European Review for Medical and Pharmacological Sciences 2016; 20: 2884-2890

Amniotic fluid stem cells: an ideal resource for therapeutic application in bone tissue engineering

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Abstract. – OBJECTIVE: Skeletal diseases, both degenerative and secondary to trauma, infections or tumors, represent an ideal target for regenerative medicine and in the last years, stem cells have been considered as good candidates for *in vitro* and *in vivo* bone regeneration. To date, several stem cell sources, such as adult mesenchymal stem cells, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have shown significant osteogenic potential.

MATERIALS AND METHODS: In this narrative review, we analyze the possible advantages of the use of AFSCs in the treatment of skeletal diseases, especially through the application of tissue engineering and biomaterials.

RESULTS: Among the different sources of stem cells, great attention has been recently devoted to amniotic fluid-derived stem cells (AFSC) characterized by high renewal capacity and ability to differentiate along several different lineages.

CONCLUSIONS: Due to these features, AFSCs represent an interesting model for regenerative medicine, also considering their low immunogenicity and the absence of tumor formation after transplantation in nude mice.

Key Words:
Bone regeneration, Amniotic fluid, Amniotic fluid-derived mesenchymal stem cells, Biotechnology.

Introduction

Skeletal diseases, like critical bone defects due to trauma, infections or tumors, as well as atrophic nonunions and osteoporosis represent a major challenge for orthopaedic reconstructive surgeons, requiring a large amount of bone regeneration for their effective treatment and resolution[1-4]. A compromised regenerative process is observed also in other clinical conditions, like osteonecrosis of the femoral head, osteogenesis imperfecta and hypophosphatasia. The current cure for bone repair is autologous bone graft, but this approach is limited by non-structural integration of autologous fragments, and cell-based therapies may be particularly effective for the treatment in patients with reduced presence of endogenous stem or progenitor cells because of advanced age.

As a consequence, the treatment of traumatic and degenerative bone defects represents one of the main targets of regenerative medicine and tissue engineering, which are novel multidisciplinary approaches aimed to reconstruct tissues or organs in order to replace damaged and injured parts of the body. Since the pioneristic studies of Haynesworth et al[5], where the stem cells (Figure 1), in particular Bone Marrow-Mesenchymal Stem Cells (BM-MSCs), were differentiated in bone, cartilage and other musculoskeletal tissues, MSCs have been largely used with three-dimensional scaffolds in order to repair or replace missing or damaged tissues to a state as close as possible to their native architecture and function[6]. However, the use of adult BM shows some limitations, such as the low frequency of MSCs (about 0.001-0.01% of nucleated cells) and the invasivity of the BM harvesting from patients. Therefore, also different cell types, such as embryonic, fetal or adult stem cells, and genetically engineered cell lines, have been tested for their ability to replace damaged cells and to restore the tissue function after transplantation[7]. Embryonic Stem Cells (ESCs) are able to

