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Stem cells in bone regeneration

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Abstract

Bone defects, including normal fracture healing as well as healing problems represent a global health problem. The need for better treatment of bone defects is one of the central issues of tissue engineering and regenerative medicine. Regenerative orthopedics has several approaches – activation of endogenous stem cells, stem cell therapy and tissue engineering. Development of new treatments is mainly focused on the tissue engineering strategies that include stem cells, bioactive signals and appropriate scaffold support.

The aim of this review is to describe a variety of stem cells that have an ability to become bone cells and therefore are of central importance for bone tissue engineering. Several cell types have been proposed as starting material - embryonic stem cells, induced pluripotent stem cells and adult stem cells. Due to ethical and safety issues, embryonic and induced pluripotent stem cells may be more suitable for studying human development and tissue formation under diverse experimental conditions, and represent an excellent base for understanding human diseases and development of innovative therapeutic solutions. Among adult stem cells, mesenchymal stem cells are the most suitable for bone tissue engineering. They can be isolated from variety of mesenchymal tissues and can differentiate into osteoblasts when given appropriate mechanical support and osteoinductive signal.

The near future of bone healing and regeneration is closely related to advances in tissue engineering. The optimization of protocols of bone graft production using autologous mesenchymal stem cells loaded on appropriate scaffolds, exposed to osteogenic inducers and mechanical force in bioreactor, should be able to solve the current limitations in managing bone injuries.

INTRODUCTION

Bone is a specialized form of connective tissue supporting the whole organism in higher vertebrates. Besides the liver, bone is the only tissue that can spontaneously heal and restore its function without leaving a scar. It is highly vascularized and subject to constant remodeling. Thus bone has the ability to regenerate after injury, however this is only the case if the injury is below the critical size. Because of prolonged lifetime, bone diseases such as infections, fractures and osteoarthritis, osteoporosis and spine diseases become a major socio-economic problem. Bone defects are one of the leading causes of morbidity and disability in elderly patients, leading to decreases in overall health and quality of life and there is an urgent need for more effective means of bone reconstruction *(1)*. Regenerative orthopedics has several approaches to bone reconstruction – activation of endogenous stem cells, stem cell therapy and tissue engineering. Tissue engineering is the 'final' option in managing bone loss *(2)*. The area of bone tissue engineering aims to repair and regenerate damaged bone. It applies the principles of biol-

