World Journal of Orthopedics

Submit a Manuscript: http://www.wjgnet.com/esps/

World J Orthop 2017 February 18; 8(2): 87-98

DOI: 10.5312/wjo.v8.i2.87

ISSN 2218-5836 (online)

EDITORIAL

Challenges of bone tissue engineering in orthopaedic patients

Enrique Guerado, Enrique Caso

Enrique Guerado, Department of Orthopaedic Surgery and Traumatology, Hospital Costa del Sol, University of Malaga, 29603 Marbella (Malaga), Spain

Enrique Caso, Research Unit, Hospital Costa del Sol, University of Malaga, 29603 Marbella (Malaga), Spain

Author contributions: Guerado E was the main author of the Introduction and the sections on RIA, the Masquelet technique and bone transportation; Caso E wrote the sections on combined stem cells therapy, tissue engineering and future directions; both authors jointly reviewed, corrected and edited the paper.

Conflict-of-interest statement: There is no conflict of interest to declare that could provoke any potential bias in the study design, the interpretation of the results obtained or the presentation of the scientific/medical content, including but not limited to commercial, personal, political, intellectual or religious interests.

Manuscript source: Invited manuscript

Correspondence to: Enrique Guerado, Professor and Chairman, Department of Orthopaedic Surgery and Traumatology, Hospital Costa del Sol, University of Malaga, Autovia A-7, Km 187, 29603 Marbella (Malaga), Spain. eguerado@hcs.es
Telephone: +34-951-976224

Received: September 14, 2016
Peer-review started: September 19, 2016
First decision: October 21, 2016
Revised: October 31, 2016
Accepted: November 21, 2016
Article in press: November 23, 2016
Published online: February 18, 2017

Abstract

Bone defects may impede normal biomechanics and the structural stability of bone as an organ. In many cases, the correction of bone defects requires extensive surgical intervention involving the use of bone-grafting techniques and other procedures in which healing is slow, there is a high risk of infection and considerable pain is provoked - with no guarantee of complete correction of the defect. Therefore, the search for surgical alternatives continues to present a major challenge in orthopaedic traumatology. The reamer-irrigator-aspirator (RIA) system, which was devised to avoid the problems that can arise with autograft harvesting from the iliac crest, consists of collecting the product of the femoral canal after reaming. The RIA technique improves osteogenic differentiation of mesenchymal stem cells, compared to bone marrow aspiration or cancellous bone harvesting from the iliac crest using a spoon. Another approach, the Masquelet technique, consists of reconstructing a long bone defect by means of an induced membrane grown onto an acrylic cement rod inserted to fill the defect; in a second surgical step, once the membrane is constituted, the cement rod is removed and cancellous autograft is used to fill the defect. Both in RIA and in the Masquelet technique, osteosynthesis is usually needed. Bone transportation by compression-distraction lengthening principles is commonly implemented for the treatment of large bone loss. However, complications are frequently encountered with these techniques. Among new techniques that have been proposed to address the problem of large bone loss, the application of stem cells in conjunction with tissue engineering techniques is very promising, as is the creation of personalised medicine (or precision medicine), in which molecular profiling technologies are used to tailor the therapeutic strategy, to ensure the right method is applied for the right person at the right time, after determining the predisposition to disease among the general population. All of the above techniques for addressing bone defects are discussed in this paper.

Key words: Bone loss; Mesenchymal stem cells; Reamer-irrigator-aspirator; Autograft; Personalised medicine; Bone transportation; Precision medicine; Masquelet technique

Core tip: This paper discusses the problems created by large bone loss, especially after major trauma, and considers current alternatives to autograft or allograft, such as the reamer-irrigator-aspirator system, the Masquelet technique, bone transportation, or the combination of stem cell therapy and tissue engineering. Future Directions addressed mainly concern the new concepts of personalised medicine and precise medicine.

Guerado E, Caso E. Challenges of bone tissue engineering in orthopaedic patients. *World J Orthop* 2017; 8(2): 87-98 Available from: URL: http://www.wjgnet.com/2218-5836/full/v8/i2/87.htm DOI: http://dx.doi.org/10.5312/wjo.v8.i2.87

INTRODUCTION

High-speed traffic accidents and injuries in the workplace continue to present major orthopaedic trauma challenges, often requiring tissue reconstruction. Worldwide, more than 4.5 million reconstructive surgical procedures are performed annually, in response to accidents, cancer surgery or cosmetic needs. In many countries, too, victims of war or civil conflict must receive complex reconstructive surgery to overcome large tissue losses[1]. Although progress has been made to reduce the incidence of such events (for example, through legislation and improved road safety), orthopaedic procedures for the treatment of large bone loss have not achieved such tangible improvements.

Bone defects can be classified into two different groups: Cavity defects, when the loss does not affect limb biomechanics but nevertheless interferes with osteosynthesis or arthroplasty implantation; and segmental defects, when normal biomechanics are impeded and the structural stability of the bone as an organ may be endangered[2,3]. Reparative surgery is still the treatment of choice for these lesions, and autologous bone grafting is considered the gold standard approach in the clinical setting, in order to harness bone's natural regenerative capacity when a bone defect occurs. Large bone losses, however, are best treated by allograft, despite its less osteogenic nature. In many cases, the correction of bone defects requires extensive surgical intervention using bone-grafting techniques. Numerous surgical procedures may be needed, involving long healing times. These fairly aggressive surgical techniques can produce a high risk of infection; moreover, they provoke substantial pain and do not guarantee complete correction of the defect. The emotional impact on the patient and financial burdens on the healthcare system are further problematic issues. Moreover, substantial donor-site morbidity and limitations on the quantity of bone that can be harvested proscribe its application when large bone loss occurs. In view of these considerations, alternatives to autograft for reconstructive surgery in large bone defects continue to be sought in orthopaedic traumatology.

Cavity defects can be resolved by the application of bone autograft, morselized allograft or bone substitutes. When cavity defects are not too large, they can be treated relatively straightforwardly, and alternative approaches such as combining cavity filling with implants are usually possible. On the other hand, cavity defects - and segmental bone defects in particular - can present major problems. This kind of lesion is often provoked by high-energy trauma, as a result of which the soft tissues are severely affected. Segmental defects may provoke major functional disability and even require amputation. The pelvis is often affected in patients with long-term arthroplasty loosening and also after bone tumour resection, whereas the femur and the tibia are commonly injured by severe trauma. In addition, the long bones of the upper limbs are frequently affected in serious accidents.

In this respect, many surgical techniques have been proposed, and some success has been obtained in treating relatively minor injuries. However, they have proved less effectiveness against large tissue lesions following high-energy trauma. When a large bone defect is experienced, the treatment challenge is twofold. On the one hand, since bone cannot remain uncovered, by skin or muscle, the absence of soft tissue cover will provoke necrosis and the non-viability of any therapeutic attempt; this is very commonly the case with injuries affecting the tibia, when anterior muscle cover is absent or insufficient. Furthermore, even well-covered bone will also need suitable vascularisation of an appropriately-sized, strong graft; otherwise, bone healing will never take place. Apart from these soft tissue and bone problems, function can be severely affected by lesions to tendons and nerves. Therefore, graft size and the vascularisation of bone implantation are of crucial importance for tissue viability, tendon function and nerve physiology.

Current therapies in this field have been developed over many years. The reimplantation of extruded bone segments is uncommon, due to worries about infection and unclear guidelines regarding timing, stabilisation and sterilisation techniques, which have led this procedure to be rejected by the majority of surgeons. The few papers that have been published in this respect have encountered great difficulties in reaching useful conclusions[4]. Another approach is that of autograft harvested from the iliac crest, followed by vascularised autograft - however, the thin shape of this autograft makes it less useful in cases of large bone loss. Bone transportation procedures have also been suggested, together with the induced membrane Masquelet technique, with the creation of an artificial in-situ chamber, after inserting a temporary cement spacer

which will eventually be surrounded by a periostium-like layer. These therapies have been complemented by growth factors - including platelet-derived growth factors and bone morphogenetic proteins (BMPs) - and cell therapies. Synthetic bone has also been included in compound approaches, in a quasi-random combination involving chance as much as science[5].

All of these research lines have sought to focus on the keystone of bone synthesis: Matrix-forming cells. However, although the results of these new therapies are always said to be "promising", they still cannot be managed precisely or combined appropriately with osteosynthesis fixation.

At present, achieving a biomechanically strong, well-vascularised and physiologically-functional bone from the treatment of segmental bone defects continues to pose a major challenge.

Tissue engineering (TE) is a promising technology for secondary reconstruction after severe trauma. TE is an interdisciplinary science combining cellular, engineering, biochemical and physicochemical factors to improve or replace biological functions[6-8], either in combination with, or independently of, an osteosynthesis technique. Different types of cells and bioactive factors have been shown to play an important role during this regeneration. The ideal biomaterial is currently believed to comprise a porous three-dimensional scaffold with patterned substrates, offering vascularisation and regeneration properties[9]. However, the biochemical mediators of this process are imperfectly understood, and their biochemical properties and the sequence in which they act remain to be clarified.

In any case, creating tissue is an unavoidable necessity, as large bone loss cannot be repaired by an *in vivo* physiological mechanism. Whether TE will eventually be capable of replacing normal biological mechanisms has yet to be determined.

BMPs are known to promote cell multiplication and differentiation, but not sufficiently as to provide an alternative to currently-available therapies. Moreover, the sequence pathways of the different molecules remain unknown. Cell therapy, as currently applied, involves three sequential steps: *In vivo* extraction, *ex vivo* manipulation and *in vivo* implantation. After this long and complex procedure, the outcome is still uncertain, especially for large bone defects.

