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Kursad Turksen *Editor*

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Stem Cells and Cell Based Strategies in Regeneration

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Stem Cells and Cell Based Strategies in Regeneration

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore the potential utility of stem cells in cell-based strategies in diverse areas of regenerative medicine. Although this has been an active area of basic and translational research for many years with enormous advances in our approaches and understanding, significant challenges remain. These challenges encompass such fundamental questions as which stem cell populations are most appropriate to achieve not just a regenerative response but also restoration of original tissue and organ form and function. To address the significant advances occurring in this very active field and the considerable challenges that remain to be overcome, I have recruited several experts to provide summaries of their ongoing research studies.

I remain very grateful to Peter Butler, Editorial Director, and Meran Owen-Lloyd, Senior Editor, for their ongoing support of this series that we have embarked upon.

I would also like to acknowledge and thank Sara Germans-Huisman, Assistant Editor, for her outstanding efforts in getting the volume to the production stages.

A special thank you also goes to the production crew for their work in generating the volume.

Finally, I thank the contributors not only for their support of the series but also for their efforts to capture both the advances and remaining obstacles in their areas of research. I am grateful for their efforts and trust readers will find their contributions interesting and helpful.

Ottawa, ON, Canada — Kursad Turksen

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Biomaterials for Regenerative Medicine: Historical Perspectives and Current Trends

Maryam Rahmati, Cristian Pablo Pennisi, Emma Budd, Ali Mobasheri, and Masoud Mozafari

Abstract

Biomaterials are key components in tissue engineering and regenerative medicine applications, with the intended purpose of reducing the burden of disease and enhancing the quality of life of a large number of patients. The success of many regenerative medicine strategies, such as cell-based therapies, artificial organs, and engineered living tissues, is highly dependent on the ability to design or produce suitable biomaterials that can support and guide cells during tissue healing and remodelling processes. This chapter presents an overview about basic research concerning the use of different biomaterials for tissue engineering and regenerative medicine applications. Starting from a historical perspective, the chapter introduces the basic principles of designing biomaterials for tissue regeneration approaches. The main focus is set on describing the main classes of biomaterials that have been applied in regenerative medicine, including natural and synthetic polymers, bioactive ceramics, and composites. For each class of biomaterials, some of the most important physicochemical

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and biological properties are presented. Finally, some challenges and concerns that remain in this field are presented and discussed.

Keywords
Biomaterials · Regenerative medicine · Scaffold · Tissue engineering

Abbreviations

BCP	Biphasic calcium phosphate
BMP-2	Bone morphogenetic protein 2
CaP	Calcium phosphate
CNFs	Carbon nanofibers
CNTs	Carbon nanotubes
DLC	Diamond-like carbon
ECM	Extracellular matrix
FBRs	Foreign body responses
GAGs	Glycosaminoglycans
GAL	Galactoxylose
GLU	Glucan
HA	Hydroxyapatite
hiPSCs	Human-induced pluripotent stem cells
MCNs	Mesoporous carbon nanomaterials
Micro-CT	Microcomputed tomography
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
MWCNTs	Multi-walled carbon nanotubes.
PCL	Polycaprolactone
PEG	Polyethylene glycol
PEO	Polyethylene oxide
PGA	Polyglycolide
PLA	Polylactide
PNIPAAm	Poly(N-isopropylacrylamide)
POE	Polyoxyethylene
PRP	Platelet-rich plasma
QDs	Quantum dots
SWCNTs	Single-walled carbon nanotubes

1 Introduction

It is well known that cells are the fundamental component of most regenerative medicine or tissue engineering strategies (Mason and Dunnill 2008; Mizuno et al. 2012). Different types of cells have been successfully used for tissue regeneration applications, including primary cells and stem cells (Levi and Longaker 2011; Lindroos et al. 2011). However, it has been shown that after free cell transplantation only a small proportion of cells are engrafted at the target site and approximately 90% of cells are lost during the first few hours following delivery (Mooney and Vandenburgh 2008). To overcome cell loss and encourage cell engraftment, scientists have looked to use cells in combination with biomaterials and, eventually, specific growth factors to offer a suitable microenvironment for tissue regeneration (Braghirolli et al. 2014; Pina et al. 2015). Biomaterials are used to efficiently transport cells and/or bioactive agents, offering a suitable microenvironment to promote cell survival and growth (Ayoub and Lucia 2017). A wide range of natural and synthetic biomaterials have been identified, which allow for the natural deposition of extracellular matrix (ECM) and regeneration of damaged tissues (Pati et al. 2015; Boersema et al. 2016; Webber et al. 2016). The success of many regenerative medicine strategies such as cell-based therapies, artificial organs, and engineered living tissues, is highly dependent on the ability to design or produce suitable biomaterials (Ayoub and Lucia 2017). In addition, accurately manipulating their physicochemical properties has a significant importance in accomplishing a favourable clinical outcome. In the design of biomaterials for regenerative medicine applications it is crucial to take into account the ability of the biomaterial to support cell survival, suitable cell function after transplantation, and encouraging autologous tissue growth *in situ* (Ducheyne 2015; Sekuła and Zuba-Surma 2018). Additionally, the biological performance of the biomaterial should be carefully assessed *in vitro* and *in vivo*, to investigate the physicochemical properties, immune response, and biodegradability rate (Ducheyne 2015). A biomaterial scaffold should provide mechanical support, form, and cell-scale design to support neo-tissue formation. Recently, several fabrication methods have been devised to obtain biomaterials with physicochemical properties

matching those of the target tissue. In addition, with the aim of enhancing different properties of biomaterial scaffolds, various composite biomaterials have been introduced by combining different natural and synthetic polymers with bioactive ceramics. This chapter focuses on the research concerning biomaterials for use in tissue engineering and regenerative medicine applications. Furthermore, the basic design principles of biomaterial scaffolds will be briefly introduced. The main body of the chapter describes the main properties of each class of biomaterials for regenerative medicine and tissue engineering, which includes natural and synthetic polymers, ceramics, and composite materials. Finally, a discussion about current concerns and challenges in the field will be presented.

2 A Brief History of the Origin and Use of Biomaterials for Therapeutic Purposes

For centuries, a myriad of materials have been investigated and applied for clinical therapeutic purposes. These materials were mostly selected based on their availability and the basic knowledge about their properties. It is hard to pinpoint the exact date in time when human beings began to apply the use of biomaterials. For instance, an embedded spear point in the hip of a tall and healthy body, dated back to 9000 years ago, was found nearby Kennewick in Washington (USA). Surprisingly, the spear appeared to be well engrafted and there were no evident signs of foreign body response (FBR). Tattoos are another example of permanent insertion of a foreign material into the skin, which dates to about 5000 years ago. Dental implants are among the earliest implants that have been found in the body. The Mayan civilization shaped nacre teeth from sea shells approximately 600 AD, and seemingly accomplished what we call today osseointegration.

Historically large wounds were closed by either cautery or sutures, there is also evidence that the ancient Greeks used metallic sutures. Owing to the function of the heart as a pump, it was a rational idea to consider substituting the heart with a synthetic pump. In 1812, Le Gallois stated that organs could be preserved by actively pumping blood over them (Min and Sun 2002). Many studies on organ perfusion with pumps were carried out between 1828 and 1868. In 1881, Étienne-Jules Marey devised a synthetic heart device, which mainly focused on investigating heart beat (Braun 1994). In 1957, Dr. Willem Kolff and his co-workers studied the artificial heart by using dogs as animal models (Nosé 2009). Rene Descartes hypothesized with the concept of using corneal contact lens' and in 1827 John F. W. Herschel postulated that a glass-based lens could protect the eye (Ratner 2013).

During 1914 to 1918 novel advanced metallic, ceramic, and polymeric materials were developed for military purposes. Surgeons began to apply these long-lasting, inert materials as a substitute for injured body parts. Hence, materials such as silicones, polyurethanes, Teflon®, nylon, methacrylates, titanium, and stainless steel, which mainly were fabricated for industry applications, were used by surgeons in medical applications (Ratner 2013). In 1906 Sir Harold Ridley investigated the response of the body to implanted biomaterials. Following World War II, Sir Harold Ridley studied the eyes of pilots which had implanted in them shards of plastic from shattered canopies of planes and found that the shards of plastic did not cause any FBR and predicted that the plastic material could be used as a biocompatible material for medical applications (Apple 2007). In 1891, Theodore Gluck carried out the first hip replacement surgery by using a cemented ivory ball, but the replacement proved to be unsuccessful (Ranawat and Ranawat 2012). After many attempts between 1920–1956 McKee and Watson Farrar finally designed a "total" hip with an acetabular cup of metal that was cemented in place (Ranawat and Ranawat 2012). Charles Kite in 1788 discussed the possibility of electrical discharges to the chest for heart recovery (Elsenaar and Scha 2002). In 1930–1931, concurrently the groups of Dr. Albert S. Hyman, and Dr. Mark C. Lidwill innovated the portable pacemaker. Dale Wurster was likely the first person, who in 1949,

introduced the Wurster process which allowed drugs to be encapsulated and consequently released slowly. In 1964 Judah Folkman introduced the up-to-date concept of controlled release with wrapped isoproterenol silicone tubes followed by insertion into animal hearts (Ratner 2013). The use of biomaterials in medicine has a long history which is not the scope of this chapter. There are some thorough reviews in this regard, which readers could refer to for more information (Cortes et al. 2008; Ratner 2013; Migonney 2014).

3 General Design Requirements for Biomaterials in Regenerative Medicine

The American National Institute of Health defines a biomaterial as "any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual" (Bergmann and Stumpf 2013). When designing biomaterial scaffolds, which are intended as templates to direct the growth of new tissue a set of key requirements should be taken into consideration. These requirements involve the assessment of biocompatibility, physical and chemical properties, as well as the economic aspects related to the use of the biomaterials in clinical practice.

Biocompatibility has been defined as "the ability of a material to perform with an appropriate host response in a specific situation"(Williams 1999). Before applying biomaterials in the body, the toxicity and biocompatibility of the biomaterial in question needs to be carefully examined. It has been well-acknowledged that all implanted biomaterials could potentially stimulate the immune response, known as foreign body response (FBR) (Morais et al. 2010). However, FBR is fundamental for destroying cellular debris and subsequently inhibiting infection. Therefore, it is essential to carefully consider the mechanisms of immune response of implanted materials in the design phase. In general, when considering the tissue responses of implantation, there are three main classes of biomaterials including bio-tolerant, bioactive, and bio-inert. Bio-tolerant materials are disconnected from bone through a fibrous layer, however, bioactive materials commonly make some chemical bonds with bone tissue, identified as osseointegration. In the case of bio-inert materials, there is a possibility to make direct interaction with the neighbouring bone tissue, however, no chemical reactions occur between them (Bergmann and Stumpf 2013). For tissue engineering applications, the biomaterials must have very specific features, such as biocompatibility, bioactivity, biodegradability, and tailorable physical and mechanical properties (Hollinger 2011; Ge et al. 2012).

In the biomedical engineering industry, the production of scaffolds for tissue engineering and regenerative medicine applications have seen a tremendous growth over the recent years. Many of these novel materials have shown promise through successful pre-clinical and clinical trials. Apart from biocompatibility considerations, it is necessary to consider other design parameters which mostly depend on the target application. One of such parameters is the architecture of the scaffold which should provide an appropriate environment for the cells to promote formation of new tissue, remodelling, vascularization, and integration. The scaffold structure must be both porous and stable, so as to allow diffusion of nutrients and metabolites without risk of collapsing (Chan and Leong 2008). In terms of architecture, another key point is the selection of an optimum scaffold pore size. The pores on the scaffold ensure that cells can efficiently interact with the ligands on the surface and should be big enough to ensure cells can successfully migrate within the scaffold before binding to the ligands and allow a minimum ligand density to be achieved. Overall, optimal scaffold structure ensures that a critical amount of cells can be bound in an efficient manner to the surface of the scaffold (O'brien 2011). In addition, when fabricating a scaffold, biodegradability must be taken into consideration

as the scaffold can only be considered as a factor of support when the body is capable of replacing the construct following the production of ECM and appropriate healing. The waste products originated from scaffold degradation must be non-toxic and must be removed without causing a disturbance to the surrounding organ system (O'brien 2011). Furthermore, mechanical properties and scaffold architecture go hand in hand in the creation of the construct. A fine balance between the various mechanical properties and architecture in terms of porosity allows the scaffold to support sufficient infiltration and vascularization, meanwhile providing the correct stability upon implantation. Mechanics relative to biology and sensitivity are considered to be also key factors, as traction forces exerted upon cellular components within a substrate have adverse effects on the formation of cells (Chan and Leong 2008).

When creating a scaffold, it is important to consider factors within the manufacturing process to ensure that the construct is clinically viable. Factors include production complexity, cost effectiveness, good manufacturing processes, production rate, delivery methods, and storage of the scaffold. The scaffold must be cost effective in terms of fabrication and an easy transition should be attainable in terms of production from a small-scale aseptic laboratory procedure to high quality batch production. Furthermore, it must be determined how could deliver the scaffold to clinical stages and how clinically store the construct (Hollinger 2011; Ducheyne 2015; Ayoub and Lucia 2017).

4 Types of Biomaterials in Tissue Engineering and Regenerative Medicine

4.1 Polymeric-Based Biomaterials

Polymeric biomaterials have extensively been used for the regeneration and engineering of various tissues, such as the musculoskeletal (Sarem et al. 2013), cardiovascular (Yazdanpanah et al. 2014), neural (Zarrintaj et al. 2018), and dermal tissues (Gholipourmalekabadi et al. 2017). The selection of biomaterials for a particular application relies on the material's physical and chemical properties, which includes surface topography (Ranella et al. 2010), architecture (Chang and Wang 2011), charge (Calatayud et al. 2014), free energy (Hoefling et al. 2010), and functional groups (Meder et al. 2012). Polymers offer an exceptional flexibility in terms of tailoring their chemical and physical surface properties. Itis possible to precisely control the bulk properties of polymers, including porosity, biodegradation, and mechanical properties, which makes polymers ideal substrates for the fabrication of scaffolds (Ravichandran et al. 2010; Cao and Zhu 2014; He and Benson 2014). Polymers are generally classified as degradable or non-degradable, synthetic or natural, or a combination of both (He and Benson 2014). Synthetic biodegradable, synthetic non-biodegradable, and natural polymers are the main classes of polymers employed in tissue engineering and regenerative medicine applications (He and Benson 2014).

Natural polymers, which comprise proteins, polysaccharides, and decellularized tissue matrices, present specific molecular ligands that favour interactions with cells (DeQuach et al. 2011; Ombelli et al. 2011; Patino and Pilosof 2011). Proteins or polysaccharides can be obtained from animal or human tissues by means of chemical extraction methods (Guo et al. 2010; Zhao et al. 2010; Azmir et al. 2013). Decellularized tissue matrices are obtained from allogeneic or xenogeneic tissues or organs that have been subjected to detergent-mediated and enzymatic processes to remove most of its cellular components (DeQuach et al. 2011). More recently, a promising source for natural polymeric matrices comprises ECM matrices that are obtained after decellularization of autologous progenitor cell cultures (Hoshiba 2017; Hyldig et al. 2017). One of the utmost benefits of natural materials is that they do not typically exhibit toxicity problems that are encountered with synthetic materials (Ige et al. 2012; Khaing and Schmidt 2012). Furthermore, natural polymers have particular protein binding sites and biochemical signals, which trigger molecular and

cellular interactions leading to enhanced integration (Ige et al. 2012). Cellulose is a linear natural polymer with b-(1,4)-D-glucose as the repeating unit. It is the most plentiful polysaccharide found in nature, which is insoluble in water (Morgan et al. 2013; Dornath et al. 2015). Several studies have demonstrated the applicability of this natural polymer in tissue regeneration approaches (Salahinejad et al. 2012; de Olyveira et al. 2014; Barud et al. 2015). Chitosan, another natural polymer, is a polycationic polysaccharide comprised of glucosamine and N-acetyl glucosamine molecules through the process of deacetylation of N-acetyl-D-glucosamine to a degree higher than 60% (Rinaudo 2006; Boddohi et al. 2009). Chitin is the second most plentiful naturally derived polymer, which is existent in the external skeleton of crustaceans and insects (Sarasam and Madihally 2005). Chitosan is a biocompatible, biodegradable, bioadhesive, and haemostatic glucosamine polymer, that can be successfully and safely used for regenerative medicine applications (Singh Dhillon et al. 2013; Rahmati et al. 2016; Rahmati et al. 2017). Zhang et al. (2015) have suggested a strong, stepwise topographic strategy to stimulate human-induced pluripotent stem cells (hiPSCs) differentiation into tenocyte-lineage after consecutive culture on flat tissue culture plastic surface and well-aligned chitosan-based ultrafine fibers. The authors used chitosan-based well-aligned fiber scaffolds to stimulate tenogenic differentiation of hiPSCs. The histological analysis indicated that the chitosan scaffolds could effectively control hiPSC-mesenchymal stem cells (MSCs) differentiation and therefore support tendon regeneration (Zhang et al. 2015) (Fig. 1).

Hyaluronic acid (hyaluronan) is an enzymatically degradable sulfated-glycosaminoglycan (GAG) consisting of alternating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid (Hintze et al. 2009). Hyaluronic acid is commonly dispersed throughout the ECM of all connective tissues especially in the synovial fluid of joints (Necas et al. 2008). Hyaluronic acid and its derivatives have been widely used as scaffolds for tissue regeneration as a result of its innate biocompatibility, biodegradability (naturally degraded by hyaluronidase), and its exceptional capability to form hydrogels (Xu et al. 2012). Alginate is a linear, hydrophilic, brown algae or bacteria polysaccharide, which includes 1,4-linked β-D-mannuronic and β-L-glucuronic acid units (Tøndervik et al. 2010). It has been reported that alginate could be potentially used for improving regeneration of damaged tissue and organs (Ma 2016). Collagen is a biodegradable fibrous protein containing three polypeptide chains which forma triple-helix structure (Parenteau-Bareil et al. 2010). Because collagen is one of the major constituents of the ECM which is degraded by metalloproteases, it has been one of the most investigated natural polymers for tissue regeneration applications (Zhu and Marchant 2011; Shabafrooz et al. 2014; Mozafari et al. 2018). Additionally, gelatin is a partial derivative of collagen, which can be simply achieved by a controlled hydrolysis of collagen (Guillén et al. 2011). Gelatin is the major component of skin, bones and connective tissues (Ha et al. 2013). A number of studies have reported the successfully regeneration of different damaged tissues through the use of gelatin scaffolds. Moreover, xyloglucan is a polysaccharide derived from tamarind seed composed of a (1–4)-b-D-glucan (GLU) backbone chain that offerings (1–6)-α-D-xylose branches (XYL) partially replaced by (1–2)-β-D-galactoxylose (GAL) (Choudhary et al. 2010). Xyloglucan has currently attracted the attention of scientists as a capable polymer for tissue regeneration applications.

Although, natural polymers have many advantages, they usually suffer some drawbacks including immunogenicity and risk of contamination, which may cause an undesirable immune response followed by an immune rejection (Mano et al. 2007). In addition, the instability of natural polymers could potentially affect the biodegradation and biomechanical properties, given that biodegradation is generally dependent on enzymatic processes (Kim 2017). Contrary to natural polymers, the synthetic counterparts (such as polyglycolide (PGA), polylactide (PLA), poly (N-isopropylacrylamide) (PNIPAAm), polyethylene glycol (PEG), polycaprolactone (PCL), polyurethane (PU)), are easy and cost-effective to synthesize, have great homogeneity, and possess

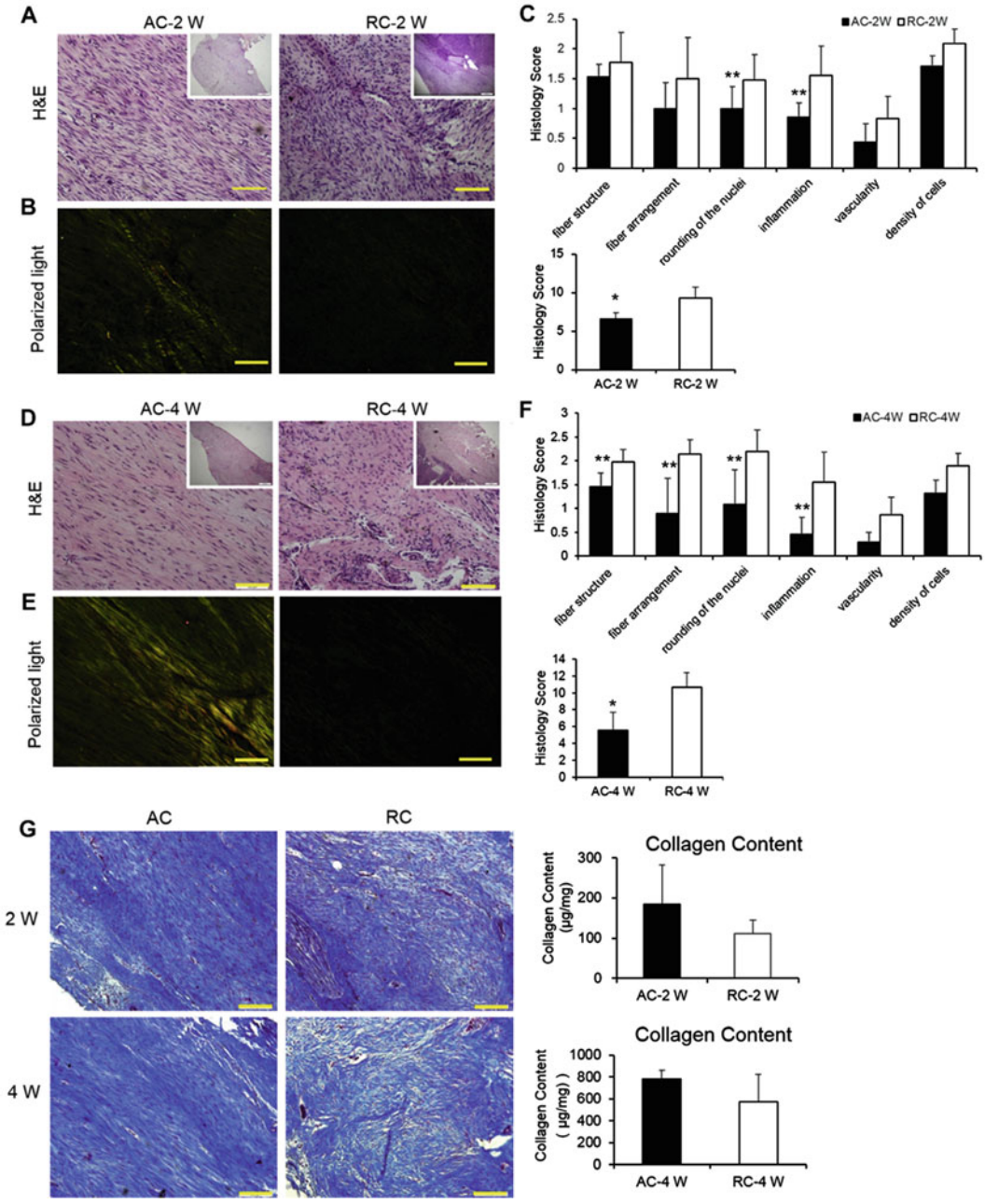

Fig. 1 A stepwise topographic strategy to stimulate hiPSCs differentiation into tenocyte-lineage after consecutive culture on flat tissue culture plastic surface and well-aligned chitosan-based ultrafine fibers. Histological morphology and ECM placed at the regenerated position 2 and 4 weeks after implantation. H&E staining and polarized light microscopy exhibited the morphology of the regenerated position along the axis of the tendon in the AC-treated and RC-treated groups at 2 and 4 weeks after surgery (**a** &**b**) and (**d** & **e**). Six factors (fiber construction, fiber organization, rounding of nuclei, inflammation, vascularity, cell population) were semi-quantitatively evaluated. The whole histology score was the totality of 6 factors to evaluate development of the regenerated tissue. Masson's trichrome staining presenting the deposited collagen in the regenerated parts and measurable tests of collagen concentration. Reprinted from (Zhang et al. 2015) with permission from Elsevier (**g**)

reproducible physicochemical, mechanical, and degradation characteristics (Kean and Craig 2012; Tian et al. 2012; He and Benson 2014). Several commercially accessible synthetic polymers have demonstrated physicochemical and mechanical properties similar to natural tissues (Gunatillake and Adhikari 2003; Sahoo et al. 2013). For instance, PLGA has been among the most attractive polymeric biomaterials which has been approved by Food and Drug Administration (FDA) for synthesizing tissue engineering scaffolds owing to the excellent biocompatibility and biodegradation properties. Kwak et al. (2017) have recently exhibited that PLGA mesh scaffolds containing human articular chondrocytes and platelet-rich plasma (PRP) have a potential ability to augment meniscal healing capacity. PEG is a polyether compound which depending on its molecular weight is also known as polyethylene oxide (PEO) or polyoxyethylene (POE). It has been well-acknowledged that PEG could be successfully used in tissue regeneration applications. For example, Stevens and co-workers have recently designed a hydrogel system based on PEG-diacrylamide (PEGDAAm) containing primary hepatocytes and supporting non-parenchymal cells and matrix metalloproteinase sensitive (MMP-sensitive) peptide as a suitable degradable scaffold for liver regeneration. The results indicated that hepatic PEGDAAm-based tissues were fully functional for over 3 weeks' post-surgery in nude mice models (Fig. 2). The results of this study provide evidence supporting the concept of using synthetic degradable materials with well-controlled cues for tissue regeneration applications (Stevens et al. 2015). Nevertheless, synthetic polymers have some limitations. One of the main limitations of synthetic polymers is their lack of specific molecular elements for interaction with cells and proteins, which often requires surface treatment of the polymer to promote integration with cells and tissues. An incomplete integration of the polymer could eventually lead to undesirable inflammatory responses within host tissues (Gunatillake and Adhikari 2003; He and Benson 2014). Hence, it has been suggested that the optimal approach for synthesizing scaffolds for tissue regeneration applications would be the use of composite biomaterials which take advantages of the benefits of both natural and synthetic biomaterials.

4.2 Ceramic-Based Biomaterials

In the last few decades, different types of biomaterials have been employed in orthopaedic and dentistry applications; nevertheless, many of them employed despite certain limitations. For instance, metals and their alloys have not reached the acknowledged aesthetic degree, and porcelain-fused-to-metal fails to have the general clearness, which could compromise for the aesthetic characteristics of dental biomaterials (Liang et al. 2008). Additionally, inadequate mechanical strength of polymers could make them unsuitable for skeletal regeneration applications (Sarkar and Lee 2015). In comparison with metals and polymers, bio-ceramics have been widely suggested as further promising candidates for orthopaedic and dentistry applications due properties including greater density, wear resistance, biocompatibility, and shinier surfaces (Best et al. 2008; Wang et al. 2012; Dorozhkin 2015). There are three types of bio-ceramics including bio-inert high strength ceramics (such as alumina (Al_2O_3), zirconia (ZrO_2) carbon), bioactive ceramics (such as Bioglass and glass ceramics) and bioresorbable ceramics (Best et al. 2008). Several studies have utilized carbon, bioactive, and bioresorbable ceramics in different regenerative medicine applications. Scaffolds containing calcium phosphate (CaP), a fundamental constituent of native bone tissue, have been shown to enhance osteogenic differentiation of progenitor cells and stem cells and stimulate *in vivo* bone regeneration (Vaquette et al. 2013; Shih et al. 2014; Surmenev et al. 2014). Kim et al. (2015) have recently investigated the effects of biphasic calcium phosphate (BCP) scaffolds containing bone morphogenetic protein 2 (BMP-2) and/or MSC on bone regeneration by using a rabbit calvarial defect model. Two and eight weeks following implantation, microcomputed tomography (micro-CT) and histological analysis was carried out (Fig. 3), showing that maximum

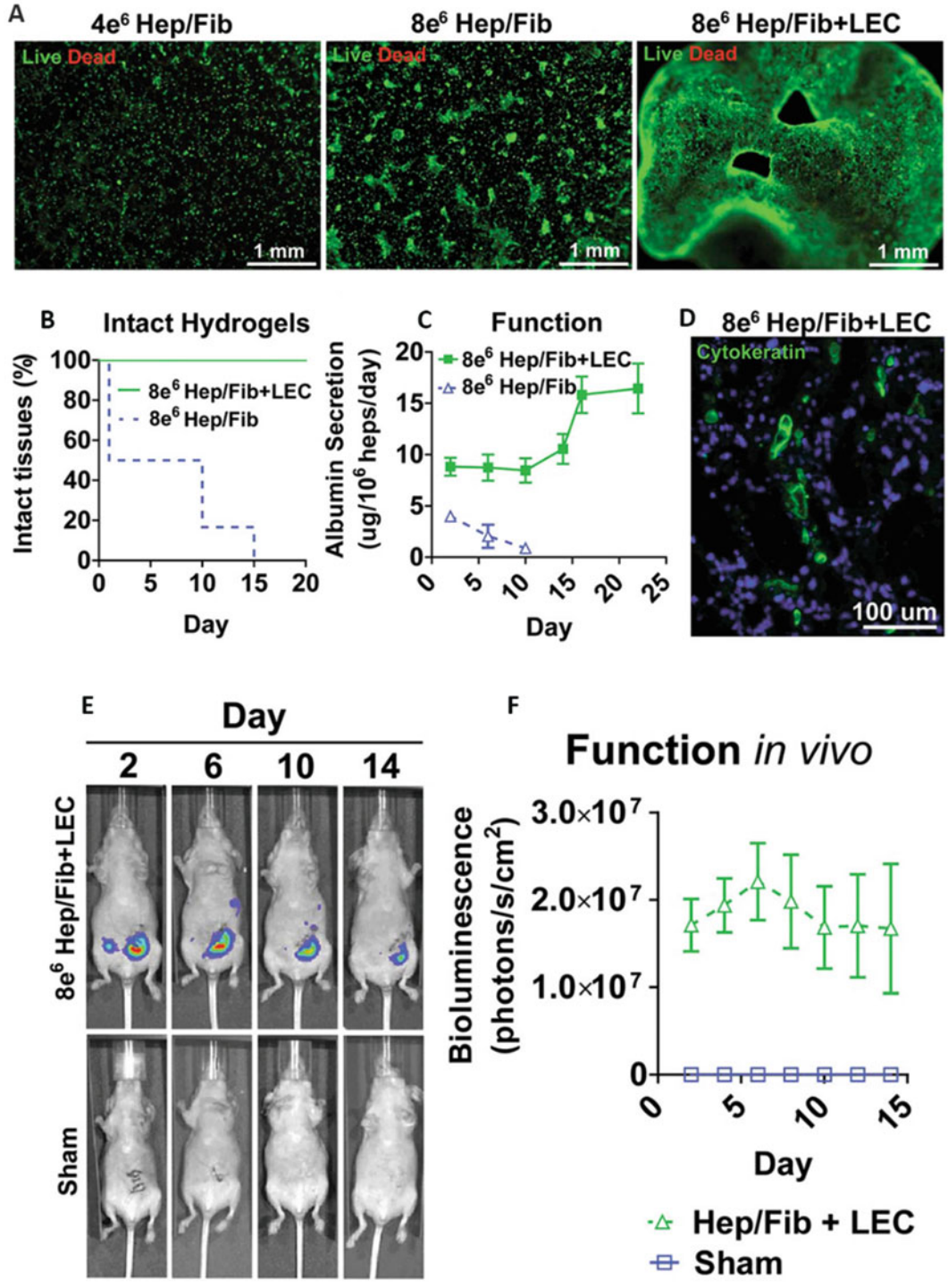

Fig. 2 A hydrogel based on PEGDAAm containing primary hepatocytes and supporting non-parenchymal cells and MMP-sensitive peptide as a suitable degradable scaffold for liver regeneration. (**a**) Insertion of LECs in the hydrogels consequences in interrelated cellular network (green, calcein; red, ethidium homodimer, 5% PEGDAAm, 10 mmol RGDS. UV 10 mW cm22 for 210 s, 1LEC 6 3 106 heps/mL) and extends the system lifetime (**b**) and liver tissue function (**c**). Hepatic hydrogels with LECs have cytokeratin-positive hepatocytes (green) after 3 weeks in culture (**d**). The treated tissues were functional following implantation *in vivo* up to 2 weeks. (5% PEGDAAm, 10 mmol RGDS). UV 10 mW cm22 for 210 s, 8 3 106 Hep/J2 1LEC 6 3 106 heps/mL in all mice w/cells. Sham models had blank MMP-degradable PEGDAAm systems (**e**, **f**). Reproduced from (Stevens et al. 2015)

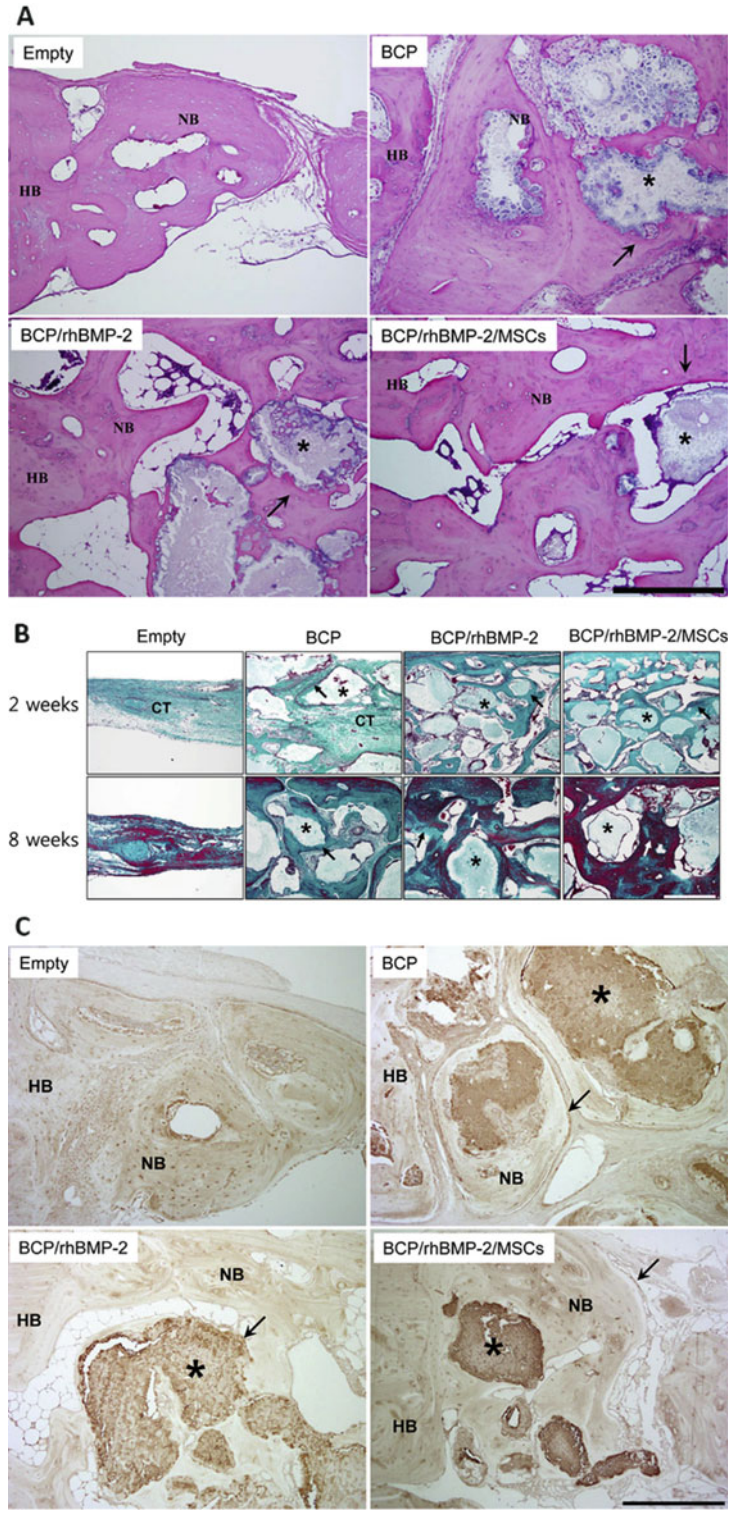

Fig. 3 The effects of BCP scaffolds containing BMP-2, and/or MSC on bone regeneration by using a rabbit calvarial defect model. (**a**) Hematoxylin and eosin stained greater magnification images at 8 weeks after surgery, in

bone development was accomplished with the BCP/rhBMP-2/MSCs combination. In addition, the extent of neo-tissue formation after 8 weeks was superior than earlier time points in each group. Several other studies have suggested using hydroxyapatite (HA)-based scaffolds as promising candidates for bone regeneration applications (Hao et al. 2015; Chou et al. 2016). For example, Chou et al. (2016) have recently investigated the efficacy of HA scaffolds containing zinc ions on bone regeneration by using a rat calvarial defect model over a six-week period. The micro-CT and histological analysis showed that 6 weeks post-surgery, the Zn-HA group induced augmented bone development in comparison with the collagen membrane and control groups.

Bioactive glasses, which have a great ability of forming a HA-like layer in both *in vitro* and *in vivo* conditions. These materials are fabricated from glass formers including silica (SiO_2), boric acid (B_2O_3), and phosphoric oxide (P_2O_5), network modifiers, and intermediate oxides. In 1969 Hench and co-workers for the first time introduced silicate-based bioactive glass (with the formulation of 45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_5 known as 45S5 bioactive glass), as a biomaterial which could potentially connect to bone tissue in biological conditions (Hench et al. 1971). Silicate-based bioactive glass is the major type of bioactive glass which has been widely suggested as a promising candidate in tissue engineering applications (Mozafari et al. 2010; Mozafari and Moztarzadeh 2014). Borate glass is another main class of bioactive glass which with a more complex network is currently used in tissue regeneration applications (Jung and Day 2009; Jung 2012). It has been shown that borate glass structure is made of trigonal planar BO_3 and/or tetrahedral BO_4 components, and the addition of metal oxides converts the planar units into tetrahedral units, increasing the grade of network connectivity (Stanić 2017). Phosphate based glasses have increased solubility, biodegradability, biocompatibility, and also chemical resemblance with the inorganic phase of natural bone tissue (Erasmus et al. 2018; Kargozar et al. 2018). Nommeots-Nomm et al. (2017) have studied the use of porous melt-derived bioactive glass foam substrates with low silica concentration on new bone formation following implantation in a lapine model. As it can be observed in Fig. 4, the X-ray micro tomography images demonstrate that the bioactive glass-based substrates had a potential ability to repair bone defects. Furthermore, other studies have reported that bioactive glass containing controllable amounts of different ions could significantly encourage osteogenesis and angiogenesis (Bari et al. 2017).