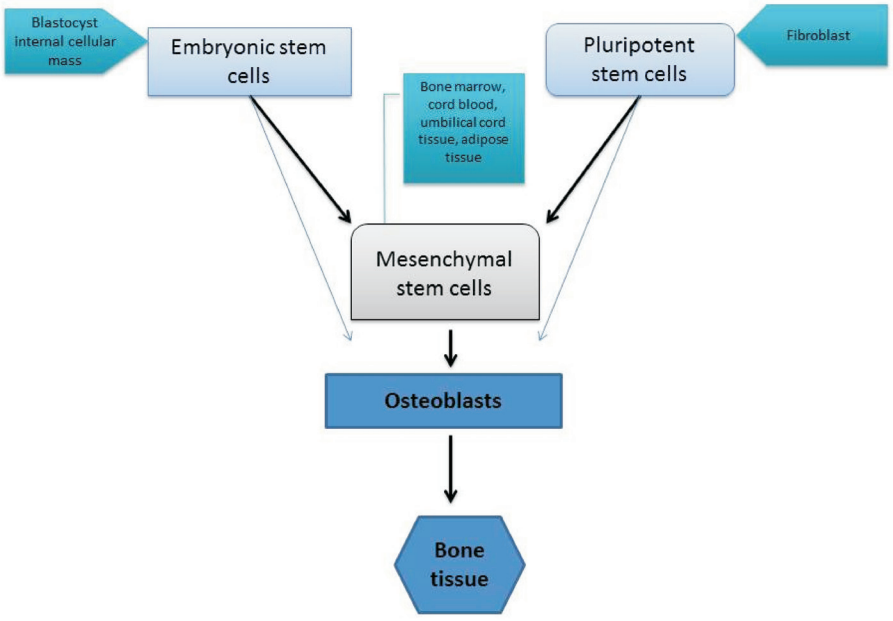

Figure 1. *Stem cells with bone regeneration potential.*

ogy and engineering to develop functional replacements for damaged tissue. Approximately 600,000 bone graft procedures are performed each year in the United States, and about 2.2 million of such procedures are performed annually worldwide *(3, 4)*. It is based on three components and includes the successful interaction of these components: (i) *cells* that are responsible for the production of tissue, (ii) a *carrier* that holds cells together and creates a physical, three-dimensional shape of the tissue, (iii) *growth factors* that direct the cells to the desired bone phenotype tissue *(5)*. It has been proven that osteoblasts, human mesenchymal stem cells (hMSCs), human embryonic stem cell-derived MSCs (hESC-MSCs) and human induced pluripotent stem cell-derived MSCs (hiPSC-MSCs) all successfully produce bone grafts when attached to proper mechanical support *(6)* with the addition of osteogenic supplements (Figure 1).

The aim of this review is to describe the current state of the art in our understanding of different types of stem cells that can participate in bone regeneration and seeks to describe the challenges that scientists face in finding a successful protocol for the bone healing.

CELLS

Bone is a dynamic biological tissue consisting of metabolically active cells. The cell component of bone consists of the precursor cells (progenitors), osteoblasts, osteoclasts, osteocytes and bone marrow hematopoietic elements. Osteoblasts are metabolically active mature bone-forming cells *(7)*. They secret osteoid, non-mineralized organic corpuscle which in turn undergoes mineralization process. Osteocytes are mature osteoblasts trapped within the bone matrix. Every osteocyte extends network of cytoplasmic tubules to the blood vessels and other cells. These cells are involved in the control of extracellular calcium. Osteoclasts are large multinucleated cells that degrade bone *(8)*. Beside cells, bone is also composed of organic and inorganic elements. Approximately 20% of the weight of bone is water until the weight of dry inorganic bone makes calcium phosphate (65-70%) and the organic matrix of fibrous proteins and collagen (30-35%)*(8)*.

Bone formation models *in vitro* are based on the fact that cell differentiation and function can be modeled according to factors that are important for embryonic development. The formation of the skeletal elements starts with cell condensation process where scattered mesenchymal stem cells migrate, proliferate and are connected by adhesion molecules. Stem cells represent the building blocks of our bodies, functioning as the natural units of embryonic generation during development and adult regeneration following tissue damage *(9)*. Owing to their unique regenerative capacity, stem cells have generated great enthusiasm worldwide and represent an invaluable tool with unprecedented potential for biomedical research and therapeutic applications *(10)*.

Stem cells are undifferentiated cells that can, under certain influence differentiate into specialized cells and tissues. During development, potency of stem cells decreases from totipotent stem cells (morula stage), capable of differentiating into all embryonic and extra embryonic tissues, to pluripotent stem cells (blastocyst stage), forming all embryonic tissues and to multi- or unipotent adult stem cells, forming tissues within their germ layer. Here, we

discuss three types of stem cells: embryonic stem cells isolated from the inner cell mass of blastocysts, induced pluripotent stem cells generated from somatic cells by introduction of key transcription factors *(10)* and adult stem cells found in various adult tissues. Adult stem cells, also called somatic stem cells, in adult organism act as repair system for the body, replenishing adult tissues, prompt tissue homeostasis throughout life and ensure tissue regeneration following damage and they have great potential in regenerative medicine *(10)*. Mesenchymal stem cells replenish connective tissues including bone. Therefore, they are the first choice among adult stem cells for regeneration of bone tissue. Adult stem cells have ability to differentiate in *in vitro* conditions and following differentiation can be implanted back into the patient *(11)*. They can also be passaged *in vitro* until they achieve the sufficient number of cells and then implanted in the patient *(12)*. Osteogenic differentiation *in vitro* is induced by ascorbic acid, β-glycerophosphate and dexamethasone *(13)*. This approach avoids stem cells differentiation into unwanted forms of tissue and also prevents the risk of malignant transformation. Regardless these advantages they are still difficult to passage *in vitro* and they often lose their phenotype.