In view of these considerations, the following techniques have been proposed, incorporating the knowledge accumulated from cell therapy principles.

REAMER-IRRIGATOR-ASPIRATOR

The reamer-irrigator-aspirator (RIA) technique is designed to avoid the problems that arise with autograft harvesting from the iliac crest, and consists of collecting the product of the femoral canal after reaming[10-13]. The cells thus collected and cultured present the same properties as those from the iliac crest[14-17]. Studies have shown there are no phenotypical differences between mesenchymal stem cells (MSCs) collected from the pelvic bone and RIA, and that the gene expression alteration found in RIA can be owned to the isolation technique employed[18]. Cell characterisation is similar for adipose-MSCs, bone marrow-MSCs and RIA-MSCs, and the osteogenic potential is similar with *in vitro* and *in vivo* approaches[19,20].

The RIA technique enhances the osteogenic differentiation of MSCs, in comparison with bone marrow aspiration or cancellous bone harvesting with a spoon from the iliac crest[17,18]. A recent study[18] compared harvesting by RIA with iliac crest aspiration and collection with a spoon, and reported that a greater concentration of colony-forming unit-fibroblasts of MSCs was obtained by RIA. Better results were also obtained by RIA for calcium tissue fixation as well as the gene expression of BMP2, SMAD5, runt-related transcription factor 2, osteocalcin and collagen type I alpha 1. Calcium fixation and osteogenic gene expression diminished considerably with higher passage numbers, in every specimen. The authors concluded that the harvesting procedure is critical for MSC differentiation *in vitro*. On the other hand, the CD271 selection of MSCs in RIA also produces a significant rise in MSC pureness and an increase expression of the transcripts implicated in bone synthesis, vessels formation and chemical attraction[20].

Revascularisation takes place within three months of reaming, and bone thickness restoration of the cortex appears normal after 14 mo, allowing the opportunity for further reaming[21].

Although RIA has achieved very promising results with respect to cavity defects, this technique is less useful for segmental ones, for which osteosynthesis supplementation is required. Furthermore, complications can arise in relation to the learning curve, to over-reaming and, in some patients, to cardiac problems produced by rapid blood loss; the latter complication is closely related to previous cardiopathy[22,23].

RIA produces less pressure than intramedullary reaming and nailing, and a lower incidence of micro-embolism, according to studies of animals[24,25] and of humans[26]. However, one clinical study reported different findings from those obtained in animal experimentation, observing no differences in healing complications between intramedullary reaming and RIA, although there was a statistically non-significant tendency for the RIA group to present more complications[27].

Both in conventional reaming for intramedullary nailing and in RIA, the coagulation and fybrinolytic response consists of higher cytokine levels, together with increased IL-6 levels, particularly in intramedullary reaming[28]. However, other authors found no differences between these groups in relation to complications and IL-6 levels[29]. In a biomechanical study of cadavers, under ideal conditions, it was found that RIA did not greatly reduce femoral cortical strength but that careful attention was needed to avoid the catastrophic failure that can occur using this eccentric reamer[30]. In fact, femoral fracture can occur[31], and complications have been reported to affect

31% of cases, including postoperative pain, bone defects, lung embolism, myocardial infarction and iatrogenic fracture[32].

RIA has similar outcomes among all human races[13], and can be performed either antegradely or retrogradely[33,34].

MASQUELET TECHNIQUE

In the original Masquelet technique, a long bone defect is reconstructed by an induced membrane grown onto an acrylic cement rod inserted to fill the defect; in a second surgical step, once the membrane is constituted, the cement rod is withdrawn and the gap is filled with cancellous autograft[35]. In a modification of this technique for tibial fractures, new surgical steps were added, such as the transfer of the soleus muscle island flap, vascularised with retrograde flow on the posterior tibial artery[36]. Further research, on large animals, has shown that the membrane compartmentalises the bone defect, protecting it from the humoral and cellular environment of the muscular layer[37]. The Masquelet technique has become increasingly popular in recent years for the treatment of large bone defects[38]. Good results have been achieved in a large-scale study of bone defects in which autograft harvesting from the iliac crest was replaced by RIA[39], with reduced morbidity in the second step of graft collection.

New research into the Masquelet technique has been conducted in animal studies, but the cell biology of animals is radically different from that of humans[40-43]. In this respect, the paper by Aho *et al*[44] is particularly significant because these authors histologically characterised the induced membrane in humans, finding that greatest vascularisation took place in 30 d old specimens, and that levels diminished by sixty per cent during the following ninety days. Thirty day-old membranes presented the highest expression of vascular endothelial growth factor, interleukin 6 and collagen 1, while sixty day-old membranes expressed less than 40% of these levels. Specific alkaline phosphatase activity, the production of aminoterminal propeptide of type-Ⅰ procollagen and calcium concentration all increased in co-cultures in the presence of a membrane sample. Furthermore, in thirty day-old cultures membranes, the formation of aminoterminal propeptide of type-Ⅰ procollagen was more than twice as high, and calcium fixation was four hundred per cent greater, than in cultures of sixty day-old membranes. The authors concluded that induced membranes present osteogenesis-improving competences but that outcomes gradually worsen, and that the ideal period for carrying out the second step operation is before the second month following the implantation of foreign material[44].

Although many studies have been conducted since the Masquelet technique was first presented in 2000, it is still difficult to predict the outcome of this approach to bone defect reconstruction, as complications are likely among most patients; at the outset, the surgical field is not optimal and the course of reconstruction is long and difficult[45].

Refinements of the Masquelet technique have recently been published by the original authors[46], and further research has been carried out on the basic science for human patients, with multicentre recruitment[47]. It has been shown that effective osseous formation *via* the Masquelet technique only incompletely emulates the cytokine expression of normal biological bone regeneration[47]. Abundant expressions of insulin-like growth factor 1 are associated with successful Masquelet therapy, whereas transforming growth factor β appears to have low contribution. Consequently, the appropriate examination of a successful non-union treatment and of cytokine expression can be made even with a lesser number of cases. Therefore, further research in this field should be aimed at finding a method, based on a small population of patients, for predicting the success or otherwise of treatments for bone loss defects, including the Masquelet technique.

BONE TRANSPORTATION

In 1969, a paper appeared in MEDLINE on the Ilizarov technique aimed to treat "long tubular bones defects by means of one of their fragments"[48]. However, it was published only in Russian and had little impact in Western orthopaedic science. Four years later, an Australian nursing journal published a paper by another Russian author on the Ilizarov technique[49], and during the 1970s more papers appeared on the biomechanics of the Ilizarov apparatus[50]. However, it did not become known worldwide until the 1980s, when Italian authors gave it major prominence[51,52]. The approach described by Ilizarov was more than a single apparatus or technique; it became a new paradigm of the cell biology of bone regeneration, and was amply referred to as such in Russian publications during this decade[53]. By creating a fracture only in the bone cortex ("bone corticotomy"), thus minimising surgical trauma, a callus consolidation process is triggered, and then maintained by means of immobilisation for 7-10 d. Thereafter, continuous slight distraction of less than 1 mm/d is exerted, and over time the gap becomes filled in.

Since its introduction, the Ilizarov technique, with its associated compression-distraction lengthening principles for the treatment of large bone loss, has been applied worldwide, and is now known as bone transportation. It has been shown that during this bone lengthening, the soft tissues also undergo stretching and subsequent physiological metaplasia[54].

However, from the outset it has been apparent that the results obtained with the Ilizarov technique are excellent in some cases, good in others, and only fair in many. Consequences such as persistent infection, deformity, limb shortening, resultant limping, impacts on other joints (for example, equinus), dystrophy and severe pain have led some patients to request amputation. The duration of this treatment and its many negative consequences discourage many therapists from considering bone

transportation in patients with severe osseous loss, and these complex cases are often referred to specialised centres[55]. Outcomes are also compromised by variables such as age older than 20 years, a larger gap magnitude, and diaphyseal rather than metaphyseal loss location[56].

More is now known about the biology underlying Ilizarov bone transportation, and greater experience and better fixators have enabled surgeons to better apply this technique. In addition, new approaches have been tested, mainly in animal experimentation, combining Ilizarov's principles with TE[57-63]. Nevertheless, the technique is still subject to complications and cannot systematically ensure a satisfactory outcome following large bone loss.

COMBINED STEM CELL THERAPY AND TE

Along the last decade, the combined treatment with immature MSCs and growth factors has been considered another promising therapy for bone synthesis. Nonetheless several terminally-differentiated cell lines (keratinocytes, osteoblasts, fibroblasts, osteocytes, chondrocytes and hepatocytes) cannot be used for artificial tissue constructs. Stem cell candidates to build artificial tissues comprise embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and postnatal adult stem cells[64,65]. There are still some limitations to the practical use of ESCs and iPSCs, including the cytogenetic regulation of teratoma development, ethical issues, immune uncertainties in relation to ESCs, and the requirements for genetic manipulation of iPSCs. Multipotent MSCs derived from postnatal adult stem cells (Wharton's jelly cells, adipose tissue, bone marrow and dental pulp) are potentially useful because of their immunocompatibility and the absence of ethical concerns. Bone marrow (BM) and adipose tissue are also good sources of stem cells for clinical use[66,67]. MSCs are cells of mesodermal derivation - different from the hematopoietic linage- existing in various infant and adult organs and conjunctive tissues. Pluripotent MSCs in the BM stromal tissue are capable of differentiating to multiple mesenchymal lines, including osseous and chondral cells. Therefore, it follows that these MSCs could be employed in the restoration of large bone loss caused by traumatisms, surgical procedures or maladies. MSCs from tissue sources such as human dental pulp, exfoliated deciduous teeth (SHED) and periodontal ligaments have similar characteristics to BM-MSCs but are commonly liable to problems such as a short collection of cells and a reduced quantity of collected tissues[68,69]. Other significant drawbacks to the use of MSC in tissue repair include, firstly, the ache and problems associated with BM collection and, secondly, the low income (1 MSC/10^4-10^6 stromal cells), which makes *ex vivo* amplification a necessity[70-72].