An important subclass of ceramic biomaterials comprises the carbon-derived materials. Carbon-based materials such as carbon nanotubes (CNTs) (Harrison and Atala 2007; Touri et al. 2013), graphene (Alasv and Mozafari 2015; Chauhan et al. 2016), fullerenes (Goodarzi et al. 2017), quantum dots (QDs) (Lim et al. 2015), nanocrystalline diamond films (Pennisi and Alcaide 2014), diamond-like carbon (DLC) (Wachesk et al. 2016), mesoporous carbon nanomaterials (MCNs) (Kim et al. 2008), and carbon nanofibers (CNFs) (Yang et al. 2007)

Fig. 3 (continued) the peripheral defect. The defects tended to combine with new tissue. Arrow, neo-bone nearby a BCP granule; arrowhead, lamella of developed bone; asterisk, BCP granule; HB, host bone; NB, new bone. (**b**) Histological analysis of the formed bone 2 and 8 weeks post-surgery, in the central part of bone defects. Two weeks post-surgery, undeveloped bone was detected in the fibrous connective tissue in the BCP/rhBMP/MSCs group. After 8 weeks, further newly formed bone in the BCP/rhBMP/MSCs group was detected than in the BCP/rhBMP group. Arrow, newly formed bone nearby a bTCP/HA granule; CT, connective tissue; asterisk, b-TCP/HA granule; Goldner's trichrome stain. (**c**) The immunohistochemical localization of osteocalcin in the border of bone defects 8 weeks after surgery. Osteocytes and the lamellar bone matrix demonstrate noticeably progressive responses to osteocalcin. OC immunoreactivity exhibited that the active osteoblast-like cells infrequently were nearby the hard tissues. Arrow, osteoblast-like cells surrounding lamellar bone matrix; asterisk, BCP granule. Reprinted from (Kim et al. 2015) with the permission from Elsevier

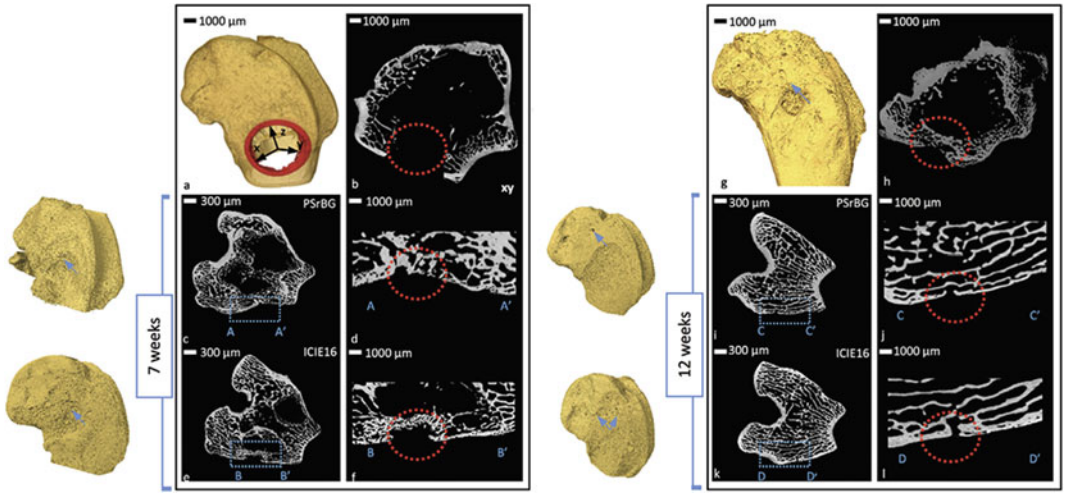

Fig. 4 3D reconstructions and 2D slices of mCT images of bone regeneration in rabbit models: (**a**, **b**) control groups at week 0, (**a**) 3D rebuilding and (**b**) 2D slice; (**c**–**f**) 2D slices, 7 weeks after ICIE16 and PSrBG substrates implantation; (**g**, **h**) 2D slices of defects after 10 weeks in control groups; (**i**–**l**) 2D slices, 12 weeks after ICIE16 and PSrBG substrates implantation. 2D slices were all over the center of the unique defect. Sections marked A-A0-DD0 demonstrate the original defect location. Conforming 3D restorations are also revealed, that the blue arrows exhibit the original defect location. Reticulated trabecular bone was detected at 7 weeks followed by bone formation. The morphology of the cortical region repaired its arrangement at 12 weeks after surgery when bioactive glass scaffolds were employed. Reprinted from (Nommeots-Nomm et al. 2017) with the permission from Elsevier

have shown promise for various tissue engineering and regenerative medicine applications. Fullerenes and nanodiamonds (NDs) have recently gained attention in the biomedical field, in particular in the fields of cancer diagnosis and therapy (Liu et al. 2010; Lichota and Krokosz 2016). NDs are derived either from processing high-pressure high-temperature diamond or by detonation synthesis. NDs possess exceptional mechanical, chemical and optical properties; such as for instance intrinsic fluorescence, which has been exploited for bioimaging purposes (Mochalin et al. 2012). CNTs, which include single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are another key type of carbon nanomaterial that have gained a great attention among biomedical scientists since their discovery (Harrison and Atala 2007; Ahadian et al. 2016, 2017). Tanaka et al. (2017) have demonstrated that when MWCNT blocks containing rhBMP-2 were inserted into murine muscle, ectopic bone was formed. Moreover, graphene, as a single 2-dimensional sheet of carbon, has been reported as a promising candidate in various tissue regeneration applications (Goenka et al. 2014; Kumar and Chatterjee 2016). Some studies have reported the success of applying graphene in drug delivery systems and tissue regeneration applications, owing to its large surface area, exceptional mechanical behaviour, and easy functionalization (Goenka et al. 2014; Shin et al. 2016). The effects of graphene substrates on human osteoblasts has been reported, as well as MSC behaviour and it has been identified that graphene-based scaffolds could be potentially used as biocompatible materials which provide favourable microenvironments for cell proliferation and differentiation (Kalbacova et al. 2010; Crowder et al. 2013). It has also been reported that graphene as a conductive biomaterial could provide cues to developing cells that support the cells electrical connections, thus suggesting a novel promising scenario for neural tissue regeneration (Gardin et al. 2016).

4.3 Composite Biomaterials

A composite material is a heterogeneous arrangement, on the macroscopic scale, of two or more materials with dissimilar physicochemical properties. The benefits of using biomaterial composites are to exploit the best merits of their components, to minimize the drawbacks of using the components separately and, in many cases, to reveal some properties that each component does not have. In addition, there is a flexibility in designing composite systems so that the properties of the final product could be easily manipulated by changing the concentration and properties of their components. In the field of biomaterials, composites emerged as a need to enhance the mechanical properties of polymers and ceramics. In consequence, the most common types of composites in tissue engineering applications are those combining polymeric and ceramic materials. While the ceramic component acts against the polymer plasticity, the polymer network protects the ceramic against a brittle fracture. One of the earliest attempts to improve the mechanical properties of polymers explored the inclusion of carbon fibers in the polymer matrix. Carbon-fiber reinforced polyether-etherketone (PEEK) is an example of a composite, which has been successfully applied for the fabrication of screws for orthopaedic use (Wintermatel et al. 1993) and bone plates (Fujihara et al. 2001). Later, the work on composite biomaterials has been focused on enhancing the mechanical properties of polymers by inclusion of small isotropic microparticles. Examples of ceramic-microparticle polymer composites include several combinations of biodegradable polymers (mainly polyesters) and bioactive ceramics (bioactive glasses and HA), which have shown significant success in orthopaedic tissue engineering applications (Rezwan et al. 2006; Boccaccini et al. 2012). Interestingly, studies have shown that as compared to microparticle composites, the inclusion of nanoscale particles in a composite scaffold had a more significant effect on the mechanical and biological properties of the system (Wei and Ma 2006; Misra et al. 2008). This subclass of composite materials comprising one of their components in the nanometer scale, also known as nanocomposites, has recently gained increased attention for tissue engineering owing to their unique tunability of physicochemical and biological properties (Cattalini et al. 2016; Dm Follmann et al. 2017). A notable example within nanocomposite biomaterials comprise the nanocomposite hydrogels, which have recently emerged as promising scaffolds not only due to their easily tailorable properties, but also because they can more closely mimic the extracellular matrix microenvironment, providing a hydrated 3D network that supports nutrient transport and enhances cell growth and maturation (Gaharwar et al. 2014; Mehrali et al. 2017). Notably, some of these nanocomposite hydrogels exhibit inherent electrical properties and biological activity that are instrumental in supporting growth and maturation of cells in electrically active tissues, such as muscles and nerves. Furthermore, nano-reinforced hydrogels have demonstrated ability to control the overall assembly of cells within the scaffold, as for instance the parallel alignment of cells in tissue engineered skeletal muscles (Ramón-Azcón et al. 2013). Another important feature of offered by some nanocomposite hydrogel systems is their ability to self-heal in response to mechanical damage, which is rapidly emerging as an exciting asset for the development of novel tissue engineering approaches (Appel et al. 2015; Jing et al. 2017).

5 Current Challenges

In recent decades, a considerable progress has been achieved in the field of regenerative medicine thanks to the use of several types of biomaterials. It becomes evident that the life of millions of patients has been saved since the introduction of biomaterials to support regenerative medicine applications. However, some challenges still remain, which demand a more thorough collaboration between biomedical scientists, engineers and surgeons. Tissue engineering and regenerative medicine strategies

involve multidisciplinary concepts that need a profound knowledge on the diverse mechanisms behind the regeneration of tissues and organs. It should be noted that most of the fundamental healing pathways of tissues in the human body are still poorly understood, which demands further research efforts. Many biomaterials have been introduced into clinical trials perhaps too early in their development phase, without a deep knowledge about their biological performance. It is evident in the literature that many studies report the biocompatibility of materials according to the results of other reports, without considering the specific conditions and applications of their own research. Therefore, for a successful application of biomaterials in regenerative medicine, it would be reasonable to avoid generalizations about the properties of biomaterials, especially their biodegradability and biocompatibility. The successful tissue response to a material does not guarantee its use as a suitable material in other tissues, as different tissues respond differently to foreign materials. In addition, many of the studies have decided about the suitability of biomaterials properties for *in vivo* and clinical use without precisely investigating their properties through valid *in vitro* and *ex vivo* experiments. Additionally, for *in vivo* testing of biomaterials properties, animal models should be carefully chosen, considering the specific properties of targeted tissue. Furthermore, researchers should consider precisely investigating the biological responses of biomaterials after combining them for designing composite biomaterials, owing to the possibility of changing their specific physicochemical properties following combination. Additionally, a vast number of novel biomaterials for regenerative medicine applications has been reported in the literature in the recent years. It would be desirable to focus the research on understanding the biological responses of these novel materials instead of continuing the innovation route without considering the ultimate goal. While some of the introduced current materials have shown excellent *in vitro* and even *in vivo* results, their clinical use remains challenging due to the cost of fabrication in large scales. Therefore, during the design of biomaterials for tissue regeneration applications, it is important to take in account the reproducibility and scalability of the fabrication approaches. Apart from physicochemical and biological properties, biomaterial scientists should bear in mind that the efficacy, ease of use, and costs, play a crucial role for the adoption of these new technologies by medical practitioners and ultimately for the successful therapeutic use of biomaterials.

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The Great Harmony in Translational Medicine: Biomaterials and Stem Cells

Evren Erten and Yavuz Emre Arslan

Abstract

Thanks to novel approaches and emerging technologies, tissue engineering and regenerative medicine have made a great effort to regenerate damaged tissue or organ with no donor needed. The approaches involve two fundamental components: bioengineered scaffolds and stem cells. Bioengineered scaffolds which can also be enriched with bioactive molecules such as cytokines, growth factors, and so on have been fabricated using a wide range of synthetically or naturally derived biodegradable and biocompatible polymers. These scaffolds should support cell attachment, migration, proliferation, and/or differentiation by mimicking the duty of native extracellular matrix. Stem cells are the other significant players in formation of the neotissue. Stem cells, bone marrow, or adipose-derived mesenchymal stem cells, in particular, have been widely used for this purpose. Recently, investigators have preferred to use progenitor cells including cardiac and neural cells in tissue engineering and regenerative medicine applications. The synergy of the bioengineered scaffolds and autologous stem cells is crucial for the successful reconstruction of damaged or missing tissues.

This review summarizes a number of excellent studies conducted on current applications of bioengineered scaffolds, novel fabrication methods, stem cells used in tissue engineering and regenerative medicine, and the future of the tissue-engineered products.

Keywords

Stem cells · Bioengineered scaffolds · Industry · Translational medicine · Regenerative therapy · Tissue engineering

Abbreviations

3D	Three-dimensional
AMSCs	Adipose-derived mesenchymal stem cells
CSCs	Cardiac stem cells
ECM	Extracellular matrix
ECSs	Embryonic stem cells
FDA	Food and Drug Administration
FDM	Fused deposition modeling
hAMSCs	Human adipose-derived mesenchymal stem cells
HUVECs	Human umbilical vein endothelial cells
iPSCs	Induced pluripotent stem cells
MSCs	Mesenchymal stem cells
NCSs	Neural stem cells
NPCs	Neural progenitor cells

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PFP	Powder-fusion printing
SCI	Spinal cord injury
SLA	Stereolithographic Apparatus

1 Introduction

Every year millions of people, unfortunately, experience tissue loss or end-stage organ failure throughout the world. This not only reduces the life quality of each patient during the wait for a proper donor but also increases the healthcare budget immensely (Langer and Vacanti 1993). Regenerative medicine and tissue engineering are the multidisciplinary and emerging fields offering hopeful therapeutic approaches in treating relentless diseases. Outstanding solutions have been presented by several studies since the mid-1980s (Langer and Vacanti 1999; Vacanti 2006).

This review aims at summarizing the fundamental dynamics of tissue engineering and regenerative medicine by mentioning the latest scientific improvements. Advanced scaffold fabrication techniques, novel approaches on stem cell practices, and also future perspectives in the field have been highlighted within recent academical findings.

1.1 The Concept of Tissue Engineering and Regenerative Medicine

Tissue engineering is an interdisciplinary field which follows the main principles of chemistry, biology, medicine, engineering, etc. with the goal of renewing, maintaining, and improving organ or tissue functions. A "neo-tissue formation" may be constituted by taking advantages of these techniques and biomaterials including biocompatible synthetic or natural polymers which can serve as a niche for stem or somatic cells (Langer and Vacanti 1993). Regenerative medicine is a more sophisticated field compared to traditional therapeutic approaches due to its unique perspective which is *utilization of human cells as a therapeutic agent*. Human cells could be somatic cells, adult stem cells, embryonic-derived stem cells, and in particular induced pluripotent stem cells (iPSCs) which were first used in 2006 (Mason and Dunnill 2010; Takahashi and Yamanaka 2006).

As tissue engineering and regenerative medicine have similar goals; they have been combined in recent years. Biocompatible and porous scaffolds are frequently used as templates in the construction of "neo-tissue" structures since frameworks have a better tendency to supply the necessary niche design enabling oxygen and medium penetration for the homogeneous growth of cells. The porous bioengineered scaffolds which can be supplemented with growth factors, chemokines, cytokines, and/or stem cells have the great potential to regenerate the target tissue by mimicking the native extracellular matrix (ECM). The integration of tissue engineering and regenerative medicine is promising for both the replacement and repairing of damaged tissues or organs in clinical applications (Atala 2004; Lui et al. 2017; Nemeno-Guanzon et al. 2012) (Fig. 1).

1.2 The Importance of Signaling Molecules: "Hermes Gods" of Cellular Life

Stem cells are able to differentiate into various cell lineages due to the marvelous genetic potential they possess. As a source of stem cells, mesenchymal stem cells (MSCs) in particular are one of the most favored cells used by scientists studying on tissue engineering and regenerative medicine due to their numerous advantages such as immunocompatibility, a remarkable proliferation rate and multipotency features, which in turn makes them significant players in clinical arena (Tuan et al. 2003).

However, determining the signaling mechanism of these cells is crucial in understanding the outcome of cellular lineage (Lv et al. 2017). The signaling molecules such as cytokines,

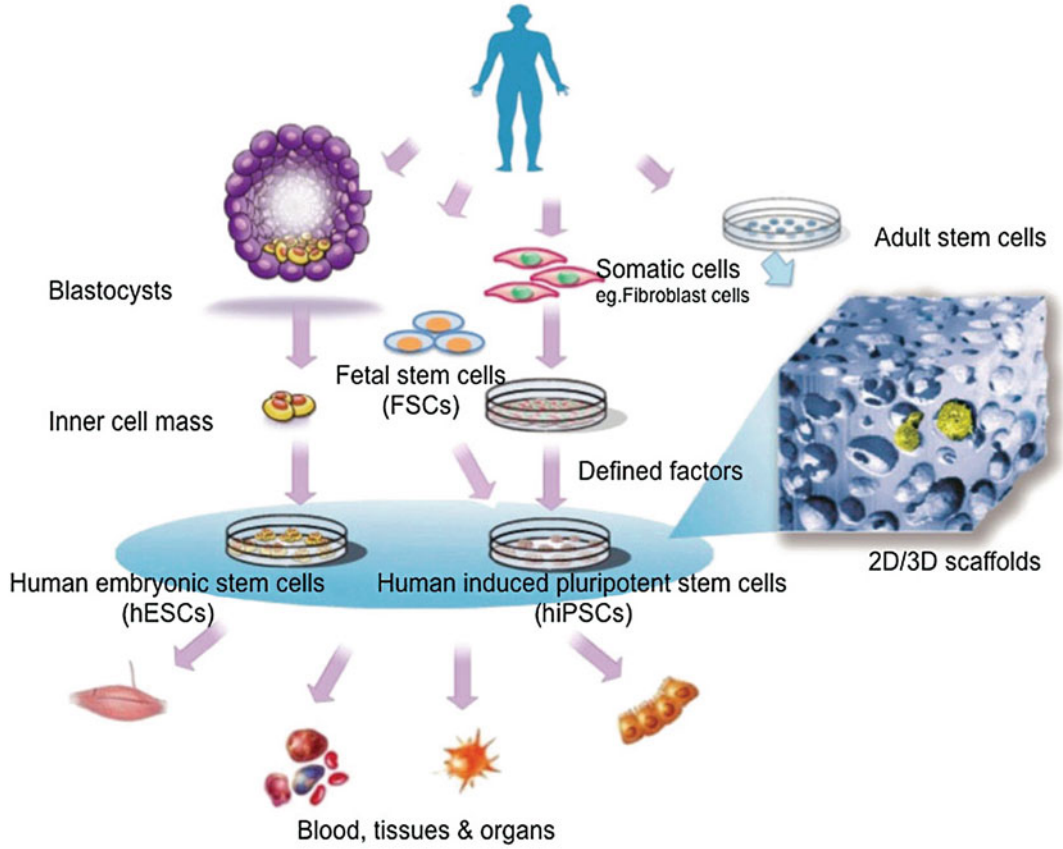

Fig. 1 The strategy of tissue engineering and regenerative medicine. (Adapted from Hu et al. (2016))

growth factors, other biochemical agents, etc. and their receptors that have been discovered and/or are waiting to be discovered regulate vital functions of stem cells. Especially vascular endothelial growth factors, fibroblast growth factors, epithelial cell growth factors, keratinocyte growth factors, hepatocyte growth factors, platelet-derived growth factors, transforming growth factor-β, and bone morphogenetic proteins are well-known ones which are frequently used in the differentiation of MSCs or therapeutic purposes (Badylak 2004; Hu et al. 2016). In addition, optogenetics which combines both the genetics and optics approach is actually quite an amazing and promising research technique which is said to have potential in both the cell signaling and differentiation processes (Kolar and Weber 2017; Repina et al. 2017).

1.3 A Sophisticated Approach: Exosomes in Regenerative Therapy

Extracellular signaling provides intercellular communication among cells. Exosomes are one of the potential carriers of intercellular signaling which consist of ß-catenin, notch ligands, as well as cellular communication proteins such as tumor necrosis factor-α and interleukin 1ß. These cellular nanovesicles are surrounded with a lipid membrane with a diameter in the range of 50–100 nm (Derkus et al. 2017).

Exosomes were firstly described in the 1970s as cellular debris and nonfunctional structures. Nevertheless, today these vesicles have led to new horizons related to cell-free treatment for several diseases as an alternative to other

conventional methods (Derkus et al. 2017; Riazifar et al. 2017). Recent studies have indicated exosomes to be outstanding tools for cardiac (Safari et al. 2016) and myocardial regeneration (Santoso and Yang 2017), cancer therapy (Di Rocco et al. 2017), neurodegenerative disorders (Kim et al. 2013), angiogenesis promoters (Gong et al. 2017), immunomodulatory therapeutic agents (Börger et al. 2017), and osteoarthritis treatment (Toh et al. 2016).

Exosomes obtained from MSCs have the potential to be used as cell-free therapeutics in addition to biomarkers, drug delivery vehicles, and vectors for stem cell-based therapy (Vizoso et al. 2017). They can also be derived from other stem cell sources such as embryonic stem cells, iPSCs, human bone marrow MSCs, human umbilical cord MSCs, and various progenitor cells (Derkus et al. 2017). Using viable cells in regenerative therapies can carry inherent risks such as inappropriate cell types and immune rejection. The other important points that cannot be ignored in stem cell-based therapy are unlimited cell growth and tumor formation risk (Gomzikova and Rizvanov 2017). Exosomes on the other hand have the potential in offering new opportunities in the elimination of these and many other concerns (Gong et al. 2017). In terms of signaling features, exosomes are considered as valuable candidates for tissue engineering and regenerative medicine as well (Bruno and Camussi 2013).

1.4 Immune Response Defines the Rules

Immunity, a marvelous response created by living creatures in order to survive against infectious diseases is vital. This, however, becomes a challenging issue that needs to be overcome for tissue or organ transplantation, which is one of the most applied medical procedures when suffering from organ dysfunction and/or failure (Badylak and Gilbert 2008). After the surgical operation, organ rejection may be the case due to the strong immune response created by the host even though immunosuppressive agents are used (Gilbert et al. 2006). Furthermore, suppression of the immune response may cause mortality and/or morbidity in patients (Wiles et al. 2016).

In order to eliminate this ambiguity, the tissue engineering and regenerative medicine perspectives may be a useful option. Whole tissue or organ decellularization is a promising technique for patients waiting for a compatible organ as the main goal of this standpoint is to reduce or suppress the adverse immune response (Wang et al. 2016). Nevertheless, the antigens that trigger the host immune response should be reduced and not completely removed, because minimal immune response may regulate the regeneration and/or healing processes of the tissues (Badylak and Gilbert 2008). Thus, acellular ECM is considered as an important opportunity to overcome some adverse immune effects (Sutherland et al. 2015).

2 Maestro of Tissues: The Extracellular Matrix

ECM is a complex and tissue-specific three-dimensional (3D) framework consisting of structural and functional molecules that are secreted by cells in this ultrastructure. It is one of the primary reservoirs of signaling molecules that modulate cellular behaviors in addition to their significant roles in the regulation of hemostasis and regeneration of tissues and organs. Phenotypic changes in the embryonic period, created by the cells in which they reside, could be given as a good example for cell-ECM interactions. Scientists have believed that numerous vital activities such as maintenance of tissues, response to injury, cancer development, etc. are based on ECM. Thus, these attributes define the ECM as crucial for physiological structures unlike ordinary products secreted by cells. In fact, any change of the ECM composition or structure could affect several events such as disease state, ageing, cellular micro-environments, etc. (Chan and Leong 2008; Fitzpatrick and McDevitt 2015; Hussey et al. 2017).

This part gives the fundamental dynamics on how tissue engineering and regenerative medicine mimic the micro-architecture of native ECM using natural or synthetic polymers to construct bioengineered scaffolds.

2.1 Bioengineered Scaffolds from Synthetic and Natural Polymers

Scaffolds play a vital role in tissue engineering and regenerative medicine applications as they provide niches and/or structural reinforcement to cells, leading to the availability of several scaffold fabrication techniques. These techniques can be categorized as decellularization, 3D-bioprinting, electrospinning (novel ones), and others such as solvent casting, gas foaming, phase separation, freeze drying (traditional ones), etc. (Chan and Leong 2008; Subia et al. 2010).

Natural ECM may be xenogenic (interspecies), allogenic (same species), and autogenic (the same organism) (Flanagan and Pandit 2003). Xenogenic and allogenic sources have the possibility of carrying pathogens as well as cellular antigens, which have the potential to create undesirable effects (also known as immune-related adverse events) for the host tissue and organ. On the other hand, allogenic cell and/or tissue transplantation via surgical procedures may result in the trauma of healthy tissues during the tissue excision process. In addition, autogenic vein (i.e., *vena saphena*) transplantation which is the golden standard for coronary bypass surgery is limited because many patients have dysfunctional vessels due to preexisting vascular diseases (Atala 2004; Chan and Leong 2008; Cho et al. 2005; Zhang et al. 2009). Therefore, application of polymers in the attempt to repair damaged or missing tissues and organs has been the case.

Synthetic and natural polymers which have biodegradable and biocompatible features can be easily processed in accordance with the purpose (Wang et al. 2014). While synthetic polymers provide better control for structural and physical durability, natural ones show good biocompatibility features (Badylak et al. 2009; Sadtler et al. 2016). Even if the use of these polymers reduces pathogenic problems, other important issues may arise such as less incompatibility with cells, static behavior against developing tissues, and formation of nonhomogeneous sites for cell diffusion (Chan and Leong 2008; Porter et al. 2009). Thus, recent researches focus on combining synthetic and/or different natural polymers to enhance the structural durability and biocompatibility all together (Bankoti et al. 2017; Goyal et al. 2017; Xing et al. 2017). For instance, Arslan et al. combined human hair keratin, jellyfish collagen, and eggshell-derived hydroxyapatite which are renewable natural sources in the construction of novel 3D scaffolds for differentiation of human adipose-derived mesenchymal stem cells (hAMSCs) into osteogenic lineage (Arslan et al. 2017).

2.2 A Novel Scaffold Fabrication Technique: Decellularization

Natural ECM may carry cellular antigens and also pathogens to the host. These undesired contents can be altered with a decellularization approach. There are several different methods for a successful decellularization process, the three main ones being physical, chemical, and enzymatic which can be applied individually or together, depending on the type of tissue. The enzymatic process on the other hand consists of trypsin, endonuclease, and exonuclease treatments (Gilbert et al. 2006). There are several studies in relation to the application of the decellularization process: nondestructive detergent-enzymatic decellularization of rabbit trachea (Den Hondt et al. 2017), physical-enzymatic decellularization of bovine tendon sheets for tendon reconstruction (Ning et al. 2017), enzymatic and nonionic detergent decellularization of rabbit carotid arteries for vascular tissue engineering (Xu et al. 2017), and physical, enzymatic, and detergent methods together for decellularization of a ECM to fabricate xenogenic scaffolds (Seyler et al. 2017). The drawback of the use of detergents in the decellularization technique is the high possibility of the loss of favorable native ECM components required for tissue repair (Gilbert et al. 2006;

Sutherland et al. 2015). To this end, investigators are intensively looking for detergent-free decellularization methods to obtain native ECM properties without any possible adverse immune effects (Erten et al. 2016; Vasudevan et al. 2014). Hereby, the preservation of the crucial biological and physical ultrastructure while removing cellular materials and pathogens from the tissue is an important challenge to be overcome by researchers studying in this field (Arslan et al. 2015; Wolf et al. 2015) (Fig. 2).

2.3 Drawing a Tissue: From Computer to Organism, 3D Bioprinting

Three-dimensional printing, the state-of-the-art technology, is a method based on simply an additive manufacturing approach. In this method, the digital data of a 3D structure is actually converted into the "tangible" objects. In contrast to applications of solvents or molds, 3D fabrication enables to create the data on digital platform (Kaushik et al. 2017). The bio-ink and the bioprinter are the main components for fabricating 3D-bioprinted structures. The other important factors are shape, strength, and resolution which depend on the host tissues (Donderwinkel et al. 2017). This technology has become very popular and opened new doors into the fabrication of 3D scaffolds with precise geometric accuracy on the macro- and microscales. Two main techniques are applied for the printing process: acellular and cellular printing. Among acellular techniques stereolithography (SLA), powder-fusion printing (PFP) and fused deposition modeling (FDM) can be applied, whereas for cellular techniques, inkjet-based, extrusion-based, and laser-assisted bioprinting is the case (Jokanović et al. 2017). Recently, cytocompatibility of 3D-printed objects was evaluated (Benning et al. 2017), and various experiments were performed related to this technology.

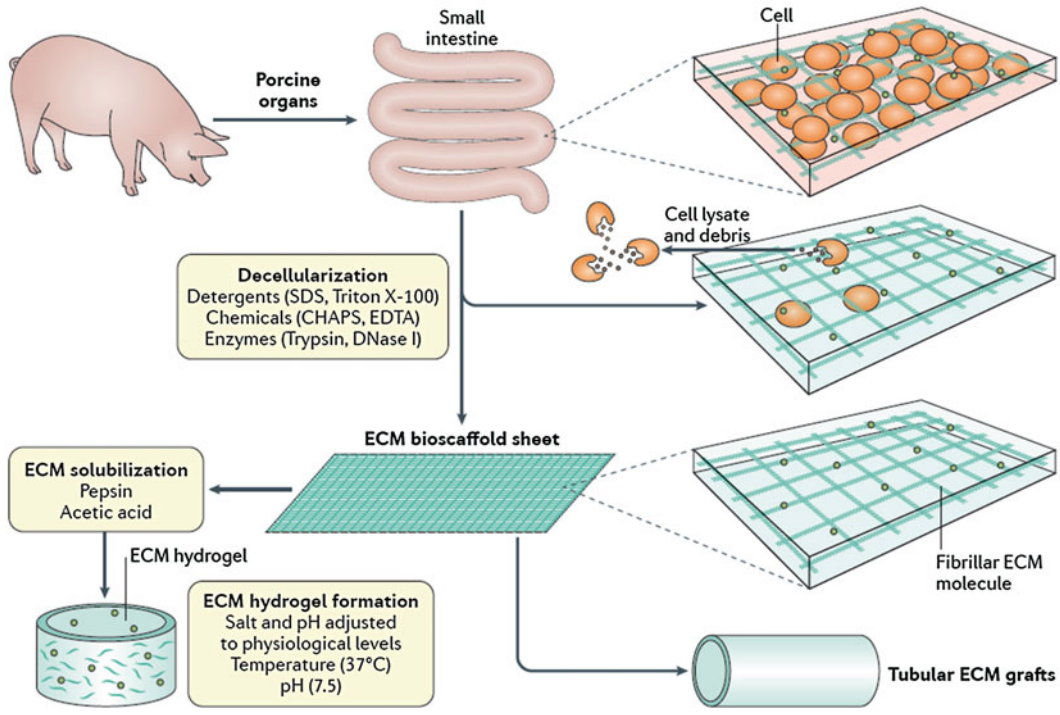

Fig. 2 An overview of bioscaffold preparation using decellularization technique. (Adapted from Hussey et al. (2017))

Three-dimensional-printed bone scaffolds and orthopedic implants (Wang et al. 2017), 3D-printed molds for personalized tissue-engineered bone regeneration (Hixon et al. 2017), 3D direct cell bioprinting for tissue engineering (Ozler et al. 2017), stereolithographic 3D printing for drug-loaded hydrogels (Martinez et al. 2017), and antimicrobial 3D-printed porous scaffolds (Vargas-Alfredo et al. 2017) are some remarkable examples of 3D bioprinting. Three-dimensional bioprinting could be integrated with electrospinning which is a favored technique, and the combination enables to prepare novel compartmented scaffolds for tissue engineering and regenerative medicine (Koudan et al. 2016) (Fig. 3).

2.4 Mimicking of the Nature: Electrospinning

Electrospinning is a unique technique used in creating nanofibrous aligned or randomly oriented meshes that could be used in tissue engineering and various applications (Seker et al. 2010). The randomly-oriented nanofibers help in supporting cells, and interconnected spaces provide cell proliferation and access to the medium. By adjusting the polymer solution concentration, applied voltage, flow rate, etc., meshes with various porosity and fiber diameters can easily be obtained. Synthetic or natural polymer-derived nano−/microfibrous scaffolds could be fabricated by electrospinning (Inanç et al. 2009; Lui et al. 2017; Xing et al. 2017). These electrospun nanofibers may mimic the native ECM networks (Tuan et al. 2003).

There are numerous studies in the literature related to the electrospinning technique, for instance, electrospun nanofibrous meshes for the peripheral nerve (Quan et al. 2016), electrospun scaffolds for osteogenic differentiation of mesenchymal stem cells (Pournaqi et al. 2017), a bilayered elastomeric scaffold for small diameter vascular grafts (Soletti et al. 2010), electrospun scaffolds for wound-healing applications (Gazzarri et al. 2013), and periodontal ligament cellular structures engineered for periodontal tissue engineering (Inanç et al. 2009).

No matter what technique is used for scaffold fabrication, the most important fact is that the prepared scaffolds should support cell adhesion,

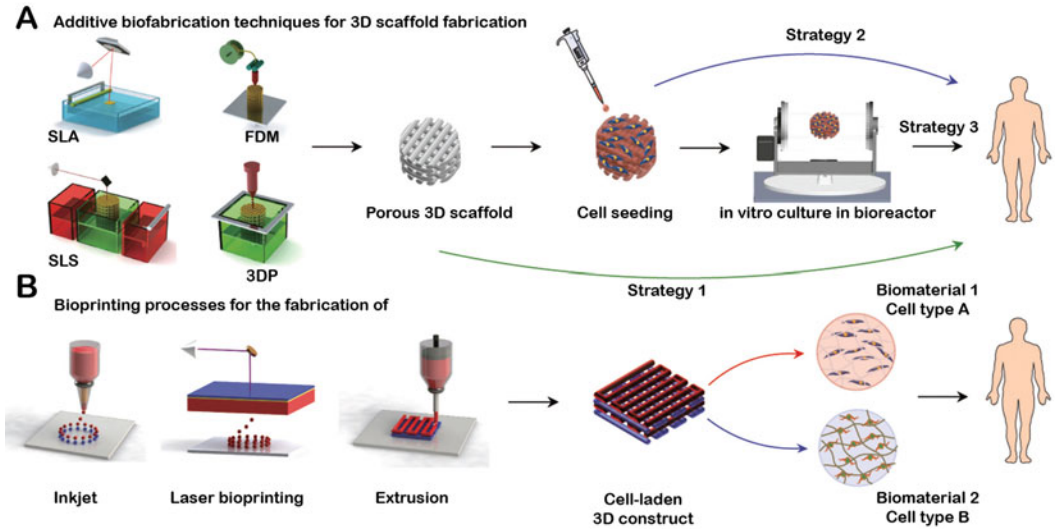

Fig. 3 (**a**) Additive biofabrication techniques can be used for 3D scaffold fabrication. Scaffold-based therapy can be performed following three strategies. Strategy 1: scaffolds can be implanted without cells. Strategy 2: fabricated scaffolds can be implanted after cell seeding. Strategy 3: scaffolds can be manipulated in vitro culture in bioreactor. (**b**) Cell-laden 3D constructions can be printed and implanted. (Adapted from Pereira and Bártolo (2015))

proliferation, and growth. Successful scaffolds have to be "friendly" toward cells by offering structural support and enough space for intercellular media. The fabricated scaffolds should also support the cells during formation of new tissues with appropriate structures and functions (Atala 2004; Chan and Leong 2008) (Fig. 4).

3 The Big Bang of an Organism: Stem Cells

Stephen Hawking in his infamous book of *A Brief History of Time* had stated that: "The universe is infinitely small and dense" (Hawking 1988). In fact, the Big Bang theory was the manifestation of his theory. Just like the universe has emerged by infinitely small and dense form, an organism is being created by a tiny stem cell as well. The certain conditions with all the laws of biology make the stem cells architects of the whole organism. As a mother of all cells, they proliferate, differentiate, and become specific cells in order to develop an organism. Stem cells divide symmetrical or asymmetrical to generate daughter cells with developmental potentials and other properties of the mother cells. This is a self-renewal process which is indispensable feature of stem cells so as to expand their numbers during development of an organism. This process also has vital role to maintain adult tissues and repair injuries (Lin 2002; Shenghui et al. 2009).

These undifferentiated cells were found in the nineteenth century; however, their existence was proved in the 1960s. Since then we have witnessed various researches and studies on them. Stem cells basically could be divided into two groups: embryonic and non-embryonic stem cells. While embryonic stem cells are pluripotent as they can generate all cell types, non-embryonic stem cells (also known as adult stem cells), like mesenchymal stem cells, are multipotent and differentiate into limited cell types. These cells in both groups are still being researched in various fields (Bianco et al. 2008; Goradel et al. 2018; Shenghui et al. 2009).

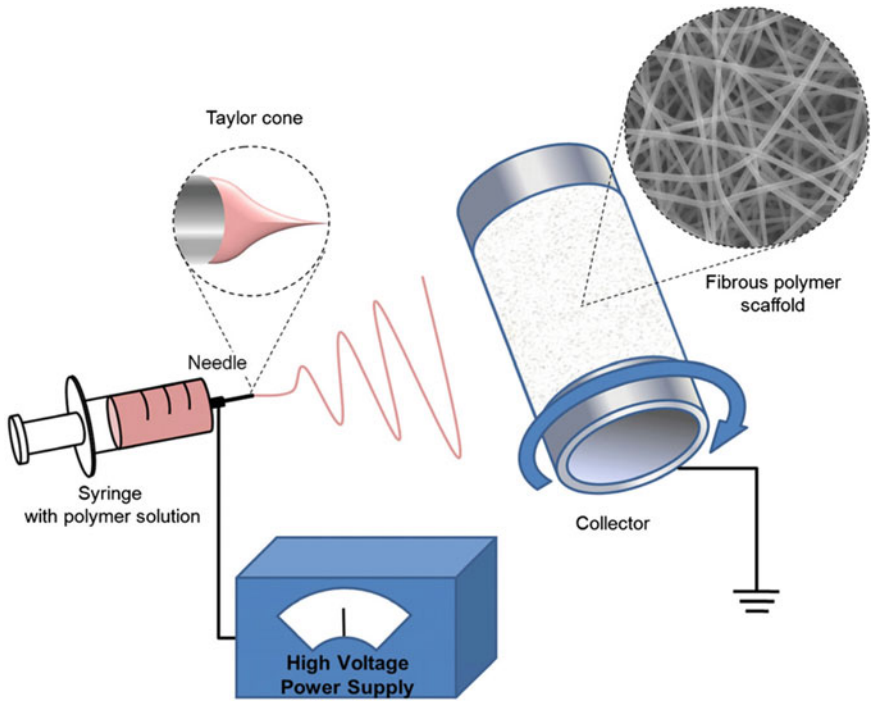

Fig. 4 Schematic illustration of the basis of electrospinning components. (Adapted from Rim et al. (2013))

This part explains how bioengineering utilize the stem cells, recent improvements, and innovative approaches to deal with diseases.

3.1 Which Type of Stem Cells Is Favorable in Regenerative Medicine?

Various stem cells are already available to use. One of them which can be obtained from inner cell mass of blastocysts stage embryos is embryonic stem cells (ESCs). Due to their rapid differentiation ability into various somatic cells at the developmental stage, those cells can be only obtained from inner cell mass. These cells are pluripotent; they can expand infinitely. Another utmost value of them, ESCs can differentiate into all germ layers of the organism in vivo and in vitro. However, these magnificent potentials have biological and ethical concerns (Prajumwongs et al. 2016; Thomson et al. 1998).

Induced pluripotent stem cells (iPSCs) were presented in 2006 by Kazutoshi Takahashi and Shinya Yamanaka. These cells were generated via somatic cells. Four transcription factors were overexpressed to acquire them: Oct4/3 (octamer-binding transcription factor 4/3), Sox2 (sex-determining region Y), Klf4 (Kruppel-like factor 4), and c-Myc (avian myelocytomatosis virus oncogene cellular homologue). iPSCs were considered a new method rather than ESCs because of their self-renewal, proliferation, and ESC-like properties without ethical concerns. Nevertheless, this method still needs to overcome genetic instabilities (Takahashi and Yamanaka 2006; Ullah et al. 2015).

The stable and highly accessible stem cells are very important for stem cell-based therapies and applications. Therefore, spotlights were focused upon MSCs. The reason of the popularity of MSCs is their relatively tolerance immunological reaction, in vivo immunosuppression effects, and differentiation abilities to mesoderm-based tissues such as chondrocytes, osteoblasts, and adipocytes. Moreover, MSCs can be autologous which means these cells can be taken from patient and implant back with the desired properties to individual. This method also prevents immunogenic adverse effects. Finally, MSCs have less ethical concerns than ESCs, and relatively, MSCs can be obtained abundantly compared to ESCs. Adipose-derived mesenchymal stem cells (AMSCs) are multipotent stem cells, and they can differentiate into various cells such as cardiomyocytes, pancreatic ß-cells, hepatocytes, and more. These cells have mesenchymal phenotype, and their one of the most important features is that these cells can be found abundant and harvested via little invasive operations. On the other hand, these cells show positive effects with cell-graft interaction. Unfortunately, MSCs also have few drawbacks like ESCs and iPSCs. Some of them could be summarized with generation and handling time delays and abnormal tissue formation. Besides, long-term culturing can cause senescence, and declining differentiation abilities with high passages are other possible problems. Also, phenotypically stable cell supply is considered an important necessity for such cell type (Godara et al. 2008; Konno et al. 2013; Lou et al. 2017; Ullah et al. 2015).

Bellei et al. have found a new method to collect AMSCs. More than 10 years, AMSCs were harvested with two methods: one of these methods was enzymatic digestion as a standard procedure, relatively expensive technique for plastic surgery, and regenerative medicine, and the other one was nonenzymatic dissociation methods that can be collected with mechanical forces to break the adipose tissues. But, this new method is offering innovative, inexpensive, and nonenzymatic process. This research presents clinically useful regenerative cells from adipose tissue, and the cells could be collected with centrifugation of the infranatant fraction of the lipoaspirate and enriched with fat shaking and wash. The method was compared with the lipoaspirate samples which was processed with collagenase, and the results showed that new procedures as an alternative for fat grafting treat stem cell-depleted tissues (Bellei et al. 2017).

Multilineage differentiation and proliferation of these stem cells were considered as early candidates for regenerative therapy. Albeit these cells have promising abilities, utilization

of iPSCs and ESCs were limited by their biological and ethical concerns. The limited differentiation capacity of adult stem cells was also proposed in 2003. These cells weren't sustainable and they also caused contraindications. However, cardiac stem cells were found in 2003, and this discovery has offered great opportunities. Cardiac stem cells (CSCs) are clonogenic, self-renewing, and multipotent both in vitro and in vivo, and also these cells are capable of generating the three major cell types of the cardiogenic cell lineages: myocytes, smooth muscle, and vascular endothelial cells. CSCs have significant regenerative potential in vivo, and these cells can attach and differentiate into beating cardiomyocytes in vitro (Torella et al. 2007; White et al. 2016).