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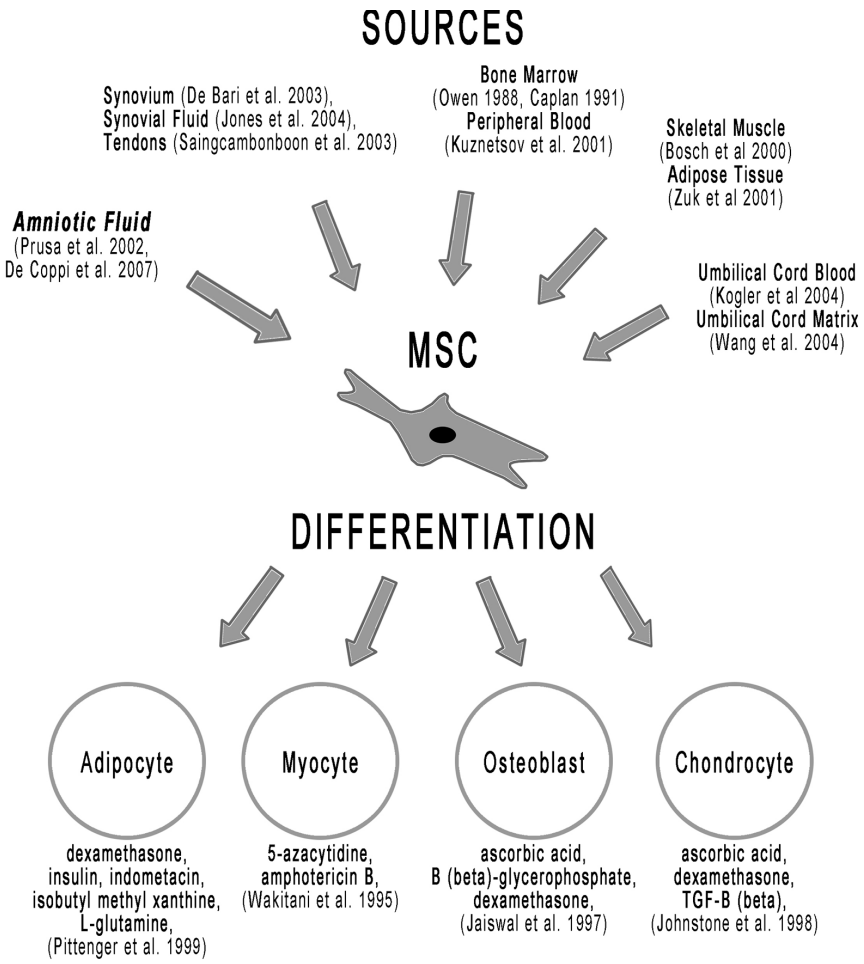

Figure 1. Sources and differentiation of MSCs.

differentiate in cells belonging to all the three embryonic layers, being thus able to produce virtually each different cell type. However, ESCs induce the formation of teratomas when injected in nude mice, and their use raises several ethical concerns. On the other hand, adult stem cells have been largely used in the clinical setting, despite their limited proliferation and differentiation ability. Autologous cells would represent the ideal transplantation source, being not rejected by the immune system and allowing to avoid the use of immunosuppressant drugs. However, these cells show limited "*ex vivo*" expansion abilities, particularly when collected from patients with end-stage organ disease[7]. The use of allogenic cells would be preferred in these cases, but this may require the creation of cell banks containing a large number of samples from different donors immunologically matched with the potential patients.

In recent years, different reports have demonstrated the presence in human amniotic fluid (AF) of stem cells (AFSCs) able to differentiate into multiple lineages and to produce cell types inclusive of all embryonic germ layers[8-11], suggesting the possible usefulness of these cells for therapeutic purposes[12-15].

Amniotic Fluid-Derived Stem Cells

Human AF samples can be easily obtained during the process of amniocentesis from women undergoing prenatal diagnosis (16^{th}-19^{th} week of pregnancy) and contains a heterogeneous population of cell types originating from embryonic and extra-embryonic tissues, classified as epithelioid (E-type) cells, amniotic fluid specific (AF-type) cells, and fibroblastic (F-type) cells[8-16]. For this reason, cell populations in AF show great diversity and variation among amniocentesis samples from different donors, time of gestation, and cultivation[17].

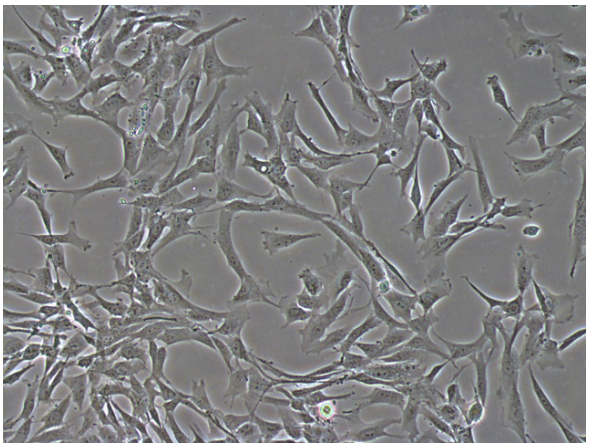

Figure 2. AFSCs of second passage.