The adipose compartment appears to have a rich population of stem cells and, like BM, has a large cellular stroma, constituted of fibroblastic-like cells (the stromal vascular fraction - SVF). This cell segment, obtained from human aspiration of fat, in turn has cells with multiline capabilities, called adipose stem cells (ASCs), which experience adipogenesis, osteogenesis, chondrogenesis and myogenesis *in vitro*. Some experiments have started to study the osteogenic potential of ASCs *in vivo*, in amalgamation with a great diversity of scaffolding materials[73]. The use of human ASCs (hASCs) in scaffolds for osseous TE has been indicated as the alternative approach of the current century to substitute or repair the normal physiology of traumatised, injured or lost bone. The biological relationship between osteoblasts and adipocytes is reflected in their common MSC origin. The accumulation of marrow adipocytes in bone loss may be caused by a shift in the commitment of MSCs from the osteogenic to the adipogenic pathway. hASCs have several characteristics that make them compatible with currently-available strategies for creating new tissue, including cell transfer, induction and the generation of tissue constructs. The MSCs located within adipose tissue are effortlessly harvested in wide amounts, with slight donor site injury or general alterations. Furthermore, human adipose tissue is ubiquitous. Subcutaneous fat tissue fragments can commonly be obtained without general or regional anaesthesia. Present techniques for extracting ASCs are based on collagenase proteolysis after which centrifugal isolation of the SVF from primary adipocytes[74] is performed. Among other features, ASCs present a fibroblast-like phenotype and lack the intercellular lipid precipitations observed in adipocytes[75].

The proliferation capability of ASCs appears to be superior than that of BM-derived MSCs. Studies have revealed that the doubling times of ASCs along the logarithmic phase of growth range between 40 to 120 h, and it changes according to donor age, the nature of fat tissue (white or brown), its placement (subcutaneous or visceral), the harvesting procedure employed, the culture circumstances, the plating concentration and media preparations[76]. Younger donors, have superior proliferation and cell adhesiveness of the ASCs. Cells progressively miss their multiplication capability with passaging. According to the β-galactosidase action, senescence in ASCs is comparable to that seen in BM-derived MSCs. The multiplication of ASCs can be encouraged by a solitary growth factor such as fibroblast growth factors (FGF)-2, EGF, insulin-like growth factor (IGF)-1 or tumor necrosis factor (TNF)-α. FGF-2, in particular, is an effective growth-stimulating factor that is needed for the long-term proliferation and self-renewal of ASCs *via* the extracellular signal-related kinase (ERK) 1/2 signalling pathway[77]. The multiplication of ASCs can likewise be activated by platelet-derived growth factor *via* c-Jun amino-terminal kinase (JNK) activation and by oncostatin M *via* activation of the microtubule-associated protein kinase/ERK and the JAK3/STAT1 pathways. ASC multiplication has also been published to be enhanced by numerous growth factors, which can contain any of the particular growth factors formerly mentioned, complemented by thrombin-activated platelet-rich

plasma, human platelet lysate and human thrombin[78].

ASCs have the capability to differentiate toward a diversity of cell lines, both *in vitro* and *in vivo*. Though ASCs are of mesodermal origin, it is now well known that they can commit themselves into ectoderm and endoderm, as well as mesoderm, lineage cells[79]. Concerning differentiation into cells of the mesodermal line and the regeneration of mesodermal tissues, ASCs may differentiate into adipogenic, osteogenic, chondrogenic, myogenic, cardiomyogenic, angiogenic, tenogenic and periodontogenic lineages. Very little is known about how cell differentiation is affected by aging.

When used combined with a carrying scaffold, the directed osteogenesis of hASCs confirms that adipose tissue is a hopeful autologous font of osteoblastic cells for bone production. This approach provides support for hASC colonisation, migration, growth and differentiation. Few descriptions have been made of purified hASCs in bone engineering, and varying degrees of success have been reported[80-87]. It has not been reported whether cellular free scaffold controls immersed in an osteogenic medium are also capable of achieving bone healing, to any degree[88,89]. Nevertheless, the use of autologous hASCs, managed in the absence of animal-derived materials, following appropriate work in standard unpolluted places, has shown that these cells can be considered safe for uses in tissue engineering, according to European Union standards for clinical cell therapy safety.

Current limitations of hASC for bone TE include the following issues: (1) transitioning from preclinical *in vivo* models to the clinical setting signifies a foremost stride; (2) appropriate serum-free media for these cells must be developed, as foetal bovine serum (FBS) is not suggested for clinical treatments, ought to contamination and infection risk; (3) the *ex vivo* multiplication of cells for two or three weeks renders them vulnerable to possible genomic unpredictability in culture; and (4) appliances that would allow sole-step recruitment, manipulation and grafting are consequently required, to avoid the necessity for cell culture and the associated hazards of utilizing FBS.

Among the challenges to be addressed in hASC bone tissue-engineering for clinical applications, it should be emphasised that the main aim of the ASC TE strategy is to define the real osteogenic capability of ASCs independently of their association with growth factors. Further key challenges to be addressed include the standardising of techniques for recruitment, separating, cultivating and managing hASCs and the publication of procedures for the correct utilisation of carrier materials. Moreover, prospective randomised clinical trials should be conducted to categorize appropriate suggestions for hASC therapies and to validate the clinical results thereby achieved. Finally, ethical and security worries must be determined previous to human use, as the first step in new scaffold usage[90].

As yet, there is little consensus regarding the efficacy of cell-based therapies in skeletal regeneration, or the most effective cell origin type, number, combination or method of delivery[91]. However, better regeneration results have been observed when cells are administered intravenously, subcutaneously or directly to the defect[92-96]. Bone cell progenitors provide bone with its distinctive capacity for repair and regeneration[97], and so their inclusion within a carrier is favoured by most surgeons. Nevertheless, the results obtained in this respect during the last 20 years have been only "promising". Experimental delayed-injection models utilising BM stromal cells have been shown to enhance the repair of injured tissue in relation to "time-of-trauma" cell uses. Time is allowed to elapse between the lesion/bone loss and the injection in order to avoid the early stages of tissue lesions, when the release of cytomodulatory peptides - including TNF-α, interleukins and interferons - and increased concentrations of acute-phase protein in serum appear to diminish the efficacy of stem and precursor populations. Although studies based on experimental spatiotemporal manipulation of cell delivery after the acute inflammatory response have achieved promising results in the field of segmental osseous tissue production[92], it remains apparent that the media and moment of cell delivery significantly influence therapy effectiveness[98].

FUTURE DIRECTIONS

The following main principles of tissue-engineering application in humans are generally accepted: (1) The manipulation of human stem cells for clinical treatment has to be carried out rendering upright laboratory techniques and the guidelines of the Food and Drugs Administration (in United States) or the European Medicines Agency[99]. In this respect, the standardisation of separation and culture processes might raise quality regulations; (2) TE constructs must be considered as medicinal products and their intended use for clinical investigation purposes are subject to European regulations for clinical trials of medical devices and advanced therapies[100]; and (3) Engineered tissue must be structurally and functionally comparable to natural tissue, be of the required size and shape, be able to continue developing after implantation into the body and be able to achieve full integration with the host.

Three components are usually necessary in TE: Cells, extracellular matrices and growth factors to provide molecular signals. The extracellular matrix-scaffold construction is a crucial aspect of bone defect repairing. Recent advances in TE have made available a large number of materials suitable for healing of bone defects and lost bone. Both *in vitro* and *in vivo* formation of bone tissue, using MSCs and 3D scaffolds has been shown[101]. Several scaffolds such as HA/chitosan composites, chitosan or gelatin/TCP constructs, electrospun collagen nanofibres, honeycomb collagen scaffolds and titanium meshes have been used with MSCs. Newly designed scaffolds, resembling the effect of growth factors on adhesion-based mechanisms, need to be further implemented by analyses of the specific "pro-osteogenic" signal

transduction pathways. Osteogenic differentiation relays on cell adhesion and the substrate interaction, which are under the control of integrin complexes interactions. Integrin-matrix interactions can induce numerous signalling pathways, including the MAPK cascade. Although few studies with hASCs have been published, their results show that alternative methods for growth factor stimulation may be fostered to induce hASCs to make and heal bone[102,103].

Regarding signalling systems, it has been suggested that soluble factors produced by ASCs (secretome) are the responsible for the potential clinical impact on different organs/tissues instead of the differentiation capability of hASCs[104]. Analyses from primary hASCs cultures have shown the release of a large series of soluble factors including growth factors such as HGF, VEGF, β-TGF, IGF-1, bFGF, GM-CSF, TNF-α, interleukins (6, 7, 8 and 11), adiponectin, angiotensin, cathepsin D, pentraxin, pregnancy zone protein, retinol-binding protein and CXCL12)[105]. Indeed, HGF expression is increased after the cells have been exposed to bFGF, EGF or ascorbic acid, reinforcing the idea that soluble factors secreted by ASCs can be modulated by exposure to different agents. Thus, transplanted hASCs into inflammatory or ischaemic regions, actively secrete these growth factors, becomes a relevant strategy to promote wound healing and tissue repair. As mentioned previously, the increased bone formation attributed to BMP2-treated ASCs is derived from the osteoconductive and osteoinductive effects of BMP2 or from the ASCs themselves, although this remains to be demonstrated by means of appropriate controls.

