MSCs in bone marrow constitute the cells with different multilineage potential e.g. tri-, bi-, and uni-potential MSCs (33).

Multilineage differentiation potential of MSCs

MSCs differentiation is a complex process with a rather unknown molecular mechanism. It is believed that various parameters may involve in commitment of MSCs to differentiate into a particular cell lineages. These factors include the molecular composition of the serum, various treatments, the type of the plastic used in culture plate and the cell interactions. Providing the appropriate conditions, MSCs are able to generate variety of cells primarily including bone, cartilage and fat (34-39).

Moreover, MSCs have been reported to enjoy more differentiation potential than that was originally expected, in that they can give rise to various cells such as neurons, keratinocytes, lungs, and intestines (40, 41). An investigation by Herrera et al (2004) has indicated that MSCs engrafted in the damaged kidney, even are able to differentiate into tubular epithelial cells and consequently promote the recovery of morphological and functional alterations (42). Such property is referred to as MSCs' plasticity or transdifferentiation.

Plasticity of MSCs

This property is defined as an ability of the cell to give rise to differentiated progeny other than the tissue cells of their origin. All the studies documenting plasticity have used models of tissue injury to induce homing and differntiation of MSCs. To explain this property, being referred by others as transdifferentiation, three hypotheses have so far been proposed. One hypothesis states that, in contrast to previous notion considering the adult stem cells as multipotent, it seems that these cells are indeed pluripotent. According to another hypothesis, however, stem cell plasticity is a consequence of their dedifferentiation into more immature state and then differentiation into a tissue cells other than those they belong to. Finally, the third hypothesis indicates that plasticity occurs because stem cells simply fuse to fully-matured cells, called differentiated progeny of the stem cells (43-45).

Recently, a subclass of immature MSCs has been isolated from bone marrow aspirates and has been reported to possess much more *in vitro* differentiation potential than the ordinary MSCs described elsewhere. The cells, referred to as multipotent adult progenitor cells, (MAPCs) have indicated to be able to undergo indefinite proliferation owing to high telomerease activity and posses the potential of differentiation into cells of three germ layers. It has been shown that these cells are not positive to hematopoietic surface antigens such as Sca1 and c-kit and CD45 and express Oct-4 transcription factor similar to embryonic stem cells (46). The interesting point is that the methodology suggested for the isolation of MAPC cells was not applied by many other labs attempting to replicate the procedure.

Osteogenic differentiation of MSCs

The main function of bone in skeletal system is to provide structural support for the body as well as its vital organs. The bone is a main source of minerals and plays a main role during the muscle contraction. Considering these functions, the main changes in bone structure following the wound or any disease could influence the body homeostasis and compromise the individual life (47).

Most defects in bone tissue heal

spontaneously with minimal treatment but in certain cases it requires further treatment for compromised healing due to interposition of soft tissue, improper fracture fixation, loss of bone, metabolic disturbances, impairment of blood supply and infection. In such instances, bone grafts and or metallic implants are recommended (48). Obtaining of the bone is accompanied by probable risk of contamination (49-51) and the main concern regarding metal implant is that they would release harmful ions increasing the risk of cancer (52). The ability of MSCs in differentiatining into osteoblastic lineages is appealing promise for cell-based treatment of bone defects especially those with large tissue loss. It is also believed that in bone defects, MSCs should be transplanted as fully differentiated cells, otherwise it may produce non-osteoblastic cells in defect sites. This notion may emphasize the importance of the studies considering *in vitro* bone differentiation of MSCs which has already gained rather considerable attention.

In vitro conditions required for MSCs osteogenesis

For bone differentiation of MSCs, the same procedure is followed in different labs. Usually, after isolation and purifying of MSCs, passageed-2-3 cells are grown into confluency using proliferation medium containing 10-15% fetal bovine serum. Then, the proliferation medium replaces with an osteogenic medium, containing 50µg/ml ascorbic acid 2-phosphate, 10nM dexamethasone and 10mM ß-glycerol phosphate. The cultures subsequently were placed in an incubator at 37°C and 5% CO_2 for 21 days, with media changes of 3 times a week. To evaluate the occurrence of bone differentiation, at the end of the cultivation period, the cells fix with 10% formalin for 10 minutes and stain with alizarin red for 15 minutes at room temperature in order to examine the mineralized matrix. For further confirmation of differentiation, RNA extraction and RT-PCR analysis of osteocytic gene expression have also been proposed.