1. Embryonic stem cells (ESCs)

ESCs were first isolated from mouse embryo by Nobel Prize Martin Evans and his team *(14)*. ESCs are considered fully pluripotent, which means that they can differentiate into all three germ layers. After while embryonic stem cells were also isolated from human embryo in the late 1990s *(15)*. Human ESCs (hESCs) grow in compact colonies on feeder layers of murine embryonic fibroblasts or human cells, which produce the extracellular matrix for cell attachment and condition the culture medium with paracrine factors. hESCs culture media were supplemented with fetal bovine serum of selected lots, serum replacement and with growth factors which activate the intracellular signaling networks maintaining pluripotency (10). Although they present many advantages for regenerative medicine and tissue engineering, many ethical issues limit their use and many countries prohibit ESCs isolation from human embryo. Because these particular cells have created an ethical debate, other researchers have begun to use embryonic or fetal cells derived from voluntary interruption of pregnancy between 5 and 8 weeks *(16)*. Darja Marlot and her team demonstrated that human ESC-derived mesenchymal progenitors can be induced to form compact, homogenous and phenotypically stable bone-like tissue by cultivation on 3D osteoconductive scaffolds in bioreactors with interstitial flow of culture medium. They developed a stepwise protocol to engineer bone-like constructs from hESCs. The engineered bone tissue was stable for 8 weeks *in vivo* and exhibited signs of continued bone development, indicating a potential for bone defect regeneration *(17)*. Vunjak-Novakovic G and her team focused on the effects of the matrix architecture and mineral content in scaffolds on bone formation by hESC. Mesenchymal progenitors derived from hESCs were cultured for 5 weeks in decellularized bone scaffolds with three different mineral densities. They conclude that minerals were beneficial for the higher expression of bone markers in cultured cells and more robust accumulation of the new bone matrix *(17)*. Besides ethical controversy, there are more problems with human embryonic cells as they can easily form a tumor tissue and they are not immunologically compatible.

2. Induced pluripotent stem cells (iPSCs)

Studying the transcription factors involved in human embryonic development, scientist found out that when fibroblasts are exposed to defined cocktail of transcription factors (Oct3 /4, Sox2, KLF4 andc-Myc), they become pluripotent and have the ability to differentiate into other tissues. Reprogramming of already differentiated cells bypass the ethical problems that have occurred with human embryonic stem cells. Discovery of these pluripotent stem cells contribute greatly to the development of regenerative medicine and opened many new areas in the stem cell biology and their use in disease treating *(18–20)*. Moreover, induced pluripotent stem cells (iPSCs), which are ESC-like pluripotent cells have brought new hope to tissue engineering and regenerative medicine because of their full pluripotent differentiation potential and excellent performance in bone regeneration *(21)*. Earlier last year a group of scientists led by the M. Tang examined what will happen with iPS cells when they were cultured in a calcium phosphate scaffold (CPC) *(22)*. iPSCs were cultured to form embryoid bodies (EBs) and MSCs migrated out of EBs. Their aim was to produce iPS cell-derived mesenchymal stem cells (iPS-MSCs), seed them on calcium phosphate scaffold and observe cell attachment and proliferation. iPSC-MSCs showed good viability and osteogenic differentiation on CPC scaffold for the first time, but the process was quite complex. To generate osteoprogenitor cells from iPSCs, the most widely used protocol relies on an intermediate embryoid body (EB) formation, but recently Dogaki Y. with his team hypothesized that an osteoprogenitor cell population could be efficiently generated from iPSCs without the EB formation step and they tried 'direct-plating' method *(23)*. They isolated a murine iPSCs colony and grew it into a medium for the MSC, after 14 days of osteogenic induction, cells proved to have a high osteogenic differentiation capacity, significant increase in alkaline phosphatase activity, expression of osteogenic genes and mineralization. When compared with ESCs, iPSCs bypass the problems with ethical controversy and immunogenicity, but there is still the possibility of tumor tissue formation.