About 1% of cells from AF (approximately 2.7 x 10^5 cells from each sample) are considered stem cells[18] and in the majority of studies so far reported, AFSCs (2) have been isolated using a selection based on the expression of the surface antigen c-Kit (CD117) present on ESCs, primordial germ cells and many somatic stem cells (c-Kit+ AFSCs)[9]. However, other authors[18] demonstrated that AFSCs can be obtained also by cultivating AF cells without any kind of previous selection (unselected AFSCs).

The interest raised by AFSCs is related to the presence of specific features making these cells different both from pluripotent ESCs and multipotent adult stem cells. About 90% of AFSCs express specific markers of ESCs, such as Oct-4 and TERT[19,20]. However, unlike ESCs, AFSCs are not tumorigenic after transplantation in mice. Thus, AFSCs represent a new class of stem cells with properties of plasticity intermediate between embryonic and adult stem cell types. In order to verify the presence of features of pluripotency in human second trimester AFSCs, Antonucci et al[21] have investigated the ability of these cells to form in vitro three-dimensional aggregates, known as embryoid bodies (EBs), and to express specific genes of embryonic stem cells (ESCs) and primordial germ cells (PGCs). EBs showed positivity for alkaline phosphatase (AP) staining and specific markers of pluripotency (OCT4, NANOG and SOX2) and the three germ layers. The most important result concerns the presence of specific markers of ESCs (such as FGF4 and DAPPA4), as well as markers typical of PGCs and, in particular, genes involved in early stages of germ cell development (Fragilis, Stella, Vasa, c-Kit, Rnf17). Finally, inactivation of the X-chromosome in female samples was also demonstrated. Taken together, these data suggest a germ cell origin of AFSCs. It has also been hypothesized that during early development, PGC-derived from epiblast could be the founder of these cells. Similar results were obtained by Moschidou's group[22], that confirmed the presence of cells of germ origin in human first-trimester AF and the same group suggested that also mid-trimester AFSCs c-KIT+, show features of pluripotency when cultured in appropriate media[23]. In last years, AFSCs have been used by several groups in preclinical studies to treat different pathological conditions such as ischemic brain injury with encouraging results[24-29]. In particular, the outcome of Tajiri et al studies[30,31] supported the idea that immunomodulatory and paracrine effects are predominant in the therapeutic properties of these cells. Finally, the discovery of several MSC-like cell in AF has allowed to isolate and select amniotic fluid mesenchymal stem cells (AFMSC) by means of the culture methodology[32]. AFMSC present multipotency, self-renewal, low immunogenicity, anti-inflammatory, non-tumorigenicity properties, have a normal karyotype and minimal ethical problem. Taken together, these cells may be appropriate sources of mesenchymal stem cells for regenerative medicine, as an alternative to embryonic stem cells (ESCs)[18,33-36].

AFS as available Source of Osteogenic Progenitor Cells

The ability of AFSCs to differentiate in osteogenic precursors has been demonstrated by several authors, and their functional activity was confirmed by the capacity to produce *in vivo* mineralized matrix and bone tissue[11,36-42]. The efficiency of this process has been demonstrated by clonogenic mineralization assays which have evidenced that 85% of AFSCs versus 50% of MSCs are capable of forming osteogenic colonies. Osteogenic differentiation of AFSCs can be improved through the use of specific culture protocols. Antonucci et al[39] have demonstrated that osteogenic differentiation of AFSCs can be obtained in a very short time through a single step protocol and after 30 days from the withdrawal of AF samples, osteoblasts progenitor display a complete expression of osteogenic markers (COL1, ONC, OPN, OCN, OPG, BSP, Runx2). Interestingly, differentiated AFSCs cells are able to growth on scaffolds and surfaces commonly used in orthopedic surgery, as evidenced by several reports; in particular, these cells