Improving the ability of hASCs to generate large quantities of bone to repair bone defect without growth factors represents a major challenge. For that reason signal transduction pathways in adult ASCs need to be explored. Osteogenesis induced by hASCs might employ an alternate signalling pathway for adipogenic and osteogenic fates. Moreover, directed manipulation of downstream signalling paths rather upstream growth factors might be also responsible for stem cell-directed bone regeneration. In this respect, ERK pathways and MAPK signalling in ASC proliferation, migration and apoptosis have been analysed. Bone regeneration has been observed in rabbits with implants of MSCs transduced with Sonic Hedgehog (Shh)-a key protein involved in bone morphogenesis. Furthermore, BMP signalling in ASCs can be modulated by downregulating noggin, using rat ASCs transduced with noggin shRNA, and thus to enhance the differentiation of cells to a osteogenic terminal linage. This noggin suppression + BMP-2 strategy has been confirmed in 3D *in vitro* experiments using complex scaffolds (consisting of chitosan, chondroitin sulphate and an apatite layer) designed to slowly release BMP-2. Wnt signalling pathways are involved in regulation of embryologic patterning, mesenchymal differentiation and stem cell fate[106]. The association of LRP5 gene mutation and the osteoporosis-pseudoglioma syndrome strongly suggests the participation of Wnt signalling in bone formation. Wnt3a induced signalling has been associated with the *in vitro* and *in vivo* inhibition of bone formation[107]. In contrast, increased bone regeneration in bone defects has been observed in MSCs from bone tissues overexpressing Wnt4. This effect may be due to a specific increase in p38 MAPK phosphorylation, which mediates the promotion of bone formation.

TE is considered an advanced therapy medicine product (ATMP), the characterisation of which requires its characteristics (identity, potency, purity and safety) to be defined and measured during product development. ATMP manufacturing activities are mainly focused on the following areas; Pre-Production Activities (patient and donor selection, biopsy procurement, cell/tissue extraction, testing, storage and distribution to Good Manufacturing Practice-GMP-laboratories for production); Production Activities (manufacturing, packaging, labelling, testing, storage and distribution); and Post-Production Activities (testing, storage and administration/implantation of the manufactured product). In the European Union, these activities are mainly regulated by Directive 2004/23EC of the European Parliament[108] which sets quality and safety standards for the main process involved in TE intended for human use (donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells). Other applicable legislation includes Directive 2006/17/EC, Directive 2006/86/EC, Directive 2012/39/EU, Commission Directives (EU) 2015/566 and 2015/565, Regulation (EC) 1394/2007, Directive 2009/120/EC and Directive 95/46/EC[109-116], in addition to EuroGTP guidelines[117].

In ATMPs, preclinical safety/toxicology assays are mandatory for sterility, mycoplasma contamination, endotoxins, aerobic/anaerobic micro-organisms, tumorogenecity and genetic stability. Their design requires specific pre-GMP laboratory activities for the selection and recruitment of stem cell donors and patients. In addition, there are specific regulations for stem cell donors[117]. The clinical problem to be solved with ATMP has two related aspects: On the one hand is the question of individual genetic susceptibility to DNA single nucleotide variants (SNV) related to several pathological conditions, especially tumorigenesis, neoangiogenesis, lymphangiogenesis and cell capacities such as cell adhesion and migration. On the other hand, account must be taken of the gene instability of the cultured and manipulated MSCs used in manufacturing ATMP products.

Among other current limitations to this technique, the potential risk of genomic instability of cells is clearly a main limitation for clinical purposes. This risk appears to increase when e*x vivo* expansion of cells are maintained for more than three weeks. Therefore, much remains to be done to standardise methods and techniques for preparing hASCs for clinical applications and this also must be carried out following GMP, FDA and EMA regulations[118,119]. Indeed, procedures for cells expansion in culture must be according to GMP guidelines for cell manipulation, and their standardisation will facilitate the quality controls, comparative studies,

maximising the reliability and reproducibility of results. In fact, discrepancies have been observed from different studies and from different laboratories, due to variability of the methods and quality of hASC isolation and of the composition of the initial cell culture. hASCs are generally stable (normal diploid karyotype) in long-term cultures, even when they have undergone more than 100 population doublings[120]. However a single report suggests malignant transformation of hASCs cultured for more than four months[121]. Yet, this spontaneous transformation of MSCs may also be due to cross-contamination with malignant cell lines (fibrosarcoma and osteosarcoma)[122]. This controversy on spontaneous hASC transformation requires further experiments and discussion, bearing in mind the needs for a careful manipulation of hASCs, together with long-term follow-up of patients.

As most cells intended for engineering tissues have been subjected to mechanical or enzymatic dissociation, and to rapid proliferation in culture with growth factors and media, among other operations, there is always the possibility that some kind of alteration might be generated within the genetic burden of the cell. Any alteration of these genes could result in tissue dysfunction and a loss of function of the affected tissue.

In this context, the quality control of cell/tissue-engineering should be focused on histomorphology patterns, 3D perfusion seeding, cellular assessments of cell sterility and endotoxins, *in vitro* cellular toxicity, proliferation, adhesion in constructs, genetic quality control for DNA and gene expression and the rheological analysis of scaffolds and new cell/TE. At present, the analysis of tumorogenecity and genetic stability, with respect to chromosomal integrity and mutations of tumour-related genes, is mainly achieved by means of genetic and epigenetic quality controls, to verify at DNA level the absence of any alteration that could lead to malignant transformation, and to ensure that gene expression levels correspond to the functions of native tissues, *via* gene expression analysis of mRNA and proteins.

TE is a novel, complex and specific technology with unexpected risks to public health and to patients. There are three main types of risks to be considered.

Risks to patients arising from the quality of the ATMP product, in particular its components, stability, activity and purity (regarding non-physiological proteins). In the characterisation of a final ATMP product, genetic stability testing is of crucial importance to avoid the risk of clinical side effects due to tumorogenecity, inadequate cell adhesion and/or the increased cell migration capability of expanded/differentiated MSCs seeded onto scaffolds.

Risks derived from the interaction between the ATMP product and the effects on molecular systems of the patient. In this sense it is important to know the immunogenicity, the risks related to genetic modification of cells driving the apoptosis, any change of function, modification of growth and/or differentiation and malignancy. Early and late consequences of homing, grafting, differentiation, migration and proliferation need also to be explored.

Risks related to persistence of the ATMP product in the patient responsible for late complications, such as cancer and autoimmune disorders.

EU legislation requires the genetic analysis of cells to ensure the absence of chromosomal instability and mutations, deletions or translocations in all tissues generated by TE and intended for clinical use.

Personalised medicine/precision medicine (PM) uses molecular profiling technologies to tailor therapeutic strategies, ensuring the right one is delivered to the right person at the right time, and determining the predisposition to disease among the population. Now days, next-generation sequencing (NGS) technologies are more accessible by cost, analytic validity and rapidity. Whole exome sequencing (WES) together with bioinformatics allows the analysis of single nucleotide variants of 85% of coding protein genes (20000 genes, 180000 exons, 1% of the whole genome)[123]. WES sensitivity for known mutations and benign variants reach up to 98.3% and its main clinical use is for the diagnosis of genetic disorders, however, WES also allows phenotype expansion and makes it possible to identify newly mutated genes, undetectable by other techniques.

Taking into account the genetic instability risk of the *ex vivo* expansion of MSCs, we suggest that the standardisation of pre-implant testing of tumorogenic burden, neoangiogenesis and cell adhesion and migration capacities, by means of NGS analysis throughout the differentiation culturing of hASCs, would improve the quality control of artificial bone tissues used for bone repair and help achieve a valid prognosis of full integration within the host of *ex vivo* differentiated hASCs.

Joint exome and transcriptome analysis will help identify a panel of genes involved in hASC proliferation, differentiation, adhesion, migration, and also telomere length control, among other questions, thus constituting a standard genetic stability cell analysis for tissue-engineered bone. This analysis will reinforce the clinical criteria applied in selecting participants for clinical trials with TE, and hence reduce the risk of adverse effects arising from an accumulation of tumour-related gene mutations.

In summary, the clinical reconstruction of large bone defects is a highly challenging procedure, and will probably remain so for the foreseeable future.

