CHAPTER X

SKELETAL STEM CELLS

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Mesenchymal stem cells (MSCs) are known to have the capability to differentiate into many cell types of skeletal tissues. MSCs were first identified in the bone marrow but since then they have been isolated and identified in many other tissues. Many possible therapeutic uses of MSCs require a careful review of their sources. Bone marrow, adipose tissue, periosteum, skeletal muscle, adult peripheral blood, umbilical cord blood, vascular pericytes, bone tissue, amniotic fluid, spleen, and dermis are sources of MSCs. Bone marrow is the most established source which has been investigated most, understood best and its use *in vivo* is promising. Adipose tissue, skeletal muscle and periosteum have also been proven to contain MSCs and may have possible future uses. Recently, MSCs have also been found in adult peripheral blood at low numbers. More research is needed to develop adult peripheral blood as a viable option for MSCs. If it were, the future use of MSCs would be greatly facilitated with the ease of its collection. Evidence of MSCs in the other tissues, such as umbilical cord blood, vascular pericytes, bone tissue, amniotic fluid, spleen, and dermis also existed but they are of limited use.

1. Introduction

In an adult, the production of new cells usually involves a chain of processes that begins with cell proliferation and involves migration, differentiation and maturation. The first cell in this chain is termed a

stem cell, which has clonogenic and self-renewing capabilities and can differentiate into multiple cell lineages. The bone marrow is the site of two separate and distinct stem cell lineages – the haemopoietic stem lineage, from which blood cells and osteoclasts develop, and the stromal /Osteoblast (OB) cell lineage from which OBs develop. The name most commonly attributed to stromal stem cell in current literature is "mesenchymal stem cell" (MSC). Other names include connective tissue stem cells, stromal stem cells, and stromal fibroblastic stem cells.[i] Contrary to what the name "MSC" may suggest, it does not give rise to all tissues derived from the embryonic mesenchyme. The mesenchyme is a specialized tissue in the embryo that gives rise not only to the muscle, bone, and other connective tissue, but also to the blood and other cells. MSCs are so named because they develop from the mesenchyme.[1] Until recently, it was assumed that adult stem cells were committed to differentiate into the tissues in which the stem cell resides. However, recent investigations have shown that this assumption was incorrect. For example, haemopoietic stem cells have been differentiated into hepatocytes,[2, 3] and neural stem cells have been observed differentiating into blood cells.[4] Similarly, MSCs not only have the ability to differentiate into OBs, but also adipocytes,[5] chondroblasts,[6] myoblasts,[7] astrocytes,[8] and fibroblasts.[9]

Recognizing that MSCs may differentiate into a number of skeletal cells, it is interesting to investigate if MSCs are present in a number of skeletal tissues, i.e. adipose tissues or muscle tissues etc. Indeed one would hypothesize that MSCs may travel in the peripheral blood, in which way enable them to spread in a wide range of tissues. A critical review of potential sources of MSCs is worthwhile due to the promising therapeutic potentials of MSCs.

2. MSC differentiation in the osteoblast lineage

The differentiation of a MSC into an OB has four main stages and five main cell types in the lineage. This is summarized in Figure 1.

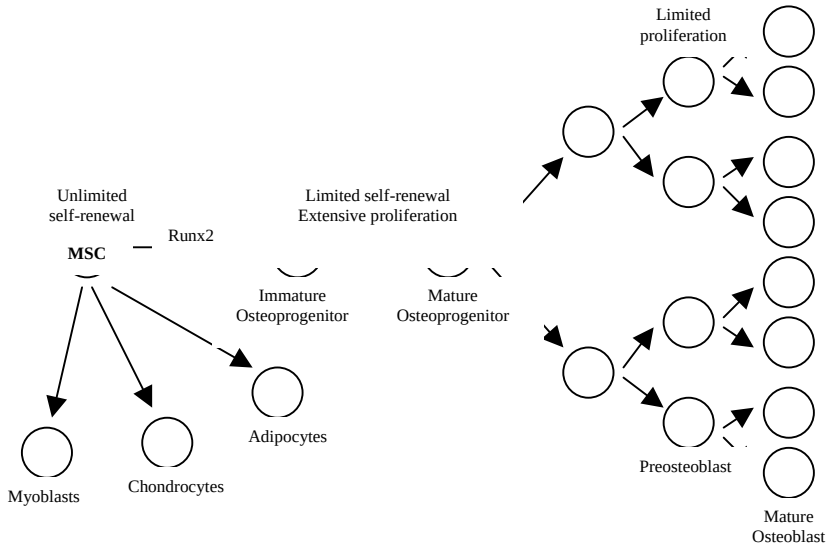

Figure 1. The differentiation pathways of MSCs. Adapted from: Aubin JE, Triffitt JT. Mesenchymal stem cells and osteoblast differentiation. In: Bilezikian JP, Raisz LG, Rodan GA, eds. Principles of Bone Biology. 3rd Edn. San Deigo: Academic Press 2002