During osteogenesis in cultures, a few cells of monolayer culture usually become detached and float in the medium. Meanwhile, in some areas of the culture dish, nodule-like structures form. Alizarin red stains red the mineralizing areas (the nodules) of the cultures. In these nodules, bone marker including collagen I, osteocalcin, parathyroid hormone (PTH) receptor, osteopontine, and bone sialoprotein can be detectable using RT-PCR analysis. Our recent morphologic investigation has shown that, in osteogenic nodules, collagen I fibers arrange in perpendicular bundles around the cells (unpublished data).

Molecular regulation of MSCs in vitro osteogenesis

The induction of osteogenesis is a highly programmed process, best illustrated *in vitro*.Treatment with the synthetic glucocorticoid dexamethasone stimulates MSC proliferation and supports osteogenic lineage differentiation (43-54). Organic phosphate, such as β-glycerophosphate, also supports osteogenesis by playing a role in the mineralization and modulation of osteoblast activities (55, 56). Free phosphates can induce expression of osteogenic markers such as osteopontin. The phosphates also exert specific effects on the production and nuclear export of a key osteogenesis regulatory gene, Cbfa1 (core binding factor alpha 1) (57-59). Other supplements, such as ascorbic acid phosphate and 1,25-dihydroxyvitamin D_3, are commonly used for osteogenic induction, with the latter involved in increasing alkaline phosphatase activity in osteogenic cultures and promoting the production of osteocalcin (60).

In addition to established supplements, members of the bone morphogenetic protein (BMP) family of growth factors are also routinely used for osteoinduction. BMP-2 alone appears to increase bone nodule formation and the calcium content of osteogenic cultures *in vitro*, while concomitant application of BMP-2 and basic fibroblast growth factor increases MSC osteogenesis both *in vivo* and *in vitro* (61, 62).

BMPs have been postulated to play a role in the selective differentiation of

mesenchymal precursors into either the osteoblastic or adipogenic lineages (63). Selective blocking of the BMP receptor type 1B (BMPR-1B) results in differentiation into adipocytic lineage rather than osteoblastic differentiation, suggesting that expression of BMPR-1B is required for mesenchymal stem cell commitment to the osteoblastic lineage. Conversely, over expression of BMPR-1A blocks adipogenic differentiation and promotes osteoblastic differentiation, suggesting that the temporal expression or loss of BMP receptor may play a key role in determining the lineage commitment of the mesenchymal precursors into osteoblasts or adipocytes (64).

There is a reciprocal relationship between adipocyte and osteoblast differentiation. Some investigations indicate that peroxisome proliferator-activated receptor γ (PPARγ), a key transcription factor involved in adipocyte differentiation (65), negatively regulate osteoblast differentiation by repressing the osteoblast specific transcription factor Runx2 (66-75). PPARγ exists in two isoforms PPARγ1 and PPARγ2 as a result of alternative splicing. PPARγ2 is expressed at high levels in fat tissue and is essential for adipogenesis *in vitro* and *in vivo*.

Signaling pathway involved in MSCs bone differentiation

The analysis of MSCs differentiation potential has been aided by the discovery of specific *in vitro* conditions for differentiation along different mesenchymal lineage. Osteogenic factors usually used in cultures are a combination of dexamethasone, β-glycerolphosphate and ascorbic acid phosphate.

Members of Wnt signaling pathway have been shown to participate in MSC osteogenesis (76, 77).

Wnts are a family of secreted cysteine-rich glycoproteins that have been implicated in the regulation of stem cell maintenance, proliferation, and differentiation during embryonic development. In the presence of the Wnt signal, the signaling pathway starts with the binding of the Wnt factor to the membrane bound receptor. The receptor transfers the signal into cell and several other intracellular proteins activated. The best characterized effect is the inhibition of the GSK3β.