On the other hand, recent studies have shown that multipotent neural stem cells are derived within the ventricular zone of the adult brain. These cells are located in the subependymal layer of the ventricular wall. Neural stem cells have self-renewal capacity and multilineage competence, and they could be especially abundant in the forebrain. These cells were reported in species ranging from mice to human. Neural progenitor cells have great expandability and are graft friendly, and also, they have shown safe and well-tolerated effects with functional traits in recent studies. One of the latest researches that was conducted by Rosenzweig et al. showed that human neural stem cell grafts can survive up to 9 months and express neuronal and glial markers under three-drug immunosuppression. This graft was implanted into sites of cervical spinal cord injury in primate. The axons were regenerated into grafts and formed synapses which means these grafts could be used for neuronal and glial milieu in the site of spinal cord injury (Goldman and Sim 2005; Rosenzweig et al. 2018). There are many other researches that were conducted with stem cells including cardiac MSCs (de Paula et al. 2017), neural progenitor stem cells (Harris et al. 2018), and induced pluripotent stem cells (iPSCs) (Saito et al. 2018).

Such investigations will lead researchers in the selection of proper stem cell source which has potential to be used in tissue engineering and regenerative medicine applications.

3.2 Great Meeting with Biomaterials and Stem Cells

End-stage organ failures, heavy injuries, congenital disorders, and cancer are life-threatening diseases for human life. Every year thousands of people only in the United States are suffering end-stage liver disease, chronic lung disease, coronary heart disease, and end-stage kidney disease. These patients who have such diseases need organ transplantation as golden standard. However, this method needs immunosuppression drugs for lifelong term and also has organ rejection risk, unfortunately.

Recent studies are promising to reduce or treat similar problems. Engineered tissues like blood vessels, the urinary bladder, and the trachea are some of the examples to be given for these studies. The target tissues are usually fabricated with biomaterials which provide 3D structure for supporting cells and tissues. These scaffolds define the place in which the target tissue will form. Also, these structures can be fabricated to support the attachment and proliferation of cells to affect the desired tissue formation. A degradable or resorbable scaffold serves as a transient structure and thus replaced with the tissue of interest.

Biomaterials have a great importance for repair of tissue or organ defects. These ultrastructures provide necessary substrate for cell adhesion, proliferation, and differentiation. Moreover, these materials also modulating cell activities and functions. An ideal biomaterial should provide niches that proper biological and mechanical effects, suitable microenvironment, and the material should dictate the stem cells into the desired cells of the tissue.

There are numerous efforts for tissue engineering applications based on optimized cell population and promoted tissue repair. Those cells could be autologous stem or progenitor cells in order to reduce or avoid immunogenic adverse effects. These cells can be isolated as primary cells from

a biopsy of the tissue or organ of interest and expand in cell cultures to obtain adequate cell population. After the procedure, these cells together with engineered biomaterials could be implanted to patient in order to repair tissues or organs (Atala et al. 2012; Badylak et al. 2011; Gao et al. 2017; Lv et al. 2017; van de Kamp et al. 2017).

Nowadays various tissue-engineered biomaterials were fabricated and interacted with cells. Melhem et al. have designed a MSC-laden hydrogel patch with multiple microchannels. They used a 3D bioprinter with SLA technique in order to control the diameters of microchannels ranging from 500 to 1000 μm, and the cells were suspended in poly(ethylene glycol) dimethacrylate solution for in situ cross-linking. The results have demonstrated that this method could be useful to prevent abnormal fibrosis resulting from acute ischemic injury (Melhem et al. 2017).

Demyelination could be caused by traumas or injuries. Spinal cord injury (SCI) also could be the reason of demyelination. Scientists have tried to minimize demyelination by improving oligodendrocyte availability in vivo. However, this approach has not been successful. Recently, a new biomaterial has emerged to optimize differentiation of neural progenitor cells (NPCs) toward oligodendrocytes as a cell delivery vehicle after SCI. The biomaterial was modified with collagen-based hydrogel to mimic target tissue. The ECM components of neonatal spinal cord were preserved in the material in order to direct NPCs into oligodendrocytes. Cell-loaded hydrogels were transplanted into model of SCI to evaluate functional recovery. *In vivo* responses were tested with several methods, and results showed that these hydrogels could direct differentiation in vivo to encourage regeneration of the central nervous system (Geissler et al. 2018).

The cartilage has a dense structure which prevents solutions to penetrate into deep tissue areas in decellularization process. Detergent-based decellularization processes damage the desired ECM components; thus decellularization of cartilage tissue was considered one of the main challenges in cartilage tissue engineering. Erten et al. have demonstrated a novel detergent-free technique to decellularize cartilage tissue. In this technique, the decellularization process was conducted during decalcification process, and vital ECM components were preserved. Bovine costal cartilage was homogenized, molded, and cross-linked in order to prepare intact cartilage ECM-based scaffolds (CEbS). Results demonstrated that 84% of nuclear material was removed with desired ECM components. Cell culture study and scanning electron microscope analyses have shown that the prepared scaffolds successfully directed stem cells into the chondrogenic differentiation without any cytotoxic effect (Erten et al. 2016).

Besides to tissue compatibility, abundant material sources are an important factor for biomaterials. A source which doesn't need a donor, is cost-effective, and prevents immune reactions is desired biomaterial components. Therefore, Arslan et al. have developed novel osteoinductive biocomposite scaffolds for bone tissue engineering. They have used human hair keratin/jellyfish collagen−/eggshell-derived hydroxyapatite substances in order to fabricate the scaffold. The keratin, collagen, and nano-sized spherical hydroxyapatite (nHA) were characterized with various methods and assays such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis, modified Lowry method, and thermal gravimetric analysis. The isolated hAMSCs were also characterized via flow cytometry. These characterizations are important to understand the quality of biomaterials and stem cells. The results demonstrated that fabricated scaffolds were not having cytotoxic effect and it could be used in bone regeneration in terms of its osteoinductive features. This research is also proving that daily materials which were considered as "waste" could be trendsetter for future biomaterials (Arslan et al. 2017).

Tissue engineering and regenerative medicine are not limited with these applications. For instance, wound and retinal researches were conducted with the biomaterial-cell interaction techniques (Kamao et al. 2017; Murphy et al. 2017). The regenerative effects of the biomaterial-cell interactions were also researched

to treat bladder defects on patients (Atala et al. 2006). These researches are a few examples of such field. We hope that this field will offer solutions in the future for tissue and organ diseases that cannot be treated.

3.3 The Regenerative Potential of Stem Cell-Derived Exosome/Scaffold Constructs

MSCs have an excellent potential to heal a defect or tissue damage. However, these cells divide very low at injury site. MSCs are also complex cells, and they have a possibility for iatrogenic tumor formation or cellular rejection. Recently, a novel cell-free approach has been offered which is based on stem cell-derived exosomes. Due to cell-to-cell communication role of the exosomes, these vesicles can be utilized instead of MSCs to promote tissue repair by means of delivering MSC trophic secretions. Another important feature of exosomes is that they can be produced by cells in large quantity. Although they are small in size and less in complexity than MSCs, they are easy to produce and store. The extracellular vesicles secreted by MSCs has lowest immunogenic effects and also contain therapeutically effects like parent cells in pathological conditions such as ischemic heart disease, kidney injury, wound healing, etc. Therefore, exosomes are considered an important source for the future of tissue engineering approaches (Alcayaga-Miranda et al. 2016; Lou et al. 2017; Yu et al. 2014).

Utilizing of exosomes is increasing and various researches in the field are promising. In order to investigate exosome effects on neural stem cell (NCS), primary NSCs were isolated from embryonic mouse brain. The isolated cells were cultured with human umbilical vein endothelial cells (HUVECs) or HUVECs-derived exosomes. The HUVECs-derived exosomes increased proliferation of NCSs and reduced the apoptosis of the NSCs. These exosomes also maintained their stemness. This research has shown that exosomes could be vital player to expand NCSs ex vivo for treatment of neurodegenerative diseases and traumatic injury of CNS (Zhang et al. 2018).

Therewithal, a few pioneer studies on exosome-loaded scaffolds are being developed and researched. Li et al. have used exosome techniques for restoration of mouse calvarial defects. The exosomes were derived from hAMSCs and combined with polydopamine-coating PLGA (PLGA/pDA) scaffolds. The exosomes were immobilized on PLGA scaffold with mild chemical procedures. Exosome-loaded scaffold was optimized osteoinductive effects by releasing slowly. This research indicates that the novel approach has promising effects to repair bone defects (Li et al. 2018).

Another study was conducted with exosomes from human iPSCs-derived MSCs. These exosomes were combined with beta-tricalcium phosphate (β-TCP) to repair critical-sized calvarial bone defects. Scientists used histological examinations, cell-counting method, scratch tests, and real-time PCR techniques in order to examine proliferation, migration, and osteogenic differentiation of human bone marrow MSCs. Additionally, bioinformatic analyses were conducted to detect the underlying mechanisms in the repair. As a result, reseachers have found that exosome combined with β-TCP scaffolds have shown better effects than pure β-TCP scaffolds (Zhang et al. 2016).

Lastly, a review published in 2017 indicated that exosome-laden scaffolds could be utilized in cancer and also offers “onco-materials” in order to detect early metastatic events. This review also proposes that these “onco-materials” could be used for inhibition of the formation of the pre-metastatic niches (Aguado et al. 2017). Numerous researches and studies based on exosome/scaffold combinations are expected in the future (Fig. 5).

4 Tissue Engineering Market

Tissue engineering basically encompasses the replacement of failed tissues or organs with cost-effective human-made biomaterials. When we consider thousands of people’s demand for organ or tissue transplantation, this field reasonably is important and developing. Critical

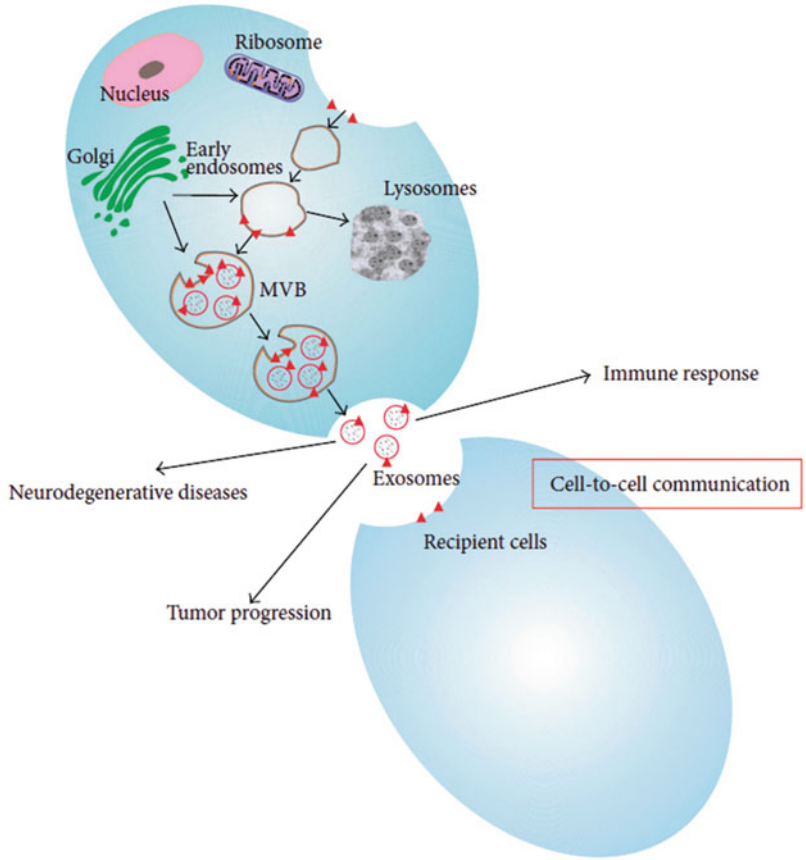

Fig. 5 Potential duties of exosomes. (Adapted from Han et al. (2016))

technologies and methods are using to fabricate functional tissues. Solvent casting and particulate leaching are used to create porous and limited thickness structures, self-assembled nanofibers to deploy and persist at target site, textile technologies for nonwoven polymers and mesh design, lithography to mold fabricated gels or ECM-derived solutions, bioreactors as a large-scale or 3D cell culture device, computer-aided designs, and many more (Berthiaume et al. 2011). These techniques are expected to increase gradually in order to find optimized biomaterial fabrication method.

Tissue engineering is a growing field in scientific progress as well as market values. There was a market in which more than 70 companies were spending $600 million per year to create a new product in 2002. As of 2007, there were approximately $1.5 billion sales; the capital values were $4.7 billion with 170 companies. In 2011, only MSC-related therapies were valued $2.7 billion and there had been predicted $4.65 billion for 2016. The popularity of MSCs made these cells as a major product type compared to other stem cells. When the market values considered only osteobiologic products that were worth $1.6 billion in 2009–2010, these product values were increased by 15% in the same term. On the other hand, Mason et al. reported that if major diseases were excluded such as end-stage renal failure, Alzheimer, heart failure, stroke and insulin-dependent diabetes, etc. from economic costs of major illnesses in the United States, approximately $250 billion annually could be saved (Griffith and Naughton 2002; Mason and Dunnill 2008; Plagnol et al. 2009; Wei et al. 2014). These studies have shown the importance of the tissue engineering and regenerative medicine as

industrial and scientific aspects. Recent reports indicated that tissue engineering and regenerative medicine market value will be expanded up to $50 billion around the globe (NASDAQ Globe News 2017).

Unfortunately, this field has major challenges to serve patients with various diseases. Building a tissue-engineered product needs essential components such as healthy expandable cells, optimized tissue-specified scaffolds, and biomaterials (Griffith and Naughton 2002). More than 20 years ago, tissue engineering and regenerative medicine emerged as an industry. The authorities like the Food and Drug Administration (FDA) are important criteria for this industry. A clearance or approval by these institutions is also a challenging part for the field in order to commercialize activities of developed products (Mao and Mooney 2015).

4.1 Approved Products at the Showcase

The tissue engineering and regenerative medicine market accelerated in the 1990s. From that day to today, various scaffolds were built for commercial purpose in tissue engineering market. In the term, there were more than 40 companies with $246 million private sector activities. One of the first products in the market was produced by Integra LifeSciences as skin substitute area. The product was described in a publication in 1980–1981, approved by the FDA in 1996, and reviewed in 1998. Aside from this, there were also other products like TransCyte and Dermagraft. These products were approved by the FDA in 1997 and 2001, respectively. While TransCyte was an acellular product, Dermagraft used polymers in order to seed foreskin-derived dermal fibroblasts. Another product, Appligraf, was a skin substitute graft, and it was based on allogenic cells (Nerem 2010).

On the other hand, the first FDA-approved biologic product in the orthopedic field, Carticel, uses autologous chondrocytes in order to repair focal cartilage defects. This application method with Carticel is based on harvested cells which were harvested from articular cartilage, expanded ex vivo, and implanted at the site of injury. The results were observed using microfracture and mosaicplasty techniques in order to compare. Liver Dialysis Unit™ is a bioartificial liver device. This hemodialysis system was approved by the FDA, and it was designed as membrane-separated device for liver tissue engineering. Decellularization of the liver is also demonstrated for the future of liver tissue engineering applications. In this field, a cell-free injectable or implantable support products like Advanced Biopolymers, Baxter, Cook Biotech, and Fidia are reported as approved products in 2009. Such-like, cell-based products were made by sheet and encapsulated cell products. These are Advanced BioHealing, ArthroKinetics, BioTissue Technologies, and Organogenesis, and those were also approved in 2009. Tissues like the cartilage, blood vessels, and bones have approved products. Japan Tissue Engineering, Karocell Tissue Engineering, and MatTek have been approved companies in 2009. These three group of tissue engineering products' manufacturers are also working on new products (Berthiaume et al. 2011; Khademhosseini et al. 2009; Mao and Mooney 2015) (Fig. 6).

5 A Journey from the Petri Dish to Human Clinic

5.1 Comparison: Pros and Cons

Tissue engineering and regenerative medicine are the interdisciplinary fields which are constantly developing. The fields mainly focus on scaffold fabrication techniques and stem cell technology to treat various incurable diseases with innovative and futuristic perspective. For this purpose, a wide range of biomaterials including synthetic or natural polymers have been successfully used to fabricate scaffolds for repairing the tissue or organ. There are numerous scaffold design and application methods to enhance the success of such fields. Today, investigators are better able to understand the underlying mechanisms of the cellular behaviors and thus develop the new

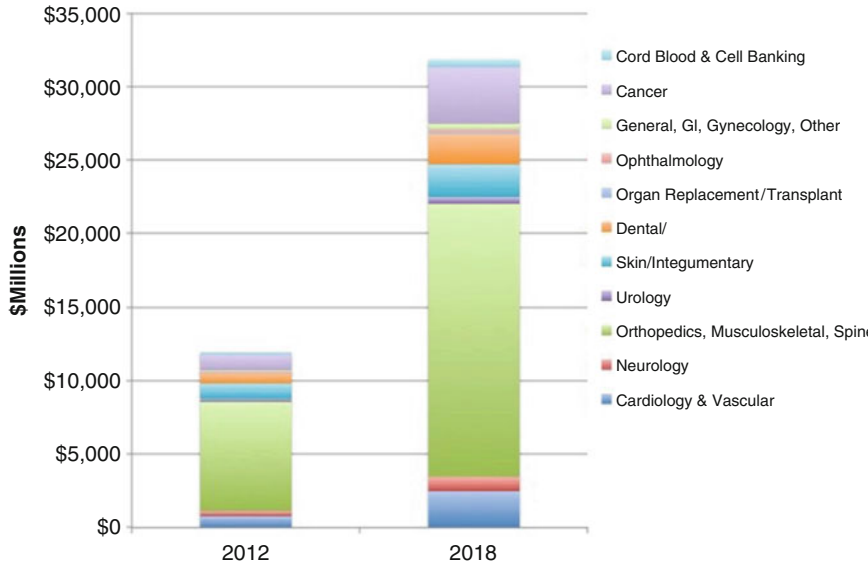

Fig. 6 Worldwide tissue engineering and cell therapy industry between years 2012 and 2018. (Adapted from Bhat and Kumar (2013))

generation treatment approaches. However, despite the successful outcomes in these areas, there are still challenges to be overcome such as providing of reliable biological sources, decreasing side effects, accessing to a sufficient number of cells, etc.

In conclusion, investigators in the field have come a long way in their understanding of the phenomena of bioengineered scaffolds for stem cell applications in tissue engineering and regenerative medicine, but more than this, it is necessary to reveal most sophisticated biomaterials for the translation of this knowledge from petri dish to human clinic.

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Adult Stem Cell-Based Strategies for Peripheral Nerve Regeneration

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Abstract

Peripheral nerve injuries (PNI) occur as the result of sudden trauma and can lead to lifelong disability, reduced quality of life, and heavy economic and social burdens. Although the peripheral nervous system (PNS) has the intrinsic capacity to regenerate and regrow axons to a certain extent, current treatments frequently show incomplete recovery with poor functional outcomes, particularly for large PNI. Many surgical procedures are available to halt the propagation of nerve damage, and the choice of a procedure depends on the extent of the injury. In particular, recovery from large PNI gaps is difficult to achieve without any therapeutic intervention or some form of tissue/cell-based therapy. Autologous nerve grafting, considered the "gold standard" is often implemented for treatment of gap formation type PNI. Although these surgical procedures provide many benefits, there are still considerable limitations associated with such procedures as donor site morbidity, neuroma formation, fascicle mismatch, and scarring. To overcome such restrictions, researchers have explored various avenues to improve post-surgical outcomes. The most commonly studied methods include: cell transplantation, growth factor delivery to stimulate regenerating axons and implanting nerve guidance conduits containing replacement cells at the site of injury. Replacement cells which offer maximum benefits for the treatment of PNI, are Schwann cells (SCs), which are the peripheral glial cells and in part responsible for clearing out debris from the site of injury. Additionally, they release growth factors to stimulate myelination and axonal regeneration. Both primary SCs and genetically modified SCs enhance nerve regeneration in animal models; however, there is no good source for extracting SCs and the only method to obtain SCs is by sacrificing a healthy nerve. To overcome

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such challenges, various cell types have been investigated and reported to enhance nerve regeneration.

In this review, we have focused on cell-based strategies aimed to enhance peripheral nerve regeneration, in particular the use of mesenchymal stem cells (MSCs). Mesenchymal stem cells are preferred due to benefits such as autologous transplantation, routine isolation procedures, and paracrine and immunomodulatory properties. Mesenchymal stem cells have been transplanted at the site of injury either directly in their native form (undifferentiated) or in a SC-like form (transdifferentiated) and have been shown to significantly enhance nerve regeneration. In addition to transdifferentiated MSCs, some studies have also transplanted *ex-vivo* genetically modified MSCs that hypersecrete growth factors to improve neuroregeneration.

Keywords
Peripheral nerve regeneration · Neuroregeneration · Neuroprotection · Mesenchymal stem cells · Schwann cells · Genetic modification · Transplantation · Transdifferentiation · Brain-derived neurotrophic factor · Clinical trials

Abbreviations

AMD	age-related macular degeneration
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMMC	bone marrow mononuclear cell
CNTF	ciliary neurotrophic factor
CNV	choroidal neovascularization
CREB	cAMP-response-element-binding protein
DRG	dorsal root ganglia
ELISA	enzyme linked immunosorbent assay
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
iPSC	induced pluripotent stem cell
MBP	myelin basic protein
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NGF	nerve growth factor
NT-3	neurtrophin 3
NT-4/5	neurotrophins 4 and 5
PDGF	platelet-derived growth factor
PNI	peripheral nerve injury
PNS	peripheral nervous system
RGC	retinal ganglion cell
SC	Schwann cell
TDM	transdifferentiation media
TENG	tissue engineered nerve graft
Trk	tropomyosin receptor kinases
tMSC	transdifferentiated mesenchymal stem cell
uMSC	undifferentiated mesenchymal stem cell
VEGF	vascular endothelial growth factor

1 Introduction

Mesenchymal stem cells (MSCs) which have been altered to resemble and act like Schwann cells (SCs) have key beneficial properties within the context of peripheral nerve trauma such as enhancing neuron survival and improving return to function. The prevalence of peripheral nerve trauma remains surprisingly high and current treatment options such as nerve graft have several pitfalls. The traditional gold standard requires the sacrifice of a healthy nerve, thus alternative remedies, such as cell transplants, are in high demand. In particular, SCs are essential to Wallerian degeneration (Salzer and Bunge 1980; Stoll et al. 1989; Lee et al. 2006), and nerve regeneration (Hadlock et al. 2000; Mosahebi et al. 2001; Schlosshauer et al. 2003; Goto et al. 2010), making excellent transplant candidates (Hadlock et al. 2000; Mosahebi et al. 2001; Zhang et al. 2002; Goto et al. 2010). However, SCs are difficult to culture *in vitro* and require harvest from a healthy nerve in order to establish a mature cell line (Moreno-Flores et al. 2006). Studies within the last 20 years have instead searched for easily harvested cells such as MSCs that can be reprogrammed or transdifferentiated

into a SC-like phenotype. Transdifferentiated MSCs are capable of expressing SC marker proteins, promoting neural tissue survival, and improving return to function in peripheral nerve injuries (Cuevas et al. 2002; Ni et al. 2010; Dadon-Nachum et al. 2011; Oliveira et al. 2013; Thoma et al. 2014). In addition to mimicking SCs, MSCs have additional benefits, such as secreting neurotrophic factors and serving as targets for genetic modification (Keilhoff et al. 2006; Pereira Lopes et al. 2006; Ribeiro-Resende et al. 2009; Wang et al. 2009; Wyse et al. 2014). The following review will provide the reader with a more in-depth perspective of current treatment options and their pitfalls; the use of cell transplants, especially SCs and MSCs; and, finally, the use of transdifferentiation to create Schwann-like cells from MSCs and their benefits to peripheral nerve regeneration.

2 Peripheral Nerve Injuries-Causes & Prevalence

Peripheral nerve injuries (PNIs) may be caused by a variety of etiologies including trauma, metabolic disorders such as Diabetes mellitus, or iatrogenic surgical complications. The most common cause of PNI is trauma, during which nerves may suffer from traction, ischemia, crushing, or penetrating wounds (Campbell 2008). Other less common causes may include thermal, electric shock, radiation, or vibrational injuries (Robinson 2000, 2004). The majority of incidents are stretch-related injuries, especially in motor vehicle accidents (Stanec et al. 1997) 30% of injuries are due to lacerations by knife, glass, saw, or long bone fractures (Campbell 2008), and about 6% are related to sports injuries (Hirasawa and Sakakida 1983). In a retrospective study by Kouyoumdjian (2006), 456 cases of PNIs showed upper limb injuries to be the most common, with the ulnar nerve most often injured (Kouyoumdjian 2006). Again, these injuries were most often due to motor vehicle accidents, particularly motorcycle crashes. In addition to affecting civilians, PNIs can commonly occur in a combative setting, where nerve injuries are commonly caused by shrapnel or blast injury from bombs or improvised explosive devices (Maricevic and Erceg 1997).

After suffering from a peripheral nerve injury, a patient's prognosis depends on the type of functional injury they have experienced. At the anatomic level, nerve injury can be divided into neurapraxia, axonotmesis, and neurotmesis (Seddon et al. 1943). In neurapraxia, the nerve remains intact but can no longer transmit impulses. Neurapraxia is typically due to segmental demyelination and is the mildest form of nerve injury. Distally, the nerve conducts normally but there is impaired conduction across the lesion due to the focal demyelination. Axons are typically anatomically intact but nonfunctional, which renders a body part paralyzed. There is sensory and motor loss due to demyelination but no Wallerian degeneration occurs. Clinically, muscle atrophy does not develop. Recovery time is typically rapid and ranges from hours to a few months. Full function is usually expected without any sort of intervention by approximately 12 weeks (Campbell 2008).

In axonotmesis, the axon is damaged but most of the surrounding connective tissue is intact. Wallerian degeneration does occur, a process which will be covered in Sect. 2. Axonotmesis is usually seen in stretch or crush injuries. Recovery and reinnervation depends upon the distance from nerve to muscle and the degree of internal axonal disorganization.

In neurotmesis, the nerve trunk is severed and most of the connective tissue is lost or distorted. Neurotmesis occurs with massive trauma, nerve avulsions, and sharp, cutting injury. There is loss of nerve trunk continuity and reinnervation typically does not occur. Without surgical intervention, the prognosis is poor. Recovery from this sort of trauma when there is significant axon loss and stromal disruption is usually prolonged and incomplete (Sunderland and Williams 1992).

When suffering from neurotmesis or axonotmesis, injuries can cause total or partial loss of motor, sensory, or even autonomic function. When left to repair itself, the peripheral nervous system can attempt one of three mechanisms: reinnervation by axonal

regeneration, reinnervation by collateral branching of uninjured surrounding axons, or remodeling of the nervous system circuitry; however, left to only these mechanisms, a full functional recovery is often not achieved (Sunderland and Williams 1992; Drake 1996; Lundborg 2000). Failure can be attributed to three problems: First, axons stop elongating and result in neuroma formation. Second, axon sprouts innervate more than one peripheral nerve branch and cause weak or contradicting muscle movements. Third, regeneration into the wrong nerve can occur if, for example, a sensory axon grows into a motor nerve or vice versa (Klimaschewski et al. 2013).

It is important to understand that while the peripheral nervous system retains the ability to reconstruct itself, only 60% of patients suffering from a PNI regain useful function (Klimaschewski et al. 2013). The occurrence of postparalytic syndromes such as paresis, synkinesis, and dysreflexia are common (Kerrebijn and Freeman 1998). Additionally, patients can experience chronic neuropathic pain, health care issues, and long periods of sick leave (Jaquet et al. 2001; Rosberg et al. 2005).

Due to the high incidence of unsatisfactory return of function, further improvements in peripheral nerve repair and regeneration have become an area of much interest. Today, PNIs have become the focus of new innovations which revolve around many different scientific disciplines. The following section will focus on the two most common areas of clinical treatment: surgery and transplantation. Other disciplines involved such as biomaterial sciences, physical therapy, and pharmacotherapy are outside of the realm of this review, though may be mentioned in the context of important interdisciplinary work.

2.1 Current Treatment Options for PNIs

The most common medical treatments rely largely on reconstructive microsurgery. Although nerve reconstruction has been attempted for centuries, techniques have improved drastically within the past few decades (Siemionow and Brzezicki 2009). Procedural options include nerve autografts, neurolysis, nerve transfers, and direct suture (end to end neurorrhaphy) (Geuna et al. 2013). The nerve transfer method has seen widespread application in recent years and is used in severe nerve trauma, including brachial plexus avulsions (Tung and Mackinnon 2010; Zhang and Gu 2011).

Although advances in microsurgical techniques have plateaued, a few interesting technological advances have occurred within the past 10 years. For example, the use of glue rather than sutures has been tested in animal models, and results indicate that glue may be equal or even superior to epi- and perineural microsuturing (Whitlock et al. 2010, Sameem et al. 2011). Another area of advancement is robotics assisted surgery. Results from experimental studies are encouraging, and robot technologies may be favored by neurosurgeons in the future (Latif et al. 2008; Nectoux et al. 2009).

Microsurgical treatment alone has relatively low success rates, which is why transplantation is drawing the most interest in regenerative medicine (Geuna et al. 2013). The current "gold standard" includes transplantation of an autologous nerve segment which has been harvested from another healthy, less important nerve such as the sural nerve. The procedure was first developed by Millesi (1981) and later deemed the standard of care (Siemionow and Brzezicki 2009). Although autografts are the "gold standard," the harvesting of another healthy nerve represents obvious limitations, which is why veins are sometimes used as an alternative (Chiu and Strauch 1990). Although vein autografts may lead to satisfactory return of sensation, comparable to nerve grafting, they are only useful for short distances as longer veins tend to collapse (Chiu 1999).

In addition to nerve and vein grafts, skeletal muscle used as guiding fibers has also been tested with relative success. Various studies have shown that muscle conduits may potentially bridge peripheral nerve defects (Meek and Coert 2002) and that grafts may even gain some functional recovery (Pereira et al. 1991, 1996; Rath 2002).

Apart from tissue transplants, cell transplants are a large area of research. MSCs and glial cells,

specifically, SCs, are commonly studied for transplantation. The purpose for use and clinical studies of each cell type will be further discussed in Sects. 4, 5 and 6. The following section will explain the process of nerve breakdown and regeneration following a traumatic nerve injury and the essential role that SCs play.

3 Wallerian Degeneration

After damage to a peripheral nerve, a complex system of molecular and cellular events take place for nerve regeneration to begin. In 1850, August Waller first described Wallerian degeneration, a process characterized by degeneration in the distal nerve stump, with elongation and regeneration in the proximal nerve stump (Stoll et al. 1989, 2002).

Soon after a PNI, SCs in the distal nerve rapidly initiate detachment of their myelin sheaths (Guertin et al. 2005). The surrounding myelin and axonal tissue begin to break down. Within hours of injury, histological changes have occurred as neurotubules and neurofilaments become disarrayed (Burnett and Zager 2004). Within 24 h of injury, SCs are stimulated to proliferate by proteins released from the disintegrating axons (Karanth et al. 2006), and later, by macrophage-derived cytokines. The SCs exhibit an increased mitotic rate, nuclear and cytoplasmic enlargement, and rapid division to form daughter cells (Burnett and Zager 2004). These daughter cells produce cytokines and trophic factors which assist in degeneration and repair (Gaudet et al. 2011). During this time, local macrophages (Mast cells) interact with the SCs to remove degenerated axonal and myelin debris. SCs and macrophages work together to phagocytose and clear the site of injury. By 36–48 h, myelin disintegration is quite advanced (Burnett and Zager 2004). The elimination of myelin sheaths is important as it clears certain growth inhibitory factors such as myelin-associated glycoproteins (Huang et al. 2005). While the distal nerve is degenerating, the nerve cell body is undergoing a process known as chromatolysis. Within 6 h of injury, the nucleus of the nerve cell body migrates to the periphery of the soma and the rough endoplasmic reticulum (Nissl bodies) breaks up and disperses (Lieberman 1971; Kreutzberg 1995). In this state, the neuron increases RNA synthesis and cellular protein content, and reduces DNA repression, in order to increase synthesis of growth-associated proteins and membrane structural components (Watson 1974).

Within 2 days, Schwann cell daughter cells have undergone rearrangement into a structure known as Bünger bands (Tetzlaff 1982). These bands act as a guidance skeleton for regenerating axon sprouts. Within a week, factors produced by SCs and injured axons leads to recruitment of hematogenous monocytes (Tofaris et al. 2002). The new macrophages continue to clear debris and produce factors which facilitate SC migration (Gaudet et al. 2011).

After weeks to months, axon sprouts begin to form, each with a specialized growth cone at the tip containing multiple filopodia. These filopodia adhere to the basal lamina of the Schwann cells within the Bünger bands, which serve as a guide toward potential new targets of innervation. Both physical and chemotactic guidance from the SCs are important in directing advancement of the growth cone (Gundersen and Barrett 1980; Dodd and Jessell 1988). Individual filopodia respond to environmental alterations in calcium (Lin and Forscher 1993) and different filopodia can react independently via local changes to actin metabolism (Kerrebijn and Freeman 1998). Once contacted by regenerative sprouts, SCs re-differentiate, express myelin mRNAs, and begin the process of remyelinating and ensheathing newly regenerated axons (Campbell 2008). If axonal sprouts are able to cross the injury site and contact a new peripheral target, then reinnervation may occur. The regeneration and repair phase may last for many months and is not always successful. Regenerating axons may enter surrounding tissue instead of the target organ or may enter the incorrect endoneurial tube, failing to reinnervate the correct target.

After nerve injury and repair, the conduction velocity of regenerated axons, their diameter, and their excitability remain below previous levels for a considerable period of time (Fields and Ellisman 1986).

In addition to the complex cellular response, PNIs induce the release of many neurotrophic factors and cytokines to create a favorable environment for axon regrowth. These polypeptides assure that the regenerating axons are growing towards the distal nerve stump and stimulate axonal sprouting. The following section will review the role of neuronal growth factors, particularly brain-derived neurotrophic factor, during Wallerian degeneration.

4 The Importance of Neurotrophic Factors During Peripheral Nerve Regeneration

In response to a peripheral nerve injury, many neurotrophic factors are upregulated. These molecules may be classified either as neurotrophic factors or neuropoietic cytokines (Lewin and Barde 1996). This review will discuss neurotrophic factors and will focus primarily on the role of brain-derived neurotrophic factor (BDNF).

Neurotrophic factors are vital to healthy neuron function for the course of the cell's life. They are important for neurite outgrowth during embryonic development, maintenance of adult neurons, and regeneration following a PNI (Klimaschewski et al. 2013). The specific neurotrophins involved in regeneration include nerve growth factor (NGF), BDNF, and neurotrophins 3 (NT-3), 4, and 5 (NT-4/5). Several growth factors are also released, including glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factors, insulin-like growth factors, neuregulins, and neuropeptides (galanin, vasoactive intestinal peptide, etc.) (Boyd and Gordon 2003; Gordon 2009). All neurotrophic factors are believed to be synthesized in target organs and then delivered via retrograde transport to the neuronal soma (Purves 1986; Oppenheim 1991). The neurotrophin members (NGF, BDNF, NT-3/4/5) share a common low-affinity receptor p75 (Chao et al. 1986) to which they all bind equally. It is thought that p75 interacts with the tropomyosin receptor kinases (Trk) to assist in transport of neurotrophins within the neuronal terminals (Gargano et al. 1997). Each neurotrophin has a specific high affinity receptor: TrkA for NGF, TrkB is specific for BDNF, and NT-4/5, and NT-3 bind to TrkC (Terenghi 1999). Every Trk receptor is located in a discreet population of primary sensory neurons (McMahon et al. 1994; Wright and Snider 1995) and TrkB and C are also present in spinal motoneurons (Ernfors et al. 1993). The following section will focus on the TrkB receptor and the various roles that BDNF plays in neuronal regeneration.

4.1 Promotion of Neuron Survival

Activation of each neurotrophin is dependent on the type of neuronal damage (motor, sensory, or autonomic). BDNF, in particular, is upregulated in motor neurons, as is its receptor, TrkB, for 48 h following an axotomy lesion (Kobayashi et al. 1996). During this time, BDNF acts as a neuroprotectant. It has been shown to rescue motor neurons from natural cell death, as well as prevent their death following axotomy (Oppenheim et al. 1992; Yan et al. 1992, 1994). This ability of BDNF to rescue motor neurons is carried out through its TrkB receptor. Once BDNF binds to TrkB, several signal transduction cascades are activated. These include insulin receptor substrate-1, Ras, protein kinase C, and many other intermediate proteins. BDNF signaling pathways activate one or more transcription factors (cAMP-response-element-binding protein (CREB), and CREB-binding protein) which regulate the expression of genes encoding proteins that are involved in neural plasticity, stress resistance, and cell survival (Bonni et al. 1999; Brunet et al. 1999; Bathina and Das 2015).

Indeed, external application of BDNF following axotomy or ventral root avulsion reduces motoneuron death (Yan et al. 1992; Novikov et al. 1995) and continuous dose-dependent

administration of BDNF shows long-term survival effects on adult motoneurons after sciatic nerve avulsion (Kishino et al. 1997). Additionally, a few studies found that application of NGF, BDNF, and NT-3 can reverse detrimental changes induced by axotomy in adult and neonatal sensory neurons (Verge et al. 1992, 1995; Eriksson et al. 1994).

4.2 Remyelination

After Wallerian degeneration occurs, the next important step in peripheral nerve recovery is remyelination, in which BDNF plays an important role. Several studies have added exogenous BDNF to a peripheral nerve injury model and examined the effects on myelin protein synthesis and myelin sheath thickness. The first study to examine this phenomenon observed that when applied in combination with ciliary neurotrophic factor (CNTF), exogenous BDNF increases myelin thickness of regenerating sciatic nerves (Lewin et al. 1997). This work was continued by a study (Chan et al. 2001) that used a SC and dorsal root ganglion (DRG) cell co-culture model, as well as a sciatic nerve *in vivo* model, to test the effects of exogenous BDNF addition following an injury. Immediately following injury, BDNF caused an enhancement in the expression of myelin protein MAG and P0. This effect was seen in both the co-culture and sciatic nerve *in vivo* model. Furthermore, when endogenous BDNF levels were reduced in the co-culture via addition of the receptor scavenger TrkB, myelin protein synthesis was inhibited as was the formation of myelin, verifying that BDNF is indeed beneficial during remyelination.

Several studies found that BDNF increases myelination during peripheral nerve regeneration. With the use of electron microscopy, Chan et al. demonstrated that the addition of BDNF increased the number of myelinating axons and the thickness of the myelin sheath *in vivo* (Chan et al. 2001). A similar study (Zheng et al. 2016) created a mouse sciatic nerve injury model and administered exogenous BDNF injections to examine the effects on myelin sheaths in the distal nerve stump. Their results showed that mice receiving BDNF administration had an increased number of myelinated fibers and that myelin sheaths were thicker when compared to control mice. Additionally, mice receiving BDNF blocking antibodies showed significant myelin deterioration in the distal sheath. Furthermore, a study by Zhang et al. 2000, demonstrated that treatment with BDNF antibody reduced the number and density of myelinated axons by 83%, and found that sensory reinnervation was impaired (Zheng and Kuffler 2000). Combined, these results demonstrate that BDNF is critical for preparing nerves for remyelination by increasing myelin proteins such as P0 and MAG, as well as protecting the distal nerve portion from atrophy by promoting remyelination.

4.3 Axonal Sprouting, Regeneration, and Functional Recovery

In addition to examining neuronal survival, regeneration, and re-myelination, several studies have looked at BDNF's role in axonal sprouting. It has been shown that following severe trauma such as ventral root avulsion, exogenous BDNF significantly increases axonal sprouting (Gordon 2009). In support of Gordon's findings, another study found that application of BDNF blocking antibodies on a transected facial nerve trunk significantly reduced axon sprouting up to 18% (Streppel et al. 2002). Axonal sprouting may increase in part, due to BDNF's role as a guidance molecule for the growth cone at the end of each axonal sprout. Studies in *Xenopus* spinal neuron models show that BDNF and NT-3 can attract or repulse growth cones based on concentration gradients (Song and Poo 1999; Zheng and Kuffler 2000).

Although BDNF may increase axonal sprouting, the data is controversial in regard to increased functional return upon application of BDNF. For example, using the sciatic function index (Bredesen and Rabizadeh 1997), gait analysis (Shirley et al. 1996), and force recovery, several studies failed to demonstrate a return to function with exogenous BDNF. One study even

showed that local long-term continuous infusion of low dose BDNF had no effect on tibial motoneurons after immediate microsurgical repair (Boyd and Gordon 2002).

On the other hand, a more recent study found that exogenous BDNF administration accelerates the recovery process in a mouse sciatic nerve injury model, while BDNF antibody treatment delayed it (Zheng et al. 2016). After the crush injury, control mice took 12 days to show initial improvement using the toe spreading score of gait analysis, and 24 days for a full recovery. Mice receiving the BDNF treatment required only 7 and 18 days, respectively. Conversely, BDNF antibody treatment delayed the processes to 17 and 30 days.