3. Adult stem cells (ASCs)

Adult stem cells are found in the post-natal body within their specific niches and they have limited differentia-

tion capacity. ASCs are applied extensively in regenerative medicine because they can be harvested from the individual, cultivated *in vitro,* exhibited to appropriate factors depending on the tissue we want to get and then implanted back into the patient. Since the cells are autologous, the immune response is avoided and all ethical questions are circumvented. Adult stem cells also represent a less risk of malignant transformation. In a living organism, adult stem cells are capable to divide when needed and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue. *Hematopoietic stem cells* give rise to all the types of blood cells *(24)*. *Neural stem cells* in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of non-neuronal cells—astrocytes and oligodendrocytes *(25)*. *Mesenchymal stem cells* have been reported to be present in many tissues. Those from bone marrow give rise to a variety of cell types: bone cells (osteoblasts and osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes) and stromal cells that support blood formation *(26–28)*.

3.1. Mesenchymal stem cells (MSCs)

Among stem cells, mesenchymal stem cells (MSCs) seem to be more suitable for bone engineering compared to ESCs and iPSCs due to several characteristics that they possess. Mesenchymal stem cells are multipotent cell population present in the bone marrow and other tissues, including adipose tissue. MSCs can differentiate into osteoblasts, chondrocytes, adipocytes and myocytes *in vitro (29)*. They have proliferation and differentiation ability, making them a great candidates for tissue engineering and regenerative medicine *(30)*. For regeneration of bone, MSCs were isolated from bone marrow and they can be used alone or in combination with the osteoinductive signal to facilitate bone healing. MSCs is often isolated based on their properties to attach on the plastic surface on which they are growing. The number of these cells in the bone marrow varies (from 0.001 to 0.1%) between different patients and it is considered to decrease with increasing age of the patient. However, cells can proliferate and achieve up to 50 population distribution *in vitro (31)*. Successful cultivation of MSCs *in vitro* requires an understanding of signaling pathways that lead the proliferation and differentiation of these cells. Many chemical, biological and mechanical factors determine which path cells will follow and whether they will stay multipotent or differentiate into a specific cell type *(29)*. MSCs differentiation to specific cell lines may be accomplished *in vitro (32)*. Osteogenic differentiation *in vitro* is induced by ascorbic acid, β-glycerophosphate and dexamethasone *(13)*. Ascorbic acid is essential for the development of osteoblasts, serves as a cofactor in the synthesis of collagen and stimulates the production of extracellular matrix, proliferation and differentiation of cells. β-glycerophosphate serve as a source of phosphate for the formation of calcium phosphate *in vitro*. It is also responsible for the formation of three-dimensional bone nodules between cells as proof of realized osteoblast phenotype. Dexamethasone (DEX) is composed by a synthetic glucocorticoid, which regulates the expression of osteoblastic genes, *in vitro* enhances differentiation, alkaline phosphatase activity and mineralization of bone *(32)*. When the MSCs are cultured in osteogenic medium they differentiate into osteoblasts that play the role in bone formation and formation of extracellular matrix and minerals. Osteogenic differentiation is divided into three phases (Figure 2). The first phase of proliferation lasts 4 days. After that in the period from 5 to 14 day the cells start to change and express alkaline phosphatase, type1collagen and they excrete extracellular matrix. The final phase lasts from 14th until 28th day and results in high expression of osteocalcin, osteopontin and mineralization *(13)*. The fate of MSCs is dependent on their microenvironment particularly the extracellular matrix that regulates the balance between self-renewal and differentiation through different cytokines, growth factors and mechanical stimuli *(33)*. Mesenchymal stem cells have been used in bone regeneration for quite some time, but their disadvantages such as reduced proliferation rate or osteogenic potential decrease during aging greatly limit their application in tissue engineering and regenerative medicine.