REFERENCES

1 **Grasman JM**, Zayas MJ, Page RL, Pins GD. Biomimetic scaffolds for regeneration of volumetric muscle loss in skeletal muscle injuries. *Acta Biomater* 2015; **25**: 2-15 [PMID: 26219862 DOI: 10.1016/j.actbio.2015.07.038]

2 **Paprosky WG**, Perona PG, Lawrence JM. Acetabular defect classification and surgical reconstruction in revision arthroplasty. A 6-year follow-up evaluation. *J Arthroplasty* 1994; **9**: 33-44 [PMID: 8163974]

3 **Paprosky WG**, Cross MB. CORR Insights®: Validity and reliability of the Paprosky acetabular defect classification. *Clin Orthop Relat Res* 2013; **471**: 2266 [PMID: 23613087 DOI: 10.1007/s11999-013-2938-2]

4 **Shanmuganathan R**, Chandra Mohan AK, Agraharam D, Perumal R, Jayaramaraju D, Kulkarni S. Successful reimplantation of extruded

long bone segments in open fractures of lower limb--a report of 3 cases. *Injury* 2015; **46**: 1389-1392 [PMID: 25943294 DOI: 10.1016/j.injury.2015.04.006]

5 **Guerado E**, Fuerstenberg CH. What bone graft substitutes should we use in post-traumatic spinal fusion? *Injury* 2011; **42** Suppl 2: S64-S71 [PMID: 21839997 DOI: 10.1016/j.injury.2011.06.200]

6 **Langer R**, Vacanti JP. Tissue engineering. *Science* 1993; **260**: 920-926 [PMID: 8493529]

7 **MacArthur BD**, Oreffo RO. Bridging the gap. *Nature* 2005; **433**: 19 [PMID: 15635390]

8 **Smith JO**, Aarvold A, Tayton ER, Dunlop DG, Oreffo RO. Skeletal tissue regeneration: current approaches, challenges, and novel reconstructive strategies for an aging population. *Tissue Eng Part B Rev* 2011; **17**: 307-320 [PMID: 21615329 DOI: 10.1089/ten.TEB.2011.0143]

9 **Qazi TH**, Mooney DJ, Pumberger M, Geissler S, Duda GN. Biomaterials based strategies for skeletal muscle tissue engineering: existing technologies and future trends. *Biomaterials* 2015; **53**: 502-521 [PMID: 25890747 DOI: 10.1016/j.biomaterials.2015.02.110]

10 **Hartsock LA**, Barfield WR, Kokko KP, Liles LL, Wind T, Green J, Giannoudis PV. Randomized prospective clinical trial comparing reamer irrigator aspirator (RIA) to standard reaming (SR) in both minimally injured and multiply injured patients with closed femoral shaft fractures treated with reamed intramedullary nailing (IMN). *Injury* 2010; **41** Suppl 2: S94-S98 [PMID: 21144938 DOI: 10.1016/S0020-1383(10)70018-8]

11 **Cox G**, McGonagle D, Boxall SA, Buckley CT, Jones E, Giannoudis PV. The use of the reamer-irrigator-aspirator to harvest mesenchymal stem cells. *J Bone Joint Surg Br* 2011; **93**: 517-524 [PMID: 21464493 DOI: 10.1302/0301-620X.93B4.25506]

12 **Cox G**, Jones E, McGonagle D, Giannoudis PV. Reamer-irrigator-aspirator indications and clinical results: a systematic review. *Int Orthop* 2011; **35**: 951-956 [PMID: 21243358 DOI: 10.1007/s00264-010-1189-z]

13 **Han F**, Peter L, Lau ET, Thambiah J, Murphy D, Kagda FH. Reamer Irrigator Aspirator bone graft harvesting: complications and outcomes in an Asian population. *Injury* 2015; **46**: 2042-2051 [PMID: 26253387 DOI: 10.1016/j.injury.2015.07.027]

14 **van der Bel R**, Blokhuis TJ. Increased osteogenic capacity of Reamer/Irrigator/Aspirator derived mesenchymal stem cells. *Injury* 2014; **45**: 2060-2064 [PMID: 25458067 DOI: 10.1016/j.injury.2014.10.009]

15 **Devine DM**, Arens D, Thalhauser M, Schiuma D, Zeiter S, Nehrbass D. Healing pattern of reamed bone following bone harvesting by a RIA device. *Eur Cell Mater* 2015; **29**: 97-104; discussion 104 [PMID: 25633308]

16 **Sinclair SS**, Horton CO, Jeray KJ, Tanner SL, Burgl KJ. Fat Layer from Medullary Canal Reamer Aspirate for Potential Use as a Supplemental Osteoinductive Bone Graft Material. *J Stem Cells* 2015; **10**: 79-90 [PMID: 27125136]

17 **Crist BD**, Stoker AM, Stannard JP, Cook JL. Analysis of relevant proteins from bone graft harvested using the reamer irrigator and aspirator system (RIA) versus iliac crest (IC) bone graft and RIA waste water. *Injury* 2016; **47**: 1661-1668 [PMID: 27269417 DOI: 10.1016/j.injury.2016.05.030]

18 **Henrich D**, Nau C, Kraft SB, Zollfrank M, Kontradowitz K, Oppermann E, Schultheiss J, Meier S, Frank J, Marzi I, Seebach C. Effect of the harvest procedure and tissue site on the osteogenic function of and gene expression in human mesenchymal stem cells. *Int J Mol Med* 2016; **37**: 976-988 [PMID: 26935410 DOI: 10.3892/ijmm.2016.2489]

19 **Kuehlfluck P**, Moghaddam A, Helbig L, Child C, Wildemann B, Schmidmaier G. RIA fractions contain mesenchymal stroma cells with high osteogenic potency. *Injury* 2015; **46** Suppl 8: S23-S32 [PMID: 26747914 DOI: 10.1016/S0020-1383(15)30051-6]

20 **Cuthbert RJ**, Giannoudis PV, Wang XN, Nicholson L, Pawson D, Lubenko A, Tan HB, Dickinson A, McGonagle D, Jones E. Examining the feasibility of clinical grade CD271+ enrichment of mesenchymal stromal cells for bone regeneration. *PLoS One* 2015; **10**: e0117855 [PMID: 25760857 DOI: 10.1371/journal.pone.0117855]

21 **Rankine JJ**, Hodgson RJ, Tan HB, Cox G, Giannoudis PV. MRI appearances of the femur following bone graft harvesting using the Reamer-Irrigator-Aspirator. *Injury* 2015; **46** Suppl 8: S65-S67 [PMID: 26747921 DOI: 10.1016/S0020-1383(15)30057-7]

22 **Calori GM**, Colombo M, Mazza EL, Mazzola S, Malagoli E, Mineo GV. Incidence of donor site morbidity following harvesting from iliac crest or RIA graft. *Injury* 2014; **45** Suppl 6: S116-S120 [PMID: 25457330 DOI: 10.1016/j.injury.2014.10.034]

23 **Donders JC**, Baumann HM, Stevens MF, Kloen P. Hemorrhagic-Induced Cardiovascular Complications During Reamer-Irrigator-Aspirator-Assisted Femoral Nonunion Treatment. *J Orthop Trauma* 2016; **30**: e294-e298 [PMID: 27124827 DOI: 10.1097/BOT.0000000000000612]

24 **Miller AN**, Deal D, Green J, Houle T, Brown W, Thore C, Stump D, Webb LX. Use of the Reamer/Irrigator/Aspirator Decreases Carotid and Cranial Embolic Events in a Canine Model. *J Bone Joint Surg Am* 2016; **98**: 658-664 [PMID: 27098324 DOI: 10.2106/JBJS.14.01176]

25 **Wang RY**, Li R, Zdero R, Bell D, Blankstein M, Schemitsch EH. The physiologic and pathologic effects of the reamer irrigator aspirator on fat embolism outcome: an animal study. *J Orthop Trauma* 2012; **26**: e132-e137 [PMID: 22377507 DOI: 10.1097/BOT.0b013e318238b22b]

26 **Volgas DA**, Burch T, Stannard JP, Ellis T, Bilotta J, Alonso JE. Fat embolus in femur fractures: a comparison of two reaming systems. *Injury* 2010; **41** Suppl 2: S90-S93 [PMID: 21144937 DOI: 10.1016/S0020-1383(10)70017-6]

27 **Streubel PN**, Desai P, Suk M. Comparison of RIA and conventional reamed nailing for treatment of femur shaft fractures. *Injury* 2010; **41** Suppl 2: S51-S56 [PMID: 21144929 DOI: 10.1016/S0020-1383(10)70010-3]

28 **Husebye EE**, Opdahl H, Røise O, Aspelin T, Lyberg T. Coagulation, fibrinolysis and cytokine responses to intramedullary nailing of the femur: an experimental study in pigs comparing traditional reaming and reaming with a one-step reamer-irrigator-aspirator system. *Injury* 2011; **42**: 630-637 [PMID: 20650454 DOI: 10.1016/j.injury.2010.06.025]

29 **Giannoudis PV**, Tan HB, Perry S, Tzioupis C, Kanakaris NK. The systemic inflammatory response following femoral canal reaming using the reamer-irrigator-aspirator (RIA) device. *Injury* 2010; **41** Suppl 2: S57-S61 [PMID: 21144930 DOI: 10.1016/S0020-1383(10)70011-5]

30 **Silva JA**, McCormick JJ, Reed MA, Morse AS, Heffernan MJ, Lavado RD, Billiar K, Wixted JJ. Biomechanical effects of harvesting bone graft with the Reamer/Irrigator/Aspirator on the adult femur: a cadaver study. *Injury* 2010; **41** Suppl 2: S85-S89 [PMID: 21144935 DOI: 10.1016/S0020-1383(10)70016-4]

31 **Giori NJ**, Beaupre GS. Femoral fracture after harvesting of autologous bone graft using a reamer/irrigator/aspirator. *J Orthop Trauma* 2011; **25**: e12-e14 [PMID: 21245702 DOI: 10.1097/BOT.0b013e3181e39bf4]

32 **Jakma TS**, Röling MA, Punt B, Reynders-Frederix P. More adverse events than expected in the outcome after use of the reamer-irrigator-aspirator. *Eur J Trauma Emerg Surg* 2014; **40**: 337-341 [PMID: 26816069 DOI: 10.1007/s00068-013-0345-8]

33 **Mansour J**, Conway JD. Retrograde reamer/irrigator/aspirator technique for autologous bone graft harvesting with the patient in the prone position. *Am J Orthop* (Belle Mead NJ) 2015; **44**: 202-205 [PMID: 25950533]