Another study created control and heterozygote BDNF knockout mice that received a left sciatic nerve crush (Takemura et al. 2012). Nerve function was evaluated using a rotarod test, sciatic function index, and motor nerve conduction velocity simultaneously with histological nerve analysis. Impaired nerve repair was observed in the BDNF heterozygote mice, which was consistent with attenuated function of BDNF. In contrast, the BDNF homozygote mice showed complete functional and histological recovery. These observations support the view that BDNF may play a pivotal role in functional return following a peripheral nerve injury.

Unlike other neurotrophic factors, BDNF is unique in that it regulates and maintains neuronal function, and when given exogenously, it counteracts degenerative changes in both sensory and motor axons. Unlike NGF, BDNF supports motoneuron survival *in vitro*, rescues from naturally-induced apoptosis, and prevents *in vivo* axotomy-induced cell death (Yin et al. 1998). While there are benefits of exogenous BDNF application to peripheral nerve lesion sites, its abilities to increase functional return are still controversial. Therefore, recent research has focused on the adjunct use of BDNF in combination with other therapies such as stem cell therapy, biomaterial conduits, pharmacotherapy, etc. A more in-depth discussion of BDNF therapy combined with stem cell use will be included in Sects. 6 and 8.

5 Cell-Based Therapy for Improving Nerve Regeneration

As discussed above, the gold standard of peripheral nerve repair continues to be the use of nerve grafting combined with direct nerve repair, and occasionally, the use of conduits to bridge larger nerve gaps. Recent research, however, has focused on cell therapy as a promising therapeutic approach for promoting nerve regeneration. Particularly, cell-based therapy has been widely studied as a delivery system for growth-promoting molecules and as a graft replacement. This section will focus briefly on the past use of glial cells such as SCs and then discuss the promising potential of bone marrow-derived MSCs.

5.1 Use of Schwann Cells

Schwann cells (SCs) play a key role in axonal regeneration, making them an attractive cell type to use for transplantation. During Wallerian degeneration, SCs remove necrotic tissue and myelin debris together with macrophages (Geuna et al. 2009). In the regeneration phase, SCs form the Bünger bands and increase synthesis of surface cell adhesion molecules and basement membrane proteins such as laminin and fibronectin to physically guide axons to distal innervation targets (Fu and Gordon 1997). SCs also produce neurotrophic factors, cytokines, and other compounds which promote neurite growth (Funakoshi et al. 1993; Hall 2001). Experimental evidence shows that addition of SCs to nerve conduits *in vitro* support axonal outgrowth (Schlosshauer et al. 2003), and improves the quality and rate of axon regeneration (Hadlock et al. 2000; Mosahebi et al. 2001; Goto et al. 2010). SCs combined with a vein conduit have even been used in bridging long nerve gaps (Strauch et al. 2001; Zhang et al. 2002). The Miami Project, a program for the investigation of brain and spinal cord injury has used SCs in a phase I clinical trial. Previous methods for SC culture were adapted for the manufacture of clinical

grade human SC products that meet FDA standards (Bunge et al. 2017), and the autologous transplant of SCs into individuals with spinal cord injury was deemed safe and feasible with no complications (Anderson et al. 2017).

Although SCs appear be an ideal source of cell for regenerative therapy, there are several technical limitations. In order to obtain a source of autologous SCs, another healthy nerve must be sacrificed for harvesting, making donor site morbidity an additional concern. Use of SCs is thought by some to be impractical since the time requirement for expanding autologous cells in culture is lengthy (Moreno-Flores et al. 2006). There is also a risk of fibroblast contamination which would lead to unwanted scarring of the nerve (Mosahebi et al. 2001). SCs require stimulation by axons or specific growth factors that mimic axonal signals in order to proliferate, and do not proliferate in response to serum factor unlike other cell types. All of these limitations have led researchers to seek an alternative to SCs for cell transplantation, and stem cells have been posed as better candidates.

5.2 Mesenchymal Stem Cells

Stem cells are a distinct population of undifferentiated cells which are characterized by potency, the ability to differentiate into a wide variety of specialized cell types, and the ability to undergo numerous rounds of mitosis while remaining undifferentiated. There are embryonic, induced, fetal, and adult stem cells, of which this review will focus on adult stem cells.

Of these different categories, adult stem cells are thought to be the most limited in their potency and are generally considered multipotent in nature since their primary role is to repair damaged tissue in which they are found (Oliveira et al. 2013). Unlike fetal and embryonic stem cells, adult stem cells raise fewer ethical concerns as they do not require human embryo destruction. Additionally, adult stem cells have a lower risk of tissue rejection as auto-transplantation is a possibility, and the small risk of teratoma formation that pluripotent embryonic or induced stem cells presents is almost null with adult cells (Bjorklund et al. 2002). Common sources of adult stem cells include mesenchymal, hematopoietic, or umbilical cord-derived. In particular, bone marrow-derived stem cells are known as mesenchymal stem cells and can differentiate into connective tissue types such as chondrocytes, adipocytes, myocytes, osteocytes, fibroblasts, and tenocytes (Muraglia et al. 2000). There is also extensive additional research to suggest that MSCs have the ability to transdifferentiate into ectodermal and endodermal lineages such as glial cells, neurons, hepatocytes, etc (Fig. 1) (Woodbury et al. 2000; Dezawa et al. 2001; Kim et al. 2002).

In addition to being a source for many cell types, MSCs are easily accessible and have the ability to rapidly divide under culture, allowing them to meet the requirements of an *in vitro* cell system. Additionally, MSCs are excellent candidates for allogenic transplantation as they are immune privileged cells and often do not require the use of immune suppressive drugs (Oliveira et al. 2013). Besides their high safety and efficacy, MSCs release paracrine factors, survive and integrate into host tissue, and concentrate in injured tissues. (Keilhoff and Fansa 2011).

6 Mechanisms Behind Nerve Regeneration Potential of MSCs

Although MSCs are highly regarded for their plasticity and ability to differentiate into many cell types, there are other mechanisms by which MSCs are thought to promote and support nerve regeneration. Such mechanisms include immunomodulation, transdifferentiation into SCs, paracrine activity, genetic manipulations, and mitochondrial transfer/cellular fusion (Fig. 2).

6.1 Secretion of Neurotrophins

As discussed already, neurotrophins promote neuronal survival, reverse the negative effects of PNIs, and lead to SC proliferation and

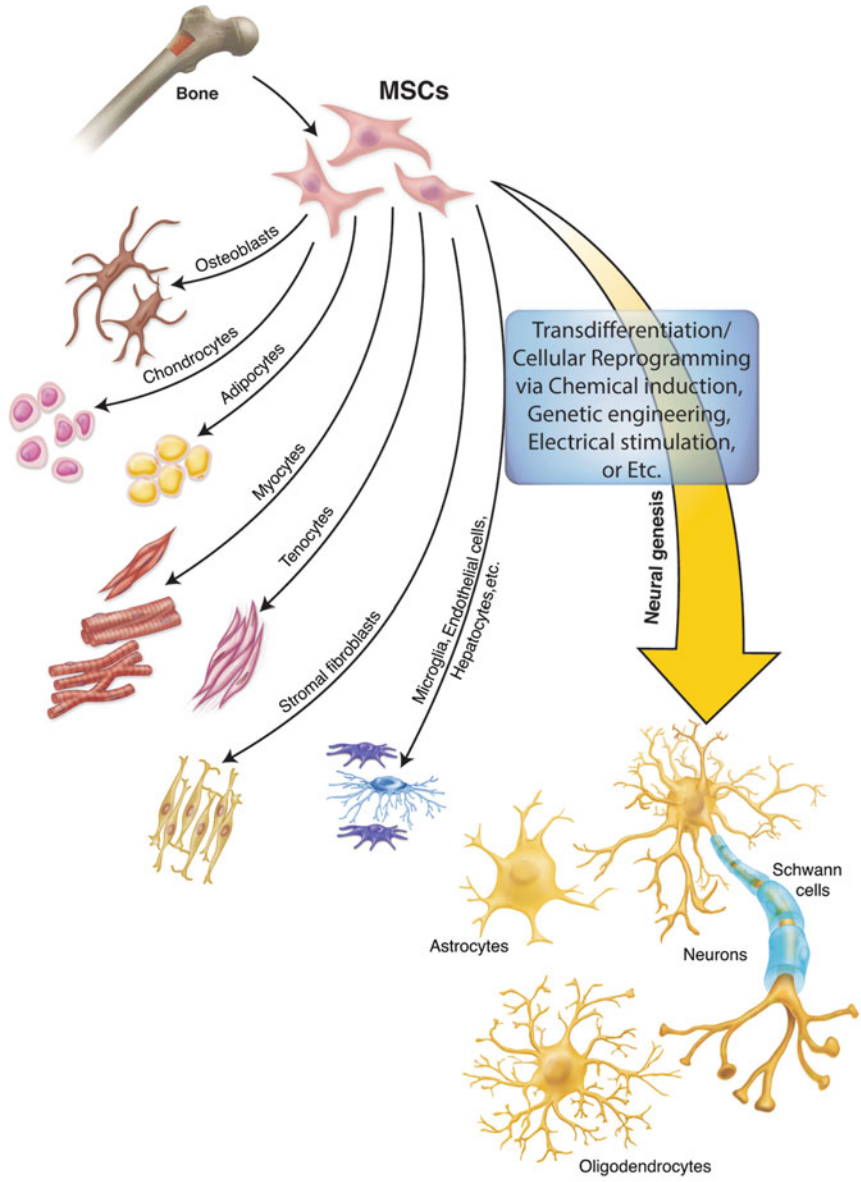

Fig. 1 Differentiation potential of multipotent bone marrow-derived mesenchymal stem cells (MSCs). Mesenchymal stem cells are capable of differentiating into mesodermal lineages including osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes and stromal fibroblasts. A number of studies have also demonstrated that MSCs can be transdifferentiated or reprogrammed into endodermal and ectodermal lineages including, microglia, endothelial cells, hepatocytes and neural cells (neural genesis into: Schwann cells, neurons, astrocytes and oligodendrocytes). (Illustration modified from Sandquist et al. 2016)

differentiation. MSCs can produce neurotrophic substances for paracrine signaling, which is likely one of the key ways that MSCs are thought to help in regeneration. Gu et al. investigated DRG explants and neurons co-cultured with MSCs and showed enhanced neurite outgrowth and neuronal cell survival due to the production of NGF, CNTF, BDNF, and basic fibroblast growth factor

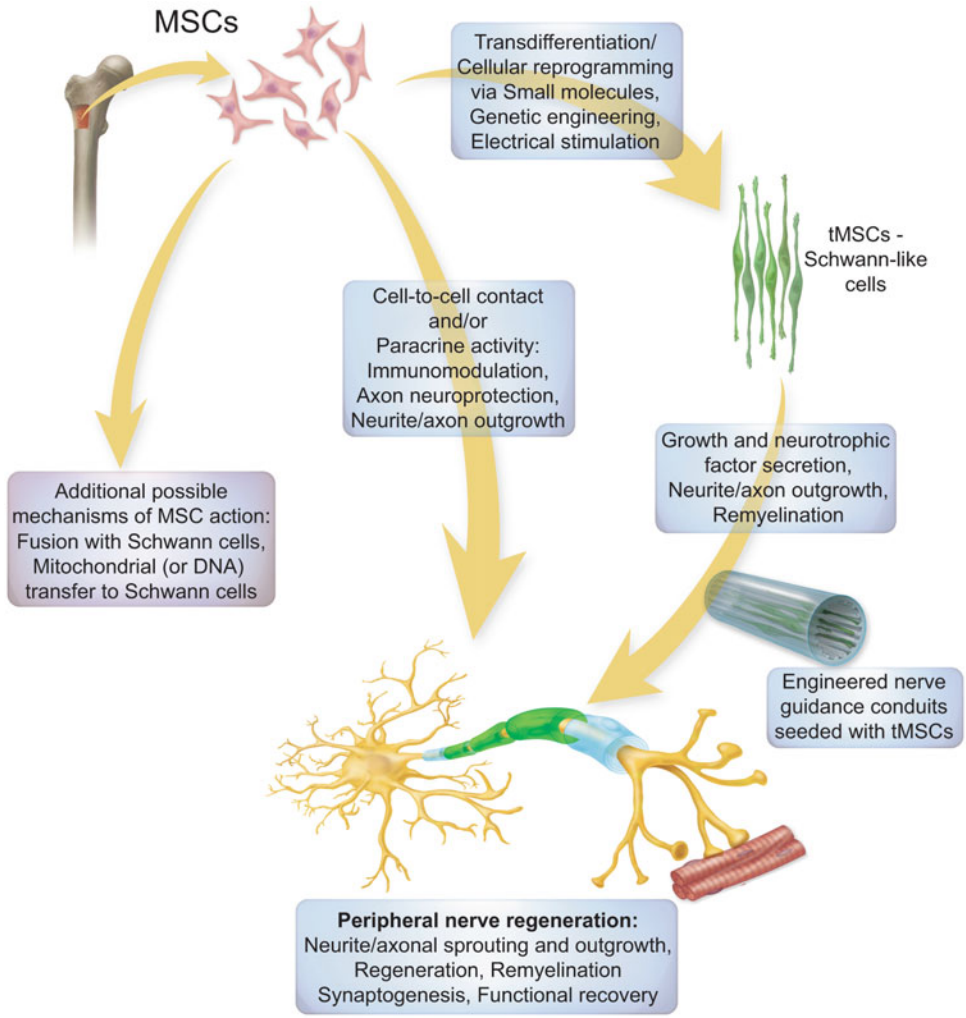

Fig. 2 Mechanisms and strategies using MSCs to promote and support nerve regeneration. Abbreviations: MSCs, mesenchymal stem cells; tMSCs, transdifferentiated MSCs; DNA, deoxyribonucleic acid. (Illustration prepared by S. Mientka, K. Moss, D. S. Sakaguchi, Biological Pre/Medical Illustration (BPMI) program, Iowa State University)

(bFGF) by MSCs (Gu et al. 2010). In the culture system, there was no direct contact between the neurons/explants and MSCs, leading researchers to believe that positive effects were due to the release of soluble growth factors. Other studies have found that DRG explants or adult neural progenitor cells treated with MSC-conditioned media also showed increased neurite outgrowth, presumably due to the presence of released growth factors in the media (Ribeiro-Resende et al. 2009; Ye et al. 2016). In addition to their direct paracrine effects, MSCs can induce SCs to produce neurotrophic mediators as well. In co-culture studies of rat SCs and MSCs, increased survival and proliferation rates of SCs was noted as well as high expression of mRNA and protein levels for NGF, BDNF, and Trk/p75^{NTR} receptors (Wang et al. 2009). The same group also examined the effect of MSCs on SCs in a rat peripheral nerve repair model, and showed that MSCs increased the generation of SCs and promoted SC-mediated neurotrophic functions. Thus,

MSCs are considered beneficial for regeneration due to their production and secretion of neurotrophic factors.

After *in vitro* co-culture studies, the next step was to determine whether MSCs continued to produce growth factors *in vivo* after transplantation, and whether these factors were biologically active. Fortunately, this inquiry returned positive results. Several studies were able to document expression of GDNF, CNTF, FGF, and even BDNF by MSCs *in vivo,* allowing for survival and elongation of neuronal growth cones (Pereira Lopes et al. 2006; Chen et al. 2007; Pan et al. 2007; Yang et al. 2011). A similar study (Ribeiro-Resende et al. 2009) transplanted MSCs at a rat sciatic nerve lesion and the results demonstrated improved regeneration of motor and sensory axons due to the production of growth factors. Other studies incorporated conduits filled with MSCs to test models of long sciatic nerve gaps (Wang et al. 2011; Yang et al. 2011; Hu et al. 2013). For example, one group implanted a collagen conduit filled with MSCs at a mouse sciatic nerve transection lesion and saw enhanced axon regeneration and remyelination (Pereira Lopes et al. 2006). Additionally, high levels of NGF and BDNF were detected, suggesting that MSCs were expressing these neurotrophins *in vivo*.

Combined, these results demonstrate that MSC-based therapy improves peripheral nerve regeneration through direct secretion of neurotrophic factors which may act locally as well as on glial cells further away.

6.2 Immuno-modulatory Effects

One of the most interesting features of MSCs is their ability to modulate the immune system making it a popular candidate for transplant therapy. When transplanted into tissues, MSCs decrease tissue inflammation and can have immunosuppressive effects by restraining T-cell proliferation and inhibiting natural killer T cell signaling (Di Nicola et al. 2002).

Additionally, MSCs promote anti-inflammatory T helper 2 cells (Aggarwal and Pittenger 2005). MSCs also suppress monocyte differentiation into dendritic cells, thus decreasing the amount of antigen presentation to T cells (Jiang et al. 2005). In a spinal cord injury model, MSC transplantation favored the development of M2 macrophages and suppressed M1 activation (Nakajima et al. 2012). M2 macrophages have anti-inflammatory activity while the classic M1 phenotype has pro-inflammatory effects in damaged tissue (Nakajima et al. 2012). The complex mechanisms behind MSCs immunomodulatory properties are still being uncovered, but they are thought to be facilitated by cell-cell contact and the secretion of soluble factors such as indoleamine-pyrrole 2,3-dioxygenase (IDO) and nitric oxide that are known to inhibit T-Cell proliferation (Mazzoni et al. 2002; Terness et al. 2002; Wang et al. 2014). A recent review by Gao et al. further details immunomodulatory properties of MSCs (Gao et al. 2017). Though exact mechanisms of MSC immunomodulation are not fully understood, their ability to decrease inflammation has been widely described, supporting the therapeutic merits of stem cells.

6.3 Cellular Fusion

In addition to the various nerve regeneration mechanisms discussed, a few studies have documented the spontaneous transfer of mitochondria from MSCs with a variety of other cell types. MSCs can form tunneling nanotubes through which mitochondria and nuclear DNA can be transferred. Several studies have utilized MSCs in acute pulmonary damage models to demonstrate mitochondrial transfer from MSCs to alveolar cells and airway epithelial cells (Spees et al. 2006; Islam et al. 2012; Li et al. 2014). Mitochondrial transfer has also been demonstrated between MSCs and cardiomyocytes, causing increased proliferation and, in Acquistapace's study, reprogramming towards a progenitor-like state (Plotnikov et al. 2008; Acquistapace et al. 2011; Vallabhaneni et al. 2012). The majority of these studies involved use of epithelial or muscle cells; however, one study found that bone marrow-derived MSCs were able to fuse with neuronal cell types,

including Purkinje cells (Weimann et al. 2003). To date, there is no evidence of mitochondrial transfer or MSC fusion with SCs, but this could represent an alternative mechanism by which MSCs support SC activity and regeneration.

7 Clinical Trials with MSCs for Neurological Disorders

Autologous cell transplantation has been investigated extensively as a therapeutic strategy for neurological disorders. Extensive *in vitro* and *in vivo* data suggest that MSCs secrete several trophic factors, support neuritogenesis and neurite growth, and promote survival and elongation of damaged peripheral nerves. An even larger body of work exists demonstrating the benefits of MSCs within the context of central nervous system disorders and spinal cord trauma. Combined, the data has proven the safety and efficacy of MSCs and allowed their use in human clinical trials – a key stepping stone to their common use as a clinical therapy.

A large number of studies have reported the use of MSCs in treatment of neurological disease and trauma (Harrop et al. 2012). Clinical trials include treatment of Multiple Sclerosis (23%), Amyotrophic Lateral Sclerosis (14%), Alzheimer's disease (5%), Duchene muscular dystrophy (3%), Parkinson's Disease (3%) to treatment of traumatic injury, with spinal cord injury models having the largest number of trials (29%) (Squillaro et al. 2016).

Fewer clinical trials have utilized MSCs within a peripheral nerve context. One retrospective study reports the use of bone marrow mononuclear cells (BMMCs) as a source of MSCs to treat patients with a median or ulnar nerve severed by knife or glass. Cells were collected from the patient's iliac crest and injected into a silicone conduit used to bridge the nerve gap. Patients implanted with the BMMC-filled conduits scored higher for motor function, sensation, and effect of pain on function than those who received empty conduits (Braga-Silva et al. 2008). Though these results are promising, the two groups of patients (with and without BMMCs) were studied decades apart; furthermore, it is unclear whether the improvements were mediated specifically by MSCs within the BMMC fraction. Nonetheless, this study provides a basis for future clinical trials.

Most clinical trials related to peripheral nerve repair with MSCs focus on diabetic peripheral neuropathy patients. For diabetic patients, MSCs are an effective therapeutic agent due to secretion of bFGF and vascular endothelial growth factor (VEGF) (Shibata et al. 2008). Evidence suggests that the effects are not mediated through differentiation into neural cell types, but rather through the secretion of these beneficial factors. (Siniscalco et al. 2011). One clinical trial was conducted using MSCs to treat patients with diabetic foot disease – a complication in which hyperglycemia induces peripheral nerve damage. Human umbilical cord blood-MSCs were injected into the patient's impaired limb resulting in obvious improvement 12 weeks after treatment. This result was attributed to the MSC's bFGF and VEGF production and also to their immune cell modulatory effects (Li et al. 2013). Current clinical trials are in stage II and III and revolve around change of nerve conduction velocities before and after stem cell intravenous transfusion (clinicaltrials.gov; NCT02387749). Results indicate that autologous bone marrow stem cells have increased bFGF and epidermal growth factor (EGF) levels at time of transfusion. Patients with MSC transfusion had greater sural nerve conduction velocities 90 days after treatment.

Other conditions with MSC-based clinical trials involving the peripheral nervous system include hemifacial spasm and burn wound healing (clinicaltrials.gov; NCT02394873, NCT03183622). One clinical trial in early phase I aims to inject autologous adipose-derived MSCs into patients with hemifacial spasm in hopes to increase facial nerve function and evaluate changes in facial nerve electrophysiology (clinicaltrials.gov; NCT02853942). While peripheral nerve regeneration is not the primary focus of some of these studies, transplanted MSCs and their secreted factors are likely to facilitate the overall repair of damaged nerves.

Unfortunately, there are no current clinical trials examining the use of MSCs in traumatic peripheral nerve damage, but important pre-clinical studies are underway. Xue et al. transplanted autologous bone marrow-MSCs into a 60 mm-long canine sciatic nerve gap using a tissue-engineered nerve graft (TENG) with improved repair and regeneration (Xue et al. 2012). The same group then moved on to implanting the MSC-TENGs into a rhesus monkey median nerve gap. Animals with MSC-TENG implants recovered motor function comparable to autologous nerve grafted animals and with greater recovery than the animals with tissue-engineered scaffold alone. Transplanted MSCs were found to express SC marker S100 and neurotrophic factors BDNF, CNTF and bFGF. Thus, it is likely that the autologous MSCs contribute to the peripheral nerve regeneration via cell replacement and secretion of beneficial factors. Lastly, after extensive safety evaluations, it was concluded that autologous MSCs could safely be used in a primate (Hu et al. 2013). Altogether, these studies provide strong support for the future clinical use of MSCs for traumatic peripheral nerve damage.

The data obtained from clinical trials, as well as *in vitro* and *in vivo* studies shows that unaltered MSCs offer many benefits for nerve regeneration, mainly by secretion of neurotrophic factors, as well as by support of SCs. However, MSCs may hold even greater potential when transdifferentiated into another cell type, such as Schwann cells. The various benefits and methods of transdifferentiated MSCs will be discussed below.

8 Transdifferentiation

Bone mesenchymal stem cells were once thought to be fairly restricted in their differentiation patterns but more studies are demonstrating that they are capable of versatility and greater plasticity. In response to a variety of culture conditions, specialized *in vivo* microenvironments and genetic manipulations, MSCs can turn into different phenotypes such as glial cells (Fig. 3). In particular, turning MSCs into a SC-like phenotype is of high interest due to the beneficial effects on nerve regeneration. MSCs can be transdifferentiated with a variety of methods, including the use of transplantation, co-cultures, small molecule cues, genetic manipulation, or as most recently described, through electric stimulation. Each method will be discussed in greater detail below.

8.1 Transdifferentiation via Transplantation

During Wallerian degeneration and nerve regeneration, a wide variety of cytokines and growth factors are released, creating a specialized microenvironment with the capacity to greatly influence cell differentiation patterns. Although controversial, these environmental signals have been utilized to transdifferentiate MSCs in response to injury or inflammation. Bone marrow-derived MSCs injected at the site of a rat sciatic nerve transection were capable of surviving and migrating, as well as differentiating into an SC-like phenotype, based on S100 immunoreactivity patterns (Cuevas et al. 2002). In this study, it was presumed that MSC transdifferentiation occurred in response to physiological environmental cues, as no MSC medium changes were made. Although transdifferentiation may have occurred, the percentage of cells positive for S100 was so low that this may not be a very efficacious method. Another 2010 study demonstrated similar results, with few numbers of transplanted MSCs at an injury site converting to an SC-like phenotype (Oliveira et al. 2010).

8.2 Transdifferentiation via Co-culture

A more simplistic approach to changing a cell's microenvironment is to adjust its neighboring interactions using co-culture methods. One study showed that direct contact co-cultures of DRG neurons and MSCs could cause a phenotypic and morphological change in MSCs to resemble

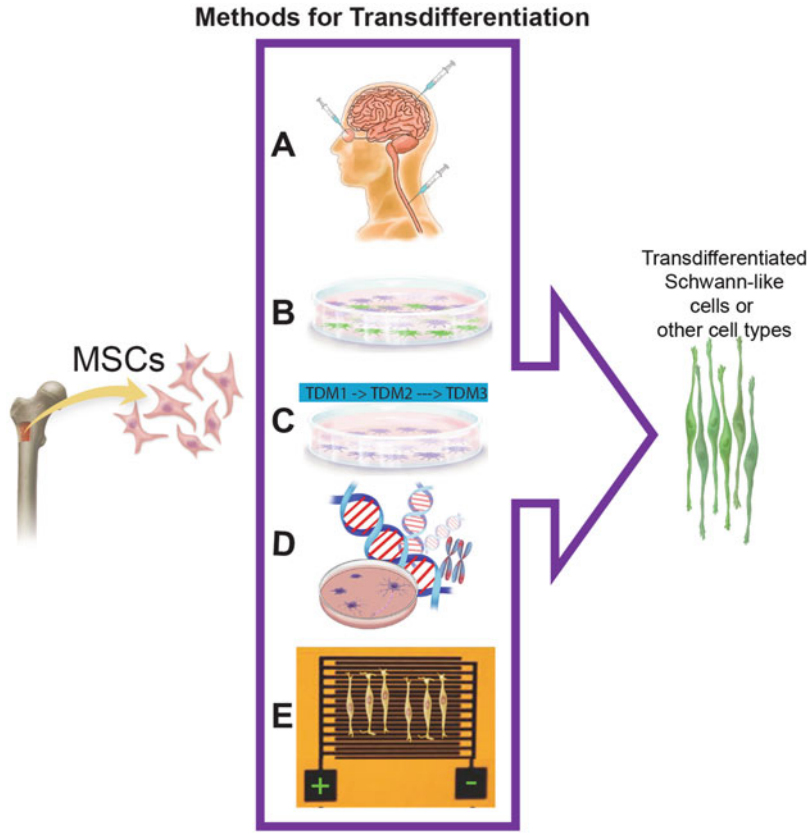

Fig. 3 Methods for MSC transdifferentiation. MSCs can be transdifferentiated with a variety of methods, including the use of transplantation (**a**), co-cultures (**b**), small molecule cues (**c**), genetic manipulation (**d**), or through electric stimulation (**e**). (Illustration prepared by S. Mientka, K. Moss, D. S. Sakaguchi, Biological Pre/Medical Illustration (BPMI) program, Iowa State University; ((**a**) adapted from Sakaguchi 2017, (**e**) adapted from Das et al. 2017)

SCs (Yang et al. 2008). Researchers suggested that the release of cytokines and other neuronal molecules on the axonal surface may play a role in the transdifferentiation process. However, this method alone did not allow transdifferentiated MSCs (tMSCs) to form compact myelin, suggesting that further molecular cues are necessary for a complete transdifferentiation process. Another study looked at co-culture of MSCs with olfactory ensheathing cells and saw a dramatic increase in the number of MSCs resembling a neural morphology which were immunoreactive to various neural markers such as GFAP, $p75^{NTR}$, and MAP 2 (Ni et al. 2010). These studies demonstrated that co-culturing methods may be sufficient to begin the transdifferentiation protocol, but additional small molecules may be needed to affect a functional change in tMSCs.

8.3 Use of Small Molecules in Media

Although transdifferentiation via transplantation and co-culture has shown some success, this method is not as successful or efficient as the addition of small molecules to culture medium. These specific molecules can trigger cell-signaling pathways and rapidly modulate cell phenotype. In 2001, Dezawa et al., discovered a cell medium protocol for transdifferentiation of MSCs into an SC-like morphology requiring incubation with beta-mercaptoethanol, then retinoic acid for 3 days, followed by forskolin, bFGF, platelet-derived growth factor (PDGF) and heregulin (Dezawa et al. 2001). After induction, these cells physically resembled SCs and expressed several SC markers including p75, S100, GFAP and O4. Bierlein De la Rosa et al.

used Dezawa et al's protocol to transdifferentiate MSCs which had been genetically modified to hypersecrete BDNF. The cells were morphologically similar to SCs and expressed SC markers S100 and p75 even after 20 days in transdifferentiation media. Additionally, secreted BDNF levels increased after 20 days and the BDNF secreting MSCs actually showed more SC markers after 8 days compared to the control, green fluorescent protein (GFP) expressing MSCs, indicating the BDNF itself may facilitate faster conversion to SC phenotype (De la Rosa et al. 2017).

Biomaterial-based scaffolds are also being investigated as a means to augment the transdifferentiation of MSCs into Schwann-like cells. For example, gelatin-based 3D conduits with different microstructures (nanofibrous, macroporous and ladder-like) have been fabricated for peripheral nerve regeneration applications (Uz et al. 2017). Their results indicated that 3D macroporous and ladder-like 3D microstructures enhanced MSC attachment, proliferation and spreading, creating interconnected cellular networks. This type of approach investigates the effects of 3D conduit microstructure and mechanical properties and may provide a better understanding of how material-cell interfaces can influence the transdifferentiation process.

Recently, the first comparative proteomic evaluation of MSC transdifferentiation was undertaken to uncover the protein contents that affects SC formulation (Sharma et al. 2017). This study identified a number of MSC proteins that were significantly regulated during SC transdifferentiation. Many of these proteins support axonal guidance, myelination, neural development and differentiation. These results provide clues to unraveling the molecular events that underlie the transdifferentiation process and may ultimately serve to facilitate nerve regeneration and repair.

Other studies have utilized compounds such as valproic acid and histone deacetylase inhibitors, along with neural inducing signaling molecules to create mature neural cells (Sandquist et al. 2016). A 2014 study used a two-step method to first create neural precursor cells, and then induced SCs from human foreskin fibroblasts (Thoma et al. 2014). These cells may potentially be used to treat peripheral nerve injuries in the future.

8.4 Genetic Modification for Transdifferentiation

A newer transdifferentiation method can now convert adult differentiated cells to a specific terminal cell type without going through pluripotency. This methodology is based on the idea of 'master control genes' in somatic cells which can be overexpressed to induce a cascade of cell fate changes (Lewis 1992; Nizzardo et al. 2013; Prasad et al. 2016). The earliest evidence of this possibility was provided by Weintraub et al, who confirmed conversion of fibroblasts to myogenic lineage by transfection of a master regulatory gene (*MyoD*) (Weintraub et al. 1991). Later, *Pax6* was recognized as a master gene responsible for neuronal differentiation. Vierbuchen et al. identified the combination of *Asc11*, *Brn2* and *Myt11* as able to convert mouse embryonic fibroblasts into mature neurons (Vierbuchen et al. 2010). Cells transdifferentiated in this manner exhibited similar functionality to cells differentiated from induced pluripotent stem cells (iPSCs) or wild-type analogs and show no tumorigenicity when transplanted *in vivo* (López-León et al. 2017). Unfortunately, this method of generating target cells through transdifferentiation relies on viral expression of exogenous transcription factors which makes demonstration of safety for clinical trials difficult; however, the method holds promise for direct cellular conversion.

8.5 Electrical Transdifferentiation

A recent study by Das et al. 2017 described a novel procedure for transdifferentiation of MSCs through the application of electrical stimuli via graphene-based electrodes (Fig. 4) (Das et al. 2017; Uz et al. 2018). Rat MSCs were seeded on a graphene interdigital electrode and subjected to either electrical or chemical

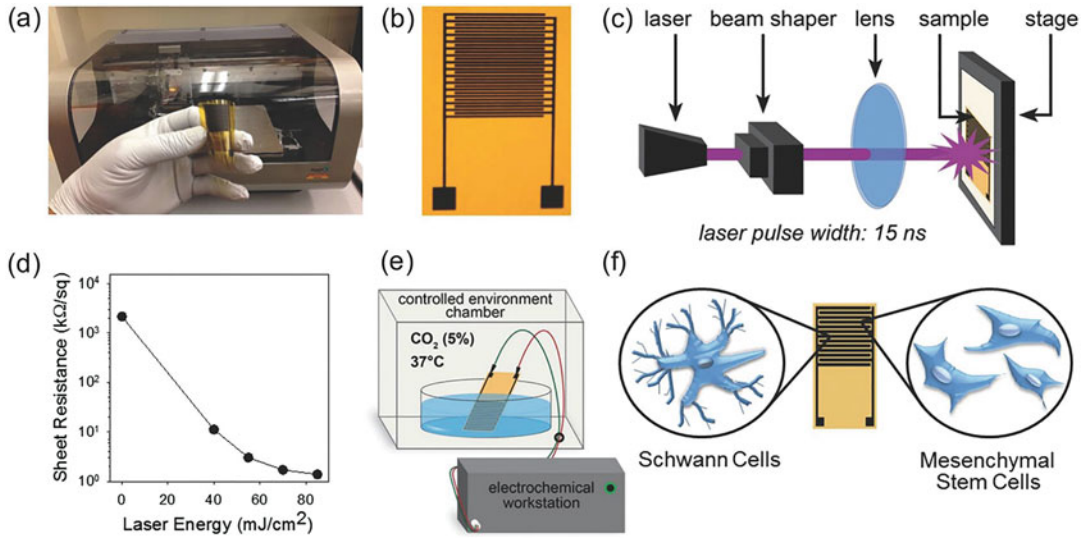

Fig. 4 Fabrication and MSC differentiation protocol on graphene interdigitated electrodes (IDEs). (**a**) The inkjet printing of the graphene IDE on a flexible and bendable polyimide substrate (Fujifilm Dimatix Materials Printer is shown in the background). (**b**) An optical image of the graphene IDE circuit with 400 μm finger width, pitch of 250 μm, and printed graphene thickness of 5–7 μm. (**c**) Schematic diagram of the pulsed-laser processing setup used for postprint annealing. (**d**) Electrical sheet resistance measurements of the printed graphene IDE irradiated with distinct laser energies. (**e**) Schematic diagrams dis-playing the cell culture medium and application of sole-electrical stimulation to the IDE circuit while (**f**) displays schematic magnified views of MSCs and postelectrical stimulated differentiated SCs. (Adapted from Das et al. 2017)

transdifferentiation, then expression of cell surface markers such as p75, S100, and S100β were analyzed with immunocytochemistry after 15 days. The results for electrical tMSCs were compelling, showing the highest degree of preferential immunolabeling, with more than 85% of cells demonstrating labeling for SC markers vs. 75% for chemically transdifferentiated MSCs. Additionally, electrically stimulated cells secreted significantly higher levels of NGF as compared to their chemically transdifferentiated counter-parts. Although not statistically significant, higher levels of BDNF and GDNF were also noted. While other reports have shown that electrical stimuli can increase growth factor level production in SCs (Huang et al. 2010; Koppes et al. 2011, 2014), this paper is the first to describe such effects in transdifferentiated MSCs. Furthermore, Das et al., demonstrated that electrical stimuli alone can transdifferentiate MSCs to a SC-like phenotype without the need for chemical growth factors, thus saving researchers time, labor, and money, while providing a novel platform for an artificial neural network circuit.

8.6 Beneficial Properties of tMSCs

Once methods of transdifferentiation had been discovered, scientists moved on to *in vivo* studies to determine the effect of tMSCs on models of peripheral nerve injury. After Dezawa et al. performed their *in vitro* cell characterization, tMSCs were transplanted into the cut end of a rat sciatic nerve. Results showed that the transplanted cells remained in a Schwann-cell like state and were capable of forming myelin sheaths, as well as supporting nerve fiber regrowth (Keilhoff and Fansa 2011). Additionally, Dezawa and collaborators also showed that tMSCs co-localized with the myelin-associated glycoprotein antibody signal, suggesting that MSCs may be able to differentiate into

myelinating cells. After this initial trial, many labs followed suite by implanting transdifferentiated MSCs into a variety of peripheral nerve and spinal cord injury models. In 2004, Mimura et al. supported Dezawa's work by showing that human and rodent MSC-derived SCs expressed myelin-related markers and contributed to re-myelination when transplanted into a rat sciatic nerve injury (Mimura et al. 2004). Using a similar transdifferentiation protocol, Keilhoff et al. (2006) also demonstrated that transplanted tMSCs within a muscle conduit promoted remyelination, and electron microscopy showed that single tMSCs were even capable of wrapping more than one axon, similar to an oligodendrocyte (Keilhoff et al. 2006).

In addition to providing functional support, transdifferentiated MSCs are capable of producing trophic factors at even higher levels than SCs. When transdifferentiated MSCs were placed in a DRG co-culture system without direct contact, tMSCs showed upregulation of BDNF and NGF. Additionally, neurite outgrowth was observed even in the presence of NGF and BDNF blocking antibodies, suggesting that other trophic cytokines or factors may be produced by tMSCs (Mahay et al. 2008). Another interesting study used a combination of two different mediums to transdifferentiate MSCs, facilitating production of large amounts of BDNF and GDNF. Interestingly, cells resembled an astrocyte morphology and expressed certain astrocyte markers. When transplanted, the cells improved muscle reinnervation and restored motor function in a rat sciatic nerve crush model (Dadon-Nachum et al. 2011). Combined, these results support the idea that MSCs display functional characteristics similar to SCs by secreting bioactive neurotrophic factors.

Soon after the introduction of tMSC transplants, scientists began to question the duration of a SC-like state once cells were placed in an *in vivo* environment. Shimizu et al. transplanted MSC Schwann-like cells within a transpermeable tube into a rat sciatic nerve gap (Fig. 5) (Shimizu et al. 2007). After 3 weeks, tMSCs continued to express SC markers such as p75, GFAP and increased S100 expression. Most importantly, the MSCs expressed myelin-associated markers such as MAG and myelin basic protein (MBP) even after 3 weeks *in vivo*, which the authors contend supports the premise that MSCs may retain SC-like characteristics even after transplantation. It is important to note however, that remeylination was not seen via immunohistochemistry or electron microscopy, as in other studies. A different study by Ishikawa et al. 2009 transplanted MSC-derived SCs within chitosan gel sponges and found that cells were able to form myelin sheaths 1 month after transplant (Ishikawa et al. 2009). The mean diameter of myelinated fibers increased continuously, even out to 4 months post-transplant. This study, along with the work by Dezawa et al. 2001, demonstrates that rat tMSCs may contribute to remyelination after transplantation into an injured PNS model. Similar results have been found in spinal cord injury models (Someya et al. 2008; Wakao et al. 2010; Kamada et al. 2011), indicating that MSC-derived SCs are effective for both PNS and CNS regeneration. Thus, MSCs are capable of expressing SC biomarkers, may express myelin markers, and even physically form myelin sheaths. Moreover, these effects may last well past the time that MSCs were last exposed to transdifferentiation media, suggesting that the acquired SC-like state is at least semi-permanent and allows cells to persist well into the acute phase of Wallerian degeneration.

Unfortunately, there have never been clinical trials involving the use of chemically transdifferentiated MSCs for the nervous system. However, a non-human primate study has been completed as an important pre-clinical step. Wakao et al. 2010, used a monkey model and followed subjects out to a year post transplant (Wakao et al. 2010). MSCs were chemically induced to resemble SCs and cell marker expression patterns were confirmed with both immunocytochemistry and reverse transcription-PCR. Cells were transplanted for 1 year in a median nerve transection model. During this year, no major health abnormalities were observed in the monkeys. Immunohistochemistry with Ki-67 revealed no signs of massive proliferation and the ^{18}F-FDG-PET scan, which detects neoplastic cells, demonstrated no abnormalities.