There are many factors, which control this differentiation process. Runx2 (previously named Cbfa1) is crucial for OB development.[10] In mice, the deletion of Runx2 leads to animals which have a skeleton comprising only of chondrocytes and cartilage; OBs and bone are not evident.[11] In addition to Runx2, Indian Hedgehog (a secreted growth factor) is required for the differentiation of MSCs to OBs in endochondral bone, but not in intramembranous bone.[12] Bone morphogenetic proteins (BMPs) are also important regulators of OB development. They tend to promote OB differentiation and bone matrix formation in the more mature OB cell lineage but having inhibitory effects in the earlier differentiation process.[10]

2.1 Markers of Osteoblast

There are a large number of markers for MSCs and more specific markers for more differentiated cells along the OB lineage.

Surface markers: There are a variety of surface markers which are detectable in very immature osteoprogenitors but which are also detectable in MSCs. Antibodies which react with such surface markers include STR0-1, SH-2, SH-3, SH-4, SB10 and HOP-26.[1] Therefore, the use of antibodies cannot be used to confirm the presence of committed osteoprogenitors but can be used to identify MSCs which may potentially differentiate into OBs. **Cellular and Molecular markers**[13]**:** The expression of the bone/kidney/liver isoform of alkaline phosphatase (ALP) is seen in the more mature cells of the OB lineage i.e. mature osteoprogenitors, preosteoblasts and mature OBs. Its expression increases with differentiation. It is not expressed by MSCs or by more immature osteoprogenitors. Type I collagen is expressed in all the cells in the OB differentiation lineage succeeding the immature osteoprogenitor. Although it is not expressed in MSCs, other cells differentiated from MSCs outside the OB lineage express it. Hence, it is not a definitive test for the presence of OBs. Osteocalcin is expressed (in varying amounts) by mature OBs but not all mature OBs express it. Therefore, osteocalcin is perhaps useful qualitatively but its use quantitatively is questionable. Cells express bone sialoprotein at various stages in the OB lineage including preosteoblasts and OBs, but in varying proportions also. The mineralized matrix secreted by OBs contains calcium phosphate. It can be detected by Alizarin Red stain.[14]

2.2 Culture condition to maximise OB lineage differentiation

Different investigators use slightly different culture methods to favor differentiation of MSCs into the OB cell lineage. Figure 2, used by Wickham et al[14] and Zuk et al[15] is a typical osteogenic culture condition.

Dulbecco's modified eagle medium (DMEM) 10% fetal bovine serum 0.01 μM 1,25-dihydroxyvitamin D3 *or* 0.1μM dexamethasone 50 μM ascorbate-2-phosphate 10 mM β-glycerophosphate 1% antibiotic (e.g. penicillin, streptomycin)

Figure 2. The most used osteogenic culture conditions.

3. Established and potential sources of MSCs

3.1 Bone Marrow

The bone marrow (BM) was the first source of MSCs identified. Friedenstein was the first to describe MSCs in the BM, although he did not call them MSCs.[16] Since then the BM has become the most established source of MSCs. Although majority of research into MSCs has been performed on MSCs from BM, one cannot assume that MSCs collected from other tissues will behave in exactly the same way as the BM-MSCs. Therefore, using BM-MSCs, as a standard to study and compare with MSCs of other tissues is necessary. The number of MSCs found in the BM was relatively high in comparison to other sources, with one in 3.4×10^4 cells in BM aspirate is a MSC.[17] BM contains more committed osteoprogenitors in addition to uncommitted MSCs. Assessment of the committed progenitors showed that 30% had osteo/chondro/adipo potentials and the remainder had osteo/chondro or pure osteogenic potential.[18] The disadvantages of using BM as a source of MSCs are largely practical. The procedure of BM aspiration requires a highly skilled professional and lasts 20-30 minutes. A local anesthetic (e.g. Lidocaine) must be used and the patient must be supine for one hour after the procedure.[19] The majority of patients experience pain during aspiration and over a third patients experience moderate to severe pain for a prolonged period afterwards.[20] The amount of BM that can be aspirated at one site is usually less than 2 ml.[14] Therefore; culture expansion of BM-MSCs is usually required before they are used for therapeutic purposes.