34 **Davis RL**, Taylor BC, Johnson N, Ferrel JR, Castaneda J. Retrograde Versus Antegrade Femoral Bone Graft Harvesting Using the Reamer-Irrigator-Aspirator. *J Orthop Trauma* 2015; **29**: 370-372 [PMID: 25635359 DOI: 10.1097/BOT.0000000000000300]

35 **Masquelet AC**, Fitoussi F, Begue T, Muller GP. [Reconstruction of the long bones by the induced membrane and spongy autograft]. *Ann Chir Plast Esthet* 2000; **45**: 346-353 [PMID: 10929461]

36 **Levante S**, Masquelet AC, Nordin JY. [Coverage of chronic osteomyelitis of the ankle and the foot using a soleus muscle island flap, vascularized with retrograde flow on the posterior tibial artery. A seven cases report]. *Ann Chir Plast Esthet* 2009; **54**: 523-527 [PMID: 19195753 DOI: 10.1016/j.anplas.2008.10.003]

37 **Klaue K**, Knothe U, Anton C, Pfluger DH, Stoddart M, Masquelet AC, Perren SM. Bone regeneration in long-bone defects: tissue

compartmentalisation? In vivo study on bone defects in sheep. *Injury* 2009; **40** Suppl 4: S95-102 [PMID: 19895960 DOI: 10.1016/j.injury.2009.10.043]
38 **Apard T**, Bigorre N, Cronier P, Duteille F, Bizot P, Massin P. Two-stage reconstruction of post-traumatic segmental tibia bone loss with nailing. *Orthop Traumatol Surg Res* 2010; **96**: 549-553 [PMID: 20605548 DOI: 10.1016/j.otsr.2010.02.010]
39 **Stafford PR**, Norris BL. Reamer-irrigator-aspirator bone graft and bi Masquelet technique for segmental bone defect nonunions: a review of 25 cases. *Injury* 2010; **41** Suppl 2: S72-S77 [PMID: 21144933 DOI: 10.1016/S0020-1383(10)70014-0]
40 **Henrich D**, Seebach C, Nau C, Basan S, Relja B, Wilhelm K, Schaible A, Frank J, Barker J, Marzi I. Establishment and characterization of the Masquelet induced membrane technique in a rat femur critical-sized defect model. *J Tissue Eng Regen Med* 2016; **10**: E382-E396 [PMID: 24668794 DOI: 10.1002/term.1826]
41 **Gouron R**, Petit L, Boudot C, Six I, Brazier M, Kamel S, Mentaverri R. Osteoclasts and their precursors are present in the induced-membrane during bone reconstruction using the Masquelet technique. *J Tissue Eng Regen Med* 2014; **11**: 382-389 [PMID: 24919776 DOI: 10.1002/term.1921]
42 **Christou C**, Oliver RA, Yu Y, Walsh WR. The Masquelet technique for membrane induction and the healing of ovine critical sized segmental defects. *PLoS One* 2014; **9**: e114122 [PMID: 25461340 DOI: 10.1371/journal.pone.0114122]
43 **Bosemark P**, Perdikouri C, Pelkonen M, Isaksson H, Tägil M. The masquelet induced membrane technique with BMP and a synthetic scaffold can heal a rat femoral critical size defect. *J Orthop Res* 2015; **33**: 488-495 [PMID: 25639666 DOI: 10.1002/jor.22815]
44 **Aho OM**, Lehenkari P, Ristiniemi J, Lehtonen S, Risteli J, Leskelä HV. The mechanism of action of induced membranes in bone repair. *J Bone Joint Surg Am* 2013; **95**: 597-604 [PMID: 23553294 DOI: 10.2106/JBJS.L.00310]
45 **Giannoudis PV**, Faour O, Goff T, Kanakaris N, Dimitriou R. Masquelet technique for the treatment of bone defects: tips-tricks and future directions. *Injury* 2011; **42**: 591-598 [PMID: 21543068 DOI: 10.1016/j.injury.2011.03.036]
46 **Mauffrey C**, Hake ME, Chadayammuri V, Masquelet AC. Reconstruction of Long Bone Infections Using the Induced Membrane Technique: Tips and Tricks. *J Orthop Trauma* 2016; **30**: e188-e193 [PMID: 26618666 DOI: 10.1097/BOT.0000000000000500]
47 **Fischer C**, Doll J, Tanner M, Bruckner T, Zimmermann G, Helbig L, Biglari B, Schmidmaier G, Moghaddam A. Quantification of TGF-ß1, PDGF and IGF-1 cytokine expression after fracture treatment vs. non-union therapy via masquelet. *Injury* 2016; **47**: 342-349 [PMID: 26775211 DOI: 10.1016/j.injury.2015.11.007]
48 **Ilizarov GA**, Lediaev VI. [Replacement of defects of long tubular bones by means of one of their fragments]. *Vestn Khir Im I I Grek* 1969; **102**: 77-84 [PMID: 4904826]
49 **Styazhkin N**. Surgeon Ilizarov and his method. *Australas Nurses J* 1973; **2**: 8 [PMID: 4488965]
50 **Liberman SB**, Semenova VD. Investigation of the strength and rigidity of Kirschner spokes in the apparatus of G. A. Ilizarov. *Biomed Eng* (NY) 1974; **7**: 226-227 [PMID: 4457167]
51 **Landini A**, Confalonieri N, Bagnoli G, Penna G, Pietrogrande V. [Consolidation time in fresh fractures of the leg treated by the Ilizarov method]. *Arch Putti Chir Organi Mov* 1984; **34**: 269-273 [PMID: 6544083]
52 **Confalonieri N**, Penna G, Bagnoli G, Landini A, Pietrogrande V. [Treatment of pseudoarthrosis with the external Ilizarov fixator. Initial experiences]. *Arch Putti Chir Organi Mov* 1984; **34**: 303-308 [PMID: 6544089]
53 **Lavrishcheva GI**, Mikhaĭlova LN. [Histogenesis of skeletogenic tissue in bone regeneration during distraction]. *Biull Eksp Biol Med* 1985; **99**: 198-201 [PMID: 2578836]
54 **Paley D**, Catagni MA, Argnani F, Villa A, Benedetti GB, Cattaneo R. Ilizarov treatment of tibial nonunions with bone loss. *Clin Orthop Relat Res* 1989; **(241)**: 146-165 [PMID: 2924458]
55 **Paley D**. Problems, obstacles, and complications of limb lengthening by the Ilizarov technique. *Clin Orthop Relat Res* 1990; **(250)**: 81-104 [PMID: 2403498]
56 **Fischgrund J**, Paley D, Suter C. Variables affecting time to bone healing during limb lengthening. *Clin Orthop Relat Res* 1994; **(301)**: 31-37 [PMID: 8156692]
57 **Marcacci M**, Kon E, Moukhachev V, Lavroukov A, Kutepov S, Quarto R, Mastrogiacomo M, Cancedda R. Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. *Tissue Eng* 2007; **13**: 947-955 [PMID: 17484701 DOI: 10.1089/ten.2006.0271]
58 **Sunay O**, Can G, Cakir Z, Denek Z, Kozanoglu I, Erbil G, Yilmaz M, Baran Y. Autologous rabbit adipose tissue-derived mesenchymal stromal cells for the treatment of bone injuries with distraction osteogenesis. *Cytotherapy* 2013; **15**: 690-702 [PMID: 23522867 DOI: 10.1016/j.jcyt.2013.02.004]
59 **Nomura I**, Watanabe K, Matsubara H, Hayashi K, Sugimoto N, Tsuchiya H. Uncultured autogenous adipose-derived regenerative cells promote bone formation during distraction osteogenesis in rats. *Clin Orthop Relat Res* 2014; **472**: 3798-3806 [PMID: 24711135 DOI: 10.1007/s11999-014-3608-8]
60 **Ando Y**, Matsubara K, Ishikawa J, Fujio M, Shohara R, Hibi H, Ueda M, Yamamoto A. Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms. *Bone* 2014; **61**: 82-90 [PMID: 24389414 DOI: 10.1016/j.bone.2013.12.029]
61 **Dehghan MM**, Baghaban Eslaminejad M, Motallebizadeh N, Ashrafi Halan J, Tagiyar L, Soroori S, Nikmahzar A, Pedram M, Shahverdi A, Kazemi Mehrjerdi H, Izadi S. Transplantation of Autologous Bone Marrow Mesenchymal Stem Cells with Platelet-Rich Plasma Accelerate Distraction Osteogenesis in A Canine Model. *Cell J* 2015; **17**: 243-252 [PMID: 26199903]
62 **Harada Y**, Nakasa T, Mahmoud EE, Kamei G, Adachi N, Deie M, Ochi M. Combination therapy with intra-articular injection of mesenchymal stem cells and articulated joint distraction for repair of a chronic osteochondral defect in the rabbit. *J Orthop Res* 2015; **33**: 1466-1473 [PMID: 26174695 DOI: 10.1002/jor.22922]
63 **Morcos MW**, Al-Jallad H, Hamdy R. Comprehensive Review of Adipose Stem Cells and Their Implication in Distraction Osteogenesis and Bone Regeneration. *Biomed Res Int* 2015; **2015**: 842975 [PMID: 26448947 DOI: 10.1155/2015/842975]
64 **Mechiche Alami S**, Gangloff SC, Laurent-Maquin D, Wang Y, Kerdjoudj H. Concise Review: In Vitro Formation of Bone-Like Nodules Sheds Light on the Application of Stem Cells for Bone Regeneration. *Stem Cells Transl Med* 2016; **5**: 1587-1593 [PMID: 27458265 DOI: 10.5966/sctm.2015-0413]
65 **Medhekar SK**, Shende VS, Chincholkar AB. Recent Stem Cell Advances: Cord Blood and Induced Pluripotent Stem Cell for Cardiac Regeneration- a Review. *Int J Stem Cells* 2016; **9**: 21-30 [PMID: 27426082 DOI: 10.15283/ijsc.2016.9.1.21]
66 **Lo Furno D**, Mannino G, Cardile V, Parenti R, Giuffrida R. Potential Therapeutic Applications of Adipose-Derived Mesenchymal Stem Cells. *Stem Cells Dev* 2016; Epub ahead of print [PMID: 27520311 DOI: 10.1089/scd.2016.0135]
67 **Du WJ**, Reppel L, Leger L, Schenowitz C, Huselstein C, Bensoussan D, Carosella ED, Han ZC, Rouas-Freiss N. Mesenchymal Stem Cells Derived from Human Bone Marrow and Adipose Tissue Maintain Their Immunosuppressive Properties After Chondrogenic Differentiation: Role of HLA-G. *Stem Cells Dev* 2016; **25**: 1454-1469 [PMID: 27465875 DOI: 10.1089/scd.2016.0022]
68 **Tan J**, Xu X, Lin J, Fan L, Zheng Y, Kuang W. Dental Stem Cell in Tooth Development and Advances of Adult Dental Stem Cell in Regenerative Therapies. *Curr Stem Cell Res Ther* 2015; **10**: 375-383 [PMID: 25274563]
69 **Chadipiralla K**, Yochim JM, Bahuleyan B, Huang CY, Garcia-Godoy F, Murray PE, Stelnicki EJ. Osteogenic differentiation of stem cells derived from human periodontal ligaments and pulp of human exfoliated deciduous teeth. *Cell Tissue Res* 2010; **340**: 323-333 [PMID: 20309582 DOI: 10.1007/s00441-010-0953-0]
70 **Mattioli-Belmonte M**, Teti G, Salvatore V, Focaroli S, Orciani M, Dicarlo M, Fini M, Orsini G, Di Primio R, Falconi M. Stem cell origin differently affects bone tissue engineering strategies. *Front Physiol* 2015; **6**: 266 [PMID: 26441682 DOI: 10.3389/fphys.2015.00266]