In the normal cells, this inhibition leads to an accumulation of the protein β-catenin which is normally regulated by the APC protein. Next, β-catenin can form a complex with a transcription factor of TCF family. This complex enters the nucleus and activates transcription and proliferation. In the absence of Wnt signal, the kinase GSK3β is not inhibited. GSK3β phosphorylates protein APC; axin and β-catenin complex. The result is the decrease of the intracellular amount of the β-catenin. The transcription and thus the cell proliferation are not activated. The simplified representation of the wnt signaling pathway is shown in Fig 1.

Two members of Wnt family namely wnt3a and Wnt5a are known to involve in MSC proliferation and differentiation respectively. When MSCs are exposed to Wnt3a, a prototypic canonical Wnt signal (i.e. through intracellular β-catenin), under standard growth medium conditions, they show markedly increased cell proliferation and a decrease in apoptosis, consistent with the mitogenic role of Wnts in MSCs (77). Interestingly, exposure of MSCs to Wnt3a conditioned medium or overexpression of ectopic Wnt3a during osteogenic differentiation inhibits osteogenesis *in vitro* through β-catenin mediated down-regulation of TCF activity (77). In this regard, the interesting point is that the expression of several osteoblast specific genes including alkaline phosphatase, bone sialoprotein, and osteocalcin, is dramatically reduced, while the expression of cbfa1/ Runx2, an early osteoinductive transcription factor was not altered, implying that Wnt3a-mediatyed canonical signaling pathway is necessary, but not sufficient, to completely block MSC osteogenesis. On the other hand, Wnt5a, a typical non-canonical Wnt member, have been shown to promote osteogenesis *in vitro* (77).