The use of BM-MSCs to regenerate bone *in vivo* has been well documented in animals. In combination with a 3D scaffold, cultured BM-MSCs have been shown to form highly vascularised primary bone tissue in mice.[21] Clinical trials are at phase II in using BM-MSCs for osteogenesis in humans. Phase I has reportedly produced successful

results where MSCs were implanted into the alveolar region of the jaw in preparation for dental implants.[22] Overall, it can be seen that BM is an excellent source of MSCs and it is relatively well understood. But practical problems of harvesting BM are a significant disadvantage to its use.

3.2 Adipose Tissue

A considerable amount of research in recent years has supported adipose tissue as a source of MSCs. Adipose tissue derived MSCs (A-MSCs) have been collected from animals[23] and numerous human sources, including the infrapatellar fat pad of the knee,[14] and lipoaspirate.[15] However, only a fraction of the cultured A-MSCs under osteogenic conditions will differentiate into osteogenic cells, and the rest remain as adipogenic cells. This fact is not detrimental to the use of A-MSCs for osteogenesis, as the differentiated OBs can be selected from the culture for therapeutic use. Adipose tissue has a number of advantages over BM as a source of MSCs. From a practical perspective, human adipose tissue is plentiful and can be removed more easily than BM (although anesthetic is usually required), larger amounts can be collected than from BM, and less pain is experienced by the patents.[24] Under some culture conditions, A-MSCs were found to produce more ALP than BM-MSCs; however, the difference was only significant in the early stages (four days).[25] Although bone formation from A-MSCs has been observed in rats,[24] it has been noted that the MSCs found in adipose tissue have several distinctions. A-MSCs do not undergo chondrogenic or myogenic differentiation under the same conditions as BM-MSCs, no osteocalcin is expressed by A-MSCs without 1,25-dihydroxyvitamin D3 unlike BM-MSCs and there are discretions in a small number of surface markers.[15] Therefore, it may not be assumed that the OBs derived from adipose tissue will act identically to BM derived OBs.

3.3 Periosteum

It has been shown as early as 1962 that the cells at the outer layer of the periosteum differentiate into osteoblasts.[26] The periosteum has been used as a source of osteogenic cells in two distinct ways – grafting of periosteal tissue, and use of periosteum as a source of MSCs (P-MSCs). Grafts of periosteal tissue have been observed to regenerate the damaged mandibular head of rabbits,[27] and the zygomatic arch of rats.[28] However, this is not dissimilar to standard bone grafting because the entire tissue is used, not just the osteoprogenitors. As a source of MSCs, the periosteum had excellent osteogenic potential.[29] 100% of equine tibial P-MSCs differentiate into osteogenic cells when cultured in osteogenic conditions. There has also been some success in using cultured human P-MSCs for bone regeneration. Osteogenic cells from biopsies of human calvarial periosteum seeded into nude mice stained positive for osteocalcin after six weeks.[30] In another study, P-MSCs were collected from rabbits and cultured to multiply and differentiate, and were subsequently seeded into a calvarial defect. This resulted in newly formed bone and repair.[31] In comparison to BM-MSCs and A-MSCs, there is relative little research performed on P-MSCs. It is unclear if the cells should 'earn' the name MSCs because they have only been observed differentiating into OBs and chondroblasts so far. Removal of periosteum also presents similar practical difficulties to BM aspiration. Local anesthetic is required and the amount can be removed is small. However, the high osteogenic potential of the P-MSCs means that they may have a possible future use in orthopaedics and tissue engineering.