71 **Li M**, Zhao Y, Hao H, Han W, Fu X. Mesenchymal stem cell-based therapy for nonhealing wounds: today and tomorrow. *Wound Repair Regen* 2015; **23**: 465-482 [PMID: 25877885 DOI: 10.1111/wrr.12304]

72 **Roubelakis MG**, Pappa KI, Bitsika V, Zagoura D, Vlahou A, Papadaki HA, Antsaklis A, Anagnou NP. Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. *Stem Cells Dev* 2007; **16**: 931-952 [PMID: 18047393 DOI: 10.1089/scd.2007.0036]

73 **Choi JW**, Park EJ, Shin HS, Shin IS, Ra JC, Koh KS. In vivo differentiation of undifferentiated human adipose tissue-derived mesenchymal stem cells in critical-sized calvarial bone defects. *Ann Plast Surg* 2014; **72**: 225-233 [PMID: 23221992 DOI: 10.1097/SAP.0b013e31825f70f5]

74 **Markarian CF**, Frey GZ, Silveira MD, Chem EM, Milani AR, Ely PB, Horn AP, Nardi NB, Camassola M. Isolation of adipose-derived stem cells: a comparison among different methods. *Biotechnol Lett* 2014; **36**: 693-702 [PMID: 24322777 DOI: 10.1007/s10529-013-1425-x]

75 **Yu G**, Floyd ZE, Wu X, Halvorsen YD, Gimble JM. Isolation of human adipose-derived stem cells from lipoaspirates. *Methods Mol Biol* 2011; **702**: 17-27 [PMID: 21082391 DOI: 10.1007/978-1-61737-960-4_2]

76 **Forcales SV**. Potential of adipose-derived stem cells in muscular regenerative therapies. *Front Aging Neurosci* 2015; **7**: 123 [PMID: 26217219 DOI: 10.3389/fnagi.2015.00123]

77 **Kakudo N**, Morimoto N, Ogawa T, Taketani S, Kusumoto K. Hypoxia Enhances Proliferation of Human Adipose-Derived Stem Cells via HIF-1α Activation. *PLoS One* 2015; **10**: e0139890 [PMID: 26465938 DOI: 10.1371/journal.pone.0139890]

78 **Cervelli V**, Scioli MG, Gentile P, Doldo E, Bonanno E, Spagnoli LG, Orlandi A. Platelet-rich plasma greatly potentiates insulin-induced adipogenic differentiation of human adipose-derived stem cells through a serine/threonine kinase Akt-dependent mechanism and promotes clinical fat graft maintenance. *Stem Cells Transl Med* 2012; **1**: 206-220 [PMID: 23197780 DOI: 10.5966/sctm.2011-0052]

79 **Gnanasegaran N**, Govindasamy V, Musa S, Kasim NH. Different isolation methods alter the gene expression profiling of adipose derived stem cells. *Int J Med Sci* 2014; **11**: 391-403 [PMID: 24669199 DOI: 10.7150/ijms.7697]

80 **Dragoo JL**, Choi JY, Lieberman JR, Huang J, Zuk PA, Zhang J, Hedrick MH, Benhaim P. Bone induction by BMP-2 transduced stem cells derived from human fat. *J Orthop Res* 2003; **21**: 622-629 [PMID: 12798061 DOI: 10.1016/S0736-0266(02)00238-3]

81 **Dragoo JL**, Lieberman JR, Lee RS, Deugarte DA, Lee Y, Zuk PA, Hedrick MH, Benhaim P. Tissue-engineered bone from BMP-2-transduced stem cells derived from human fat. *Plast Reconstr Surg* 2005; **115**: 1665-1673 [PMID: 15861072]

82 **Peterson B**, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P, Lieberman JR. Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. *Tissue Eng* 2005; **11**: 120-129 [PMID: 15738667 DOI: 10.1089/ten.2005.11.120]

83 **Chang SC**, Chuang H, Chen YR, Yang LC, Chen JK, Mardini S, Chung HY, Lu YL, Ma WC, Lou J. Cranial repair using BMP-2 gene engineered bone marrow stromal cells. *J Surg Res* 2004; **119**: 85-91 [PMID: 15126087 DOI: 10.1016/j.jss.2003.08.003]

84 **Lieberman JR**, Daluiski A, Stevenson S, Wu L, McAllister P, Lee YP, Kabo JM, Finerman GA, Berk AJ, Witte ON. The effect of regional gene therapy with bone morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. *J Bone Joint Surg Am* 1999; **81**: 905-917 [PMID: 10428121]

85 **Tsuda H**, Wada T, Yamashita T, Hamada H. Enhanced osteoinduction by mesenchymal stem cells transfected with a fiber-mutant adenoviral BMP2 gene. *J Gene Med* 2005; **7**: 1322-1334 [PMID: 15926193 DOI: 10.1002/jgm.777]

86 **Mesimäki K**, Lindroos B, Törnwall J, Mauno J, Lindqvist C, Kontio R, Miettinen S, Suuronen R. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. *Int J Oral Maxillofac Surg* 2009; **38**: 201-209 [PMID: 19168327 DOI: 10.1016/j.ijom.2009.01.001]

87 **Lendeckel S**, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. *J Craniomaxillofac Surg* 2004; **32**: 370-373 [PMID: 15555520 DOI: 10.1016/j.jcms.2004.06.002]

88 **Dong SW**, Ying DJ, Duan XJ, Xie Z, Yu ZJ, Zhu CH, Yang B, Sun JS. Bone regeneration using an acellular extracellular matrix and bone marrow mesenchymal stem cells expressing Cbfa1. *Biosci Biotechnol Biochem* 2009; **73**: 2226-2233 [PMID: 19809195 DOI: 10.1271/bbb.90329]

89 **Zuk PA**. Tissue engineering craniofacial defects with adult stem cells? Are we ready yet? *Pediatr Res* 2008; **63**: 478-486 [PMID: 18427291 DOI: 10.1203/PDR.0b013e31816bdf36]

90 **Lim ML**, Jungebluth P, Ajalloueian F, Friedrich LH, Gilevich I, Grinnemo KH, Gubareva E, Haag JC, Lemon G, Sjöqvist S, Caplan AL, Macchiarini P. Whole organ and tissue reconstruction in thoracic regenerative surgery. *Mayo Clin Proc* 2013; **88**: 1151-1166 [PMID: 24079685 DOI: 10.1016/j.mayocp.2013.03.011]

91 **Black CR**, Goriainov V, Gibbs D, Kanczler J, Tare RS, Oreffo RO. Bone Tissue Engineering. *Curr Mol Biol Rep* 2015; **1**: 132-140 [PMID: 26618105 DOI: 10.1007/s40610-015-0022-2]

92 **Berner A**, Henkel J, Woodruff MA, Steck R, Nerlich M, Schuetz MA, Hutmacher DW. Delayed minimally invasive injection of allogenic bone marrow stromal cell sheets regenerates large bone defects in an ovine preclinical animal model. *Stem Cells Transl Med* 2015; **4**: 503-512 [PMID: 25834121 DOI: 10.5966/sctm.2014-0244]

93 **Orlic D**, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; **410**: 701-705 [PMID: 11287958 DOI: 10.1038/35070587]

94 **Dreger T**, Watson JT, Akers W, Molligan J, Achilefu S, Schon LC, Zhang Z. Intravenous application of CD271-selected mesenchymal stem cells during fracture healing. *J Orthop Trauma* 2014; **28** Suppl 1: S15-S19 [PMID: 24378433 DOI: 10.1097/BOT.0000000000000063]