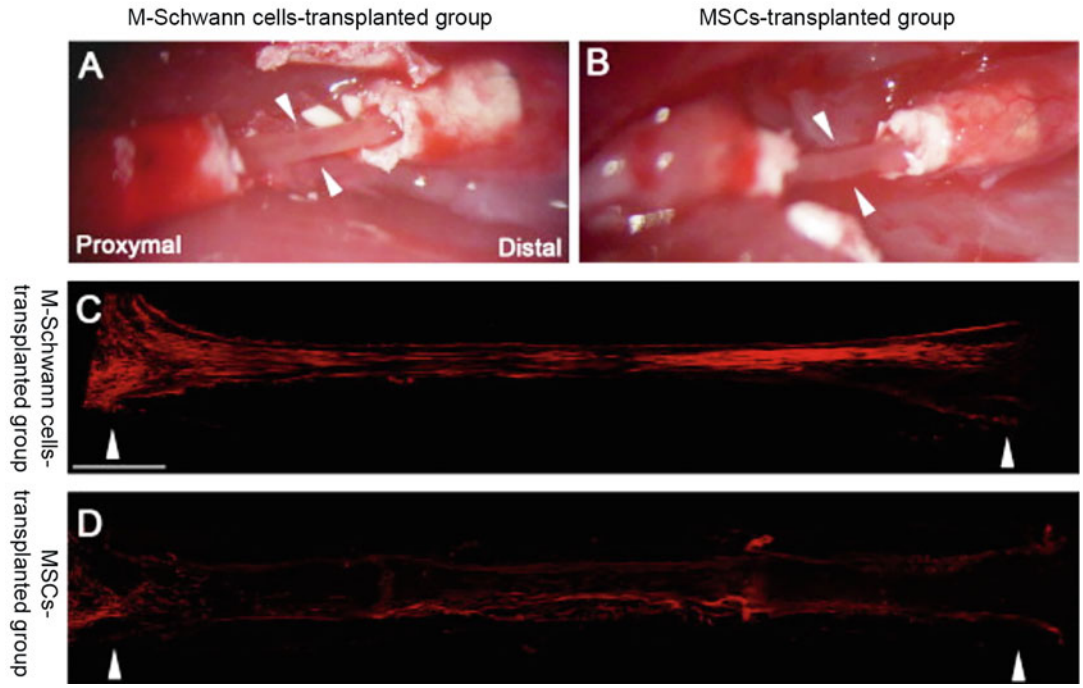

Fig. 5 Transdifferentiated MSCs bridge 10 mm nerve gap. Transected rat sciatic nerve gap bridged with graft containing MSCs transdifferentiated into SCs (M-Schwann cells, (**a**) and undifferentiated MSCs (**b**) 3 weeks after transplantation. Newly formed tissue indicated by arrowheads. Nerve fibers stained with neurofilament (red) for M-Schwann cell (**c**) and MSC (**d**) groups. Scale bar = 100 μm. (Figure adapted from Shimizu et al. 2007)

Furthermore, monkeys regained function, and electrophysiology with histology revealed restoration of the severed nerve. This study is particularly important because it demonstrated not only the efficacy of transdifferentiation, but also the safety of long-term implantation of tMSCs in nonhuman primates.

9 Genetically Modified MSCs

The literature has aptly demonstrated that undifferentiated MSCs can produce neurotrophic factors *in vitro* as well as *in vivo* and that the process of transdifferentiation can even further increase production levels. Only in recent years have researchers begun to investigate the continuous production of these proteins via functional gene insertion. As one of their novel features, MSCs are suitable for transduction and expression of exogenous genes, making them a good candidate system for genetic engineering. The most widely used systems are now either lentivirus or retrovirus-based. In regards to nervous system disorders, MSC lines have been created to over express a wide variety of neurotrophic factors such as GDNF, NGF, and BDNF (Wyse et al. 2014), as well as other growth factors. Pre-clinical studies by Sharma et al. 2015, demonstrated that MSCs genetically modified for production and secretion of BDNF, GDNF and even a hybrid MSC line hyper-secreting both BDNF and GDNF, had similar viability and proliferation rates when compared to the non-genetically modified original MSCs (Sharma et al. 2015). One 2008 study by Bauer et al., went so far as to develop an in depth biosafety model to specifically assess the risk of retro- and lentiviral

vectors (Bauer et al. 2008). Human hematopoietic stem cells and MSCs were transduced with Moloney murine leukemia virus and transplanted into 481 immunodeficient mice. There was no detectable evidence of insertional mutagenesis leading to human leukemias or solid tumors during the 18 months animals were studied. Additionally, no vector-associated adverse events were observed and in 117 serum samples analyzed, there was no detectable viral DNA. These findings indicate that virally transduced MSCs are stable and may behave biologically like the wild type MSC population, making them suitable for *in vivo* study and use in a variety of disease and injury models. Genetically modified MSCs have been used in studies ranging from the treatment of neurodegenerative disorders, including ischemic injury, retinal degeneration, spinal cord injuries and peripheral nerve transections. Studies in each of these areas will be discussed below.

9.1 Use of Genetically Modified MSCs in Neurodegenerative Disorders

9.1.1 Parkinson's Disease

Use of GDNF was first described in 1993 as a potential treatment for Parkinson's disease because of its ability to increase dopamine uptake and aid in the survival of embryonic midbrain dopaminergic neurons (Lin et al. 1993). With the challenge of administering GDNF infusions, cell-based strategies to deliver GDNF have been receiving attention. In one study, MSCs transduced with a GDNF retrovirus vector increased dopaminergic neuron sprouting (Moloney et al. 2010). A similar study found that injections of GDNF MSCs given 1 week before a lactacystin lesion of the medial forebrain also significantly increased dopamine levels (Wu et al. 2010). Furthermore, Ren et al. (2013) transplanted GDNF MSCs into the brain of non-human primates and saw increased dopamine levels and improved contralateral limb function (Ren et al. 2013). Preclinical studies provide evidence that GDNF MSCs produce high levels of a functional trophic factor, which, with further safety and efficacy data, could be used in clinical trials as adjunct treatment for Parkinson's disease.

9.1.2 Alzheimer's Disease

Treatment options for Alzheimer's disease are limited and focus on symptoms related to neurotransmitter systems, rather than targeting the underlying pathologies. Given the prevalence of the disease and lack of treatments, new strategies are being developed which focus around the use of NGF. Autologous fibroblasts engineered to express NGF were transplanted in eight patients with Alzheimer's. Patients saw an improvement of Mini-Mental Status Examination scores and a reduced decline in cognitive scores (Ren et al. 2013). A phase II clinical trial is still open for this method (Wyse et al. 2014). MSCs have not directly been used in human clinical trials, however, promising work by Li et al. (2008) demonstrated reduced memory deficits in the Morris-water-maze task in a rat model when NGF MSCs were transplanted to the hippocampus (Li et al. 2008). Future studies will likely include further *in vivo* transplantation trials with NGF MSCs in both rodent and non-human primate models, and in human trials.

9.1.3 Huntington's Disease

Compared to the other neurodegenerative diseases discussed, Huntington's disease is unique in that clinical signs may be directly correlated to reduced levels of neurotrophic factor BDNF. Low BDNF levels in the striatum are due to loss of function of the wild-type huntingtin protein. This protein modulates BDNF transcription and plays a role in BDNF transport and secretion (Zuccato et al. 2001). The Dunbar laboratory first demonstrated that murine MSCs engineered to overexpress BDNF improved disease progression on a transgenic mouse model of Huntington's (Dey et al. 2010). Important pre-clinical trials by Pollock et al. 2016 utilized a double-blind study to examine the effects of transplanted human BDNF MSCs on disease progression in a mouse Huntington's disease model (Pollock et al. 2016). Treatment with MSCs decreased striatal atrophy and significantly reduced anxiety. BDNF MSC treatment also

increased the mean lifespan of mice. This study demonstrated the efficacy of BDNF hypersecreting MSCs as a medical therapy for Huntington's disease and set the groundwork for future clinical trials.

9.2 Ischemic Brain Injury

Ischemic brain injury causes the death of various important cell types such as neurons, glial, and endothelial cells. Regain of function and brain tissue repair necessitates cell replacement and formation of a new network (van Velthoven et al. 2009). When transplanted into ischemic regions of the rat brain, MSCs reduced functional deficits after 14 days, scar thickness was decreased, and the number of proliferating cells in the subventricular zone was increased (Chen et al. 2001; Li et al. 2001; Lu et al. 2001). Improvement by MSC treatment has been attributed to decreased apoptosis, MSC differentiation into neuronal cells, and promotion of neurogenesis, angiogenesis, and synaptogenesis (Chen et al. 2003; Iihoshi et al. 2004; Mimura et al. 2004; Mimura et al. 2005; Li et al. 2006). Several groups have used genetically modified stem cells that overexpress growth factors known to enhance neuronal survival including BDNF and GDNF. When BDNF overexpressing MSCs were delivered to an ischemic brain model via injection, infarct volume was reduced and functional outcome was improved (Kurozumi et al. 2004; Nomura et al. 2005; Horita et al. 2006). Furthermore, BDNF expressing MSCs can significantly improve behavioral test results and reduce ischemic damage as indicated via magnetic resonance imaging (MRI) analysis after 7 and 14 days (Kurozumi et al. 2005; Nomura et al. 2005).

9.3 Retinal Degenerative Disease – Glaucoma

Glaucoma is a leading cause of progressive blindness and is estimated to effect over 2 million Americans (Friedman et al. 2004). Glaucoma is an optic neuropathy resulting in progressive loss of visual function due to loss of retinal ganglion cells (RGC) whose axons project through the optic nerve and terminate in visual centers. To prevent this loss of retinal cells, several research groups have investigated the neuroprotective effects of MSCs which have been genetically modified (Hou et al. 2010; Harper et al. 2011; Park et al. 2012; Machalińska et al. 2013) or chemically stimulated (Levkovitch-Verbin et al. 2010) to augment secretion of neurotrophic factors as a strategy for retinal neuroprotection. In these studies, modified MSCs were successfully transplanted whether intravitreally or subretinally, though subretinal transplant appears to yield greater neurotrophic factor mRNA and protein levels in the rat retina (Park et al. 2012).

Harper et al found that intravitreal transplant of BDNF-expressing MSCs preserved RGCs to a greater degree than unmodified MSCs and allowed for greater protection of retina and optic nerve function in a rat glaucoma model (Harper et al. 2011). Rat and human MSCs chemically stimulated to secrete BDNF and GDNF were also neuroprotective after intravitreal transplant in rats with optic nerve transection (Levkovitch-Verbin et al. 2010). Hou et al. used MSCs genetically modified to secrete an anti-angiogenic factor called pigment epithelial-derived factor (PEDF) as a strategy to inhibit choroidal neovascularization (CNV) – the underlying cause of wet AMD. The results indicate a recruitment of MSCs to sites of CNV, a reduction in the CNV proliferation and an increase in retinal pigment epithelial cells that protect photoreceptor cells (Hou et al. 2010). These studies provide a promising basis to the use of modified MSCs as a therapy for retinal degenerative diseases such as glaucoma and AMD via neuroprotection of cells vulnerable to these diseases.

9.4 Spinal Cord Injuries

In addition to various therapies within the brain and retina, modified MSCs have been used with variable success in the spinal cord. In a 2005 study by Lu, Jones, and Tuszynski, BDNF

MSCs were injected into a crushed rat spinal cord injury and the extent and diversity of axonal growth was increased (Lu et al. 2005). Additionally, SCs preferentially migrated to the BDNF secreting grafts. Unfortunately, functional recovery was not achieved for any of the studied rats. Another study was performed by Sasaki et al. 2009, in which BDNF secreting human MSCs were implanted at a T9 spinal cord lesion (Sasaki et al. 2009). After 5 weeks, locomotor improvement was observed for the BDNF group and there was increased axonal sprouting. Specifically, an increased number of serotonergic fibers were observed in the ventral horn grey matter, an area important for motor controlled movement. Unlike the 2005 Lu study, Sasaki's group demonstrated that BDNF MSCs are associated with improved functional outcome following a spinal cord injury. Due to the conflicting data reports, additional studies are necessary before the full benefits of BDNF delivery via engineered MSCs can be determined for the treatment of spinal cord damage.

9.5 Peripheral Nerve Injury

Of all the disease models discussed so far, peripheral nerve injuries have the fewest published studies involving transplantation of genetically modified MSCs. This may be because researchers are now utilizing a multi-disciplinary approach and studies often involve the use of engineered conduits, cell transplants, and now even gene therapy. One of the first studies to use a MSC gene delivery system transplanted MSC spheroids transfected with the BDNF gene (Tseng and Hsu 2014). Spheroids were combined with a polymeric nerve conduit to bridge a 10 mm rat sciatic nerve transection gap. MRI was used to track the transplanted cells. Animals receiving the BDNF MSC spheroids had the shortest gap bridging time, the largest regenerated nerve, and the thickest myelin sheath at 31 days. Furthermore, BDNF MSC spheroids significantly promoted functional recovery. A more recent study (Gao et al. 2016) combined multi-channel agarose scaffolds with BDNF MSCs to bridge a 15 mm sciatic nerve transection gap. Additionally, the distal sciatic nerve segment was injected with a BDNF lentiviral vector. Twelve weeks after injury, BDNF secreting cells significantly increased axonal regeneration and injection of the lentiviral vector at the distal segment enhanced axonal regeneration beyond the lesion. A recent study investigated the efficacy of BDNF *ex vivo* gene transfer to umbilical cord blood-derived MSCs in a rat sciatic nerve crush injury model (Hei et al. 2017). Four weeks post-surgery, the BDNF expressing MSCs exhibited more peripheral nerve regeneration than the controls. Additionally, sciatic function index, axon counts, and axon density were significantly increased for both the BDNF MSC and regular MSC groups. The results from these studies are promising and indicate that BDNF hypersecreting MSCs in particular can improve sciatic nerve regeneration. Unlike other areas of research, no pre-clinical characterization studies looking at safety and appropriate dosage ranges have been published. This would be a necessary step before testing BDNF MSCs outside of a rat model.

10 Conclusions and Future Directions

Peripheral nerve injury limits mobility and sensation in up to 2.8% of all trauma patients and often results in unsatisfactory return to function (Noble et al. 1998). Although the gold standard of microsurgery with autograft has seen advances in the last decade, there are significant drawbacks associated with the procedure. For this reason, scientists have proposed the use of an alternative transplant type, in the form of autologous stem cells. Specifically, research is directed at the conversion of mesenchymal stem cells towards a SC-like fate to aid in Wallerian degeneration, neuronal regeneration, and possibly even remyelination. Additionally, MSCs have their own unique benefits such as immunomodulatory properties, secretion of neurotrophic factors, possible mitochondrial transfer, and the ability to be easily genetically modified. In order to resemble a SC, MSCs must undergo transdifferentiation

which can be achieved through a variety of methods including incorporating specific factors into the growth media, co-culture method, i*n vivo* transdifferentiation, and others. Although these older techniques have their benefits, methods of transdifferentiation have changed drastically within the last 10 years and now include master gene modification as wells as the use of specific cell signaling molecules combined with histone deacetylase inhibitors.

As demonstrated by the more recent body of literature, scientists are beginning to investigate other somatic cell types in addition to bone marrow MSCs including but not limited to fibroblasts, adipocytes, and even hair follicle stem cells (Amoh et al. 2005; Kingham et al. 2007; Thoma et al. 2014). These studies rely largely on immunocytochemical staining, co-culture neurite outgrowth, and gene expression patterns to support transdifferentiation of cells into SCs. Unfortunately, none of these studies have measured growth factor secretion levels from transdifferentiated cells, and only Thoma's study looked at the ability of these cells to create myelin. In order to truly assess whether these transdifferentiated cells are SCs, future work should test growth factor secretion, perform patch-clamp recordings, transplant cells into rat sciatic nerve models, and examine myelin formation via electron microscopy.

In addition to testing new cell types, researchers are trying new methods of transdifferentiation and emphasizing the use of genetic control and epigenetic cues. Future research may focus on SC de-differentiation or multi-step transdifferentiation in which a less-differentiated intermediate is first created, and then the fully transdifferentiated cell type is achieved, such as in Thoma et al.'s work with fibroblasts. While these cell fate reprogramming methods are promising, they can often be time consuming, difficult to consistently reproduce, and cost prohibitive. Additionally, rigorous studies have yet to be performed which examine the tumorigenic capacity of these cells and their long term genetic stability. While the field of transdifferentiation still has many challenges to overcome, it is a promising focus in the study of regenerative medicine and offers new insight into cell fate plasticity.

Specifically, in regards to the peripheral nervous system, researchers have shown that a variety of regenerative cell types may act like SCs by secreting trophic factors, supporting re-myelination, and decreasing time to functional return of severed nerves. When transdifferentiated cells are combined with multiple neuroregenerative strategies such as *ex vivo* gene delivery, and biomaterial conduits, they may become powerful alternatives to traditional peripheral nerve regeneration therapies.

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Immunomodulatory Behavior of Mesenchymal Stem Cells

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Abstract

The use of Mesenchymal Stem Cells (MSCs) in the treatment of diseases where immunomodulation impacts therapy is increasing steadily. Recent studies aim to achieve effective use of MSCs in treatment of Graft versus Host Disease (GvHD), Crohn's disease and organ transplantations. The molecular mechanisms governing immunomodulatory properties of MSCs have not been fully understood, although current studies are indicating progress. Especially, *in vitro* studies and animal models provide a major contribution to our knowledge in clinical use of MSCs. The immunosuppressive and immune-enhancer properties of MSCs are –typically- determined with respect to type and concentrations of soluble molecules found in their physiological environment. In mammals the immune system protects the organism -not only- from certain microorganisms, but also from any entity that it recognizes as foreign, including its own cells when it is received as a threat. This protection can sometimes occur by increasing the number of immune cells and sometimes by suppressing a pathologically hyper-induced immunological response. In particular, realization of the bi-directional effect of MSCs on immune cells has placed substantial emphasis on this area of research. This chapter focuses on the interaction of MSCs with the immune cells, the bilateral role of these interactions, and whether studies that aim to understand these interactions can yield promising results in terms of developing improved use of MSCs in treatment.

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Keywords

GvHD · Immonology · Immunomodulation · Immunosuppression · Mesenchymal Stem Cells · T cells

Abbrevations

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cells
CD	Cluster of Differentiation
ConA	Concanavalin A
GvHD	Graft Versus Host Disease

MHC II	Major Histocompatibility Complex class II
MSCs	Mesenchymal Stem Cells
NK	Natural Killer (NK)
NO	Nitric Oxide (NO)
iNOS	Nitric Oxide Synthase
IDO	Indoleamine 2,3-Dioxygenase
IL	Interleukin
IFNγ	Interpheron γ
PBMCs	Peripheral Blood Mononuclear Cells
TLR	Toll-like receptors
TNFα	Tumor Necrosis Factor α

1 Introduction to Immune System

The immune system encompasses the elements in our body that have the thankless job of keeping the body healthy, protecting it from disease (Steinman 2004). The immune system fights against outside factors like pathogens, as well as internal diseases such as cancerous cells with constant vigil, utilizing immune cells, molecules, and immune organs, in a structure that is only second to the nervous system in complexity (Steinman 2004; Dantzer and Wollman 2003; Chaplin 2010).

The primary function of the immune system is to prevent infectious diseases. Pathogenic microorganisms are capable of replicating at a rate much faster than the cells of a multicellular organism, especially in human body; where the environment is favorable for their growth (Lewis 2000). Even a small number of pathogenic organisms are capable of quickly overpopulating a host cells at the site of an infection (Tribble and Lamont 2010). It is the immune system's job to develop a rapid and an efficient response against these invaders, and quickly isolate and eliminate a threat so that the organism itself is not too disturbed by the presence of the invaders (Spiering 2015). The importance of the immune system can be seen in individuals suffering from the Acquired Immunodeficiency Syndrome (AIDS) where the fatality due to the disease is not directly due to the presence of the virus itself, but because of the opportunistic infections that the immune system fail to address (Okoye and Picker 2013).

The immune-response is a multi-layered, complex reaction to an invasion (Chaplin 2010). The initial response is mediated by the elements of innate immunity, which are a group of non-specific, first responder cells, tissues and molecules that attempt to prevent diseases before they form a foothold (Nicholson 2016). If the innate immune system fails to prevent the disease, the cells of the innate immunity activate the adaptive immune system (Iwasaki and Medzhitov 2015). In contrast to innate immunity, adaptive immunity takes a longer time to respond – up to several weeks, versus the several minutes of the innate immunity. Adaptive immunity is capable of forming a disease-specific response, one that is more effective than the response of the innate immunity (Paust et al. 2010; Simon et al. 2015).

1.1 Innate Immune System

While the adaptive immune system can produce a highly effective response against invaders, it takes up to a week to develop a response against a new pathogen (Simon et al. 2015). Considering that pathogenic bacteria can have a doubling-time of 20 min, the adaptive system alone cannot handle the immune response to a new disease in such short timeframes (Conway and Cohen 2015). On the other hand, the innate immune system that is capable of responding to a new pathogen instantly provides a wide-spectrum type of response (Mogensen 2009). Innate immunity can prevent less number of infections on its own, and slows down the development of stronger ones, while the adaptive immune system develops a stronger response to eliminate them completely (Mogensen 2009; Nicholson 2016).

Innate immunity components recognize pathogens through common elements shared by all pathogens, called molecular patterns (Mogensen 2009). When the cells and molecules of the innate immunity encounter one such element, they activate other innate immunity components quickly by replicating and producing

signals that initiate and sustain the immune system activation as they migrate to the infection site (Nicholson 2016). Innate immunity response is also responsible for the activation of the adaptive immune system if an infection persists (Iwasaki and Medzhitov 2010).

As the first element of the innate immunity, the skin forms the outermost barrier of entry against the pathogens. It has many anti-pathogenic properties, such as having a low pH and water-content, providing antimicrobial lipid secretions and residence to beneficial microbiota, which limit the growth of any potential invaders (Belkaid and Hand 2014; Sanford and Gallo 2013). Protection by the skin is complemented by the so-called defensins, small antimicrobial proteins that can kill microorganisms by disrupting their cellular membranes (Yamasaki and Gallo 2008). Another group of proteins that are part of the innate immunity are the factors of the complement system, which takes various roles, including disrupting the cellular membrane of pathogens, triggering the migration of the immune cells to the site of infection, inducing phagocytosis of pathogens by phagocytic cells, and activating the adaptive immune cells (Nicholson 2016).

Cellular elements of the innate immunity respond rapidly to invaders. Phagocytes are immune cells that are capable of engulfing their targets, and digest them with powerful enzymes they produce (Selders et al. 2017). Neutrophils are the most common cells of the innate immune system, and are the first cells to respond to an infection (Selders et al. 2017). Neutrophils are a type of granulocytes that are named after the granular structures found in their cytoplasm where hydrolytic enzymes used to digest pathogens in the phagocytic process are deposited (Nicholson 2016). They proliferate rapidly, and die in a few hours (Selders et al. 2017). Other granulocytes, such as eosinophils and basophils, constitute a minority of the granulocyte population, and play key roles in defending the body against multicellular parasites and inducing allergic responses (Liaskou et al. 2012).

Macrophages are the second set of phagocytic cells of the immune system. They are a lot larger than the neutrophils, and are capable of phagocytosing invaders at a much rapid rate (Ren et al. 2003). Additionally, they can last for a longer period of time than the neutrophils (Ren et al. 2003).

Both neutrophils and macrophages have surface receptors that recognize the molecular patterns of pathogens (Nicholson 2016). Toll-Like Receptors (TLR) are one of the best known examples of pattern-recognizing receptors (Armant and Fenton 2002). Different types of TLRs detect different components, such as bacterial lipoglycans, flagella and bacterial cell wall components (Janssens and Beyaert 2003).

Final group of cells in the innate immune system are the Natural Killer (NK) cells. NK cells are responsible for the destruction of cells that are infected by intracellular pathogens, such as viruses. NK cells are activated when they encounter a non-self antigen on the surface of an infected cell whereby any antigen that does not belong to the host is considered as non-self (Holzemer et al. 2017). When activated, NK cells kill the infected cell either directly with the secretion of membrane disrupting proteins, or indirectly, by activating the apoptotic pathways of the cell (Nicholson 2016).

Both innate and adaptive immune system cells secrete signaling proteins, called cytokines, to communicate to each other. Cytokines are soluble proteins that activate immune cells, inducing their proliferation and differentiation enhancing an inflammatory response. Some of these cytokines, such as IFN-ɣ and some of the interleukins, are necessary to activate the adaptive immune response.

1.2 Adaptive Immunity

Adaptive immune system produces a tailor-made reaction to specific antigens, producing a more effective response to an infection than that produced by the innate immune system, at the cost of a greater time between exposure and response (Nicholson 2016). However, after the first encounter of an antigen, the adaptive immune system is capable of creating a "memory" of

these specific antigens with its memory cells (Spiering 2015). If the same antigen is encountered again, the adaptive immune system immediately responds, resulting in an immunity against that particular pathogen (Holzemer et al. 2017; Chaplin 2010).

T cells and B cells are the primary components of the adaptive immune system (Sullivan et al. 2016; Chaplin 2010). During their development, each of these adaptive immunity cells is designed with a structure that recognizes a specific antigen. When the innate immune system is incapable of handling an infection, dendritic cells -the master controllers of the immune response- bring antigen samples from the infecting pathogens to lymph nodes, where T and B cells are produced and stored (Nicholson 2016). Dendritic cells are classified as Antigen Presenting Cells (APC), which are capable of carrying antigens on their cell membrane via specialized membrane proteins called major histocompatibility complex proteins, Major Histocompatibility Complex class II (MHC II) (Mantegazza et al. 2013). When a dendritic cell brings an antigen that is recognized by a T cell that responds to that specific antigen, that particular T cell becomes activated. Activated adaptive immunity cells undergo a process of clonal expansion during which antigen specific T cell rapidly proliferates producing many more identical copies of itself (Nicholson 2016; Chaplin 2010).

One subset of T cells, called the T-helper cells, start searching for a B cell that is also specific to the same antigen. When these cells bind to each other, the T-helper cell activates the B cell, inducing it to produce antibodies.

Antibodies are secreted proteins composed of four polypeptide chains. (Chailyan et al. 2011). When bound, antibodies make it easier for phagocytic cells to engulf their cognate pathogen cells, that are formed into tight clusters consisting of several pathogens bound together, and –thereby- allow the inactivation of pathogens (such as viruses), and even destroy pathogens with the aid of the complement system (Chailyan et al. 2011).

2 MSCs Mediated Immuno-Modulation

Despite of great hope in the treatment of many diseases using stem cells, the risk of forming teratomas and the lack of sufficient clinical research constitute a great concern (Deakin et al. 2009). Stem cells are highly regenerative cells with the ability to differentiate into multiple cell types (Frese et al. 2016). MSCs can be isolated from many types of tissues and can be transformed into many cell types in the body (Kalinina et al. 2011). This feature of stem cells has made them an attractive area of research in tissue engineering. In order for a cell to be categorized as a stem cell it must express specific surface markers, adhere to the plastic, and display a fibroblastic morphology (Ullah et al. 2015). Studies show that the administrated labeled stem cells accumulate mostly around the blood vessels in *in vivo* (Yu et al. 2016). MSCs are also involved in many processes other than differentiation, such as cellular aging, wound healing, damaged tissue repair, and regulation of inflammation (Dimarino et al. 2013; Maxson et al. 2012). Although their differentiation capacity is less than that of Embryonic Stem (ES) cells and induced Pluripotent Stem (iPS) cells, MSCs have become a matter of choice as a therapeutic tool, due to low risk of teratoma formation and lack of ethical concerns in their clinical use (Zomer et al. 2015). Moreover, MSCs act as immunomodulators underscoring their versatility and usefulness (Rivera-Cruz et al. 2017). This chapter aims to explain how MSCs function in immunomodulation and which factors influence their immune-regulatory function.

2.1 Down-Regulation of Immunity

Several of the recent studies have shown that MSCs can silence an immune response by suppressing activated immune system cells (Shi et al. 2012; Ankrum et al. 2014). The ability of MSCs to turn into different cell types as well as to suppress the immune response allow them to be

used in the treatment of many diseases, especially in transplantation applications (Herberts et al. 2011). However, this suppression varies depending on the concentration and the type of molecules secreted by immune cells. Animal studies have shown that MSCs express high levels of inducible Nitric Oxide Synthase (iNOS) that promotes production of Nitric Oxide (NO), key mediator of immune suppression (Adamiak et al. 2017). Furthermore, it has been observed that MSCs with iNOS deficiency fail to suppress the immunological response (Li et al. 2012; Klinker and Wei 2015). These findings indicate that NO plays a major role in immunosuppression in murine models (Oh et al. 2014). It should also be noted that while a peak in NO amount is an important factor in suppressing murine immunoreactivity, a decrease in tryptophan levels due to Indoleamine 2,3-Dioxygenase (IDO) activity is responsible for the same role in human cells (Dai and Gottstein 1999). The IDO enzyme breaks down tryptophan, an important amino acid for the development of the immune response, and hence, the consumption of tryptophan by IDO prevents the development of an immune response (Moffett and Namboodiri 2003; Soliman et al. 2010).

2.2 Up-Regulation of Immunity

One of the oldest studies on MSCs aimed to investigate its application in the treatment of the GvHD (Miyamura 2016; Rizk et al. 2016). Contrary to the predicted positive outcome from such studies, application of MSCs failed to prevent GvHD in most of the patients. It was proposed that the major reason underlying this failure was due to activation rather than the intended suppression of immunogenic properties of administered MSCs (Ryan et al. 2005). In other words, it was discovered that under certain circumstances stem cells do not suppress, but -rather- enhance the immune response depending on the type as well as the amount of molecules secreted by the immune cells (Pearl et al. 2012). For example, treatment of MSCs with high concentrations of Concanavalin A (ConA) or pro-inflammatory cytokines elicits an immunosuppressant effect (Chhabra et al. 2012). However, exposure of the stem cells to low concentrations of ConA and IL-10 results in loss of their immunosuppressive capacity (Ma et al. 2014; Tejedo et al. 2010). Underlying mechanism for the observed loss of immunosuppressive properties of MSCs exposed to low concentrations of Con A and IL-10 is -most likely- due to the failure of up-regulating iNOS levels in response to the cytokines which is a requirement to silence immune response (Xiao et al. 2015; Fracchiolla et al. 2017). In addition, other studies have shown that a strong inflammation is needed to occur beforehand so that MSCs can execute suppression of immunity (Gao et al. 2016). In another report, transplantation of MSCs one day prior to their administration in GvHD patients failed to generate the desired immunosuppression (Nevruz et al. 2013; Wang et al. 2016). In other words, the stronger the immunological response is, the higher the magnitude of the immunosuppression property becomes (Castillo et al. 2007; La Rosa and Diamond 2012). At the same time, the amount of inflammation hints us the level of inflammation allowing us to predict the extent of the immunological response (Zhang and An 2007). It was demonstrated that MSCs treated with Interpheron γ (IFN-γ) resulted in an activation of antigen-specific cytotoxic Cluster of Differentiation 8þ (CD8þ) T cells (Obar and Lefrancois 2010). This finding is encouraging in regards to the use of MSCs in applications where an increase rather than suppression of immune response offers a therapeutic advantage where a strong immune response can be engineered to selectively attack the tumor cells as the first line or adjuvant therapy of various cancers. Therefore, use of MSCs as enhancers of the immune response is worth pursuing and demands further studies aiming to understand the molecular mechanisms underlying this type of response (Qi et al. 2018). Studies show that stem cells given to the patient prior to tissue transplantation either induce such a strong nausea that transplantation can fail or promote more dibilitating clinical conditions (Sullivan et al. 2016). To solve this problem, the molecular mechanism of the interaction between stem cells

and immune cells needs to be elucidated in *in vitro, in vivo* and *ex vivo* studies.

This dual behavior of MSCs regarding the amount of inflammation makes it possible to use them in clinical trials for immunological diseases. Precise understanding of the molecular mechanism for this dual behavior must be obtained in future studies. T cells are the pivotal players in mediating immunomodulatory impact of MSCs.

2.3 T Cell Dependent Mechanism

T cells and their subtypes are extensively studied in terms of suppression of the immunological response by MSCs (Kyurkchiev et al. 2014). The most important feature that makes T cells more important than other immune cells is that T cells are directly or indirectly involved in the proliferation and function of other cell types from immunogenic origin (Gu et al. 2010; Zhao et al. 2009). MSCs depend on certain soluble factors, cell-cell contact mediators, and intercellular crosstalk between MSCs and T-cells in order to suppress the immune response. It has been shown that IDO synthesis becomes upregulated in IFN-γ-treated stem cells, and as a result, the proliferation of T cells is suppressed via reduction of the tryptophan levels in the inflammation area (Mbongue et al. 2015; Croitoru-Lamoury et al. 2011). Another study showed that induction of NO by MSCs blocked the proliferation of T cells via inhibiting the phosphorylation of STAT5 one of the key regulators of immune response (Zhang et al. 2017). Moreover, the same study showed that mouse cells that are unable to synthesize NO failed to suppress the immunological response (Yoo et al. 2017; Zhang et al. 2017). For example, in MSCs that are pre-treated with IFN-γ, and co-cultured with $CD4^+$ Peripheral Blood Mononuclear Cells (PBMCs), cytokines IL-3, IL-10 and IL-13 become secreted resulting in suppression of inflammation which is a case also seen in the response of Th2 cells (Croitoru-Lamoury et al. 2011). Furthermore, there was strong down-regulation of pro-inflammatory cytokine secretion, including IFN-γ, IL-1a and b, TNF-α, and TNF-β in Th1 cells (Jaffer et al. 2010). Cell-to-cell interaction and crosstalk between T cells and MSCs are required for the suppression of the T cells to by MSCs (Wang et al. 2012; Haddad and Saldanha-Araujo 2014).

Significant attempt has been made to address the question whether the immunosuppressant effect of MSCs is directly executed by these cells, -indirectly- via their impact on T cell differentiation which is typically suppressed or both. Alternatively, the crosstalk between MSCs and T cells could promote Treg-like-behavior of the T cells. In another study where MSCs co-cultured with Th17 cells from patients with inflammatory disease, it was observed that MSCs inhibited the secretion of pro-inflammatory cytokines, IL-17, IL-22, IFN-γ, TNF-α, while upregulating IL-10 (Lim et al. 2016; Kovach et al. 2015). MSCs are unique in the sense that the spectrum of immune cell types they can modulate ranges from cytotoxic T cells to pro-inflammatory Th17 and Th1 cells and to Th2 and Treg cells making them an indispensable tool for tissue or organ transplantation studies (Ma and Chan 2016). Since the majority of studies focusing on immunomodulation of T cells were in the context of transplant rejection and systemic disease, conclusions from these studies should be considered in future of tissue engineering.

3 Which Comes First Injury or Inflammation?

Among the important developments in regenerative technologies over the years, stem cell therapy has emerged both as a promising therapy in treatment of wound healing, acting as an important stimulant and regulatory factor in tissue regeneration (Wu et al. 2007). One of the most important reasons why MSCs are used successfully in tissue repair and healing is due to the fact that they can manage the immune cells, stromal cells, endothelial cells, and precursor cells in the area of damaged tissue via secreted trophic factors (Gao et al. 2016). An important factor in MSC transplantation is that cell characteristics and therapeutic potential are highly influenced by aging and pathological conditions (Bruna et al. 2016; Tokalov

et al. 2007; Kim et al. 2015). It has been reported that the aging status can adversely impact MSC division, differentiation, secretion of paracrine signals and trophic factors, and consequently, their ability to support tissue repair (Li et al. 2016). In addition to aging, disease status in patients with diabetes, obesity, and cardiovascular disorders will also significantly affect the outcome of the MSCs transplantation (Leon and Maddox 2015).

In MSCs transplants, it is also important to keep the cell deaths after transplantation at a minimal level, and thus, preserve the activation of cells in the tissue, in order to increase the success of transplantation (Baldari et al. 2017). As a result, some of the factors that will influence survival of implanted stem cells include: the interaction of cells with the Extra Cellular Matrix (ECM) in terms of adhesion, the mechanical stress and frictional forces developed during transfer, and finally, the absence of any immunologic response at site of the tissue transfer (Schwartz 2010). Depending on the reasons mentioned above, cells that cannot successfully bind to the ECM enter an apoptotic process called anoikis (Su et al. 2015). In general, we see that there is a significant relationship between therapeutically ineffective transplantation and number of MSCs transferred that is too low to sustain the survival and viability of stem transplanted cells (Parekkadan and Milwid 2010). Although factors affecting the successful transfer of MSCs that are delivered exogenously are generally well-studied, further work in this area at the molecular level is under way (Kollar et al. 2009; Leibacher and Henschler 2016).

Although the cellular processes regulating migration and tissue localization of transferred MSCs are not fully known at the molecular level, it is likely that these steps are orchestrated via intercellular communication and organization among cells that is operated by the chemokines and their receptors, similar to the situation seen in immune cells (Hocking 2015; Nitzsche et al. 2017). In recent years, intracellular and membrane-bound chemokine analysis revealed their substantial role in regulating the migration of MSCs in terms of the presence or absence of specific chemokines in a certain pattern (Baek et al. 2011). However, it is of utmost importance that these analyses are performed under such experimental conditions that are highly mimetic of the physiological environment of the MSCs, because although MSCs isolated from the tissue are initially positive for important chemokine factors, these factors may be lost in the advancing passages (Honczarenko et al. 2006). For example, alterations in CCR1, CCR7, CCR9, CXCR4, will reveal severe deficiencies in the chemotactic responses of the cells. In addition, studies have shown that the chemokines that are deposited in the membranes of MSC populations from different tissues determine the ability of that population in migrating to and regulating the targeted tissues (Albersen et al. 2013). For example, CCR9 chemokine-positive MSCs normally provide and control immigration to the intestines, while those carrying CCR1 migrate to the areas of inflammation in the joints or brain tissue (Kholodenko et al. 2013).

Another possible parameter that affects the migration and homing abilities of MSCs is growth factors secreted by these cells (Zachar et al. 2016). Studies have shown that one of the important reasons underlying the failures in stem cell transplantation is that MSCs lose their ability to migrate to different tissues. In recent years, *in vitro* studies have shown that many growth factors, i.e. TNF-α, IGF-I, PDGF, SDF-I and IFN-γ induce migration of MSCs (Naaldijk et al. 2015). When the all the factors that affect MSC transplantation success are put in perspective, recent preclinical and clinical studies confirm how each parameter is contingent on another such as stem cell source, transfer method, pathological state of the patient, timing and culture conditions (Werner et al. 2014).

4 Safety Concerns and Challenges

Although the recent stem cell studies are promising, there is more to be done to reconcile the findings from these studies effectively as a meaningful contribution to the development of

bioengineering technologies that will allow routine use of these cells in the diagnosis and treatment of patients (Hirschi et al. 2014). MSCs are one of the most intensively used types of stem cells today due to a number of reasons (Ullah et al. 2015). First, a large part of the MSCs do not create a problem ethically and can be obtained from the waste biological material (Oliveira and Barreto-Filho 2015). Second, they do not form teratoma, and can be obtained in many different tissues (Damjanov and Andrews 2016). Furthermore, although there have been many studies on the immunosuppressive and enhancing properties MSCs, the molecular mechanism is not yet fully elucidated. Especially, -not only- the localization of MSCs to a target organ when the MSCs enter the body and their effects in the long-run, but also the potential effect on other organs and tissues be taken into account (Caplan and Hariri 2015). In addition, the relationship of stem cells to other diseases should be examined and the underlying mechanism should be elucidated.

5 Future Perspective

MSCs are the most promising type of stem cells that have been widely used in recent times due to many of their features such as differentiation potential to other cell types, capability of immunological modulation, enhancing tissue regeneration are just a few to mention. Due to these properties, many studies about MSCs have been carried out and a lot of information has been collected. More efforts are certainly in need to address the reasons for the number of failed clinical attempts. Nonetheless, the number of successful works are encouraging to implement new therapy methods that utilize MSCs. The effects of MSCs on the immune modulation are still being investigated. In the near future, these promising cells are likely to be used effectively in the treatment of diseases. And this use will be a hope for many patients waiting for treatment.

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Gene Therapy Strategies in Bone Tissue Engineering and Current Clinical Applications

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Abstract

Gene therapy provides a promising approach for regeneration and repair of injured bone. Application of gene therapy has displayed increased efficiency in various animal models and preclinical trials in comparison with traditional bone grafting methods. The objective of this review is to highlight fundamental principles of gene therapy strategies in bone tissue engineering and solutions of their current limitations for the healing of bone injury. Vector types are debated for the repair of defected site due to demonstration of constraints and applications of the protocols. In recent years, the combination of gene therapy strategies and bone tissue engineering has highly gained attention. We discussed viral and non-viral mediated delivery of therapeutic protein by using scaffolds for bone tissue engineering. Although pre-clinical studies have showed that gene therapy has very promising results to heal injured bone, there are several limitations regarding with the usage of gene delivery methods into clinical applications. Choice of suitable vector, selection of transgene and gene delivery protocols are the most outstanding questions. This article also addresses current state of gene delivery strategies in bone tissue engineering for their potential applications in clinical considerations.