3.4 Skeletal Muscle

Cases of ectopic bone formation are seen clinically in conditions such as heterotrophic ossification and during fracture healing.[32] This suggests that there may be osteoprogenitors present in the muscle. Indeed, it has been found that muscle satellite cells have the multipotential properties similar to BM-MSCs. These muscle satellite cells (which are only found in skeletal muscle) have been observed to express myogenic, adipogenic and osteogenic potential.[33,34] Levey et al[32] collected healthy adult

skeletal muscle and cultured it to enrich satellite cell numbers in osteogenic conditions. Greater than 70% of the cultured cells expressed ALP and osteocalcin. The majority of recent research into osteogenesis using muscle satellite cells concerns gene therapy. One approach is gene therapy using BMPs to stimulate osteogenesis.[35] Muscle, as a source of osteoprogenitors, has an advantage because its removal is a little more convenient than BM aspiration although anesthetic is required and there may be some pain experienced for up to a few weeks. However, the osteogenic potential of the muscle-MSCs cells appear not to be as osteogenic as those derived from bone marrow, periosteum, or adipose tissue at this stage of investigation and more research into this area is required.

3.5 Adult Peripheral Blood

A small number of investigations have examined the possibility of adult peripheral blood as a source of MSCs with somewhat mixed results. There have been several investigations of note recently, one of which detected MSCs in patients with breast cancer.[36] However, if MSCs were found in the peripheral blood of healthy individuals, it would be of greater significance. Zvaifler et al[37] centrifuged peripheral blood from normal individuals in order to obtain MSC rich elutriation fractions. In the appropriate fraction, MSCs were found in over 100 individuals. It was reported that 0.3-0.7% of this blood cell fraction consisted of MSCs, i.e. 1 in 2 x 10^9 blood cells are MSCs. After culture for 20 days in osteogenic conditions, about a third of the selected cells expressed ALP, osteocalcin and other markers for OBs. However, no *in-vivo* data on bone formation potentials of these blood MSCs was presented. Kuznetsov et al[38] furthered the investigation by transplanting human osteogenic cells derived from blood on ceramic particles into the subcutis of immunocompromised mice. Bone formation was found at the transplant sites after eight weeks, and with the use of a human DNA probe the osteocytes in the newly formed bony tissues were identified as human origin. But the number of MSCs found in human blood is very rare.[38] More recently, Li et al[39] have reported that the number of MSCs in the peripheral blood of patients with long bone fractures and non-

unions increased significantly, and the BMP-2 expression was also significantly unregulated in the peripheral blood mononuclear cells in the fracture patients compared with the normal controls. Shirley et al[40] have further confirmed that in a rabbit ulna fracture model, BM-MSCs were recruited to the fracture sites from remote bone marrow sites via peripheral circulation. These findings point to a possible direction of research into the use of blood MSCs for tissue repair and engineering.

However, there were studies, which have not succeeded in obtaining MSCs from adult peripheral blood. These include Lazarus et al,[41] and Wexler et al.[17] Neither of these studies could identify MSCs in the peripheral blood. These may be due to the low prevalence of the MSCs in blood described by Zvaifler et al[37] and Kuznetsov et al[38], and the inappropriate culturing techniques used. The advantages of using peripheral blood as a potential source of MSCs are obvious, as the procedure for collecting blood is one of the most common procedures conducted in clinical practice. This would make therapy utilizing MSCs very accessible to almost the entire population. However, the numbers of MSCs in the adult peripheral blood are very low, and more research on the enrichment and recruitment of blood MSCs are needed.

3.6 Umbilical Cord Blood

Umbilical cord blood (UCB) is currently used as a source of heamopoeitic stem cells.[42] It has been suggested that it may also contain MSCs. There have been a number of investigations that support this. Rosada et al[43] successfully cultured MSCs from full-term human UCB and under osteogenic conditions, these cells differentiated and stained for ALP, osteocalcin and mineralised matrix. When transplanting the UCB-MSCs into the subcutaneous tissue of mice, it resulted in a greater amount of stroma-like tissue formation and a lesser amount of bone formation compared to BM-MSCs. It was noted that UCB-MSCs were slower to establish in culture, had a lower precursor frequency and a lower level of bone antigen expression than that of BM-MSCs.[44, 45] However, some studies[17, 46] failed to identify MSCs in UCB, which suggested that this source is difficult to work with to obtain MSCs

reliably. Use of UCB-MSCs as a source would involve allogeneic transplantation, as it is not possible to obtain autologous UCB cells. It has been reported that using UCB transplantation for haemopoietic stem cells does not require a close human leukocyte antigen match,[47] but it is not known if this is the case for MSCs transplantation. In effect, the cross matching that may be required along with the long-term storage considerations of UCB, are the two great disadvantages to use UCB as a possible therapeutic source of MSCs.

