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OPINION ARTICLE

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Post-natal "mesenchymal" stem cells: the assayable skeletal potency

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Post-natal "Mesenchymal" Stem Cells (MSCs) are fibroblastoid multipotent cells with a high capacity for self-renewal. Skeletal Stem Cells (SSCs), also known as Bone Marrow Stromal Cells (BMSCs) or MSCs from Bone Marrow (BM-MSCs), are the best known post-natal mesenchymal progenitors of mesodermic tissues in humans. Skeletal MSCs are perivascular cells in bone marrow, and progenitors of all tissues that together comprise the bone-bone marrow organ (bone, cartilage, fat and perivascular stromal cells; the latter coincide with the skeletal/mesenchymal stem cell and the cell type that provides a niche for hematopoietic stem cells)[1-4]. They can be prospectively isolated based on phenotype, generate clonal progenies *in vitro*, and replicate the development of bone/marrow organ (also defined ossicle, including bone and bone marrow) in defined experimental transplantation systems[4,5].

To date, MSCs have been isolated from several human post-natal tissues, e.g., bone marrow, adipose tissue, umbilical cord matrix, tendon, lung, periosteum, dental pulp, synovium and many more[6]. Regardless of source, these cells display markers such as CD29, CD44, CD70, CD73, CD90, CD105, and Stro-1, lacking markers for early hematopoietic lineages (CD34, CD45 and others)[7,8]. Albeit, the markers for identification of a "true" MSC remain somewhat controversial. However, the biological skeletal properties of MSCs are essentially based on canonical *in vitro* assays[9] using cultures that are chemically directed towards osteo-, adipo- and chondrogenic differentiation (Figure 1A) employing respectively osteogenic, adipogenic and chondrogenic inducers[10]. These *in vitro* assays are not stringent and specific, and often fail to predict *in vivo* differentiation potential and behavior of a given MSC strain upon *in vivo* transplantation. *In vivo* skeletogenic potential of MSCs for osteo-tissue regeneration is not predicted by *in vitro* detection of tissue-specific phenotypic markers (e.g. mRNA or protein) or by the observation of *in vitro* surrogates of *in vivo* differentiated features, such as deposition of alcianophilic matrix (cartilage), mineralization nodules (bone), or intracellular lipid accumulation [(adipocytes); Figure 1A, a-c]. In fact, the expression of osteoblastic markers can be induced in culture, in a variety of manners and in a variety of cells[10] that remain unable to make bone *in vivo*. The multipotency of MSCs derived from multiple human adult tissue sources is commonly but wrongly believed to be assayable by *in vitro* differentiation assays. It is generally assumed that MSCs, non-hematopoietic cells, isolated from multiple human adult tissues and expressing minimal criteria for defining human multipotent mesenchymal stromal cells[9], might be induced to proliferate extensively and forwarded to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* when appropriately stimulated[10-14]. However, there are many methods to induce an osteogenic phenotype *in vitro*[10]. These methods result in the generation of a tissue that does not have the structural organization of bone that is formed *in vivo*; in many cases, mineralization is due to dystrophic calcification in contrast with the true bone formation. Adipogenesis can also be induced *in vitro* in different culture conditions[10], but the adipocytes that are formed tend to be multivacuolar (immature), whereas mature adipocytes in marrow are univacuolar (mature)[15]. MSCs generate mature tissue *in vivo*, not in plastic. Therefore, many "mesenchymal" cell strains are able to "differentiation" as artificially assessed *in vitro,* however, remain unable to generate bone, cartilage or fat when implanted in conventional *in vivo* differentiation assays, resulting in the formation of fibrous tissue. Indeed, the skeletogenic capacity of a given cell strain is only proven by *in vivo* transplantation. The orthotopic bone formation assay (Figure 1B, a) is commonly used to study osteogenesis *in vivo*. For evaluation of *in vivo* osteogenic functionality of MSCs, cell-scaffold constructs are transplanted in femoral bone defects in immunodeficient mice (or rabbit)[16,17]. When cell-scaffold constructs are implanted in femoral critical size defect, we observe several weeks after transplantation, bone formation in place of fibrous tissue[18]. Compared to orthotopic assay, the ectopic bone forming assay (Figure 1B, b-c) has unique advantage since there is no requirement for bone cytokine stimulation and cell-to-cell interaction with endogenous bone-forming cells. Heterotopic subcutaneous implantation is the simplest experimental model of ectopic bone formation. However, a variety of ectopic locations can be used for cell implantation, including subcutaneous[19-21], intramuscular[22] sites and the kidney capsule[23-26]. Transplantation under the kidney capsule allows implanted MSCs to interact with a recipient environment that provides a rich vascular supply. Issues associated with skeletogenic potential of MSCs in kidney capsule transplants are that in addition to being a technically difficult surgical procedure, only small samples can be transplanted and studied, and the number of samples analyzed per recipient animal is limited. To overcome these limitations, more recent studies have utilized the ability of immunocompromised mice to accept cell transplants in a variety of anatomical sites that allow multiple transplantations[27]. The principal aim of these assays is to evaluate *in vivo* skeletogenic formation capacity of MSCs and follow the fate of implantation together with ceramic scaffold constructs [e.g., using hydroxyapatite/tricalcium phosphate (HA/TCP)] or as a cell pellet without a vehicle [e.g., growing cells as unmineralized pellets in chondrogenic differentiation medium] in immunocompromised host [5,27,28]. Indeed, for transplants, the use and nature of