Keywords

Bone tissue engineering · Gene activated matrices · Gene therapy · Viral vectors

Abbreviations

AAV	Adeno-associated virus
ASC	Adipose stem cell
BMPs	Bone morphogenetic proteins;
cDNA	Complementary deoxyribonucleic acid
COL1A1	Type I collagen
dsDNA	Double-stranded DNA
FDA	the US Food and Drug Administration
GAMs	Gene-activated matrices
GMP	Good manufacturing practice
HAP	hydroxyapatite
HIV	Human immunodeficiency virus
IGF	Insulin-like growth factor
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6

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LIMP-1	LIM mineralization protein-1
MoMLV	Moloney murine leukemia virus
MSCs	Mesenchymal stem cells
OPG	Osteoprotegerin
PDGF	Platelet-derived growth factor
PEG	Poly (ethylene glycol)
PLGA	Poly (lactide-co-glycolic acid)
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kappa-B ligand
Runx2	Runt related transcription factor 2
SCID	X-linked severe combined immunodeficiency disease
ssDNA	Single stranded-DNA
TGF- β	Transforming growth factor-β
TNFα	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

1 Introduction

Although bone has a unique repair and regenerative capacity of its own without formation of scar tissue after injury, there are various clinical conditions in which bone healing is impaired. Infection, tumor resections, fractures followed by segmental loss, trauma and developmental deformities can cause large bone defects that are lack of intrinsic bone healing potential (Verrier et al. 2016). Traditional treatment of large bone defects is the use of graft materials including autologous bone and allogenic bone grafts to ensure osteoinductive and osteoconductive stimulus to accelerate bone regeneration. Even though autologous bone graft shows a high success rate, it has restrictions including insufficient amount of existing bone for autografting, morbidity at the harvest site and post-operative complications (Atasoy and Kose 2016; Kim et al. 2009). The demand of allogenic bone grafts has increased as an alternative to the autografts. Although unlimited quantity of allogenic bone grafts is available, possible risk of the transmission of disease, safety issues, processing and preservation of allografts are primary concerns (Delloye et al. 2007). Moreover, a variety of bone graft substitutes such as ceramics, metals, bioactive glasses and polymers have been investigated as extenders, fillers and enhancers for bone grafting techniques, but usually show inadequate reabsorbabilities (Vaccaro 2002). In addition to these conventional approaches to enhance bone regeneration with remarkable clinical effects in recent years, bone repair has also employed the application of biological therapies. Various growth factors have been used to augment bone formation.

Several members of bone morphogenetic proteins (BMPs) such as BMP-2, −4, −5, −6, −7, −8, −9 and − 10 have the osteogenic potencies and osteoinductive properties. In several animal studies and preclinical trials showed that they are able to stimulate bone formation. Recombinant human BMP-2 (InductOs®, UK and Infuse™, USA) and BMP-7 (OP-1®, USA and Osigraft®, UK) have been used in clinical practice and approved by the US Food and Drug Administration (FDA) (Lissenberg-Thunnissen et al. 2011). Despite significant influences of these proteins for bone healing, they have side effects including ectopic bone formation, stimulation of cancer cells and osteolysis in clinical performance (Oryan et al. 2014). *In vitro* studies have displayed increased volumes of numerous cytokines including interleukins (IL-1α, IL-1β, IL-6) and tumor necrosis factor-α (TNFα) because of high levels of BMP-2. Besides, BMP-2 indirectly stimulates NF-κB ligand (RANKL)-mediated osteoclastogenic activity and enhances inappropriate adipogenic differentiation (James et al. 2016). Delivery of BMPs, determination of their suitable physiological concentrations, and cost-effectiveness of BMPs have still been under debated (Lissenberg-Thunnissen et al. 2011). Additionally, the relative ratio of BMPs to their inhibitors such as noggin, chordin may be a major determinant of bone healing (Kwong and Harris 2008).

Gene delivery methods offer solutions in regarding to the adverse effects of growth factor usage and controversial clinical efficacy. Gene therapy involves transfer of the target genes into appropriate cells. Delivery of nucleic acids via carriers stimulates osteogenic growth factor synthesis because of endogenous protein expressions

(Kim et al. 2016). Various animal studies showed that transfer of cDNA encoding for osteoinductive proteins inside the suitable cells induces local bone formation (Lee et al. 2001; Peterson et al. 2005). Furthermore, preclinical data have been noted for the successful bone repair by *ex vivo* and *in vivo* strategies (Baltzer and Lieberman 2004; Shen et al. 2004; Gerstenfeld et al. 2006; Carofino and Lieberman 2008). However, targeting the right gene into the appropriate cells for sufficient expression of therapeutic proteins and choosing of the suitable carrier systems for different clinical applications to accelerate bone regeneration are still the major concerns to minimize adverse effects of gene therapy.

Tissue engineering is a multidisciplinary branch based on the basic concepts of life sciences and engineering toward the advancement of biological factors for the improvement, regeneration and maintenance of tissue function (Goessler et al. 2006). Scaffolds, cells and bio-signal molecules are used to increase cells proliferation and differentiation for the stimulation of tissue regeneration (Kushibiki and Tabata 2005). In spite of safety concerns, gene therapy strategies have displayed significant promise for the repair of a wide range of bone diseases. Gene therapy strategies in bone tissue engineering for the treatment of bone involve the delivery of gene of interest via viral and non-viral vectors to the injured site. Additionally, incorporation of osteogenic genes with the biocompatible scaffold greatly accelerates the bone healing (D'Mello et al. 2017).

This review discusses recent developments of gene delivery procedures in bone tissue engineering. Main benefits and limitations of various gene delivery methods are described for bone regeneration. Additionally, the article highlights how gene therapy protocols are being used in clinic.

2 Basic Principles of Gene Delivery

Vectors are the carriers which deliver DNA inside the target cells and subsequently into the nucleus are being used for the gene delivery. They can be categorized as non-viral and viral vector delivery by *in vivo* or *ex vivo* manner.

2.1 Viral Vectors

Viruses have widely been used to transfect cells since they are able to naturally translocate their own DNA easily into host cells. Sequences of the viral genome associated with virulence and pathogenicity are generally eliminated and modified with target genes and their regulatory sequences to manufacture a recombinant viral vector. Viral vector can either be integrated into host genome followed by a long-term expression or it can stay apart from the host genome as a circular plasmid called episome, resulting in comparatively short-term expression. Most commonly used virus types for gene delivery are vaccine virus, herpes simplex virus, measles virus, pox virus, lentiviruses, retroviruses, adenoviruses and adeno-associated viruses (AAV) (Carofino and Lieberman 2008; Evans and Huard 2016; Balmayor and van Griensven 2015).

Selection of suitable gene delivery vector needs attention of variety of factors including; (i) duration of transgene expression that is necessary for efficacy, (ii) target cells which easily facilitate transduction, (iii) method of gene delivery (e.g. *ex vivo, in vivo),* (iv) immunogenicity that can be acceptable for host, (v) regulation of desired gene activity for a period of time (Phillips et al. 2007). Lentivirus, retrovirus, adenovirus and AAV are preferred to get used for gene transfer to bone. Each of these vectors has its distinctive features that depend on the targeting cell type and desired expression time. Table 1 lists properties of each vector type used in gene transfer for tissue regeneration. Nonetheless, there are safety issues with the usage of viral vectors such as retrovirus or lentivirus in orthopedic applications in terms of their insertional mutagenesis, cancer risks and life-threatening diseases. Integration into the host cell genome is useful for the treatment of genetic diseases such as X-linked severe combined immunodeficiency disease (SCID). However, osseous lesions do

Table 1 Viral vector types used in gene therapy

Viral vector types	Insert size	Infection	Gene delivery strategies	Immunogenic reactions
Lentivirus	RNA genome, ~ 8–10 Kb	Dividing and non-dividing cells	Integrated into genome	Low
Retrovirus	RNA genome, ~ 8–10 Kb	Dividing cells	Integrated into genome	Low
Adenovirus	dsDNA genome, ~35 Kb	Non-dividing cells	Episomal	High
Adeno- associated virus (AAV)	ssDNA genome, 4,8 Kb	Non-dividing cells	Episomal (~90%)	Moderate
			Integrated into genome (~10%)	

not require long-term expression. They presumably need a short-term expression of an appropriate osteogenic growth factors (Evans 2011; Hacein-Bey-Abina et al. 2010; Kurian et al. 2000). Besides, viruses can be immunogenic and stimulate a certain inflammatory response which may affect duration of gene expression (Raper et al. 2003; Rie Molinier-Frenkel et al. 2000).

Retroviral vector is a RNA-based vector whose package capacity is 8–10 Kb. Retroviruses such as Moloney murine leukemia virus (MoMLV) have frequently been used in clinical trials (Barquinero et al. 2004). They can randomly integrate into the genetic material of the host cell, which may cause disruption of endogenous cell activity such as cell cycle progression resulting in oncogenesis by insertional mutagenesis. Although they have been engineered to change their viral tropism such as reduction of competent retroviral replication, the potential risks of retroviral integration give rise to significant safety concerns. The risk of insertional mutagenesis can be tolerable to cure life-threatening disorders that require long-term expression of therapeutic proteins, but may not be tolerable for bone regeneration that is required to have better quality of life (Phillips et al. 2007; Yi et al. 2011).

Lentiviral vectors such as human immunodeficiency virus (HIV) are a specialized group of retroviruses that are capable of infecting both non-dividing and dividing cells in contrast to traditional retroviruses. The viral genome also integrates into the host genome and exhibited long-term expression. Despite of their low immunogenicity, origin of lentivirus is the main restriction for their usage in clinical practice (Sinn et al. 2005). Additionally, lentiviral genome is much more complicated and difficult to modify than MoMLV due to complexity of its genetic material. Lentiviral-based gene transfer methods may be a suitable for bone healing which needs a long-term expression of desired proteins for the treatment of large critical-sized defects occurred due to tumor resection or severe trauma (Phillips et al. 2007).

The usage of AAV vectors has significantly increased in last two decades with success and safety in clinical trials (4.9% in 2012). AAV is a non-enveloped virus with a 4.8 Kb ssDNA genome. It does not cause any known disease and replicates without a helper virus. It infects non-dividing cells and stays as an episome inside the nuclei of infected cells for extended periods. Nevertheless, this vector has a small DNA packaging capacity. It is also expensive and difficult to manufacture (Evans 2014; Schwarz 2000).

Adenovirus is the most commonly used vector type among the others (23.3% of all trials) (Ginn et al. 2013). It includes dsDNA packaged inside a non-enveloped capsid and remains in the nucleus as an episomal element. It also transduces various non-dividing cells and has a larger DNA package capacity (~35 Kb). Adenoviral-based transgene delivery has displayed remarkable promise for the treatment of bone repair. The basic benefits of adenoviral vector are their abilities to

accomplish a high efficiency of transduction and high level of transgene expression. Its transgene expression is observed at a high level within 1–2 weeks followed by a decrease or complete loss after 3–6 weeks. Duration of transgene expression may be ideal for various orthopedic applications. (Cao et al. 2004; Evans 2014). However, the main limitation with the use of adenoviral vectors is their high immunogenicity resulting in host immune response, decreasing therapeutic protein expression and interfering with repeated dosage. Adenovirus serotype 5 is the most commonly used vector to infect the patient naturally. However, most patients possess their own circulating antibodies which may prevent the usage of even first dosage of the vector. Even though *ex vivo* gene delivery method of adenovirus decreases this problem, adenoviral vectors and their alternative serotypes cause immunogenic reactions (Ersching et al. 2010; Evans et al. 2009a, b; Evans 2013).

2.2 Non-viral Vectors

Since viral based vectors are usually complicated to modify, expensive and have some safety concerns, the use of non-viral vector is particularly more attractive in comparison with viral vectors. Non-viral gene delivery is usually performed using circular, plasmid dsDNA to display constant chemical stability and can easily be generated in bacteria. However, clinical applications of non-viral gene delivery are limited due to their low level of *in vivo* transfection capacity and duration of transgene expression. The choice of a suitable non-viral vector depends on targeting cell type, desired period of gene expression and safety profile for each particular application (Carofino and Lieberman 2008). The efficacy of gene delivery is enhanced by either liposome, polymeric micro/n capsules or electroporation and sonication as physical stimuli. Besides, a gene-gun technique including particle bombardment with DNA-coated gold onto cell sheets is used as a mechanical procedure for gene transfer (Cody Bünger 2005).

3 Gene Delivery Strategies

There are two main gene delivery methods: *"in vivo"* and *"ex vivo"* (Fig. 1). *In vivo* gene delivery approach includes delivery of either viral or non-viral vector via generally percutaneous injection or local implantation directly to the site of the osseous defect. *Ex vivo* delivery needs isolation of target cells, *in vitro* culture and genetic manipulation of the cells followed by re-implantation into the osseous defect (Bleiziffer et al. 2007).

3.1 *In Vivo* Gene Delivery

Main objective of the *in vivo* systemic delivery is to propagate and express therapeutic protein in the bone and such gene delivery application is necessary for the treatment of osteoporosis or osteogenesis imperfecta that influences entire bone tissue. On the other hand, local delivery of gene presents and expresses the target genes in certain sites such as fracture and trauma which require local implantation (Evans 2013).

In vivo gene delivery reduces the risk of contamination because of one-step procedure required for the individual. Even though this method is less expensive, faster, simpler and less invasive procedure than *"ex vivo"* treatment method, they have significant limitations in terms of safety concerns (Phillips et al. 2007). Furthermore, it is not easy to transfect the target cells *in vivo* in which the desired protein is expressed at low level. Moreover, expression of the protein in non-target sites and propagation of the vectors are the other restrictions of the *in vivo* gene transfer strategy (Heyde et al. 2007).

Physical placement procedures contain direct injection of the target gene into the injured site. Gene can be delivered by a virus or forced into nuclei of cells by electroporation, sonoporation and microinjection for the gene to pass through the cells *in situ* (Pelled et al. 2010). In this context, we will review direct injection of viral-based gene delivery *in vivo*. Gene activated matrices will be reviewed under the title of gene therapy

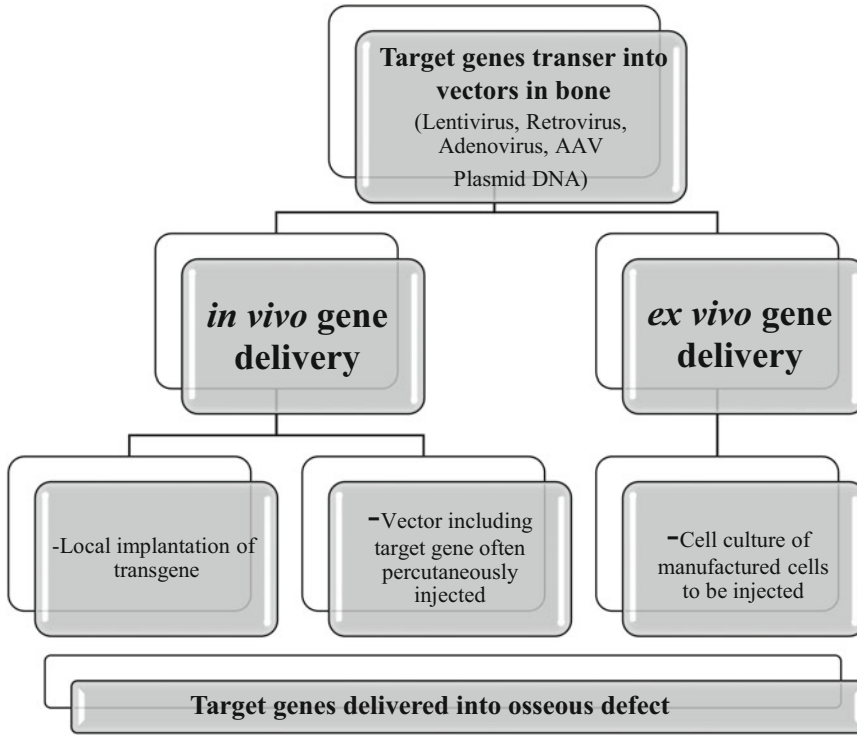

Fig. 1 Schematic representation of gene delivery strategies in bone

progress in bone tissue engineering by *in vivo*-based strategies.

Adenoviruses have been commonly used for the direct injection of viral vector and promising results have been noted in animal studies for bone regeneration. Local delivery of adenovirus carrying BMP-2 cDNA enhanced extracellular matrix mineralization in rabbits and rats having critical-sized femoral bone defects (Baltzer et al. 2000; Betz et al. 2007a, b). Similarly, percutaneous injection of adenovirus including either BMP-2 or BMP-6 cDNA as transgenes treated bone at both osteotomy and ostectomy sites in horse model by enhancing mineralized callus formation after 8 weeks of surgery (Ishihara et al. 2008). Even though results of adenoviruses encoding BMP-2 had a great promise to induce bone formation in small animal studies, the use of direct injection of adenoviral vector for stimulating bone regeneration in a large animal model failed in some studies. In one of the study, tibial osteotomy model was established in sheep (Egermann et al. 2006). After direct injection of adenoviral vectors carrying BMP-2 into defect area, callus formation was significantly reduced after 8 weeks. Moreover, high amount of antibodies for both the adenovirus and BMP-2 was observed due to immune reactions in sheep. These findings highlighted the importance of species distinctions and the innate immune system (Evans 2015).

3.2 *Ex Vivo* Gene Delivery

Ex vivo gene delivery contains several steps. Cells obtained from patient are cultivated *in vitro* for a period of time, transduced to synthesize the gene of interest and later re-implanted at the injured site in which they produce expression of therapeutic proteins. However, *ex vivo* gene delivery requires two-step invasive method including harvest of the cells in one surgical procedure and re-implantation of genetically manipulated cells in another operation (Fig. 2). This clinical model greatly raises cost and resource utilization

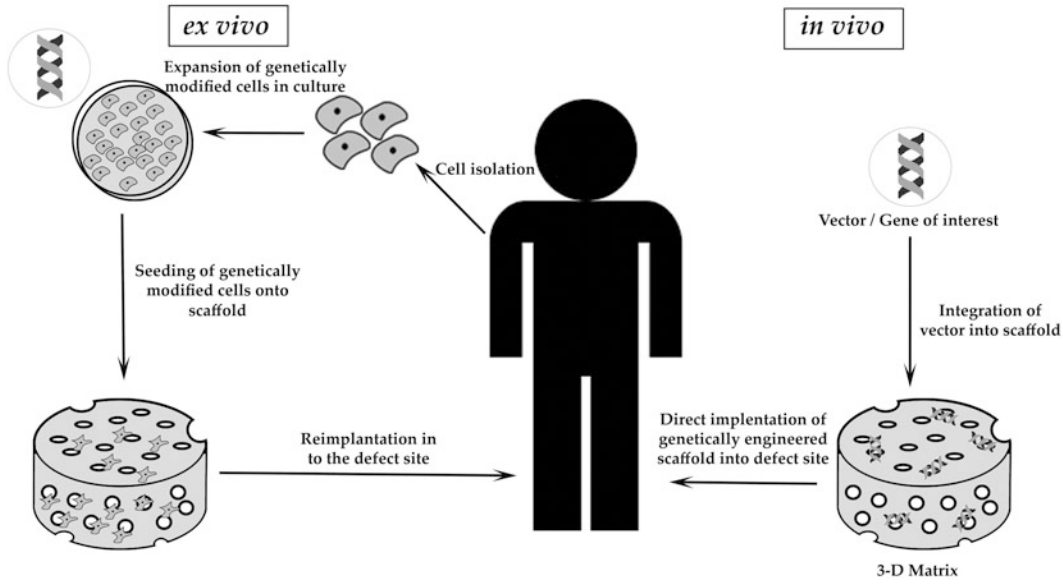

Fig. 2 Gene delivery methods for bone tissue engineering. Gene of interest that encodes therapeutic protein is added into an appropriate vector. For biomaterial-mediated *in vivo* gene delivery, the vector is integrated into a scaffold and implanted into the site of defects. Then autologous cells infiltrated the scaffold. For *ex vivo* gene delivery, cells are removed from the host and genetically modified. The cells are then seeded into suitable scaffold and reimplanted into the osseous defect

(Carofino and Lieberman 2008). Even though this delivery strategy is laborious, it has the advantages in terms of the genetic manipulation of desired cells and measurement of the transfection efficiency *in vitro* (Bleiziffer et al. 2007). Various studies have displayed the results of *ex vivo* gene therapy in which increased bone formation and repair of critical-sized defects in different animal models were observed (Table 2). Lieberman et al. (1998, 1999) used *ex vivo* gene delivery methods with the use of adenovirus expressing BMP-2 cDNA to transduce bone-marrow stromal cells. They observed heterotrophic bone formation in SCID mice and repair of large segmental femoral defects in rat models within 12 weeks. Furthermore, several investigators (Chang et al. 2009a, b; Dai et al. 2005; Ishihara et al. 2009; Xu et al. 2005) performed adenovirus-mediated BMP-2 expression in large animal models such as goats, horses and pigs by using *ex vivo* gene delivery method. Different cell sources such as periosteum, muscle, fat and fibroblasts have been used as cellular delivery vehicles for *ex vivo* gene delivery (Hsu et al. 2008; Ishihara et al. 2009; Lee et al. 2001; Peterson et al. 2005; Rao et al. 2013; Breitbart et al. 1999; Shen et al. 2004; Shin et al. 2010). However, Gugala et al. (2003) demonstrated no significant effect of the different human cell types in terms of stimulation of heterotrophic bone formation by *ex vivo* gene delivery of adenoviral vectors expressing BMP-2 cDNA.

Lentiviruses are also used for *ex vivo* gene delivery in the healing of osseous defect. Virk and co-workers (Virk et al. 2008) showed the efficacy of lentiviral vector-mediated BMP-2 cDNA expression as compared to adenoviral vector in femoral defects. Biomechanical and micro CT analysis demonstrated that lentiviral-mediated gene transfer of BMP-2 showed superior quality of bone healing with prolonged BMP-2 expression in comparison with the adenoviral vector. These results suggested that duration of the expression of therapeutic protein is an important factor to repair critical size femoral defects in rats for local gene therapy.

Non-viral based *ex vivo* gene delivery was confirmed by Gazit and colleagues. They

Table 2 Summary of selected examples of rational *ex vivo* gene therapy in animal models for bone healing

Transgene	Vector	Experimental model	Target cells	References
BMP-2	Adenovirus	Mice, goats, horses, pigs	Bone marrow stromal cells	Lieberman et al. (1998, 1999), Chang et al. (2009a, b), Dai et al. (2005), Ishihara et al. (2008) and Xu et al. (2005)
BMP-2	Adenovirus	Rat	Adipose derived MSCs	Hsu et al. (2008)
BMP-2	Plasmid DNA	Rat	Gingival fibroblast cells	Shin et al. (2010)
BMP-2	Adenovirus	Equine	Dermal fibroblast cells	Ishihara et al. (2009)
BMP-2	Adenovirus	Mice	Muscle derived cells	Bosch et al. (2000) and Lee et al. (2001)
BMP-4	Retovirus	Rat	Muscle derived cells	Shen et al. (2004)
BMP-2	Adenovirus	Rat	Adipose derived MSCs	Peterson et al. (2005) and Dragoo et al. (2005)
BMP-7	Retrovirus	Rabbit	Periosteum	Breitbart et al. (1999)
BMP-2	Lentivirus	Rat	Bone marrow stromal cells	Virk et al. (2008)
BMP-2	Plasmid DNA	Mouse	Pluripotent mesenchymal stem cells	Gazit et al. (1999)
LIMP-1	Adenovirus	Rabbit	Bone marrow derived buffy coat cells	Viggeswarapu et al. (2001)

developed genetically engineered pluripotent mesenchymal stem cells expressing BMP-2 and transfected with a non-viral vector and used it in murine segmental defect. New bone formation and induction of bone growth from the engrafted cells were observed after 8 weeks of post-transplantation according to the results of histomorphometry and X-ray analysis (Gazit et al. 1999).

Because of high cost of expansion and transduction of cells under good manufacturing practice (GMP) conditions and sequential stages of traditional *ex vivo* strategies, one-step procedure at the site of injury have been developed by Viggeswarapu et al. (2001). They used this approach in bone repair by using buffy coat cells and collagen-ceramic composite sponge as a carrier. The cells were transduced with adenovirus vector encoding LIM mineralization protein-1 (LIMP-1) and re-implanted to promote spinal fusion in rabbit models during one step operation.

Genetically modified muscle and fat tissues can be used to repair bone defects and known as "facilitated endogenous repair" since these tissues have osteoprogenitor cells and can act as intrinsic scaffolding features. Also, they can be harvested, transduced and re-implanted to the patient in the one-step operative procedure (Bosch et al. 2000; Dragoo et al. 2005; Evans 2013). This alternative method expedites the procedure. Evans and co-workers reported (2007, 2009a, b) fast healing of rat femoral segmental defects and osteochondral defects in rabbit models. Biopsied muscle and fat tissues were transduced with an adenovirus vector carrying cDNA encoding BMP-2. Genetically modified autologous tissues were then re-implanted into critical-sized defects of animal models in a single surgery. Results showed rapid healing of critical-sized defects and efficient endochondral ossification. The use of facilitated endogenous repair approach for *ex vivo* gene delivery can diminish safety

concerns regarding the immune response against adenoviral vector which is the main limitation of clinical use of gene therapy. Additionally, the complexity is decreased, because of one-step procedure (Evans 2015).

4 Gene Delivery Strategies for Bone Tissue Engineering

Bone tissue engineering via gene delivery strategies has been developed recent years with transferring a variety of nucleotides to overcome the disadvantages of direct *in vivo* delivery. Three elements are usually required for gene therapy-based bone tissue engineering including gene of interest for osteogenic growth factors, scaffolds and cells (Kim et al. 2016).

Generally, the gene of interest that is carried either by non-viral or viral vector, is settled together with an appropriate scaffold at the site of the osseous defect to provide osteoconduction and osteoinduction (Fig. 2). Thus, DNA is protected from degradation and an immune response. Incorporation of DNA into a biomaterial provides sustained delivery and appropriate internalization that may increase transgene expression while diminishing the amount of vector used (Heyde et al. 2007).

Choosing and manufacturing an appropriate biocompatible 3-D scaffold material is an important parameter for bone tissue engineering applications to provide the structural and functional properties of injured bone. A number of natural and synthetic biomaterials have been identified for the potential application in bone regeneration. Collagen, chitosan, coral, poly (ethylene glycol) (PEG), poly (lactide-co-glycolic acid) (PLGA) etc. are some of these natural or synthetic polymers to support cell migration, adhesion, ingrowth and differentiation for bone healing.

Synthetic polymers have been used for delivery of non-viral vectors. Lee et al. (2011) observed significant extracellular mineralization and osteoblast formation by implantation of PLGA-mediated transfer of Runt-related transcription factor (Runx2) and Osterix genes in adipose stem cells (ASC) resulting in enhancement of both in *vitro* and *in vivo* osteogenesis. ASC seeded PLGA scaffold increased bone formation in nude mice after 6 weeks.

Natural polymers such as collagen, coral and chitosan have been investigated for gene delivery of therapeutic proteins to facilitate osteoblast proliferation and differentiation. The 3D structures of scaffolds made from biodegradable polymers provided osteoinductive features with direct implantation *in vivo*, resulting in recruitment of osteoprogenitor cells to the injured site (Bleiziffer et al. 2007; Chakkalakal et al. 2001; Dang and Leong 2006; Zippel et al. 2010).

4.1 Biomaterial-Mediated Non-viral Gene Delivery

The delivery of desired gene within a matrix provides controlled environment where gene transfer takes place. Gene-activated matrices (GAMs) are based on the transfer of the plasmid DNA via polymeric scaffolds to the target cells. They have usually been used for non-viral gene transfer methods. They consist of plasmid DNA and a biodegradable biomaterial as a carrier. Generally, plasmid DNA is integrated into a collagen sponge and implanted into the site of damaged tissue. The concept depends on *in situ* transfections of autologous cells which infiltrate into the GAM following implantation. After that, transfected cells can excrete the target gene to begin local response (D'Mello et al. 2017). GAMs have several benefits including low immunogenicity and easy production at large scale. Also, distribution of vectors into other tissues can be avoided. Collagen, silk and chitosan are widely used for bone tissue engineering. Among natural polymers, the gene activated-collagen scaffold is the most commonly preferred biomaterial since it has a high potential of biodegradability and provides ingrowth and differentiation of osteoprogenitor cells (Yamamoto et al. 2014). Table 3 summarizes the selected examples of GAM-mediated gene therapy procedures for bone repair. Fang et al. (1996) confirmed biological response of new bone formation in

Table 3 Gene delivery with scaffolds for the regeneration of bone defect in bone tissue engineering applications

Scaffold	Vector type	Gene of interest	Animal model	Result	References
ACS/PLGA hybrids	Plasmid DNA	Runx2/ Osterix	Nude mice	Transfection of Runx2 and Osterix genes significantly increased synthesis of OCN, COL1A1, ALP and BSP resulted in enhancement of bone formation in 6 weeks after surgery.	Lee et al. (2011)
Collagen sponge	Plasmid DNA	BMP-4/ PTH 1–34	Large segmental defects of adult rat femur, dog bone defect.	Synergitic actions of BMP-4 and PTH 1–34 caused new bone formation in injured site.	Fang et al. (1996) and Bonadio et al. (1999)
Collagen sponge	Plasmid DNA	VEGF	Rabbit large segmental bone defects	VEGF-GAM led to significant enhancement of angiogenesis and osteogenesis followed by new bone formation after 12 weeks of implantation.	Geiger et al. (2005)
Collagen/ Nanohydroxyapatite	Plasmid DNA	BMP-2/ VEGF	Rat cranial defect	Dual delivery of BMP-2/VEGF stimulated vascularization and fracture healing *in vivo*.	Curtin et al. (2015)
Collagen sponge	Adenovirus	PDGF-B	Alveolar bone defects in rat	Safety profiles of GAM mediated PDGF-B was evaluated and results demonstrated appropriate safety profiles in terms of toxicity and systemic involvement.	Chang et al. (2009a, b)
Coral/chitosan	Plasmid DNA	PDGF-B	Osseous defect in mice	Results indicated that coral/ chitosan composites increased cell profileration and expressions of COL1A1 and PDGF-B proteins in periodental regeneration.	Zhang et al. (2007)
Chitosan/coral	Adenovirus	BMP-7/ PDGF-B	In dog mandible	Combinations of BMP-7/PDGF-B significantly induced in bone formation after 12 weeks of surgery.	Zhang et al. (2009)
Silk fibroin	Adenovirus	BMP-7	Calvarial defect in SCID mice	Silk fibroin carrying BMP-7 cDNA were able to stimulate promotion of new bone formation in skull defect of mice.	Zhang et al. (2011)
Cortical bone allograft	AAV	RANKL/ VEGF	Femoral defect in mouse	Combination of RANKL and VEGF were capable of providing allograft healing to form a vascularized new bone.	Ito et al. (2005)
Cotical bone allograft	AAV	caALK2 (BMP receptor)	In murine femoral allografts	Results demonstrated a significant increase in vascularization and osteogenesis because of AAV-cALK2 transduction of MSCs. Osteoclastic resorption of allograft were observed in fracture callus at day 14 and day 28 followed by endochondral bone formation.	Koefoed et al. (2005)

large segmental defects of rat femur by implantation of collagen based-GAMs encoding either BMP-4 or parathyroid hormone (PTH) 1–34. In the same way, similar results were confirmed by using a dog bone defect model (Bonadio et al. 1999). It was also shown that GAM including VEGF increased both vascularization and osteogenesis in large segmental bone defects in rabbit (Geiger et al. 2005). Furthermore, collagen-nanohydroxyapatite scaffold combined with plasmid DNA for both BMP-2 and VEGF was investigated after 4 weeks of post-implantation in a critical size cranial defect of rats (Curtin et al. 2015). In addition to that, the result of World's first clinical case of GAM consisting of collagen-hydroxyapatite (HAP) scaffold containing VEGF for the treatment of maxillofacial bone defects or sites of bone atrophy (clinicaltrials.gov: NCT02293031) was reported by Bozo et al. (2016). It was observed that collagen-HAP scaffold with the plasmid DNA encoding VEGF was found quite promising. On the other hand, due to its limited efficacy regarding a medical indication range, more human clinical trials are required to determine the best treatment choice for bone healing with regarding the use of GAMs.

4.2 Biomaterial-Mediated Viral Gene Delivery

GAMs have been improved by using different scaffolds and viral-based vector approach. It was reported that recombinant adenovirus encoding PDGF-B delivered in a GAM in periodontal osseous defect of rat shows promising safety profiles that may be used in human clinical trials for bone repair (Chang et al. 2009a, b). Coral and chitosan composite with a plasmid carrying PDGF-B cDNA was first investigated by Zhang et al. (2007). They observed that gene-activated coral/chitosan 3-D matrices were potential candidates to increase periodontal bone formation (Zhang et al. 2007). For further studies, the same group displayed an enhancement of alveolar tissue regeneration at the dental implant sites by preparation chitosan/collagen scaffolds with adenoviral vectors encoding both BMP-7 and PDGF-B in a dog model (Zhang et al. 2009). Another natural biocompatible polymer for bone regeneration, 3-D silk fibroin, was combined with adenoviruses encoding BMP-7 and then implanted in a critical size calvarial defect of SCID mice resulting in an enhancement of both *in vitro* and *in vivo* osteogenesis (Zhang et al. 2011).

Ito et al. (2005) reported a manufactured GAM procedure in which allografts were used as biomaterials and AAV as the viral vector. Allografts with the combination of recombinant AAV-RANKL and AAV- VEGF displayed significant angiogenesis and bone regeneration. The host cells were transduced by AAV surrounding the implant, resulting in bone formation around the graft in mouse femoral defect model. Based on the benefits of AAV vectors for orthopedic applications, constitutively active BMP receptor that is encoded by caALK2 cDNA was used as a gene and delivered with allografts. Osteoclastic resorption of the allograft, vascularization and prevention of immune reaction were effectively observed after 6 weeks in murine femoral allografts (Koefoed et al. 2005). Despite of clinical safety concerns about the usage of AAV, it could aid as an "off-the-shelf" product without losing its infectivity due to its stability especially in freeze-dried form (Evans 2011).

5 Clinical Applications of Gene Therapy in Bone Defects

Gene therapy strategies have been used for the treatment of clinical indications such as osteoporosis (Baltzer et al. 2001; Bolon et al. 2001; Kostenuik et al. 2004), osteogenesis imperfecta (Niyibizi and Li 2009), long bone healing (Nauth et al. 2010; Pelled et al. 2010), tumors (Lin Tan et al. 2009; Witlox et al. 2007) and osteolysis (Doran et al. 2004; Ulrich-Vinther et al. 2002). Especially, long bone healing and osteogenesis imperfecta present the most excellent potential for clinical applications of gene therapy in bone.

Osteoporosis which includes loss of mineral from the bone occurs the uncoupling of osteoclastic and osteoblastic activities. Enhancement of

bone mass can be carried out by decreasing the activities of osteoclasts or by increasing the mineral deposition of bone by osteoblasts. So, gene therapy method considers the reality that this disease influences the whole skeleton system (Evans et al. 2009a, b). RANKL is the most significant factor that increases osteoclastogenesis and osteoprotegerin (OPG) inhibits the activity of RANKL (Goater et al. 2002). The systemic delivery of adenoviral vector encoding OPG represented efficacy in treating osteoporosis ovariectomy mouse model (Bolon et al. 2001). Similarly, intramedullary injection of IL-1Ra gene in a murine ovariectomy model resulted in inhibition of bone loss and enhancement of deposition of new bone after 5 weeks (Baltzer et al. 2001). Turgeman et al. (2001) displayed the influence of *ex vivo* gene therapy strategy in patients having osteoporosis. Bone marrow-derived human mesenchymal stem cells (MSCs) from osteoporotic patients were infected with adenoviral vector encoding BMP-2. These genetically engineered cells enhanced osteogenic activity after BMP-2 gene transduction *in vivo*. There are several gene products which enhance bone formation. Among them, PTH 1-34 (peritarpide; Forteo) delivered by direct injection is under investigation and a monoclonal antibody to the osteoclast differentiation factor RANKL is in phase III trials for the treatment of osteoporosis (Evans et al. 2009a, b).

Osteogenesis imperfecta is a Mendelian genetic disease and occurs from mutations in the genes encoding proα1 and proα2 chains of type I collagen (COL1A1) (Niyibizi et al. 2004). Several pre-clinical studies have been developed so far (Horwitz et al. 2002; Niyibizi and Li 2009; Sheridan 2011). The osteogenesis imperfecta transgenic murine model in which there was no proα2 COL1A1 synthesis was used to discover gene therapy method for this disease. *In vitro* studies displayed that transduction of bone marrow stromal cells with an adenoviral vector encoding a murine proα2 COL1A1 resulted in synthesis of correct proα2 COL1A1 and correct assembly of COL1A1 that comprised of proα1 and proα2 heterodimers with the ratio of 2:1 (Cody Bünger 2005; Niyibizi et al. 2001). Moreover, several studies (Chamberlain et al. 2004, 2008; Deyle et al. 2012) showed promising results for inactivation of mutant collagen genes by using AAV-mediated gene targeted osteogenesis imperfecta. Since this disease influences whole skeleton system, *ex vivo* gene therapy method was used.

Gene transfer strategies can be used for long bone healing including fractures, non-unions, spine fusion and segmental defects since the molecular mechanism of bone repair is very well known. Generally, recombinant human BMP-2 has been used to enhance local bone repair. Pre-clinical data of delivery of BMP-2 via gene therapy methods presented much greater and safer results than protein therapy in small animal models such as mouse, rabbits and rodents because of the adverse effects such as osteolysis, malignancy and infections (Carragee et al. 2011; James et al. 2016). However, the main limitation of growth factor-based gene therapy is the inadequate large animal studies including sheep or goat (Evans 2013). Wang et al. (2003) demonstrated the effects of adenoviral vectors encoding cDNA for BMP-2 with the use of infected bone marrow stromal cells on spinal fusion in rats. Results showed an adequate stimulation of an intertransverse spinal fusion in this model.

Progressing gene therapy strategies towards clinical applications has several constraints because of the cost-effective and prolonged process. In addition to demonstration of safety and efficacy of this therapy in animal studies, several pharmacological and toxicological tests have to be done under GMP conditions before starting phase studies in humans. Furthermore, pre-clinical testing requires new drug approval from FDA (Evans et al. 2009a, b). Since the major objective of gene therapy for bone is to manufacture a system which includes a one-step procedure to deliver the target gene, either off-the-shelf product or one-step *ex vivo* gene delivery methods would be simple and cheap (Carofino and Lieberman 2008).

Safety concerns are the primary consideration for non-lethal and non-genetic diseases. Consequently, adenovirus and AAV have been usually used as the viral-vectors for clinical trials in *ex vivo* gene delivery protocols. Besides, suitable

biomaterial should support the genetically modified cell types to enhance gene delivery method (Evans 2015). Still, the selection of gene of interest, gene delivery methods and determination of therapeutic dosage are outstanding questions for clinical applications. Gene therapy in bone tissue engineering is highly adaptable and effective gene delivery method in a suitable anatomical site. Design of GAM technology can induce bone formation and regeneration by releasing and sustaining adequate availability of gene of interest to the injured site (D'Mello et al. 2017).

6 Conclusion

In recent years, gene therapy has emerged as a novel strategy for the treatment of bone-related disorders. As discussed in this review, a number of gene therapy methods have accelerated mineralization of matrix resulting in new bone formation both in *in vitro* and *in vivo* studies.

In this review, we highlighted the importance of gene therapy approaches for healing of bone. The application of gene therapy via bone tissue engineering holds a great promise. Genetic modification of the target cells provides controlling of cell function at a molecular level for bone healing. Particularly, the use of adenoviral vectors encoding BMPs displays a powerful tool for the treatment of osseous defect. However, safety concerns still remain in terms of the usage of this vector type. To overcome this limitation, the biomaterial-mediated gene delivery protocols in the defect area have been used. Moreover, allograft-mediated transduction has been developed for transgene delivery via AAV displaying highly efficient results in animal studies.

In spite of fast evolution in gene therapy approaches for bone tissue engineering, there are still numerous challenges to overcome that prevent the progress towards clinical practice. The integration of these approaches with suitable cell types and an appropriate engineered scaffolds matrices that closely resemble native bone including osteoinductivity, osteoconductivity, mechanical stability, porosity and biocompatibility are required for the achievement of optimal bone regeneration. Novel scaffolds which are able to maintain structural integrity providing infiltration of osteoprogenitor cells should be developed. Furthermore, polymer-based vectors should be improved due to their lower transfection efficiencies in comparison with viral vectors.

Although the literature defines a number of successful examples with the usage of gene therapy strategies to cure injured bone, clinical application of gene therapy for the treatment of bone is still under investigation. Long bone healing and osteogenesis imperfecta have impressive pre-clinical results in animal models. On the other hand, regulatory obligations, limitations of financial supports, insufficient animal studies, safety issues and effectiveness of gene delivery protocols are still the main limitations of bringing gene therapy into clinical considerations.