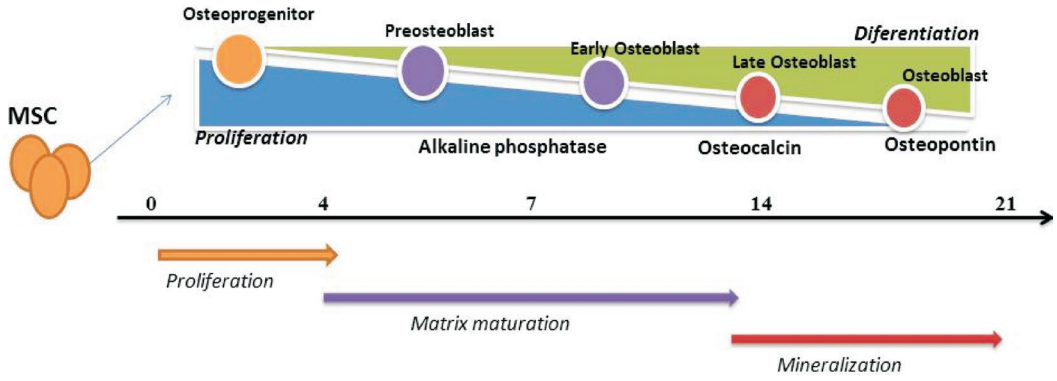

Figure 2. *Lineage-specific differentiation of mesenchymal stem cells (MSCs) through three stages of differentiation from 0-21 days.*

Sources of MSCs for bone regeneration

Stem cell niche enable homeostasis and retains the ability of stem cells to self-renew *(34).* MSCs are located next to vessels walls, on the surface of the trabecular bone, in umbilical blood, within dental tissue and synovial fluid. But the most important source of stem cells for repair of the skeleton are derived from bone marrow, endosteum and perivascular cells. Team of scientists led by Ryu H. H. *(35)* isolated MSCs from fat and monitored their osteogenic potential comparing them with MSCs from the bone marrow. There were no significant differences in functional recovery among the MSCs groups. Scientist also isolated the MSCs from other types of tissues. Bugarski D with his team tried to compare the characteristics of MSCs derived from two different human tissues: peripheral blood (PB-MSCs) and umbilical cord (UC-MSCs) *(36).* Cells were isolated according to the adherence to plastic after gradient-density separation or an explant culture method, respectively and compared regarding their morphology, clonogenic efficiency, proliferating rates and differentiation potential. MSCs derived from both sources exhibit similar morphology, proliferation capacity and multipotency. Results demonstrated that both MSCs represent good alternative sources of adult MSCs that could be used in cell therapy applications. One year after that, Polianskaia G.G. with his team analyzed the characteristics of mesenchymal stem cell lines isolated from different tissues of 5-6-weeks human embryo: bone marrow (line FetMSC) and muscle of limb (line M-FetMSC) *(37).* They confirmed MSC status for FetMSC and M-FetMSC lines and number of interlinear differences related to growth characteristics and differentiation potential were revealed. Suggesting the possible influence of different microenvironments in which the cells are in the body before their growth *in vitro*. For many years, bone marrow is still the most important source of stem cells. Although the sources of stem cells are widespread and studies have shown that they do not differ much in osteogenic potential, the ideal stem cell source for bone regeneration should be easily accessible, noninvasive and cell should be rapidly expandable by *in vitro* culture. The data collected so far have shown that adipose tissue is an abundant source of MSC and due to its wide body distribution makes it accessible by minimally invasive methods. These MSC are also easy to isolate and expand *in vitro (38).*