Chondrogenic differentiation of MSCs

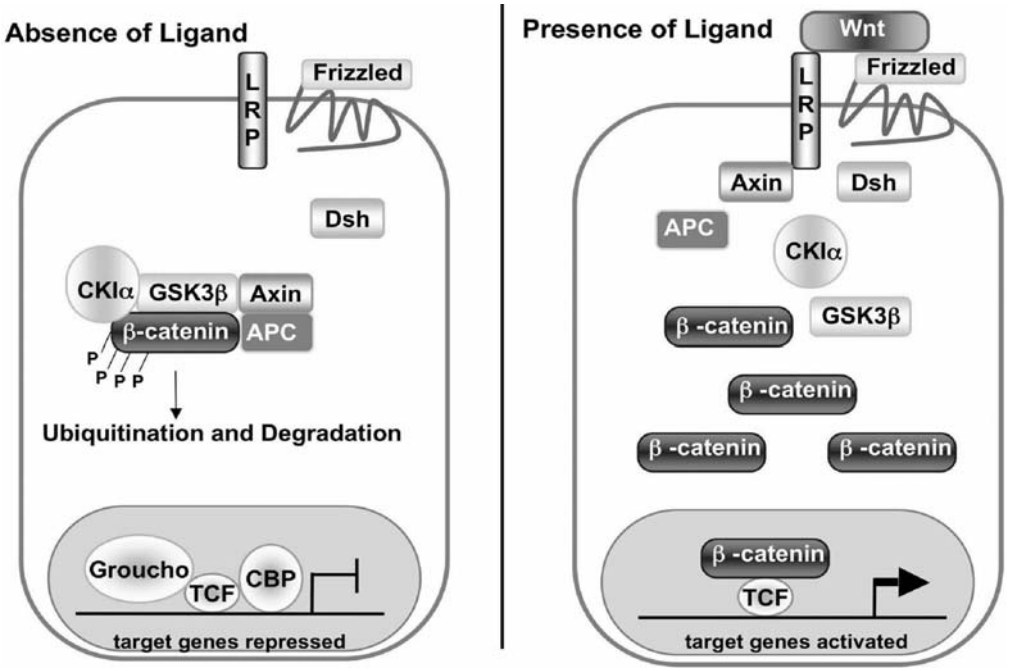

Fig 1: The simplified representation of the wnt signaling pathway

Cartilage tissue damages are a rather worldwide problem of many people and are produced by either trauma or age-dependent degenerative diseases (78, 79). Despite considerable attempts to find a way to improve cartilage regeneration, this issue still remains challenging in the field of regenerative medicine primarily due to the specific nature of cartilage biology (80, 81). The normal mechanism of tissue repair is not working on cartilage regeneration because the tissue is avascular. In natural process of tissue repair of living body, damaged tissue is regenerated by means of humeral and cellular elements brought to the spot from the adjacent areas as well as the intact cells of the tissue after injury. Regeneration of damaged cartilage tissue is impaired due to its inherent low cell density and avascularity (82-85). MSCs could be considered as an appropriate source for cell-based treatment of cartilage defects owing to their capacity in undergoing extensive self-renewal proliferation as well as the potential of giving rise to chondrocytic cell lineage.

In typical cell therapy strategies, the implanted cells are terminally differentiated cells that are intrinsic to the healing site. Therefore, one important step in preparing MSCs cells for clinical use is providing the condition in which the cells can differentiate into mature cartilage before being implanted. This will guarantee the transplantation of only chondrocytic cells and therefore avoid the unwanted bone formation when undifferentiated cell is implanted.

In vitro conditions required for MSCs cartilage differentiation

Cartilage differentiation of MSCs is usually conducted in a micromass culture system (86-94). Approximately 200,000 cells (passage 2-3) are pelleted by centrifugation at 300 g for 4 minutes, followed by incubation at 37°C and 5% CO_2 in a 0.5 ml chondrogenic medium, composed of 10ng/ml transforming growth factor ß3, 500ng/ml bone morphogenetic protein-6,100nM dexamethasone, 50µg/ml ascorbic 2-phosphate, 50 µg/ml ITS and 1.25 mg/ml bovine serum albumin. The cultures are maintained for 3 weeks with medium change of 3-day intervals. At the end of this period, the pellets are usually analyzed for differentiation using toluidine blue staining for methachromatic matrix and RT-PCR analysis for cartilage-specific markers including collagen II,

aggreacan, and collagen X.

Molecular regulation of MSCs in vitro chondrogenesis

The induction of chondrogenesis in MSCs depends on the coordinated activities of many factors, including parameters such as cell density, cell adhesion, and growth factor. Culture conditions conductive for chondrogenic induction of MSCs require high-density pelleting and growth in serum free medium containing specific growth factors and supplements. The TGF-β super family of protein and their members, such as the bone morphogenetic proteins (BMPs) are well-established regulatory factors in chondrogenesis. TGF-β1 was initially used for *in vitro* culture and can induce chondrogenesis under this conditions (95, 96), although TGF-β3 has recently been shown to induce a more rapid and thorough expression of chondrogenic markers (97, 98). Another TGF-β family member, BMP-6, appears to increase the size and weight of pellet cultures and to increase amount of matrix proteoglycan produced (99). BMP-2 and BMP-9 have also been used in three-dimensional MSC culture systems, such as those seeded in the hydrogel alginate. It is under such conditions that they can induce markers of chondrogenesis (100).