95 **Cameron SH**, Alwakeel AJ, Goddard L, Hobbs CE, Gowing EK, Barnett ER, Kohe SE, Sizemore RJ, Oorschot DE. Delayed post-treatment with bone marrow-derived mesenchymal stem cells is neurorestorative of striatal medium-spiny projection neurons and improves motor function after neonatal rat hypoxia-ischemia. *Mol Cell Neurosci* 2015; **68**: 56-72 [PMID: 25828540 DOI: 10.1016/j.mcn.2015.03.019]

96 **Gross JB**, Diligent J, Bensoussan D, Galois L, Stoltz JF, Mainard D. Percutaneous autologous bone marrow injection for treatment of delayed and non-union of long bone: a retrospective study of 45 cases. *Biomed Mater Eng* 2015; **25**: 187-197 [PMID: 25538069 DOI: 10.3233/BME-141235]

97 **Dawson JI**, Kanczler J, Tare R, Kassem M, Oreffo RO. Concise review: bridging the gap: bone regeneration using skeletal stem cell-based strategies - where are we now? *Stem Cells* 2014; **32**: 35-44 [PMID: 24115290 DOI: 10.1002/stem.1559]

98 **Watanabe Y**, Harada N, Sato K, Abe S, Yamanaka K, Matushita T. Stem cell therapy: is there a future for reconstruction of large bone defects? *Injury* 2016; **47** Suppl 1: S47-S51 [PMID: 26768292 DOI: 10.1016/S0020-1383(16)30012-2]

99 **Halme DG**, Kessler DA. FDA regulation of stem-cell-based therapies. *N Engl J Med* 2006; **355**: 1730-1735 [PMID: 17050899 DOI: 10.1056/NEJMhpr063086]

100 **The European parliament and the Council of the European Union**. Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004 10.12.2007. Official Journal of the European Union. L 324/121, Last Accessed August 2016. Available from: URL: http://ec.europa.eu/health/files/eudralex/vol-1/reg_2007_1394/reg_2007_1394_en.pdf

101 **Yousefi AM**, James PF, Akbarzadeh R, Subramanian A, Flavin C, Oudadesse H. Prospect of Stem Cells in Bone Tissue Engineering: A Review. *Stem Cells Int* 2016; **2016**: 6180487 [PMID: 26880976 DOI: 10.1155/2016/6180487]

102 **Timashev P**, Kuznetsova D, Koroleva A, Prodanets N, Deiwick A, Piskun Y, Bardakova K, Dzhoyashvili N, Kostjuk S, Zagaynova E, Rochev Y, Chichkov B, Bagratashvili V. Novel biodegradable star-shaped polylactide scaffolds for bone regeneration fabricated by two-photon polymerization. *Nanomedicine* (Lond) 2016; **11**: 1041-1053 [PMID: 27078220 DOI: 10.2217/nnm-2015-0022]

103 **Sándor GK**. Tissue engineering of bone: Clinical observations with adipose-derived stem cells, resorbable scaffolds, and growth factors. *Ann Maxillofac Surg* 2012; **2**: 8-11 [PMID: 23483030 DOI: 10.4103/2231-0746.95308]

104 **Kapur SK**, Katz AJ. Review of the adipose derived stem cell secretome. *Biochimie* 2013; **95**: 2222-2228 [PMID: 23770442 DOI: 10.1016/j.biochi.2013.06.001]

105 **Senarath-Yapa K**, McArdle A, Renda A, Longaker MT, Quarto N. Adipose-derived stem cells: a review of signaling networks governing cell fate and regenerative potential in the context of craniofacial and long bone skeletal repair. *Int J Mol Sci* 2014; **15**: 9314-9330 [PMID: 24865492 DOI: 10.3390/ijms15069314]

106 **Kokabu S**, Lowery JW, Jimi E. Cell Fate and Differentiation of Bone Marrow Mesenchymal Stem Cells. *Stem Cells Int* 2016; **2016**: 3753581 [PMID: 27298623 DOI: 10.1155/2016/3753581]

107 **Belinsky GS**, Sreekumar B, Andrejecsk JW, Saltzman WM, Gong J, Herzog RI, Lin S, Horsley V, Carpenter TO, Chung C. Pigment epithelium-derived factor restoration increases bone mass and improves bone plasticity in a model of osteogenesis imperfecta type VI via Wnt3a blockade. *FASEB J* 2016; **30**: 2837-2848 [PMID: 27127101 DOI: 10.1096/fj.201500027R]

108 **The European Parliament and the Council of the European Union**. Directive 2004/23/EC of The European Parliament And of The Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. Official Journal of the European Union. L 102/48; 7.4.2004. [accessed 2016 Aug]. Available from: URL: http://eur-lex.europa.eu/Lex-UriServ/LexUriServ.do?uri=OJ: L: 2004: 102: 0048: 0058: en: PDF

109 **The European Parliament and the Council of the European Union**. Commission Directive 2006/17/EC of 8 February 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards certain technical requirements for the donation, procurement and testing of human tissues and cells. Official Journal of the European Union L 38/40. 9.2.2006. [accessed 2016 Aug]. Available from: URL: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex: 32006L0017

110 **The European Parliament and the Council of the European Union**. Commission Directive 2006/86/EC of 24 October 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing, preservation, storage and distribution of human tissues and cells. Official Journal of the European Union L 294/32. 25.10.2006. [accessed 2016 Aug]. Available from: URL: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX: 32006L0086

111 **The European Commission**. Commission Directive 2012/39/EU of 26 November 2012 amending Directive 2006/17/EC as regards certain technical requirements for the testing of human tissues and cells Official Journal of the European Union. L 327/24. 27.11.2012. [accessed 2016 Aug]. Available from: URL: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX: 32012L0039

112 **The European Commission**. Commission Directive (EU) 2015/565 of 8 April 2015 amending Directive 2006/86/EC as regards certain technical requirements for the coding of human tissues and cells Official Journal of the European Union L 93/43, 9.4.2015. [accessed 2016 Aug]. Available from: URL: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv: OJ.L_.2015.093.01.0043.01.ENG

113 **The European Commission**. Commission Directive (EU) 2015/566 of 8 April 2015 implementing Directive 2004/23/EC as regards the procedures for verifying the equivalent standards of quality and safety of imported tissues and cells. Official Journal of the European Union L 93/56, 9.4.2015. [accessed 2016 Aug]. Available from: URL: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX: 32015L0566

114 **The European Parliament and the Council of the European Union**. Regulation (EC) No 1394/2007 of The European Parliament and of The Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004. [accessed 2016 Aug]. Available from: URL: http://ec.europa.eu/health/files/eudralex/vol-1/reg_2007_1394/reg_2007_1394_en.pdf

115 **The Commission of the European Communities**. Commission Directive 2009/120/EC of 14 September 2009 amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use as regards advanced therapy medicinal products. Official Journal of the European Union L 242/3, 5.9.2009. [accessed 2016 Aug]. Available from: URL: http://ec.europa.eu/health/files/eudralex/vol-1/dir_2009_120/dir_2009_120_en.pdf

116 **The European Parliament and the Council of the European Union**. Directive 95/46/EC of the European Parliament and of the Council of 24 October 1995 on the protection of individuals with regard to the processing of personal data and on the free movement of such data. Official Journal of the European Union L 281/31, 23.11.95. [accessed 2016 Aug]. Available from: URL: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex: 31995L0046

117 **Governing Regulations CFR - 1270 Series**. [accessed 2016 Aug]. Available from: URL: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPartFrom=800&CFRPartTo=1299

118 Commission Directive 2003/94/EC of 8 October 2003 laying down the principles and guidelines of good manufacturing practice in respect of medicinal products for human use and investigational medicinal products for human use. Official Journal of the European Union L 262/22, 14.10.2003. [accessed 2016 Aug]. Available from: URL: http://ec.europa.eu/health/files/eudralex/vol-1/dir_2003_94/dir_2003_94_en.pdf

119 **K. Good Manufacturing Practice**. EudraLex - Volume 4 Good manufacturing practice (GMP) Guidelines. [accessed 2016 Aug]. Available from: URL: http://ec.europa.eu/health/documents/eudralex/vol-4/index_en.htm

120 **Paula AC**, Martins TM, Zonari A, Frade SP, Angelo PC, Gomes DA, Goes AM. Human adipose tissue-derived stem cells cultured in xeno-free culture condition enhance c-MYC expression increasing proliferation but bypassing spontaneous cell transformation. *Stem Cell Res Ther* 2015; **6**: 76 [DOI: 10.1186/s13287-015-0030-4]

121 **Gimble JM**, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res* 2007; **100**: 1249-1260 [PMID: 17495232 DOI: 10.1161/01.RES.0000265074.83288.09]

122 **Lye KL**, Nordin N, Vidyadaran S, Thilakavathy K. Mesenchymal stem cells: From stem cells to sarcomas. *Cell Biol Int* 2016; **40**: 610-618 [PMID: 26992453 DOI: 10.1002/cbin.10603]

123 **Hedges DJ**, Burges D, Powell E, Almonte C, Huang J, Young S, Boese B, Schmidt M, Pericak-Vance MA, Martin E, Zhang X, Harkins TT, Züchner S. Exome sequencing of a multigenerational human pedigree. *PLoS One* 2009; **4**: e8232 [PMID: 20011588 DOI: 10.1371/journal.pone.0008232]

P- Reviewer: Minana MD, Nishio K, Zhou S **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com