even after osteogenic differentiation maintain a good ability to proliferate on titanium screws[40] commonly used in orthopaedic implantology. These attractive results suggest their possible application in cell therapy of bone injuries[40]. Sun et al[43] demonstrated that osteoblastic differentiation of AFSCs and their induction to generate mineralized tissue can be obtained also by the use of culture protocols based on the use of bone morphogenic protein 7 (BMP-7). Compared to hMSCs, AFSCs showed a stronger responsiveness to BMP-7, in particular when cultured on synthetic NF scaffolds providing a more favorable microenvironment and enhancing their osteoblastic differentiation in vitro and bone formation *in vivo*[43]. In this view, the usefulness of the use of specific scaffolds during AFSCs culture to improve their ability to differentiate into osteogenic precursors has been clearly demonstrated also by Peister et al[44] which demonstrated that the "*in vitro*" pre-differentiation of AFSCs in porous medical-grade poly-e-caprolactone (mPCL) scaffolds produced seven times more mineralized matrix after subcutaneous in vivo implantation in a rat model. These results demonstrate the potential of these cells to produce 3D mineralized bioengineered constructs in vitro and in vivo suggesting their usefulness for functional repair of large bone defects. Recently, it has been studied calcium-sensing receptor (CaSR) expression in hAFMSCs and the activity of calcimimetic R-568 during in vitro osteogenesis. The results demonstrated that CaSR is expressed in these cells and positively correlates with osteogenic markers. This information is important because clarifies the mechanisms of hAFMSC osteogenesis and could provide additional molecular basis for the use of calcimimetics in bone regenerative medicine[45]. In addition, it has been observed that the canonical Wnt/ß-catenin signaling pathway appears to trigger huAFMSC osteoblastogenesis, since during early phases of osteogenic differentiation, the expression of Dishevelled-2 (Dvl-2), of the non-phosphorylated form of ß-catenin, and the phosphorylation of glycogen synthase kinase-3ß (GSK3ß) at serine 9 were upregulated. The modulation of Wnt signaling may represent a novel approach to direct cells towards a more definite differentiation. However, future studies are awaited to assess the validity of this strategy[46]. Finally, Morabito's group[47] studied the effects of calcitonin on huAFMSCs during osteogenic differentiation, in terms of the physiological role of calcitonin in bone homeostasis. The data obtained showed that calcitonin receptor was expressed in proliferating and osteo-differentiated cells and huAFMSCs could be considered a potential osteogenic model to study in-vitro cell responses to calcitonin (and other members of the calcitonin family).

AFS Based Therapies for Bone Tegeneration

Due to their excellent ability to differentiate into osteogenic precursors, AFSCs can be considered as a very promising tool in the area of bone regeneration. De Coppi et al[11] early demonstrated that, after subcutaneous implantation into immunodeficient mice of osteogenically differentiated AFSCs embedded in an alginate/collagen scaffold, it was possible to observe ectopic bone formation, suggesting that these cells could be used to engineer bone grafts for the repair of bone defects. A practical approach to the use of AFSCs for postnatal sternal repair has been reported by Steigman et al[48], who isolated rabbit AFSCs, cultured on biodegradable nanofibers and then implanted them into full-thickness sternal defects. Two months after implantation, *in vivo* imaging modalities confirmed chest closure and bone formation. This study concluded that engineered bone tissues can be a viable alternative for sternal repair and that AFSCs can be a practical cell source for engineered chest wall reconstruction.