3.7 Other Sources

A number of other tissues have been investigated as potential sources of MSCs, with varying success and variable potential for therapeutic use. Vascular pericytes have been investigated intermittently over the years. In one of the more recent studies, transplanted bovine pericytes into athymic mice were found to form various tissues including bone.[48] In culture conditions to encourage OB differentiation, the pericyte derived MSCs stained for a wide host of OB markers including osteocalcin and bone sialoprotein. A number of earlier investigations also support the *in vitro* differentiation of pericytes into OB-like cells.[49, 50] The source of the pericytes in the studies were the vessels of the retina and therefore would not be a sensible option for human collection. Bone tissue has also been identified as a source of committed osteoprogenitors.[51, 52] But to remove bone from the body may be the most difficult procedure to gain osteoprogenitors. Recently, a study reported second trimester amniotic fluid to be a source of MSCs that had a greater expansion potential than BM-MSCs.[53] A more thorough investigation into this would be warranted but the therapeutic use is limited due to the possible need for cross matching, long-term storage and possible (although small) risks to the fetus. The fetal blood and liver have also been reported to contain MSCs,[54] although this is unlikely to be used for therapeutic purposes. The spleen has recently been identified as a source of MSCs in rats.[55] Although experiments failed to produce any bone matrix *in vivo*, the spleen cells cultured under osteogenic conditions stained positive for osteocalcin, ALP and bone sialoprotein. Once again, the use of spleen as a potential source of MSCs would be rather limited. Dermis has also

been shown to contain MSCs[56] and this area may also be worthwhile for further investigation as the skins are relatively easy to remove and plentiful.

4. Conclusion

A wide variety of sources, both established and novel, were discussed and their advantages and disadvantages examined (Table 1). The most established, best-understood and most reliable source of skeletal stem cells is the bone marrow (BM). However, the collection of BM is quite possible the most significant drawback to its future therapeutic use. Both adipose tissue, due to its high availability and relatively easy collection, and periosteum, due to its high osteogenic properties, have possible future potentials. Skeletal muscle, umbilical cord blood, vascular pericytes, bone tissue, amniotic fluid, spleen and dermis are all less significant sources of MSCs. With development and further investigation, there may be some possible merits to these sources.

The identification of MSCs in adult peripheral blood is a significant finding. Although not all studies have confirmed MSCs in the blood and the studies, which were successful, identified only a very small numbers of MSCs in peripheral blood, the ease of collection of peripheral blood could transform the clinical use of MSCs in the future. It is essential to further study techniques of enriching, isolating and differentiating skeletal stem cells from adult peripheral blood. Were this to be successful, clinical use of MSCs could become widespread in orthopaedics and many other lines of medicine. Until then, bone marrow will probably remain the primary source of choice for skeletal stem cells.

Table 1. Summary of sources of skeletal stem cells

Source	Advantages	Disadvantages	Comment
Bone marrow	Reliable, relatively high numbers of MSCs,[17] well established, high OB potential.[18]	Collection – time consuming, painful, small amount removable.	At present the best option for clinical use.
Adipose tissue	Reliable, plentiful supply, less difficult collection than BM, though not ideal.	Lower OB potential than BM,[14] subtle differences in A-MSCs and BM-MSCs.[15]	Fairly good therapeutic potential. More research needed.
Periosteum	Excellent OB potential.[30]	Collection – time consuming small amount removable.	Fair therapeutic potential. More research needed.
Skeletal muscle	Plentiful supply.	Low OB potential.[33, 34] Collection difficulties.	Fair therapeutic potential.
Adult peripheral blood	Ease of collection.	Low numbers of MSCs, low OB potential.[37,38]	Worthy of more research. If reliable techniques developed, it would become source of choice.
Umbilical cord blood	Convenient collection. No pain experienced.	Low OB potential,[43] slower to culture than BM.[44] Difficult to isolate, allogeneic transplantation needed.	Low therapeutic potential.
Vascular pericytes	No obvious advantages.	Difficult collection, small amounts.	Fairly low therapeutic potential. More research needed.
Bone tissue	Source of committed osteoprogenitors.[51,52]	Removal of bone is a difficult procedure.	Fair therapeutic potential.
Amniotic fluid	Greater expansion potential than BM-MSCs.[53]	Possible risks to fetus. Allogeneic transplantation.	Fairly low therapeutic potential.
Spleen	No obvious Advantages.	Little research[55] Difficulty of collection.	Low therapeutic potential.
Dermis	Easy collection.	Difficult to isolate MSCs, little research.[56]	Low therapeutic potential.

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