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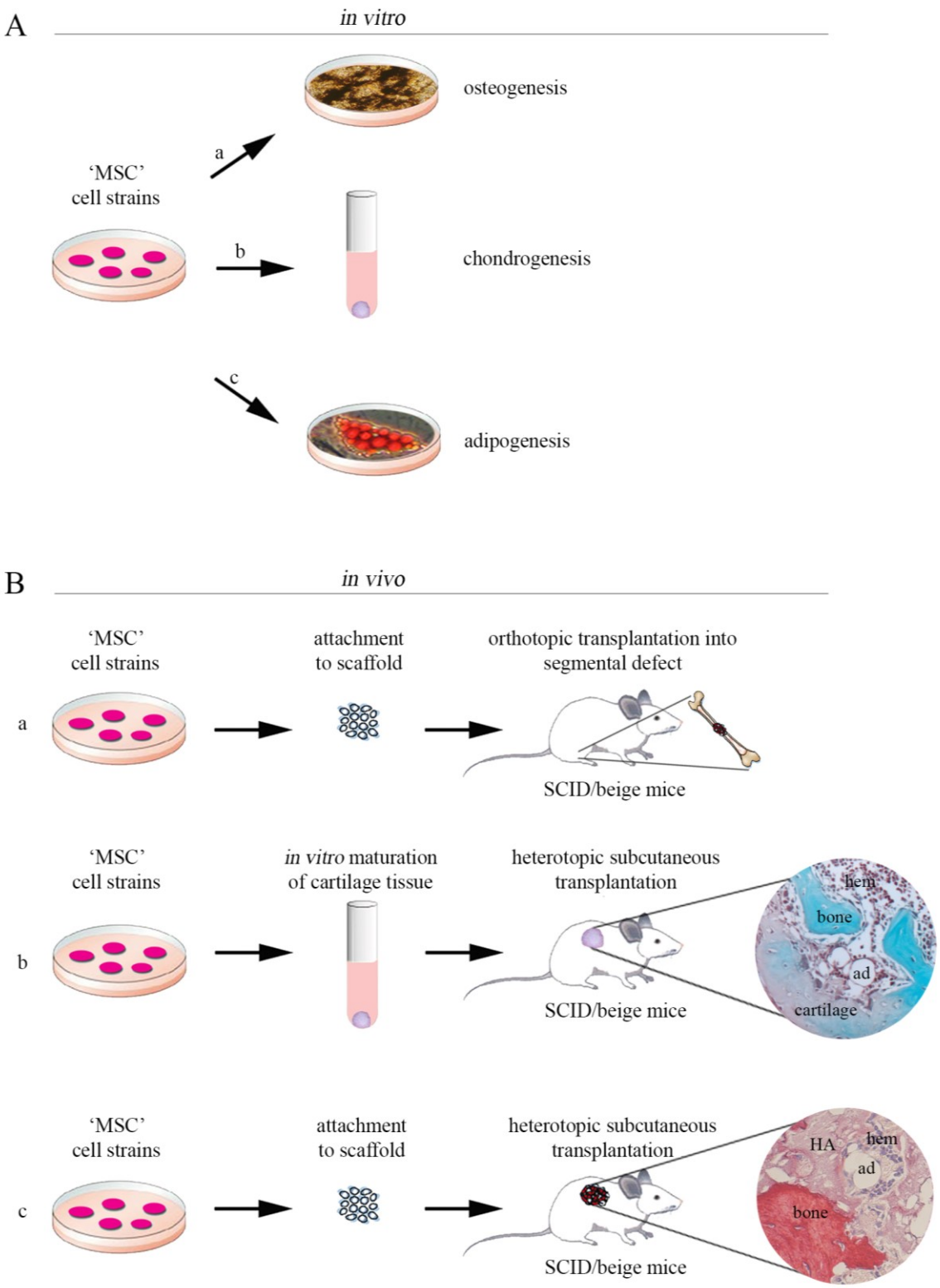