The potential of AFSCs to synthesize mineralized extracellular matrix within different porous scaffolds of collagen, poly-D, L-lactic acid (PDLLA), and silk fibroin was investigated by Maraldi et al[49], who induced osteoblastic differentiation of these cells by using both two-dimensional cultures and three-dimensional scaffolds (collagen, fibroin, and PDLLA). The effect of AFSCs pre-differentiation in scaffolds on the subsequent bone formation in vivo was determined in a rat subcutaneous model, evidencing a higher osteogenic differentiation and mineralized extracellular matrix production. Authors suggested that fibroin may represent an effective scaffold material for functional repair of critical size bone defects. Rodrigues et al[50] investigated whether the differentiation stage of AFSC could improve bone regeneration in a rat model of critical sized femoral defect. AFSCs were seeded onto a starch-poly (ε-caprolactone) (SPCL) scaffold and cultured in order to obtain undifferentiated cells, cells committed to the osteogenic phenotype (2 weeks of culture) and "osteoblast-like" cells (3 weeks of culture). Constructs composed of AFSC-SPCL

scaffolds from each differentiation stage were then implanted into critical sized femoral defects, and the formation of new bone was examined by micro-CT imaging and histological analysis of constructs retrieved at 4 and 16 weeks after implantation. The best results in terms of complete repair of the defect were showed by animals treated with SPCL scaffolds seeded with osteogenically committed AFSC, in which it was possible to evidence the presence of blood vessels in the inner sections of the scaffolds, suggesting the potential of differentiated AFSCs inducing bone regeneration and angiogenesis in non-union bone defects[50]. A further evidence of the usefulness of AFSCs in regenerative medicine has been provided by Turner et al[51] in a study carried out on New Zealand rabbits in which a full-thickness diploic nasal bone defect had been induced. Animals were treated with size-matched implants of electrospun biodegradable nanofibers with or without allogeneic AFSs cultivated in osteogenic medium. Post-mortem analysis evidenced similar levels of defect radiodensity in the two groups, but extracellular calcium levels were significantly higher in engineered grafts than in acellular implants. Authors concluded that AF derived engineered bone could represent a useful tool in perinatal craniofacial reconstruction.

Conclusions

The use of adult stem cells in regenerative medicine may alleviate ethical and availability concerns, with the additional advantages, in some cases, to allow autologous grafts. The demonstrated presence of stem cells within AF have raised great interest due to the large accessibility of these cells by means of routine amniocentesis, their ability to differentiate in several cell lineages, the absence of tumorigenicity after transplantation and the lack of ethical problems related to their use. Compared to embryonic stem cells, amniotic stem cells can be obtained without destroying human embryos, thus solving much of the ethical controversy. Stem cells from AF could be useful both for personalized cells supply for newly born children and for banking cells to be used for therapeutic cell transplantation in immunologically matched recipients. As reported above, AFSCS have demonstrated to be able to differentiate into osteogenic precursors under different culture conditions, and to produce mineral matrix even after in vitro transplantation. However, also other specific features of AFSCs suggest that these cells could represent in a next future the gold standard for regenerative medicine in the orthopaedic field. In fact, a further element supporting the usefulness of AFSCs for allogenic transplantation is provided by their low immunogenicity, since only a small fraction of these cells are slightly positive for antigens HLA-DR (MHC class II). AFSCs are resistant to rejection due to the expression of immunosuppressive factors such as CD59 (protectin), which inhibits the complement membrane attack complex and prevent complement from damaging cells, and HLA-G, which plays a key role in immune tolerance in pregnancy[52]. Moreover, AFSCs have been demonstrated as able to inhibit the proliferation of T lymphocytes[53]. As a consequence, AFSCs offer an advantage over cells derived from other sources, being able to survive after allogeneic transplant without using immunosuppressive therapy. Moreover, due to these specific features, low cost protocols to isolate AFSCs could be established, in order to create banks containing all MHC immunotypes, which could be used for allogenic clinical applications in the orthopaedic field.

Conflicts of interest

The authors declare that they have no conflicts of interest concerning this article. No financial support has been received by the authors for the preparation of this manuscript.

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