Mesenchymal stem cells-based therapies

Mesenchymal stem cells have been used in the clinic for approximately 10 years. From animal models to clinical trials, MSCs have afforded promise in the treatment of numerous diseases, mainly tissue injury and immune disorders *(39)***.** For example, in the 2010s Yoshikawa T. and his team analyzed the regenerative ability of autologous MSCs in degenerated intervertebral discs. Patients had lumbago, leg pain, and numbness. MSCs were from the patient marrow fluid and were cultured using the medium containing autogenous serum and then were grafted on collagen sponge. Two years after surgery, radiograph and computed tomography showed improvements in the vacuum phenomenon in both patients *(40).* Mesenchymal stem cell (MSC) transplantation shows exciting promise for the future regenerative approach to intervertebral disc disease. Many people worldwide suffer from bone defects due to trauma or disease. Usually small bone defects heal spontaneously but large defects cannot regenerate without intervention *(41).* When injury occurs MSCs are activated and they secrete signals that are trophic and immunomodulatory telling us the true potential of MSC-based cell therapy. The stem cells trigger or activate the body's own healing system. This MSCs immunosuppressive properties have been explored in cell/organ transplant, tissue repair, autoimmune diseases and prevention of graft versus host disease (GVHD) *(42).* For example, MSCs have been successfully applied to GvHD in patients receiving bone marrow transplantation and in patients diagnosed with severe steroid resistance *(39).* Taking into consideration the impact on the suppression of GVHD and inducing remission within the organism, MSC were investigated for the purpose of treating inflammatory bowel disease, type1diabetes and chronic obstructive pulmonary disease *(43).* Some studies in mice suggest that MSCs can promote formation of new blood vessels in a process called neovascularization. MSCs do not make new blood vessel cells themselves, but they stimulate the growth of the endothelial precursors – cells that will develop to form the inner layer of blood vessels *(44)*. Group of scientists led by Nicolas C. R cultured hMSCs from bone marrow with human umbilical vein endothelial cells (HUVECs) in differentiation medium *in vitro*. The coculture of cellular aggregates of hMSC (92%) and HUVEC (8%) supports the formation of a primitive three-dimensional vascular network. This study demonstrated that the *in vitro* development of engineered vasculatures leads to a more functional, stable vasculature *in vivo (45)*. The ability of MSCs to secrete such factors is recognized as an important component of their ability to promote tissue regeneration. It may be possible to allograft them for the purposes of bone regeneration and this would obviate the need for treating each patient with his own cells, thus simplifying and streaming the entire process.

Great success was achieved in the treatment of bone diseases with mesenchymal stem cells in animal models but a lot of gaps in our knowledge need to be filled in ordertobe able to use MSCs in clinical practice. It is still unknown mechanism of MSC-mediated bone regeneration *in vivo* and because of that it is difficult to maintain a stable phenotype of MSCs *in vitro (46).* To promote bone regeneration several treatment approaches have been used and the most common treatment is direct injection of MSCs. The MSCs can be better delivered to the site of injury bone if they are connected to three-dimensional

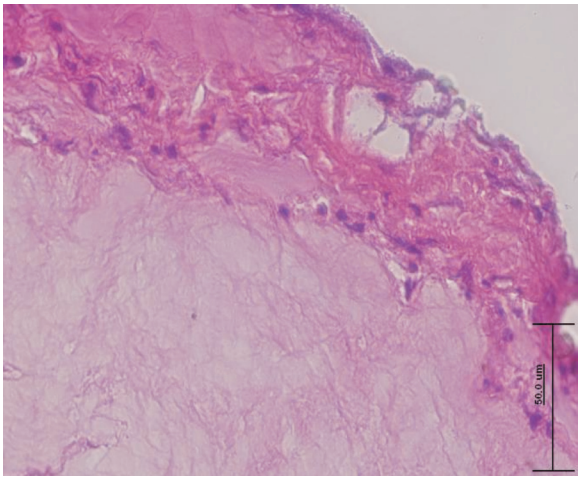

Figure 3. *Bone graft after 21 days of cultivation of human mesenchymal stem cells on peptide hydrogel in osteogenic medium. Marginal part of the image represents the dense layerof differentiated cells (osteoblasts). Cells were stained with hematoxylin and eosin (HE) dye, blue part representing the core, and the dark red part representing the cytoplasm of cells. Center of the bone graft represents a dense connective tissue consisting of matrix proteins.*

(3D) scaffold that mimics the mechanical and biological role of extracellular matrix. Therefore, the design of such scaffold is crucial for therapy with mesenchymal stem cells. Such scaffolds have the potential to provide the corresponding 3D environment for the growth and differentiation of cells *(13).*

MSC-bone constructs

Bone constructs fabricated using scaffolds, cells and growth factors could be appropriate substitutes for bone transplantation (Figure 3). The general approach to bone tissue engineering is the first selection of cells and then the scaffold that will be seeded with cells. Three-dimensional scaffolds made of biomaterials are developed as an interim basis for the growth of cells in an organized manner. It is also well known that the three-dimensional organization of cells will affect cell development *(33, 47).* Preferably they need to be biocompatible, biodegradable and mimic *in vivo* structure of the bone tissue. The cells in the body are surrounded by or embedded in an extracellular matrix, thus scaffold should have the properties that mimic the environment *(30).* These properties contribute to the cell attachments, proliferation and differentiation.