Figure 1. Skeletogenic potential of "mesenchymal"stem cells derived from different adult human tissues: diagram of *in vitro* assays and *in vivo* transplantation assay systems. A) Conventional *in vitro* skeletogenic differentiation assays are conducted using multiclonal MSC strains, derived from different tissues, which are first expanded on plastic, and then cultured/induced under osteogenic (a), chondrogenic (b) and adipogenic (c) conditions. B) Scheme of *in vivo* skeletogenic transplantation protocol. a) Orthotopic osteogenesis assay, *in vivo*, with scaffold. In this model, for investigating osteogenic differentiation *in vivo* at orthotopic sites, cells are loaded onto osteoconductive carriers and then cell/ceramic constructs are transplanted into bone defects mice with injured. b) Heterotopic subcutaneous osteogenesis assay, *in vivo*, without scaffold. In this model, for investigating osteogenic differentiation at heterotopic sites, cells are grown as unmineralized pellets in chondrogenic differentiation medium and then transplanted into the subcutaneous tissue of immunocompromised (SCID/beige) mice to generate heterotopic skeletal tissues; c) In an heterotopic subcutaneous osteogenesis transplantation assay, cell strains are grown in culture, loaded onto osteoconductive carriers [e.g. using hydroxyapatite/tricalcium phosphate (HA/TCP)] and then transplanted subcutaneously in the back of the immunocompromised SCID/beige mice. Sirius red stains bone intensely due its high collagen content (c). Safranin O stains (orange-violet) chondrocytes and cartilage due to their proteoglycan content and Fast green stains (green-blue) bone matrix (b). HA, hydroxyapatite; ad, adipocyte; hem, hematopoiesis.

transplantation substrate/vehicle are essential components for successful osteogenesis. Osteogenesis does not proceed when MSC suspensions are injected subcutaneously or intramuscularly, or when MSCs are implanted into rapidly resorbed vehicles[27]. Thus, in order to form bone, transplanted MSCs require the presence of an organized carrier in which they can adhere and proliferate for periods long enough to ensure differentiation and osteogenesis. Protocols for the isolation and culture of osteogenic progenitor cells, and the choice and design of osteoconductive carrier, represent the most important open questions in bone tissue engineering and preclinical studies. Of the mineralized vehicles used for MSC transplantation to date, hydroxyapatite-based constructs have been the most successful[27].

Conclusions

The basis for osteogenic properties of MSC population cannot be validated on the ability to develop biochemical or morphological markers of an osteoblast *in vitro*. Thus, the finding of alkaline phosphatase positive cells or even a mineralized matrix, particularly when culture cells are treated with potent inducer such as bone morphogenetic proteins (BMPs) or further cultured in osteoinductive medium supplemented with beta-glycerophosphate, dexamethasone and ascorbic acid[10], may not predict how MSCs will behave upon transplantation. While, *in vitro* assays have empirical but limited value, *in vivo* assays represent the gold standard toward definition of putative skeletal “mesenchymal” cells for showing true osteogenic potency of any tested cell population. Indeed, no identical post-natal MSCs-committed progenitors are incorporated in human connective tissues[28]; native skeletogenic potential is inherent to the system of progenitor/stem cells found in the skeleton. MSCs from BM are able to generate skeletal tissues, *in vivo* and spontaneously[4,5,28], with no pre-osteogenic induction in culture. Other MSCs derived from different tissues require reprogramming signals in order to acquire skeletogenic capacity[29]. MSCs from different post-natal tissues express the same cell surface markers[7,8], similar osteogenic differentiation properties *in vitro*[10] but different skeletogenic potential *in vivo*, as indicated using orthotopic and heterotopic *in vivo* transplantation assay systems[4,16-28], and this can be the reason for their different behavior in clinical applications. Therefore, *in vitro* studies of MSC osteogenic differentiation show several limitations. *In vitro* results need to be compared with *in vivo* pre-clinical studies in order to suggest the best MSC type for clinical skeletal regenerative therapies.

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Abbreviations

MSCs	: Mesenchymal Stem Cells
SSCs	: Skeletal Stem Cells
BMSCs	: Bone Marrow Stromal Cells
BM-MSCs	: MSCs from Bone Marrow
HA/TCP	: Hydroxyapatite/tricalcium phosphate
BMPs	: Bone morphogenetic proteins
ad	: Adipocyte
hem	: Hematopoiesis

Potential Conflicts of Interests

None

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