Signaling pathway involved in MSCs cartilage differentiation

Recent studies have demonstrated that TGF-β, BMP-2, and growth differentiation factor-2 (GDF-2) rapidly induce type II collagen expression, suggesting critical roles of signaling by the TGF-β superfamily for chondrocyte-specific gene expression (101-103). In chondrogenic differentiation of MSCs, several TGF-β-dependent signaling pathways are involved. The Smad pathway is one of them that is widely represented in most of the cell types and tissue being studied (104). Investigations have shown that other additional pathways may be activated following treatment with TGF-β in specific contexts. Activation of Ras, extracellular signal- regulated kinase ½ (ERK1/2), and c-Jun N-terminal kinase (JNK) by TGF-β signaling has been reported in primary intestinal epithelial cells and chondrogenic cell line derived from mouse teratocarcinoma (105, 106). In an attempt to define the contribution of specific TGF-β dependent signaling pathway involved in the regulation of chondrogenesis from human mesenchymal stem cells, the ERK1/2 pathway was inhibited by U0126, a specific inhibitor of mitogen activated protein kinase 1/2 (MEK1/2), which is an upstream molecule that activates ERK1/2. For inhibition studies, U0126 was added with TGF-β3 treatment at the same time. According to the results MEK inhibition resulted in complete down-regulation of type II collagen. In contrast, aggrecan expression was detected in the same level by treatment of U0126. It seems that type II collagen expression might be critically regulated by downstream signaling molecules of ERK1/2. It seems that ERK1/2 mediated signaling pathway might be one of the key signaling factors and the inactivation resulted in blocking of chondrogenesis of mesenchymal stem cells. Chondrogenesis of mesenchymal stem cell is regulated by complicated protein kinase signaling cascades. Recently, it has been reported that aggrecan gene expression was regulated by cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase (MAPK) pathway but not protein kinase A (107).

Some evidences have indicated that Wnt and Wnt-related family of signaling proteins would also involve in adult cartilage formation. It has been reported that Wnt3a have chondro-stimulatory effects in mouse C3H10T1/2 cells. The other evidence for Wnt involvement in chondrogenesis is the identification of the constitutive expression of Wnt5a in pellet culture *in vitro* (108-111).

Genes involved in bone and cartilage differentiation of MSCs

In a study, Song and Tuan used affymetrix human genome U133 array set and compared the transcriptum profiles associated with three mesenchymal lineages derived from human MSCs, namely, osteoblast, chondroblast and adipocyte, to that of uncommitted MSCs (33). Genes with 1.5 fold or higher level of increased expression during differentiation were selected and

categorized into three subclasses, depending on their upregulation in one, two, or all three lineages. Among 39,000 transcripts analyzed for osteogenesis, adipogenesis, and chondrogenesis, respectively, 914, 947, 52 genes increased their expression in one mesenchymal lineage, while 235, 3 and 10 genes shared upregulation expression between two lineages. Most interestingly, there are eight genes whose expression are increased during all three mesenchymal lineage differentiations, suggesting that they might function in all three lineages, and thus may represent the putative master control genes. These genes are identified as period homolog1 (PER1), nebulette (NEBL), neuronal cell adhesion molecule (NRCAM), FK506 binding protein 5 (FKBP5), interleukin 1 type II receptor (IL R2), zinc finger protein 145 (ZNF145), tissue inhibitor of metalloproteinase 4(TIMP4), and serum amyloid A2.The function of these genes cover a broad rage of cellular processes, including cell adhesion, protein folding, organization of actin microfilament, as well as inflammatory response, implying that initiation and commitment of adult stem cells is a complex process requiring the coordination of multiple molecules and signaling pathways.

Asymmetric division as the first event in MSCs commitment for differentiation

The key question about the MSCs differentiation is how and when MSCs tend to stop proliferation and initiate the differentiation. In response to this fundamental question, a model has been proposed that explains the regulation of adult stem cell differentiation (33). In this model, two continuous yet distinct compartments were considered for MSCs. In the first compartment named stem cell compartment, the cells remain quiescent and growth arrested in G0/G1, until stimulated for example by the supplementation of growth factors. At this time they undergo asymmetric division and , giving rise to daughter cells , one being the exact replica of the mother and maintaining multi-lineage potential and, the other daughter cell becoming precursor cell, with a more restricted developmental program. Precursor cells continue to divide symmetrically, generating more tri-potent and bi-potent precursors that were morphologically similar to multi-potent cells and differed in their gene transcription repertoire, and as a result, still residing in the stem cell compartment. The exit from stem cell compartment to the committed compartment occurs when precursor cells divide symmetrically and generate uni-potent progenitor capable to differentiate into specific lineage. Indeed, the commitment compartment includes the cells with only one differentiation potential.

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