There are two types of scaffolds, *natural* and *synthetic*. Natural biomaterials alginate, chitosan, collagen, fibrin, hyaluronic acid and hydroxyapatite are extensively used for growing tissues because of the similarity with the extracellular matrix. However, the problems in the alignment properties and the source of these materials do not eliminate the problems of immune rejection of grafts. When synthesized, polymers can be determined with precise design of physical properties, but still we have the problem with biocompatibility. In order to achieve the best possible integration into the host tissue researchers are using composite materials which are combination of natural and synthetic materials *(47).*

Group of scientists led by Prosecká E presented a new type of scaffold. They combined type 1 collagen and hydroxyapatite enriched with polycaprolactone nanofibers (Coll/HA/PCL) *(48).* They planted MSCs on scaffold in osteogenic media and thrombocyte-rich solution. Scaffold was implanted into the white rabbit and they watched bone regeneration *in vivo*. Results have shown that the installation of nanofibers in the scaffold and thrombocytes in combination with MSC represent a novel treatment for bone defects. This unique fusion between nanotechnology and biotechnology offers unprecedented possibilities in studying and modulating biological processes on a molecular and atomic scale. Later in August a group of scientists led by the Anne-Laure Gamblin presented also their type of scaffold. They made calcium phosphate (CaP) ceramics scaffold and seed them with human mesenchymal stem cells (hMSC). Scaffold was implanted in paratibial muscle of nude mice. After 8 weeks, at the site of implanted scaffold they noted a formation of bone tissue, but the mechanism of osteoinduction by hMSC with CaP stay unclear *(49, 50).*

Controlled release of small molecules, growth factors and osteoinductive signals from the scaffold is very important for osteogenic differentiation, especially for therapeutic applications. Bone morphogenetic proteins (BMPs) have a greatability to induce differentiation of osteoblasts and are often used as osteoinductive signals embedded in the scaffold for the differentiation of stem cells *in vivo*. BMPs are family members of transforming growth factor-beta (TGF-β). This is afamily of signaling proteins that regulate a large number of cellular activities, including differentiation, proliferation, migration, apoptosis and bone formation *(51).* Currently, the FDA approved technique is the implantation of the collagen carrier with BMP2 signal *(52, 53).* Collagen scaffold was soaked in recombinant BMP2. This technique, although clinically accepted, shows poorly controlled release of BMP resulting in high concentrations in the beginning, well above normal physiological conditions, that quickly decrease and wash out *(54).* Beside the bone regeneration, BMP2 is studied also in cartilage repair *(55).*

CONCLUSION

Bone defects that are due to trauma or pathological and physiological bone resorption represent a global health problem. The need for bone regeneration is one of the central issues in regenerative medicine. Cellular therapy and tissue engineeringare becoming a useful addition to medical therapies for repairing and restoring function

of tissues. Tissues may be restored with differentiated cells of the type normally found in the target tissue, or with progenitor cells, including stem cells that can differentiate into the mature cells of the tissue. Several cell types and tissues have been proposed as starting material; adult stem cells, embryonic stem cells, induced pluripotent stem cells, but some cell choices are more adaptable to cellular therapy in patients. Today, the general opinion is that mesenchymal stem cells from adult tissues are the most suitable for bone tissue engineering. ESCs and iPSCs allow the study of human development and tissue formation under diverse experimental conditions and represent an excellent base for understanding human diseases and developing innovative therapeutic solutions.

The near future of bone healing and regeneration is closely related to advances in tissue engineering. Perhaps therapy using scaffolds, healing factors and stem cells together would be able to solve the current limitations in managing bone injuries. Bone constructs elaborated with tissue engineering principles are a promising substitute for autologous bone graft and have long been considered the golden standard for repair of large bone defects. Although application of MSCs as cellular material facilitates the construct fabrication, there is still some issues with MSC preparation mainly due to expansion in culture that can affect their genome and phenotype. However, more work needs to be done to fully determine the clinical potential, efficacy, and safety of stem cell-based treatments.

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