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Promotion of Cell-Based Therapy: Special Focus on the Cooperation of Mesenchymal Stem Cell Therapy and Gene Therapy for Clinical Trial Studies

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Abstract

Regenerative medicine (RM) is a promising new field of medicine that has mobilized several new tools to repair or replace lost or damaged cells or tissues by stimulating natural regenerative mechanisms nearby cell and tissue-based therapy approaches. However, mesenchymal stem cell (MSC) based therapy has been shown to be safe and effective to a certain degree in multiple clinical trial studies (CTSs) of several diseases, in most MSC CTSs the efficacy of treatment has been reported low. Therefore, researchers have focused on efficacy enhancing of MSC to improve migratory and homing, survival, stemness, differentiation and other therapeutic applicable properties by using different approaches. Gene therapy is one of the experimental technique tools that uses genes to change cells for therapeutic and investigation purposes. In this study has been focused on genetically modified MSCs for use in RM with an emphasis on CTSs. We highlight the basic concept of genetic modifications and also discuss recent clinical studies aspects. Recently reviewed studies show that MSC therapy with assistant gene therapy can be used in cancer therapy, heart diseases, Fanconi anemia and several other diseases.

Keywords

Cell therapy · Clinical trial studies · Gene therapy · Mesenchymal stem cell

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Abbreviations

MSCs	Mesenchymal stem cells
GEMSC	Gene engineering of mesenchymal stem cells
ISCT	International Society for Cellular Therapy

CTSs Clinical trial studies
RM Regenerative Medicine

1 Introduction

Regenerative medicine is one of the modern fields of medicine that nowadays holds promise not only as recompense for donor insufficiency but also as a tool to improve the standard of treatment. Generally, RM history includes the history of several its subsets as showed in Fig. 1, but we do not want discuss about history of RM in this paper. Here we discuss the exciting recent investigations, including novel transgenic and genetic tools and the promotion of mesenchymal stem cells as a therapeutic tool that is bringing these fields closer together. Cell therapy is one branch of the RM containing bio-medicinal products, which provide different cells for transplantation or as carriers with therapeutic purposes. On the other hand, gene therapy defined as a field of biomedical research that is the goal of influencing the course of cell genetic at the DNA/RNA level.

RM has the diamond value in future medicine if achieved it. This branch of medicine due to advances in stem cell biology, Genetic, Cell/Drug delivery systems and other fields have unlocked new chance to improve existing regenerative medicine and develop novel ones. The most important goal of cell-tissue therapy is repair or replacement damaged cells, tissues and organs so that's their defective functions are restored. This target can be achieved by stimulating natural regenerative processes or by cell-tissue therapeutic techniques.

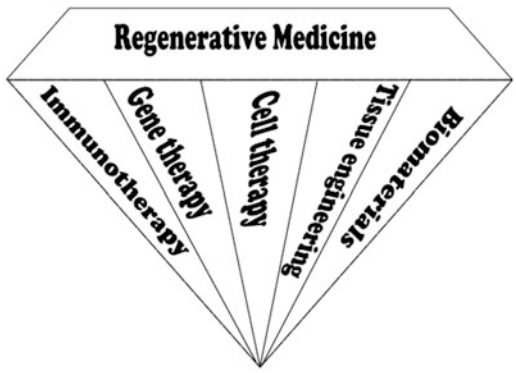

Fig. 1 Schematic illustration of RM as diamond and its branches

2 Mesenchymal Stem Cells Therapy

2.1 Mesenchymal Stem Cells and Its Clinical Using Properties

About 50 years ago, Friedenstein et al. described new fibroblasts from the monolayer cultures of guinea-pig bone marrow and spleen which now is named MSCs (Shaer et al. 2014). Mesenchymal stem cells are multipotent adult stem cells and one of the important sources of stem cells that are present in many tissues, including umbilical cord, bone marrow and adipose tissue. In 2006, International Society for Cellular Therapy (ISCT) listed the minimum criteria for defining multipotent MSCs which were included (Dominici et al. 2006): (1) Specific surface antigen (Ag) expression: positive expression for CD105, CD73, CD90, and negative for markers including CD45, CD34, CD14 or CD11b, CD79, α or CD19, and HLA-DR (2) In vitro differentiation into three cell types including osteoblasts, adipocytes, and chondrocytes, (3) Plastic-adherent cells isolated from different tissues in the standard culture conditions. However, there are some large differences in surface markers and identity of MSCs from various sources of them (Lv et al. 2014). In the following have been listed known biological properties of MSCs that these properties make them a good candidate for clinical applications:

- Differentiation and trans differentiation into other cell lineages: MSCs can directly differentiate into the endothelial cells, and fibroblast-like cells or transdifferentiate into non-mesoderm-like cells (Nie et al. 2011; Hu et al. 2014).

- Paracrine activity: they secrete crucial cytokines and growth factors for cell survival (Caplan and Dennis 2006) and proliferation of cells in injury sites (Nuschke 2014; Gnecchi et al. 2016).
- Immuno-modulatory response: they decrease pro-inflammatory cytokine and nitric oxide (NO) production and also the promotion of immunosuppressive macrophage (M2) formation (Zhang et al. 2010; Glenn and Whartenby 2014).
- Increment of cell homing and migration: MSCs migrate to the injury site in response to chemotactic signals from the damage sites, and as well as secretion of pro-migratory factors from MSCs causes the migration of other cells to the repair tissue (Li et al. 2015b; de Mayo et al. 2017).
- Promotion of neovascularization: MSCs induction neovascular network formation by increasing the endothelial cells and secretion of pro-angiogenic factors leads to a rapid healing process (Hong et al. 2013; Kosaraju et al. 2016).
- Supportive therapy: due to known and unknown details, have been reported positive therapeutic effect of MSCs therapy in clinical and preclinical studies, for instance supportive effect on hematopoiesis and enhance marrow recovery following chemotherapy or radiotherapy, and/or treatment of aGVHD (Le Blanc and Pittenger 2005; Squillaro et al. 2016).

A search of the official database of the US National Institutes of Health for registered CTSs containing the term "mesenchymal stem cells" returns nearly 810 results which approximately 47% of all MSC therapy clinical trials have been registered in the past 5 years and respectively China, United State and Spain have most clinical trial registrations in field of "clinical MSC therapy" research. (Search was done at date 3/2/2018).

2.2 Limitations and Barriers of Mesenchymal Stem Cell Therapy

Due to these promising properties, MSCs have been considered as a potential stem cell therapy for various human disorders including cancer, metabolic diseases, cardiovascular disease, wound healing and tissue engineering field. Therefore, MSCs due to themselves main potentials properties have become an interesting vehicle for cell therapy but yet there are several issues and barriers that restrict their application to clinical treatments. Frist, various studies have made evident that finding potency from MSCs in laboratory conditions are different by their potencies in the preclinical studies and are limited by natural cell niche or/and physiological conditions when administered systemically at therapeutic doses (da Silva Meirelles et al. 2008; Nombela-Arrieta et al. 2011; Muñoz Ruiz and Regueiro 2012). In other hands, investigation MSCs in the laboratory and in vitro conditions add complexity to MSCs clinical applications because the artificial conditions may introduce experimental conditions (Sandhaanam et al. 2013) and appending these outcomes to the physiological functions of the organisms is difficult. Secondly, with regard to the systemically injection of mesenchymal stem cells and tracking the injected cells in vivo revealed that only a small proportion of the stem cells was placed in the target sites. So accurate guide of MSCs to the target site is one of the purposes that need to the promotion of MSCs delivery by different methods (Golchin et al. 2017). Thirdly, contrary to good properties of MSCs, MSCs have the insufficient expression of some factors and low cell viability after transplantation, so we need some manipulations of MSCs to increase their efficiency and viability. Hence we felt, it is importantly, we discuss the underlying limitations of MSCs and review a genetic engineering guideline for clinical MSC therapy in hopes of improving their therapeutic efficacy.

3 Genetic Modification and Vectors

A transgene is defined as a "gene or genetic material which has been transferred into the genome of one organism from another origin". Transgenesis cause in changing the phenotype of cells or organisms (Manis 2007). In the beginning, the transgenesis was used to produce genetically modified bacteria and yeast. Subsequently, gene therapy with the aim of curing a defect cell and using it to the treatment of special diseases was raised. One of the most important achievements in gene modification of cells is producing of induced pluripotent stem cells (iPS) which has created a huge development and promising cure in cell therapy and regeneration medicine, so that several CTSs by using iPS has been started in recent years. However, in recent years, it was reported that iPS cells can be created by non-genetic methods (Zhou et al. 2009; Cyranoski 2013; Hou et al. 2013) but stem cells and especially MSCs are an appropriate cell candidate for gene therapy objects. While the prerequisite of MSC genetic engineering is efficient gene transfection, the genetic modification of MSC is achieved via various vectors. However, generally, Genetic engineering of MSC can be done via viral vectors although recently the use of non-viral vectors is taken into consideration (Fig. 2). An applied discussion of these methods has been provided in Table 1 (Merten and Al-Rubeai 2011; Park et al. 2015; Sage et al. 2016). Recently, the use of miRNAs has been introduced as genetic tool for manipulate of entire intracellular regulatory signaling which can communicate between several genes (Munoz et al. 2013).

3.1 MSCs and Gene Therapy

MSCs due to self-renew and powerful proliferation potency has become a good candidate cell source for genetic engineering. On the other hand, their ability to nest in metastatic tumors and in damaged tissues, have expanded their applications in the field of RM, drug delivery and gene therapy of cancers and different metabolic diseases (Muñoz Ruiz and Regueiro 2012). In the case of the cell therapy and RM, MSCs are engineered generally to increase their migration or homing, survival, stemness retention and production of specific growth/differentiation factors. In Table 2, we have summarized some main engineering factors from various studies for the promotion of MSCs.

3.2 New Strategies for Gene Engineering of MSCs in Cell Therapy

One of the main reasons of interesting in gene engineering of MSC (GEMSC) is the new investigation of these to new genetic materials such as "microRNAs (miRNA)", small interfering RNA (siRNA) and "exosomes". Currently, the genetic modification of MSCs alongside the traditional gene modification protocols continues by the use of new investigation of miRNA, alone, in combination with traditional gene modification or exosome structure based protocols. MiRNAs are small non-coding RNA molecules (contains approximately 22 nucleotides) found in higher eukaryotes and some viruses that regulate gene expression at the post-transcriptional level of different cells (Liu and Olson 2010; Mathiyalagan and Sahoo 2017). Recent findings manifest a novel mechanism that exosomes of stem cells, mediate promotion process of stem cell lifespan via transferring their unique repertoire of miRNA. For instance, studies have shown that miR-126 has the increasing effect of neo-angiogenesis after transplantation of BM-MSCs in the infarcted zones of myocardial infarction in the mouse model (Chen and Zhou 2011; HUANG et al. 2013). In 2011, Chen J and Zhou SH showed transplantation of MSCs overexpressing MiR-126 can improve angiogenesis and increase the release of angiogenic factors from MSCs in the infarcted zone of the myocardial infarction of mouse model (Chen and Zhou 2011). Afterward, Yan et al. attempted to confirm the osteogenesis enhancing the effect of antimiR-138 on the cell

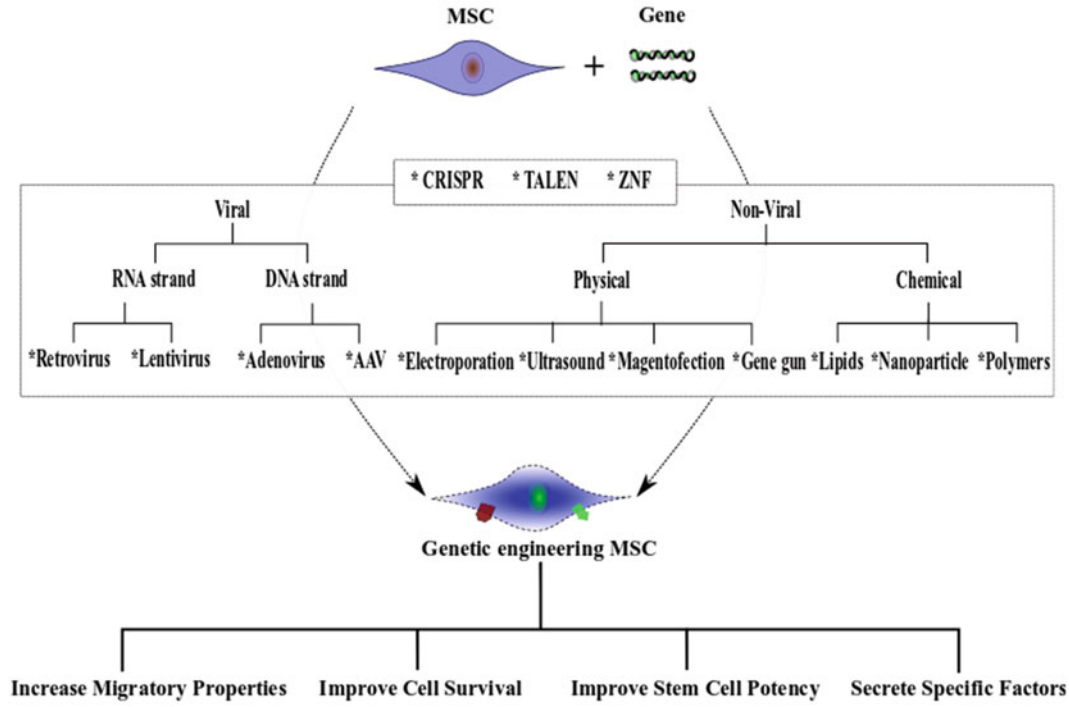

Fig. 2 Schematic representation of genetic engineering for the promotion of mesenchymal stem cell therapy

Table 1 Summary of main properties of two types of vectors for using in gene therapy

Type of vector	Advantage	Disadvantage
Viral	Do not affecting stem cell properties of host cell	None of them have been confirmed as a standard vector for all transgene aims
	Innate ability to entry into and survive within the host cell nucleus	Cytopathic effects and adverse immune reactions
	The long-term and stable production	Possibility of insertional mutagenesis and oncogene. Activation in host cells or tissues
	Can lead to 90% transduced cells	
	Each type of viral vector has especial pros and cons	High production cost
	Integration of the transgene into the host genome (stable) and/or episomal form (short-term)	
Non-viral	Able to deliver larger transgenes	Low transfection efficiencies
	Capable to scale-up manufacturing	Transient gene expression
	Low in immunogenicity	Affecting host cell viability
	The wide array of design choices	
	More cost effective than viral vectors	

sheets composed of BM-MSCs in the immunocompromised mice (Yan et al. 2014). Cui et al. demonstrated that during the hepatic differentiation of hUC-MSCs could be mentioned the basis role of microRNAs and specific microRNAs such concomitant transfection with six different miRNAs—miR-1290, miR-1246, miR-30a, miR-148a, miR-424, and miR-542-5p (Cui et al. 2012). Kim et al. indicated that transfection of miR-302d mimic increase cell proliferation and can control cell survival by protecting the cells from oxidant-induced cell death in human AT-MSCs (Kim et al. 2014). Another study has reported miR-302 family positively affect the

Table 2 Summary of the main research factors for promotion of MSCs via genetic manipulation

Properties	Factors	Functions	Ref
Migratory	SDF-1	The receptor of CXCR-4 and CXCR7 in the outer membrane of MSCs and increase engraftment of MSCs overexpressing IGF-1	Haider et al. (2008), Nakamura et al. (2013), Won et al. (2014), Liu et al. (2014)
	Aqp1	A water channel molecule which was an important regulator of MSC migration	Meng et al. (2014), Nowakowski et al. (2016)
	CCR1	The receptor protein for recruitment of immune cells to the site of inflammation which increase MSC migration induced by chemokines	Huang et al. (2010), Hodgkinson et al. (2010)
	Nur7 & Nurr1	Two nuclear receptors which can enhance cellular migration properties of MSCs	Maijenburg et al. (2012)
	IL-8	Ligand for CXCR-1 which are important for MSC in vitro migration	Hodgkinson et al. (2010), Maijenburg et al. (2012)
Survival	Akt	Enhanced MSC secretion of paracrine factors and reduced scar size and fibrosis	Gnecchi et al. (2006), LIM et al. (2006)
	SDF-1	Have a role in decrease of apoptosis and increase engraftment of MSCs	(Nowakowski et al. (2016)
	HIF-1	A protein which promote MSC survival under hypoxia condition	Lv et al. (2017)
	Bcl-2	Have a fundamental effect in anti-apoptosis of cells	Li et al. (2007), Golchin et al. (2016)
	IGF-1	A small polypeptide that regulate survival, self-renewal, and differentiation of various cells and specially MSC	Youssef et al. (2017)
	Notch	Regulates glycolysis under hypoxic conditions and enhances the MSCs lifespan	Huang et al. (2013), Moriyama et al. (2014)
	HO-1	A stress-response protein that MSC overexpression of its enhance anti-apoptotic and anti-oxidative capabilities of MSCs	Tsubokawa et al. (2010)
Stemness	Pluripotency genes	OCT4, NANOG, and SOX2 which are pluripotent markers related to embryonic stem cells and induce stemness in MSC	Tantrawatpan et al. (2013), Zomer et al. (2015)
	FGF-2	A growth factor to promote proliferation and inhibit cellular senescence through a PI3K/AKT-MDM2 pathway	(Coutu et al. (2011b)
	NRF2	As potential marker which overexpression of it increase the proliferation and reduce the rate of apoptosis in MSCs	Yuan et al. (2017)
	TERT	An RNA dependent DNA polymerase which synthesizes and extends of the telomere of DNA, so sustain the immortal phenotype of stem cells	(Li et al. (2015a), Pirmoradi et al. (2017)
	miRNA	Key RNA sequence regulators for administration of various cell type, specially MSCs	Eskildsen et al. (2011), Hass et al. (2011), Munoz et al. (2013)

Specific factors	HSV-Tk	A conditional suicide gene marker in mammalian cells that MSC by expressing HSV-tk could exert cytotoxic effect on tumor cells upon treatment	Amano et al. (2009), Niess et al. (2011)
	IFN-β	A gene which encodes a cytokine of interferon family that this cytokine decreases tumor formation and reduces tumor metastases	Xie et al. (2013), Sage et al. (2016)
	TRAIL	TRAIL is a key transmembrane protein that acts in apoptosis pathway and resulting in apoptosis of especially cancer cells	Lee et al. 2003, Li et al. (2010), Sage et al. (2014)
	HNF-4α	An orphan nuclear receptor and master regulator of hepatic differentiation that reduces tumor growth	Walesky and Apte (2015), Wu et al. (2016)
	Apoptin	A protein with an inherent ability to lyse cancer cells without any damaged for normal cells	Zhang et al. (2016)
	IL-12	A heterodimeric pro-inflammatory cytokine that can reduce ascites volume and increase survival of MSCs	HAN et al. (2014), Sun et al. (2015), Firestein and Kelley (2017)
	UPD	A suicide gene cytosine deaminase that regresses some tumors	Altanerova et al. (2012)
	VEGF	A signal released protein from various cells which can effectively prevent myocardial wall thinning, suppress myocardium fibrosis and enhance the blood vessel formation	Gao et al. (2007), Yeh et al. (2014)
	IL-18	A pro-inflammatory cytokine that its genetically modified MSCs improve cardio protection	Xu et al. (2009), Wang et al. (2009)
	HGF	Hepatocyte growth factor is a recognized angiogenic factor, and endothelial cell chemoattractant, that bone marrow-derived MSCs overexpressing HGF could be a can restore local blood stream and regenerate lost cardio-myocytes	Duan et al. (2003)
	CF-IX	Coagulation factor IX that deficiency of this factor causes hemophilia B, so genetic-engineered MSC may be used for treatment of hemophilia B and/or other plasma protein deficiencies	Coutu et al. (2011a)

Aquaporin 1 (aqp1), B-cell lymphoma 2 (Bcl-2), CC chemokine receptor 1 (CCR1), Factor IX (FIX), Fibroblast growth factor-2 (FGF-2), Heme oxygenase-1 (HO-1), Hepatocyte nuclear factor 4α (HNF4α), Hepatocyte growth factor (HGF) Herpes simplex virus thymidine kinase (HSV-TK), Hypoxia Inducible Factor (HIF-1) Insulin-like growth factor-1 (IGF-1), Interferon beta (IFN-β), Interleucin-8 (IL-8), Interleucin-12 (IL-12), Interleucin-18 (IL-18), Stromal cell-derived factor 1 (SDF1), Telomerase reverse transcriptase (TERT), TNF-related apoptosis-inducing ligand (TRAIL), MicroRNA (miRNA), Protein kinase B (Akt), Vascular endothelial growth factor (VEGF), Uracil phosphoribosyl transferase (UPD)

expression of pluripotency markers like OCT4, Nanog, and Sox2 mRNAs (Kim et al. 2014). Shaer et al. reported the human placental decidua basalis MSCs (hPDB-MSCs) could be programmed into functional insulin producing cells by transfection of miR-375 (Shaer et al. 2014). Furthermore, there are several reports to enhance survival of MSCs by miRNA, especially miR-210 in the oxidative stress environment condition that may contribute via Bcl2/Beclin-1 or/and c-Met pathway activation (Chang et al. 2013; Xu et al. 2014, 2016).

Exosomes are nano extracellular vesicles (EV) which released via different cells into the extracellular environment and can influence tissue responses (Golchin et al. 2018). In the 2010s, was determined that coding and none coding RNAs (ncRNAs) such mRNA and miRNA can be loaded as "goods" in EVs (Ma et al. 2017). Rubina Baglio et al. in 2015 demonstrate that primary BM-MSCs and AT-MSCs release various small RNAs such tRNAs and different type of miRNAs via exosomes (Baglio et al. 2015). EV-associated ncRNAs can act as new treatment targets for various therapeutic purpose. However, among different diseases, cancer is the most common candidate for EV-associated RNAs therapy (Gong et al. 2017). Xin et al. reported the exosome-mediated transfer of miR-133b from multipotent BM-MSCs to neural cells to neurite outgrowth (Xin et al. 2012). Recently, Gong et al. presented a hypothesis that exosomes secreted from MSCs deliver miRNAs into endothelial cells and mediate angiogenesis via using tube-like structure formation and spheroid-based sprouting of human umbilical vein endothelial cells (HUVECs). They reported that exosome-mediated transfer of angiogenic miRNAs can play an important role in MSC mediated angiogenesis (Gong et al. 2017). In several other studies are reported delivery of small interfering RNA (siRNA) into MSCs for improving the efficacy of MSC therapy in survival, differentiation and etc. (Otani et al. 2009; Lai et al. 2011; Zhu et al. 2014; Ma et al. 2016).

However, there are many studies which had been focused on new strategies for GEMSCs for therapeutic purposes by using ncRNAs (such miRNAs and siRNAs) and exosome delivery systems (Liang et al. 2016; Figueroa et al. 2017; O'Brien et al. 2018). In addition, recent studies show that probably can be designed new miRNAs by using novel methods that can be helped to the more genetic engineering of cells for therapeutic purposes (Senís et al. 2017; Fischer et al. 2017).

4 Therapies Use of Genetic Modification MSCs in Clinical Trials and Discussion

Clinical trials are studies that are done in clinical research and are designed to investigate specific queries about new treatments and known interventions that should be prepared data on safety and efficacy for subsequent study and possible clinical application. The clinical trial studies include five phases: Early Phase 1 or Phase 0, Phase 1, Phase 2, Phase 3, and Phase 4, which describe the stages of a clinical trial study. As mentioned, we can consider much therapeutic potentials for cooperation of MSC therapy and gene therapy. Even though, the documented clinical trial results are limited. Nevertheless, there is only a few clinical trial listed on the NIH clinical trials website (https://clinicaltrials.gov/). In case of MSCs therapy and gene therapy cooperate with each other, the first clinical trial study which started in 2003 and its result published in 2006, Ripa, et al. performed a pilot study of combined VEGF165 gene therapy and stem cell mobilization in patients with severe chronic ischemic heart disease and reported this treatment as safely approach (Muñoz Ruiz and Regueiro 2012). In this study, tow strand separately but alongside each other was used and was highlighted application of genetic engineering MSCs in CTSs.

The second clinical trial is assessing safety and efficacy of MSCs genetically modified against head and neck cancer (GX-051) to produce interleukin-12 after intra-tumoral injection (NCT02079324). In this study is expected that IL-12 expressing MSC vaccine GX-051 secretes IL-12 to activates the immune system by both

promoting the secretion of interferon-gamma (IFNγ) and inducing cytotoxic T-cell responses to activates natural killer cells (NKs) and decreased cell proliferation and increased cell death in tumor cells of these patients.

In the third study which has been started in 2017 (NCT03351868), will be used autologous hematopoietic and MSCs transduced with the lentiviral vector carrying the gene FANCA ex vivo for Fanconi anemia patients. The aim of this study has been determined the treating of Fanconi anemia by using a self-inactivating lentiviral vector to functionally correct the defective gene of hematopoietic stem/progenitor cells and evaluating the safety and efficacy of this protocol.

In addition to the studies mentioned, we found published studies, which have applied genetically modified MSC in the clinical trials, however there are three studies that one of them has used MSC as a delivery vehicle to locate oncolytic virus in cancer site. In the follow, we will briefly describe their study design and results one by one.

Neuroblastoma (NB) Clinical Trial In this study, researchers have used MSC as a delivery vehicle to locate oncolytic virus in cancer site. Neuroblastoma (NB) is the most abundant extra cranial solid tumor in children. Notwithstanding invasive treatments, there is no strong wish for the long-term survival of the patient with a metastatic tumor (Maris et al. 2007). However, new treatment method base on modified adenovirus that can replicate specifically in tumor cell is proposed (Alemany 2007). ICOVIR-5 is an oncolytic adenovirus that is controlled by the E2F-responsive promoter (Cascallo et al. 2007; Alonso et al. 2007). This transcriptional regulation, confine the replication of ICOVIR-5 to cells with an activated RB pathway. (Characteristic of cancer cells). CELYVIR is an autologous MSC that is transfected by ICOVIR-5, which have used in this clinical trial. To prepare CELVYR, MSCs are harvested from bone marrow of the patient with metastatic neuroblastoma and are inactivated by x-ray irradiation (30Gy) to prevent enhance metastasis that could be done by viable MSCs (Post et al. 2003). At the same time, radiation has no effect on adenovirus replication (Karnoub et al. 2007). Autologous MSCs are infected by ICOVIR-5 at a multiplicity of infection (MOI) of 200 plaque forming units (p.f.u.) and they were infused through a central line. Four patient with refractory stage IV neuroblastoma received CELVYR, at least two times and presence of ICOVIR- 5 was evaluated by PCR, anti-adenovirus IgG and electronic microscopy. Presence of ICOVIR- 5 was positive for all patient at least in one method. In one patient 5 days after infusion, ICOVIR-5 has detected in bone marrow aspiration that conforms carrying of ICOVIR-5 by MSC to metastasis site. Iodine 123 metaiodobenzylguanidine (123 I-MIBG) scintigraphy had no response in all patients except one, that 5 days after the third infusion for him, I-MIBG positive area was biopsied and no NB cell was there. The authors have reported that he was in complete remission 36 months after therapy. There was no hematologic, neurologic and metabolic sever side effect in patients except auto limited fever and mild increasing of transaminase. Also, there was no evidence of tumor growth or progressing disease related to infusion of radiated MSCs which are infected by adenovirus. It has been shown that MSCs will target metastasis, deliver ICOVIR-5 there and produce progressive viral infection at the site of metastasis but not in other areas. Only a few detections of viruses in urine and serum sample conform low systemic release of virus and minimum systemic toxicity (García-Castro et al. 2010).

Gastrointestinal Tumors Clinical difficulties in the area of gastrointestinal tumors are tumor recurrence, metastasis, opposition to therapy and also create an obstacle in the curative surgical process by local tumor growth (Stintzing et al. 2016). As a new point of view in cancer therapy, it is believed that emphasis on the stromal component of a tumor, make better results to inhibition of tumor growth (Stintzing et al. 2016; Soheilifar et al. 2018). In preclinical models, MSCs which are transfected by a vector containing the herpes simplex virus thymidine kinase (HSV-Tk) as suicide gene, tumor-specific

expression of thymidine kinase has been reported (Dominici et al. 2006; Rhee et al. 2015). In transfected cells, thymidine kinase convert prodrug ganciclovir (GCV) to ganciclovir triphosphate which is a competitive inhibitor of deoxyguanosine triphosphate, resulted in inhibition of DNA polymerase and guides cell to apoptosis (Niess et al. 2015). This clinical study has been performed in phase I/II clinical trial on 6 and 16 patients respectively. After preparation of investigational medicinal products (IMP), termed MSC_apceth_101 (genetically engineered MSC), In phase I, 3 patients had received IMP at the total dose of 1.5 × 106 cell/kg through IV injection, during 3 weeks (0.5 × 106 cell/kg each week). The others had received IMP at the total dose of 3 × 106 cell/kg through IV injection, during 3 weeks (1.0 × 106 cell/kg each week), 48–72 h after IMP injection, GCV has been injected in three consecutive days by the way of GCV producer recommendation. In the end, all of the patient's safety-related data have been collected, analyzed and approved as good and tolerable IMP to entire to phase II. In this phase 16 participant as two group of patients were selected, the first group which consists of patients with advanced disease, were received only MSC_apceth_101 and GCV. The second group, which consists of patients with adenocarcinoma that were qualified for surgery and neoadjuvant treatment received a single dose of MSC_apceth_101 and GCV prior to surgery and 1–3 days after surgery they received GCV. Based on results and patient follow-up, the authors have claimed that If this IMP well tolerated and have good efficacy, it could be used as a part of routine conventional therapies such as chemo and radiotherapy (von Einem et al. 2017).

Pulmonary Fibrosis Pulmonary silicosis is an incurable recurrent disease which is characterized by permanent fibrosis and an interstitial lesion in lungs that are effects of silica particle inhalation (Leung et al. 2012). MSCs will trap in pulmonary circulation after systemic infusion or they can preferential place or home in lungs by the implementation or in response to inflammation (Hori et al. 2013; Liu et al. 2015). It has been shown that hepatocyte growth factor (HGF) has to protect effect against fibrosis (Chakraborty et al. 2009) and Engineered MSCs which are transfected by HGF cDNA-vector represent immunosuppressive activity (Bian et al. 2009). Moreover, bone marrow MSCs that are transfected by HGF reduced bleomycin-induced lung fibrosis (Gazdhar et al. 2013). This study was designed to evaluate the therapeutic effect of MSC in combination with (HGF) in human lung silicosis. Four patients received 2 × 106 cell/kg through during about 30 min by IV route. This infusion was repeated in tow next weeks. 30 min after cell injection, fever and chilling were observed in tow patients that they disappeared after injection of 10 mg Dexamethasone. No sign of fever, headache, diarrhea, and vomiting have been reported. So the authors claim that the therapy is generally safe. Clinical and laboratory follow-up 6 months after cell therapy revealed that forced vital capacity (FVC) and Forced expiratory volume in 1 s (FEV1) a little increased after therapy but average of them had no significant variation before and after cell injection, arterial blood oxyhemoglobin saturation (SpO2) significantly increased and symptoms of a cough and dyspnea improved, and partially absorption of lesion in tow patient was observed after 12 months. Also 6 months after MSC therapy the ratio of peripheral blood CD4+/CD8+ T lymphocyte generally increased and the serum IgG concentration decreased in to the range of normal values (7.6–16.6 g/l), the mean level of serum ceruloplasmin as a sign of pulmonary infection (Cernat et al. 2011) and collagen deposition in the damaged lungs slightly decreased (Sauni et al. 2012). this results totally show MSC/HGF will effect by inhibition of chronic inflammation. As conclusion researcher has recommended that to ensure and fulfillment of long-term safety and effectiveness of therapy, a placebo-controlled clinical trial by more number of the patient should be performed (Liu et al. 2015).

5 Conclusion

Despite recent advances that have significantly developed in gene engineering of different stem cell type, especially MSCs, in laboratory and in vivo investigation, a few clinical trials have focused on this procedure and this procedure still has considerable limitations and complications. In food and drug administration (FDA) definition, gene therapy is "the administration of genetic material to modify or manipulate the expression of a gene product or to alter the biological properties of living cells for therapeutic application" and until the point in time under discussion 16 cell and gene product have been approved from the FDA cellular, tissue and gene therapies advisory committee. However, some limitation of gene delivery to cells have been reduced through a new generation of vectors, and researchers are increasingly interested about cell therapies, especially MSC therapy, that are proving safe and efficacious in treating untreatable diseases.

The emergence of new techniques especially such as CRISPR has created a new development in cellular genetic engineering so that in recent years several clinical studies have been started based on CRISPR. It is certain that a major contribution to the regeneration medicine includes in cell and gene therapy cooperation.

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Conflict of Interest The authors declare no conflict of interest.

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Mesenchymal Stem Cells-Derived Exosomes for Wound Regeneration

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Abstract

Wound healing is a complex process with the considerable burden on healthcare system. There are several cellular therapy methods that have been introduced to treat different types of wounds. Despite the advantages of cellular therapy, it is needed to overcome different limitations of this method such as; tumorigenicity and immune rejection. Accordingly, scientists have suggested cell-based vesicles and exosomes. Exosomes can promote proliferation, migration, and angiogenesis process in the wound environment. They have also some advantages such as the potential for drug and gene delivery, easy to storage, and stability in the body. These advantages make them as a novel approach in regenerative medicine without the limitations of cellular therapy. In this review, the authors emphasize on biological properties of MSC-exosomes and their therapeutic effects as a new strategy for wound regeneration.

Keywords

Exosomes · Mesenchymal stromal cells · Regenerative medicine · Transport vesicles · Wound healing

Abbreviations

ANGPT1	Angiopoietin 1
ASCs	Adipose-derived stem cells
BBB	Blood-Brain Barrier
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CHA	Composite collagen–hydroxyapatite
ECM	Extra-cellular matrix
DFU	Diabetic foot ulcers
EGF	Epidermal growth factor
EVs	Extra-cellular vesicles
FDA	Food and Drug Administration
FGF 2	Fibroblast growth factor 2
IGF-1	Insulin growth factor 1
IL-1	Interlukin 1
IL-6	Interlukin 6
iPS	Induced pluripotent stem cells
MSCs	Mesenchymal stem cells
PDGF	Platelet-derived growth factor
STAT3	Signal transducer and activator of transcription 3
TGF-β1	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor-alpha
UCB-MSCs	Umbilical cord blood mesenchymal stem cells
VEGF	Vascular endothelial growth factor
MSC Exo	Mesenchymal stem cells-derived exosomes
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor 1
SDF-1	Stromal cell-derived factor 1

1 Introduction

The skin is the outer soft tissue of the body which protects it against external agents such as infections. Damaging and loss of skin tissue integrity lead to wounds (Murphree 2017). There are several classification for wounds, including: acute or chronic wounds, penetrating or non-penetrating wounds, clean or contaminated wounds, and etc. (Percival 2002; Mohil 2012). Among different types of wounds, chronic ones as a considerable burden on healthcare system, affected ~6.7 million of people around the world and its healing costs ~$20 billion per year alone in the US (Järbrink et al. 2017). This type of wounds occurs when the natural wound healing process which includes three programmed stages (inflammatory phase, Proliferation phase, and Maturation phase) is impaired by several factors (Frykberg and Banks 2015). In this regard, investigators are looking for the safe and cost-effective approaches to wound management. Although various researches have concentrated on facilitating the wound healing process, currently definitive therapies are not available. In recent years progression in (stem) cell therapy have given the promise to improve the wound healing and the majority of studies have focused on the importance of applying mesenchymal stem cells (MSCs) in wound regeneration (Murphy and Evans 2012; You and Han 2014; Isakson et al. 2015; Zhang et al. 2015c). Despite the advantages of cell therapies, some limitations such as immunological rejection and genetic variation still exist (Herberts et al. 2011; Zhang et al. 2015c). More recent studies have revealed that the role of (stem) cells in wound healing and tissue regeneration have been mainly associated with their secretome and paracrine effects rather than their differentiation ability (Dittmer and Leyh 2014; Zhang et al. 2015c). Accordingly, many investigations have demonstrated that the exosomes which secreted by cells, strongly supports their paracrine effects (Rani et al. 2015; Zhang et al. 2015c). Exosomes are cell- secreted vesicles which can be applied as a biomarker of diseases and also can be potentially applied in the field of regenerative medicine including wound healing (De Jong et al. 2014; Edgar 2016; Bjørge et al. 2018; Jing et al. 2018). Under the scope of this review, we discuss the current state and feature perspective of MSC derived exosomes (MSC-EXO) for treatment of different types of wounds.

2 Current Treatment Strategies for Wound Regeneration

2.1 Wound Dressings

Generally, healing of wounds especially chronic wounds needs a long time and usually, if that possess the natural healing procedure, the severe scar will be induced (Kamoun et al. 2017). Therefore, the development of a method which provides acceleration of wound closure, reduction of scar formation, and promotion of wound repair, seems to play a crucial role in wound management. Accordingly, wound dressing is an almost old method which be used in different types of wounds. There are several types of wound dressing including rubber, foam, electro spun nanofiber, hydrogel, etc. that are usually composed of natural or synthetic biomaterial such as chitosan, hyaluronic acid, collagen, silicon based, cellulose, etc. (Tran et al. 2017; Zhao et al. 2017). Wound dressing can affect on wound management through various pathways. For instance, it can change wound environment, preserve the wound from bacterial infections, provide gas exchange, protect the wound from sever dryness, and maintain moist environment and consequently, it will be easy to remove without any pain (He et al. 2018; Zhou et al. 2018). It also can protect wound environment from infection during healing (Dreifke et al. 2015; Han and Ceilley 2017). However, despite several advantages, dressing cannot provide perfect peripheral circulation, fluid balance, sensation of environment and other desired conditions to promote complete regeneration. Therefore, developing new approaches to return natural skin construction and function seems to be critical (He et al. 2018).

2.2 Skin Substitutes

After sever disruptions such as burns and traumatic injuries, skin has a poor capacity to regenerate itself and needs to a suitable substitute for return its function (Jeschke et al. 2017). Skin substitutes have been largely used in various conditions such as grafts for surgical or burn defects. Based on the biological origin of skin substitutes they can be used as autografts, allografts, and xeno-grafts. Autografts are the most beneficial than others but requirement of adequate autologous skin is not possible in a single setting. Hence, allo and xeno-grafts are used as worthful alternatives because of their simple availability and ability to accelerate the healing process and help to reconstruct skin structure. In spite of the mentioned advantages of allo and xeno-grafts, immune rejection and also potential scar formation are serious disadvantages that need to be considered by investigators and clinicians (Yamamoto et al. 2018). Although, using of skin grafts and wound dressings are traditional methods, but their application would be more useful in combination with novel methods. Application of bioengineered skin substitute in skin grafts are examples of these new technologies (Yamamoto et al. 2018; Zeng et al. 2018). There are some serious limitations including: higher costs, risk of infection, antigenicity, time, and susceptibility to injury (Han and Ceilley 2017; Bhardwaj et al. 2018). Hence, scientists have focused on various novel strategies such as cell therapy and regenerative medicine. Accordingly, manufacturing bioengineered skin substitutes have been received considerable attention and investigated in recent years to replace the traditional healing methods.

2.3 Growth Factors and Cytokines

Wound healing consists of different overlapping phases including inflammatory, proliferative and remodeling (Cabral et al. 2018). Various of growth factors and cytokines are involved in controlling of these phases including: platelet-derived growth factors (PDGFs), granulocyte-macrophage colony stimulating factor (GM-CSF), and fibroblast growth factors (FGFs). It seems that PDGF as the most important factor is the first clinically approved growth factor for chronic non-healing ulcers. Several studies demonstrated the pivotal role of this factor in the

wound healing process (Embil and Nagai 2002; Werner and Grose 2003; Wang et al. 2018). In addition to PDGF, Platelets secrete other growth factors such as inflammatory cytokines (IL-1 and IL-6) to activate and recruit the neutrophils, macrophages, and fibroblasts. On the other hand, after initiating the clotting cascade and matrix formation, alpha granules are released by platelets secreting growth factors, including: epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF- β) (Barrientos et al. 2008). They can be used in different forms such as topical gels for example, recombinant human PDGF-BB (Regranex®) is the only FDA approved growth factor (topical gel) over the past 20 years for chronic non-healing wounds especially diabetic foot ulcers (DFUs) (Han and Ceilley 2017; Laiva et al. 2018; Nakagami et al. 2018). However, there are some concerns about the excess usage of these products, such as its probable carcinogenic effects (Fürstenberger and Senn 2002). Therefore, in recent years, more novel promising methods have been introduced to provide a safe and effective strategy for wound management. In recent decade, cell therapy and regenerative medicine have opened a new horizon for investigators to develop efficient therapeutic methods for wound healing.

3 Stem Cells and Tissue Engineering Methods for Skin Repair

Stem cells have a unique capability to differentiate into various tissue specific cells. Several cells that can be derived from different tissues, such as bone marrow, peripheral blood; umbilical cord blood, and adipose tissue have been studied in preclinical and clinical wound healing studies. For instance, many clinical studies have demonstrated that autologous or allogeneic bone marrow and adipose tissue-derived MSCs can enhance the healing process of chronic wounds by inducing angiogenesis and tissue formation (Teng et al. 2014; Dreifke et al. 2015; Han and Ceilley 2017). Moreover, cell-based strategies can introduce various bio-products for clinical use in different diseases including wounds. Hence, cell therapy and regenerative medicine has progressed with developing several techniques in isolation, engraftment, and expansion of stem cells to provide safe and cost-effective products. In recent years, induced pluripotent stem cells (iPS) have been produced with reprogramming of somatic cells to provide patient or disease specific embryonic-like pluripotent stem cells and significantly reduce in rejection rate (Wilson and Wu 2015). Although, Stem cell therapy can improve chronic wound healing quality, some fundamental questions about the optimal cell population, suitable time of cell delivery, survival of transplanted cells, and the ability of cells to preserve their characterization in new conditions need to be answered (Eming et al. 2014). Additionally, such limitations in autologous skin grafting have been proposed. Accordingly, tissue engineering is a therapeutic method which creates bio-engineered tissues for regenerative medicine (Drury and Mooney 2003). Furthermore, various tissue engineering approaches were investigated by focusing on the various types of growth factors. However, the remarkable challenge in tissue engineering is providing an environment to promote pivotal mechanisms (Sorg et al. 2017). According to different stages and types of wounds, various methods have been used such as; cell delivery into the injured site, gene modification, and using scaffolds (Yu et al. 2014). There are several synthetic and natural scaffolds which can be used in tissue engineering such as: hydrogels, nano-fibrous scaffolds, composite collagen–hydroxyapatite scaffolds (CHA), etc. These scaffolds act as extra-cellular matrix (ECM) which organize cells and stimulate their growth processes to develop specific tissues. Type of scaffold strongly depends on the properties of specific application and cell types (Drury and Mooney 2003; Liu et al. 2017). One of the most common and promising types of stem cells which have been used in tissue engineering and regenerative medicine is MSCs. They can be isolated from various tissues and organs and differentiate into multiple cell lineages (Heo et al. 2018; Womack et al. 2018).

3.1 Mesenchymal Stem Cells in Wound Regeneration

3.1.1 Overview

Healing of wounds can be affected by several factors that can possess either positive or negative results. For instance, psychosocial issues (poor quality of life, low physical activity, etc.), obesity, and diseases like diabetes are influential factors. In this regard, researchers are always looking for a proper and cost-effective treatment to overcome the limitation of wound healing such as cost and effectiveness. MSCs due to their features such as differentiating potential, secreting paracrine factors, immunomodulatory effects, and self-renewal capacity are seriously considered by researchers for application in healing of wounds (Yu et al. 2014; Bai et al. 2017a, b; Wang et al. 2017).

3.1.2 Mesenchymal Stem Cells

MSCs are multipotent cells which were extracted from the bone marrow for the first time. Today, researchers have found that they can also be isolated from adipose tissue, nerve tissue, umbilical cord blood, dermis, dental pulp, placenta, synovial fluid, skeletal muscle, hair follicles and even from the circulatory system. MSCs have some properties including self-renewal differentiation potential into mesodermal, ectodermal and endodermal lineages. Based on scientific evidences, MSC is a stem cell which can express: CD29, CD44, CD73, CD90, CD105, while there is a lack of expression of CD14, CD34, CD45, CD19, CD11b, CD79α, and HLA-DR. Additionally, the other specifications of MSCs include the ability of sticking to the plastic surfaces, immunomodulatory features, homing and in vitro long-term banking and cryopreservation neuroprotection secretion of cytokines and growth factors proliferation (Dominici et al. 2006; Teng et al. 2014; Yu et al. 2014; Ullah et al. 2015; Lopez-Verrilli et al. 2016; Spees et al. 2016; Perez-Hernandez et al. 2017). Hence, according to these remarkable characteristics, MSCs can play a special role in cell therapy, treatment of various diseases, and tissue regeneration. For instance, in the wound regeneration processes, angiogenesis, immunomodulatory properties, and anti-inflammatory effects are resulted from their multi-lineage differential potential of MSCs (Lee et al. 2012; Scott Maxson et al. 2012; Yu et al. 2014; Lee et al. 2016). In addition, they can enhance angiogenesis and accelerate re-epithelialization by releasing vascular endothelial growth factor, pro-angiogenic factors, and angiopoietin-1 (ANGPT1) as their paracrine effect (Yu et al. 2014; Yáñez-Mó et al. 2015; Lee et al. 2016). On the other hand, MSCs can reduce inflammation, granulation tissue formation and scar formation. Reducing inflammation may have an effect on reduction of scar formation by decreasing fibrosis (Scott Maxson et al. 2012; Nuschke 2014). Furthermore, based on the antibacterial properties, they can also control bactericidal activities which are regulated by immune cells and decrease the rate of bacterial infection (Duscher et al. 2016). MSCs from different sources have different effects on wound regeneration. Therefore, various sources of MSCs were used for treatment of different types of wounds. Furthermore, several studies are trying to introduce MSC-derived exosomes (MSC-exosomes) as a safer alternative. Various secretory factors such as extra-cellular vesicles are released from MSCs. Nowadays, several studies revealed that exosomes as a type of these vesicles may have therapeutic potentials (Teng et al. 2014; Yu et al. 2014; Rani and Ritter 2016).

4 Stem Cell-Derived Exosomes

4.1 Overview

Cellular communication is essential for the proper coordination, normal function of living cells, and their acts against damages and traumas. This process occurs through transmitting different signals (such as cell-surface molecules and secreted molecules) which can come from the adjacent cells and also their environment. These signals can be transferred over the cell membrane and sometimes they can operate by communicating

with receptor proteins which are in close-contact with both the inside and outside of the cell (Rossello and Kohn 2010; Raposo and Stoorvogel 2013; Turturici et al. 2014). The releasing of extra-cellular vesicles (EVs) by cells is considered as the main mechanism which makes a communication between the cells. Any cell types can be able to produce various classes of EVs including exosomes and micro-vesicles (MVs) (De Jong et al. 2014; Keshtkar et al. 2018). In contrast to micro-vesicles (which are formed from the apoptotic bodies and plasma membrane), exosomes have an endocytic origin. Additionally, exosomes are carrying active signals which can influence the function of the target cells (Marote et al. 2016). According to the body of literature, use of exosomes in the field of regenerative medicine can put it forward as a cell-free therapy with promising curative outcomes (Marote et al. 2016; Cobelli et al. 2017).

4.2 Exosomes

Exosomes are extra-cellular nano-vesicles (30–150 nm) which transfer active cargoes between the cells (Zhang and Grizzle 2014; Marote et al. 2016). They have a particular compound of lipids, RNAs, and proteins which enveloped by a phospholipid layer. These type of EVs can be found in different body fluids such as plasma, cerebrospinal fluid, breast milk, urine, amniotic fluid, and saliva (Looze et al. 2009; Zhang and Grizzle 2014; Marote et al. 2016). Exosomes were discovered about 30 years ago (in the 1980s) by the Johnstone and their colleagues, for the first time (Théry 2011; Lin et al. 2015). There are some conventional methods of exosomes isolation including ultracentrifugation, immune-affinity capture techniques, density gradient separation, chromatography, and using commercial kits such as polymer-based precipitation. On the other hand, some proteins are known as particular exosomal markers such as CD9, CD63, and CD81 which can use for exosome identification (Zhang and Grizzle 2014; Marote et al. 2016). In recent years, the biomarker role of exosomes has attracted a great interest because of their considerable potential in the diagnosis of various diseases (De Jong et al. 2014; Hessvik and Llorente 2017). One of the most common approaches of regenerative medicine is cell-based therapy in which cells are applied for tissue repair either through direct manner or paracrine effects (Dittmer and Leyh 2014). There are several pathways of cell communication in the setting of paracrine functions. One of them is performed by their secreted factors and cytokines. Most of these factors are released as cargoes of exosomes, not essentially as soluble elements (Camussi et al. 2010; Dittmer and Leyh 2014; Vishnubhatla et al. 2014). In recent decades, regenerative medicine has focused on the development of MSC and their derived exosomes in treatment of various diseases and different damaged tissues such as wounds (Lou et al. 2017; Vizoso et al. 2017).

4.3 MSCs-Derived Exosomes

Multiple studies have indicated the role of MSCs in regenerative medicine through the paracrine effects and producing different types of EVs including exosomes which carry as cargoes micro RNAs, mRNAs, and proteins (Phinney and Pittenger 2017). Although MSC-exosomes are same as other exosomes in morphology and also expression of the markers, but their RNA and protein composition are completely different. On the other hand, in contrast to other types of exosomes, MSC-exosomes play a fundamental role in altering the function of target cells through the horizontal transfer of their composition (Bai et al. 2017a, b; Phinney and Pittenger 2017). Additionally, according to several studies, MSC-exosomes from different sources are also different in function (Katsuda et al. 2013; Lopez-Verrilli et al. 2016; Bai et al. 2017a, b). In general, the composition of MSC-exosomes affects on differentiation and regenerative capacity of MSCs and give them a crucial therapeutic task (Nawaz et al. 2016).

5 Biological Properties of MSCs-Derived Exosomes

Recent investigations have indicated that MSC-exosomes due to their biological properties, are as potent as their sources (Batrakova and Kim 2015; Nooshabadi et al. 2018). These types of exosomes avoid degradation and phagocytosis by macrophages, circulate for prolonged periods of time within the body, and penetrate the blood-brain barrier (BBB). On the other hand, they attach to target cells by means of receptor ligands and cell surface proteins, and transfer their specific cargoes to target cells. Therefore, they can be suggested as appropriate vehicles for drug delivery (Lou et al. 2017). Additionally, MSC-exosomes can repress activation of T-cells and contribute to preserving immune homeostasis (Baquir and Hancock 2017; Casado et al. 2017). Hence, they can store safely and provide cell-free therapy without any risk of tumorigenicity and immunological rejection (Bai et al. 2017a, b). Moreover, they can support MSCs' functions within the preservation of homeostatic microenvironment (Lou et al. 2017). Finally, gathering all of biological properties in MSC-exosomes have changed them to a valuable cell-free therapy which can use in regenerative medicine.

6 Clinical Applications of MSCs-Derived Exosomes

As the mentioned, several studies have exhibited that the useful outcomes of MSC therapy are mainly resulted from their paracrine effects not trans-differentiation and engraftment. Accordingly, as MSC-exosomes contain of various secretory mediators derived from MSCs, they can use in cell-free therapeutic settings (Chen et al. 2017). Nowadays, scientists have paid a lot of attention to specific cargos of MSC-exosomes and their curative potential in different pathological conditions including immune disease, neurodegenerative disorders, cardiovascular and liver diseases, and also skin tissue damages. In addition, exososmes can be used as biomarkers for early diagnosis in different disease, especially cancers (Cheng et al. 2017). On the other hand, these vesicles have a low toxicity, and can tolerate the body environment –proved by ubiquitous presence in natural body fluids- compared with other curative tools such as transplanted (stem) cells. In summary, all of the mentioned properties of MSC-exosomes, have introduced them as a potential therapeutic techniques (Suntres et al. 2013).

7 Therapeutic Effect of MSCs-Derived Exosomes in Wound Regeneration

The wound healing cascade includes a series of molecular and cellular events such as angiogenesis, proliferation, cellular migration, tissue remodeling, and extra-cellular matrix deposition (Sinno and Prakash 2013). This cascade can be promoted by different types of biological molecules extracted from the exosomes (Than et al. 2017) through the various complex mechanisms (Table 1).

Composition of the exosomes can easily deliver the massage of signaling cells into target cells (e.g. endothelial, keratinocytes, and fibroblast) due to their lipid layer which can avoid proteolytic degradation (Schwab et al. 2015). Further, MSC-exosomes can activate some signaling pathways including STAT3, AKT, Wnt/β-catenin, and ERK in target cells which play an important role in wound healing process (Rani and Ritter 2016). Activation of these signaling pathways also can enhance the expression of several growth factors which involved in wound regeneration process by target cells, such as Interleukin-6 (IL-6), Signal Transducer and Activator of Transcription 3 (STAT3), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and stromal cell-derived factor – 1 (SDF-1) (Hu et al. 2016; Rani and Ritter 2016). Accordingly, these growth factors can promote the angiogenesis, cell migration, cell proliferation, and re-epithelialization (Rani and Ritter 2016). On the other hand, it has been revealed that MSC-exosomes in wound environment can

Table 1 Sources and mechanisms of MSCs-derived exosomes in wound healing

References	Exosomes source	Model	Wound type	Effect
Zhang et al. (2015b)	Human umbilical cord mesenchymal stem cell (HUC-MSCs)	Rat	Second-degree burn	Promote angiogenesis
Zhang et al. (2015c)	Human induced pluripotent mesenchymal stem cell (hiPSC-MSCs)	Rat	Created wound on the dorsal skin	Promote collagen maturity angiogenesis
Shabbir et al. (2015)	Bone marrow mesenchymal stem cell (BM-MSCs)	Human	Diabetic wound	Promote angiogenesis
				Enhance fibroblast migration and proliferation
				Increase STAT3 genes
Liang et al. (2016)	Human adipose mesenchymal stem cell (adMSCs)	Mice	_	Promote angiogenesis
				Transfer miR125a to endothelial cell
Zhang et al. (2015a)	Human umbilical cord mesenchymal stem cell (HUC-MSCs)	Rat	Second-degree burn	Promote proliferation, migration, Re-epithelialization
				Inhibit apoptosis
Li et al. (2016)	Human umbilical cord mesenchymal stem cell (HUC-MSCs)	Rat	Third-degree burn	Decrease inflammation
Zhang et al. (2016)	Human umbilical cord mesenchymal stem cell (HUC-MSCs)	Rat	Deep second-degree burn	Promote self-regulation of Wnt/b-catenin signaling at the remodeling phase
Hu et al. (2016)	Human adipose mesenchymal stem cell (adMSCs)	Mice	Inguinal wound	promote migration, proliferation and collagen synthesis of fibroblasts.
Fang et al. (2016)	Umbilical cord mesenchymal stem cell (UC-MSCs)	Mice	Remove skin	Promote angiogenesis
				Reduce immune response
				Stimulate endogenous stem cell recruitment and proliferation

transfer Wnt4 to stimulate Wnt/β-catenin pathway in skin cells, and subsequently active AKT pathway to inhibit skin cell apoptosis. β-catenin signaling pathway also can stimulate pro-angiogenic effects in endothelial cells and enhance cutaneous wound healing (Zhang et al. 2015a; Rani and Ritter 2016). In general, several signaling pathways and biomolecules can be activated by MSC-exosomes to improve the wound healing outcomes (Fig. 1).

8 Conclusion

Nowadays, many therapeutic methods have been developed for different types of wounds. Cell-based therapy is one of the promising methods which have been widely used in recent years. Variety of stem cells can be used in this era specifically for reducing scars following wound healing (Han and Ceilley 2017). Beside tremendous advantages of cell therapy, it has some serious limitations such as: tumorigenicity and immune rejection. To overcome these limitations more novel cell-free therapies have been developed by scientists which demonstrated interesting therapeutic effects. One of the considerable cell-free methods is using exosomes which can be extracted from different sources. Exosomes that contain siRNA, DNA, protein, miRNA, and peptides can moderate and regulate gene expression in target cells (Fang et al. 2016; Pham 2017). According to the capacities of exosomes especially MSC-derived exosomes in regulation and carrying signal and various pathways (inflammation, apoptosis, immune response, migration and proliferation), they can play an important role in promoting the wound healing cascade and worthful therapeutic effects. Additionaly, using of exosomes have been proposed for different applications such as: apoptosis, inflammation, cardiac remodeling, and

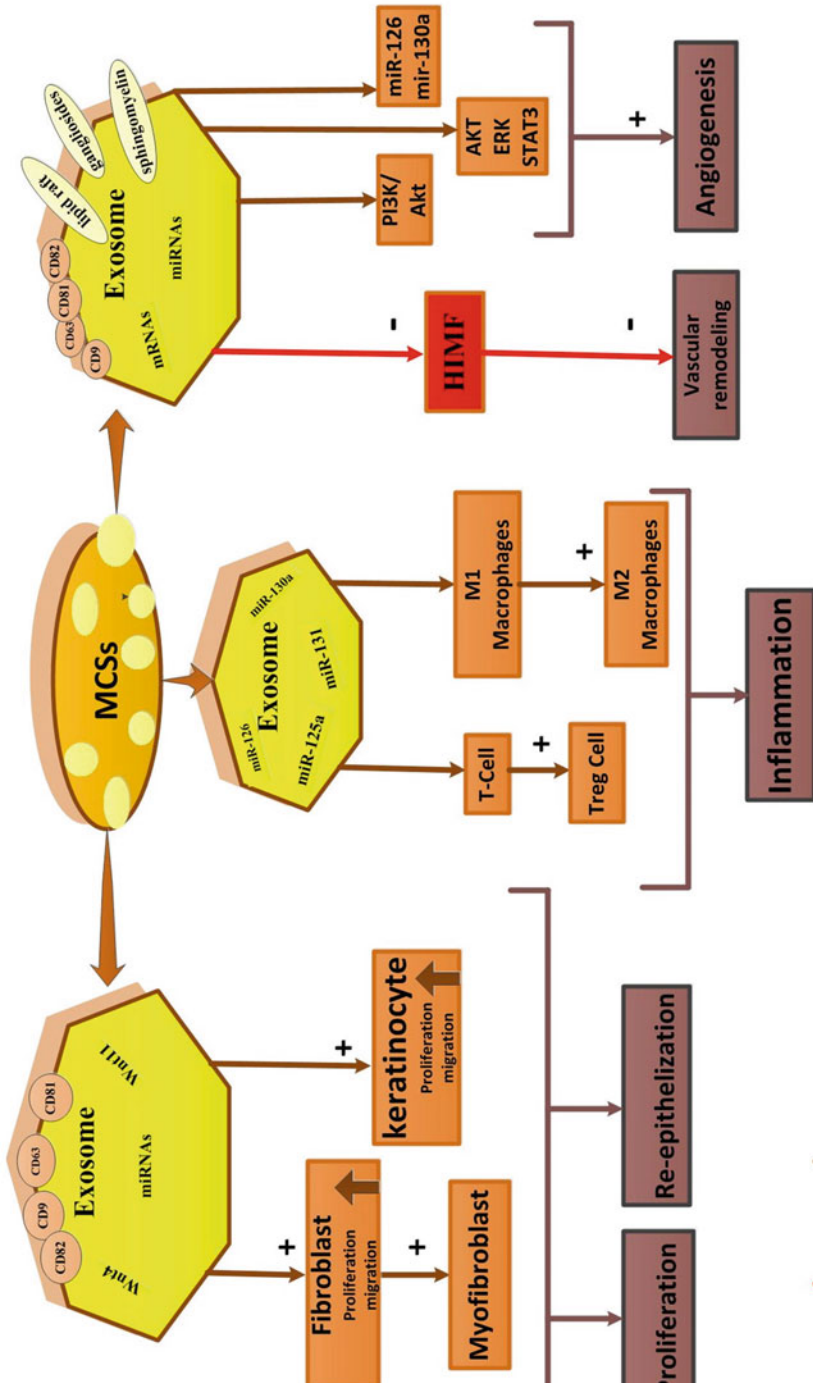

Fig. 1 MSCs-derived exosomes mediate different phases of wound healing. MSC-exosomes lead to proliferation and re-epithalization by enhancing proliferation and migration of fibroblasts and keratinocytes through mediating activation of several factors. MSC-exosomes enhance wound healing by delivering Wnt4 (Zhang et al. 2015a). MSC-exosomes can exhibit immunosuppressive effects by regulating proliferation and differentiation of lymphocytes, MSC-exosomes can repress T-lymphocyte proliferation and they exchange T lymphocytes into the T-regulatory phenotype. MSC-exosomes also enhance converting of macrophages toward the anti-inflammatory M2 phenotype in the inflammation phase (Silva et al. 2017). MSC-exosomes exhibit angiogenic effects through several mechanism (Wu et al. 2018), they cause anti-vascular remodeling by suppress HIMF (Huang et al. 2015)

cardiac regeneration in cardiovascular system, myocardial ischemia/reperfusion (MI/R) injury, and cancers (Raposo and Stoorvogel 2013; Zhang et al. 2015a, b; Rager et al. 2016; Pham 2017). Also, they are widely used in cutaneous wound healing (Monsel et al. 2016; Pashoutan Sarvar et al. 2016; Sun et al. 2016). Hence, MSC-exosomes could be candidate as the alternative of cell therapy methods (Herberts et al. 2011; Wu et al. 2018). On the other hand, many researchers drew attention to special features of exosomes on drug (Lou, Chen et al. 2017) and gene delivery (Samanta et al. 2017). Despite the several clinical trials, their safety and potency and also their task in drug/gene delivery are still unanswered (Cheng et al. 2017). In this regard, the International Society for Extracellular Vesicles was established in 2011, to develop this knowledge around the world (Raposo and Stoorvogel 2013). Nevertheless, more preclinical and clinical studies are needed to reveal unknown aspects of exosomes and their therapeutic effects.

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Adipose Tissue-Derived Stromal Cells for Wound Healing

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Abstract

Skin as the outer layer covers the body. Wounds can affect this vital organ negatively and disrupt its functions. Wound healing as a biological process is initiated immediately after an injury. This process consists of three stages: inflammation, proliferation, remodeling. Generally, these three stages occur continuously and timely. However, some factors such as infection, obesity and diabetes mellitus can interfere with these stages and impede the normal healing process which results in chronic wounds. Financial burden on both patients and health care

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systems, negative biologic effect on the patient's general health status and reduction in quality of life are a number of issues which make chronic wounds as a considerable challenge. During recent years, along with advances in the biomedical sciences, various surgical and non-surgical therapeutic methods have been suggested. All of these suggested treatments have their own advantages and disadvantages. Recently, cell-based therapies and regenerative medicine represent promising approaches to wound healing. Accordingly, several types of mesenchymal stem cells have been used in both preclinical and clinical settings for the treatment of wounds. Adipose-derived stromal cells are a cost-effective source of mesenchymal stem cells in wound management which can be easily harvest from adipose tissues through the less invasive processes with high yield rates. In addition, their ability to secrete multiple cytokines and growth factors, and differentiation into skin cells make them an ideal cell type to use in wound treatment. This is a concise overview on the application of adipose-derived stromal cells in wound healing and their role in the treatment of chronic wounds.

Keywords
Adipose tissue · Chronic disease · Mesenchymal stromal cell · Regenerative medicines · Wound healing

Abbreviations

AMPs: Antimicrobial peptides
ASCs: Adipose-derived stromal Cells
bFGF: Basic fibroblast growth factor
BM-MSCs: Bone marrow-derived mesenchymal stem cells
DFU: Diabetic foot ulcer
ECM: Extracellular Matrix
EGF: Epidermal growth factor
FGF2: Fibroblast growth factor-2
GAGs: Glycosaminoglycans
HBOT: Hyperbaric oxygen therapy
HBSs: Hypertrophic burn scars
HGF: Epidermal growth factor
HTSs: Hypertrophic scars
IGF: Insulin-like growth factor
IL-1α: Interleukin 1 alpha
IL-6: Interleukin 6
KGF: Keratinocyte growth factor
MMPs: Matrix metalloproteinases
MSC: Mesenchymal stromal cells
NPWT: Negative pressure wound therapy
NRGs: Neuregulins
PDGF: Platelet-derived growth factor
PMNs: Polymorphonuclear leukocytes
PU: Pressure ulcer
SDF1: Stromal-derived factor 1
SSI: Surgical site infection
SVF: Stromal vascular fraction
TGF-β: Transforming growth factor beta
TIMPs: Tissue inhibitor of metalloproteinases
TNF-α: Tumor necrosis factor
VEGF: Vascular endothelial growth factor
VLU: Venous leg ulcers
VPF: Vascular permeability factor
CAL: Cell-Assisted Lipotransfer

1 Introduction

The term of chronic wound is used for the wound that couldn't be treated with normal wound healing procedures and includes venous and arterial insufficiency ulcers, diabetic wounds, and pressure injuries (Frykberg and Banks 2015; Iqbal et al. 2017). This type of wounds place a significant burden on patient's life and healthcare system around the world and treatment of them is a multibillion-dollar worldwide issue that only in the united states affect 5.7 million people (Šitum et al. 2016; Järbrink et al. 2017). Generally, wound healing is a complicated natural process that occurs through three phases respectively: hemostasis and inflammation, proliferation, and remodeling (Martin 1997; Sinno and Prakash 2013; Han and Ceilley 2017). Some factors

interrupt this natural process and cause chronic ulcers (Cutting 1994; Guo and DiPietro 2010; Bereznicki 2012). Different surgical and non-surgical treatments for management of chronic wounds including hyperbaric oxygen therapy, ultrasound therapy, laser treatment and skin grafting has been used (Enoch et al. 2006; Johnston et al. 2016). These treatments have advantages and disadvantages. Some of their advantages include promoting fibroblast proliferation, down regulating inflammatory cytokines and supporting the epithelialization of the wound surface. Despite that, they are not sufficient and cost-effective for perfect chronic wound healing process (Piaggesi et al. 1998; Norman et al. 2016). Recently using stem cells to improve the treatment of chronic wound has operated as an advanced technology (Branski et al. 2009; You and Han 2014; Frykberg and Banks 2015). Stem cells are highly proliferating cells that can preserve their ability to divide and regenerate themselves for long times (Hilmi and Halim 2015, Stoltz et al. 2015). They have different types such as mesenchymal stem cells (Maharlooei, Bagheri et al.), hematopoietic stem cells (HSC), endothelial stem cells (Bertozzi, Simonacci et al.), etc. Among different type of stem cells, MSCs afford many advantages for cell therapy such as easiness of harvesting, availability, and multilineal differentiation capacity (Zuk et al. 2002). MSCs which are used in cell therapy can be isolated from various sources including: bone marrow tissue, adipose tissue, umbilical cord tissue, etc. (Kim et al. 2007, Klingemann et al. 2008; Basiouny et al. 2013). Adipose-derived stromal cells (D'andrea, De Francesco et al.) showed better properties compared with other types of MSCs includes ease of accessibility and more proliferation capacity (Kern et al. 2006). Moreover, they demonstrated some properties that could be useful in the clinical application of ASCs, consist of angiogenecity, immunomodulation, and improvement of tissue remodeling. Therefore, ASCs can be suitable stem cells to improve wound healing (D'andrea et al. 2010, Schubert et al. 2011, Vériter et al. 2011, Ferraro et al. 2013, Fromm-Dornieden and Koenen 2013, Tsuji et al. 2014, Vériter et al. 2014, Lafosse et al. 2015). The aim of this review is to discuss about wound healing processes, various wound regeneration and treatment methods, and cell therapy as a new strategy to promote these processes, and the therapeutic effect of ASCs in wound management.

2 Skin

Skin is one of the largest organs in the body in terms of surface area and weight. It carries out a number of essential functions. The primary and main function of skin is protection (Wysocki 1989; Clark et al. 2007). Skin as a first defensive barrier protects the body's internal environment from external foreign agents. Skin's protective function acts through both innate and adaptive parts of the immune system. The first one is included physical, chemical, and biochemical barrier (Antimicrobial peptides (AMPs)), acidic PH and normal micro biota. The second one, is adaptive immunity, which includes T and B lymphocytes and their secretions (Proksch et al. 2008; Harder et al. 2010; Bangert et al. 2011; Baroni et al. 2012). Skin is composed of three layers: epidermis, dermis (papillary and reticularis) and subcutaneous fat layer (Mihm Jr et al. 1976). Skin tissue includes various components such as Collagen. Among various types of collagen, type I and III is dominant in papillary dermis and subcutaneous fat layer (Weber et al. 1984; Wysocki 1989). Sometimes, chemical, physical and thermal injuries can disrupt all functions of skin, especially the protection role and lead to different types of wounds.

3 Wound

Wound is an injury which impairs the natural anatomical structure and function of skin by loss of continuity of skin layers (Wysocki 1989; Atiyeh et al. 2002). There are different systems to classify the wounds. On the basis of etiology, wounds are classified into three types: surgical, traumatic and chronic. According to the depth of tissue loss, wounds are divided into three types:

1. Superficial wounds: there is loss of epidermis and papillary dermis. Scar remaining, wound contraction is negligible. It takes 10 days to heal (short time) if there is an accurate infection prevention (Percival 2002; Rittié 2016).
2. Partial-thickness or deep dermal wounds: In these wounds, there is loss of epidermis and partial loss of dermis with exposure of basement membrane and nerve endings. Healing process will be taken 10–21 days with re-epithelialization, a degree of scar formation, and wound contraction (Percival 2002; Bryant and Nix 2015; Rittié 2016).
3. Full thickness wounds: in this type, dermis layer is lost and some other deeper layers can be damaged too. Granulation formation along with re-epithelialization is required to heal these wounds. These wounds can be classified as both acute and chronic (Percival 2002; Bryant and Nix 2015; Rittié 2016).

Generally, there are three types of wound closure: primary, secondary and delayed primary or tertiary intention. In the primary intention method, surgical wound closure is happened through joining the wound edges by sutures, staples, or tape which reduces the infection risk, tissue loss, volume of drainage (Wysocki 1989; Atiyeh et al. 2002). Secondary closure is a suitable treatment choice for wounds with considerable tissue loss or chronic wounds whit highly contamination mostly. These types of wounds can be healed with excessive granulation, re-epithelization, contraction by excessive scar remaining (Wysocki 1989; Atiyeh et al. 2002). In tertiary intention or delayed primary intention suturing the wounds occurs after a short period of time. During which time wounds are left open to clean themselves (Rittié 2016). Wound healing is a normal bio physiological process that consists of three phases: inflammation, proliferation, tissue remodeling. In normal wound healing, these phases occur in an optimal time and sequence. But sometimes, various factors such as age, gender, infection, and medications can influence the normal process and result in chronic wounds. Chronic wounds are one of the most important issues in the field of medical science because of their consequent influences on the quality of life, economic burden on health care systems and individual expenses (Kapp and Santamaria 2017). Data from developed countries have suggested 1–2% of the population will experience a chronic wound during their lifetime (Enoch and Price 2004; Boateng et al. 2008; Brackman and Coenye 2015). Globally, 25% of diabetic people suffer from chronic wounds. Chronic wounds can be classified into different groups: diabetic foot ulcer (DFU), venous leg ulcers (VLU) and pressure ulcer (Kolaparthy, Sanivarapu et al.), surgical site infection (Di Rocco, Gentile et al.), abscess, or trauma ulcers. Globally, the incidence and prevalence rate of DFU are respectively 1–4% and 5.3–10.5%. In North America, Asia, Europe, Africa, Oceania and Saudi Arabia the prevalence rate of DFU have been estimated to be 13.0%, 5.5%, 5.1%, 7.2%, 3.0%, and 16.8% respectively (Singh et al. 2005; Rahim et al. 2017; Zhang et al. 2017). All over the world, increasing aged population, obesity and its related diseases such as cardiovascular diseases and diabetes have been expected to increase the rates of chronic wounds. Finally, chronic wound as a global issue still needs novel solutions and treatments.

4 Wound Healing

Wound healing is a physiologic mechanism initiated following tissue injury to restore the function and structure. Wound healing has an equivocal meaning of repairing and regeneration. There are differences between these two terms. Although, regeneration is a precise replacement of injured tissues and functions, repair is only a physiologic adaption without care to exact replacement of tissues that is accompanied by scar formation and fibrosis (Metcalfe and Ferguson 2007; Stramer et al. 2007; Reinke and Sorg 2012; You and Han 2014). In general terms, wound healing is a complicated process in which the damaged tissue or organ is repaired and also regenerated. The quality and required time to heal and risk of infection depend on the depth of

wounds and injured or lost tissues (Li et al. 2016). This process is consisted of three sequential stages including inflammation, tissue formation (proliferation), tissue remodeling (Han and Ceilley 2017).

4.1 Inflammation

Hemostasis and phagocytosis are two important events of inflammation (Wysocki 1989). Vascular disruption and extravasation trigger platelets activation. Platelet activation and cytokine secretion induced primary platelet plug. This plug performs two main functions:

1. Stops bleeding.
2. Provides a matrix for inflammatory and other cells required for next stages (Hart 2002; Velnar et al. 2009).

A network of fibrin fibers stabilizes the platelet plug (Laurens et al. 2006). During the inflammatory phase, platelets and immune cells release growth factors and cytokines like TGF-β, IL-1α, TNF-α, PGDF, etc. These factors trigger fibroblasts to induce the collagen, glycosaminoglycan, and proteoglycans synthesis (Laurens et al. 2006). Histamine as a chemical mediator causes vasodilation and increased vascular permeability which allows the infiltration of various cells like polymorphonuclear leukocytes (PMNs) (neutrophils and macrophages) and mononuclear leukocytes. The first cell line (population) of wound healing is PMNs (Stadelmann et al. 1998). PMNs release cytokines and proteolytic enzymes involved in removal of devitalized tissues, foreign matters and organisms (Goldman 2004). The PMNs can't be active for a long time furthermore, after this stage wound macrophages start their role. Mononuclear leukocytes are the subsequent cell population to the PMNs in wounds. They release mitogens, fibroblast chemoattractants and, also clear the wound of old neutrophils (Stadelmann et al. 1998). After inflammation subsidence, fibroblasts become the predominant subsequent cells in wounds (Stadelmann et al. 1998; Enoch and Price 2004). In the next two phases, the fibroblasts functions and ECM expression play an important role in skin repair.

4.2 Proliferation

Angiogenesis, cell migration, re-epithelization, granulation tissue formation, collagen formation and contraction are as the essential events be happened during proliferation phase. Angiogenesis the essential event of this stage provides the oxygen and metabolic needs. Endothelial cells have a key role in this process and their proliferation trigger by vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2). Vascular endothelial growth factor (VEGF) is a protein expressed in keratinocytes at the surface and margins of wound triggers the proliferation of endothelial cells. It is also called vascular permeability factor (VPF) which can increase the permeability of local microvasculature (Brown et al. 1992; Dvorak et al. 1995; Frank et al. 1995). Collagen synthesis by fibroblasts is the basic process of this stage. This process requires an acidic environment hence hypoxia and anaerobic metabolites like lactic acid that trigger the release of growth factors as stimuli for this synthesis. These collagens boost the tissue tensile strength. There are 13 types of collagens in human body which type I and III are more responsible for body tensile strength. In normal skin, the ratio of type I to III is significantly high. During this phase, type III in comparison to type I shows a percentage increase. Fibroblasts provide a ground substance of fibronectin and hyaluronate which acts as a scaffold for collagen deposition, glycosaminoglycans and cells essential to wound repair. On the other hand, macrophages initiate this by releasing TNF-α and PDGF. Later fibroblasts secrete PDGF by themselves. Accumulation of fibroblasts, capillaries, wound macrophages in the scaffold of collagen and other components of ECM (glycosaminoglycans (GAGs) including HA, and the glycoproteins fibronectin and tenascin) is called "granule". Presence of capillaries causes the pink color of these granules (Stadelmann et al. 1998; Enoch and Price 2004;

Goldman 2004). Contraction as a specific feature of chronic wounds can be seen in this stage too. Myofibroblasts attained by the differentiation of fibroblasts to myofibroblasts are responsible for contraction. Transforming growth factor β (TGF-β) is the main stimulant factor of the fibroblasts to contract the collagen (Montesano and Orci 1988; Stadelmann et al. 1998; Ng et al. 2005). The fibronectin clots prepared in the last phase are essential for this stage. Activation, migration, proliferation of keratinocytes cause the wound closure by producing a new layer of epithelium. ECM deposition is conducted by mesenchymal stromal cells (MSC). (Boink et al. 2016; Choi et al. 2016; Kato et al. 2017; Na et al. 2017). β-cantenin is a factor increased in mesenchymal stem cells during the proliferative phase. This factor regulates the dermal fibroblasts proliferation and inhibits the keratinocyte's migration. Studies have shown there is a correlation between β-cantenin and TGF-β. Wound size depends on the β-cantenin expression in wound. β-cantenin also can be a structural protein as a component of cellular adherens junction (Cheon et al. 2006; Bowley et al. 2007; Zhang et al. 2009). ECM deposition and angiogenesis is regulated by transforming growth factor b1 (TGFb1) (Border and Noble 1994; Sankar et al. 1996; Gehris et al. 2003; Boink et al. 2016).

4.3 Remodeling

Remodeling will be started following equilibrium of collagen synthesis and degradation (Stadelmann et al. 1998). During this phase, ECM and collagen fibers prepared in the proliferation phase are remodeled and realigned (Boink et al. 2016). Re-arrangement of collagen network to a more tensile form is accompanied by degradation of prior collagen and ECM. Therefore, mature scars show a normal ratio of type I and III collagen which we can see in intact skins (Witte and Barbul 1997; Stadelmann et al. 1998). Matrix metalloproteinase (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are key enzymes of remodeling. An imbalance between these two enzymes can cause hypertrophic scar (Akita et al. 2010) as a significant feature of chronic wounds. TGF-β can prevent the protease function of MMPs by activating the TIMPs (Yuan and Varga 2001; Chakraborti et al. 2003; Stamenkovic 2003; Zhu et al. 2013). This process is regulated by various cytokines and growth factors. Epidermal growth factor (EGF) family has eleven members: EGF, heparin binding EGF-like growth factor, transforming growth factor-α, amphiregulin, epigen, epiregulin, β-cellulin and the neuregulins (NRGs) NRG-1, NRG-2, NRG-3 and NRG-4. These family members are very important in wound healing and re-epithelialization because the interactions between these factors can affect the final scar formation (Enoch and Price 2004; Gomathysankar et al. 2014; Boink et al. 2016; Chae et al. 2017). Wounds heal in the three forementioned stages. The result of healing will depend on the time and quality of the process. The wound healing society has defined some criteria to distinguish acute and chronic wounds from each other. If the process occurs timely and orderly with a return to relative natural structure and function these wounds will be considered as acute wounds (Robson 1997; Stadelmann et al. 1998). Wounds which have not completed their repairing process timely and orderly or their result is not sufficient enough in terms of structure and function are referred as chronic wounds (Robson 1997; Boateng et al. 2008; Werdin et al. 2009). These wounds seem to get "stuck" in one of the healing phases especially in the inflammation phase and usually healing takes more than 12 weeks (Enoch and Price 2004; Agren and Werthen 2007; Boateng et al. 2008). Sometimes, the presence of foreign objects in the deep wound areas can be the cause of chronic inflammation (Boateng et al. 2008). Chronic wound healing follows the same process of acute wound healing but with little differences in formation of abundant granulation tissue and often with excessive fibrosis leading to scar contraction and loss of function. Wound contraction and the excessive volume of granulation tissue are the special traits of chronic wounds which make them distinguished from the acute wounds (Stadelmann et al. 1998). As described above, various factors

can be the causes of chronic wounds through altering the states of healing processes or personal health state. Some of the mentioned altered states will be discussed generally. High levels of pro-inflammatory cytokines such as TNF-αa, interleukin-1β (IL-1β), and TGF-β1 impairs the proliferation and normal morphologic of skin fibroblasts during the healing process and causes the chronic wound (Enoch and Price 2004). Studies have been reported that fibroblasts in chronic ulcers are similar to controlled ones and both of them can produce all of the ECM components such as fibronectin. Although there is a fibronectin lack in chronic wounds and this lack is more relevant to its degradation by serine proteinases rather than the shortage of its synthesis. The materials derived from fibronectin degradation lead to the MMPs activation. Increased levels of various MMPs and serine proteases and decreased levels of tissue inhibitor of metalloproteinases (TIMPs) cause impaired healing process especially the remodeling phase by inactivating the growth factors and fibronectin. Researches have been suggested by adjusting the concentration of TIMPs as a treatment, we can prevent further degradation of endogenous and exogenous growth factors (Stadelmann et al. 1998; Enoch and Price 2004; Agren and Werthen 2007). Studies have reported the correlation between the overexpression of IL-6 as an immunoregulatory cytokine and Hypertrophic burn scars (HBSs) that is characterized by a collagen accumulation (Xue et al. 2000). Adequate expression of α5β1 integrin by migratory keratinocytes which performs its function in re-epitheliazation results in normal wound healing while low expression of this protein has been reported as one of the main causes of chronic wounds (Enoch and Price 2004; Margadant and Sonnenberg 2010). There are several methods which we offered to overcome chronic wound problems. For instance, antimicrobials, negative pressure wound therapy (NPWT), topical usage of growth factors, hyperbaric oxygen therapy (HBOT), bioengineered skin substitutes, ultrasound and finally the stem cells are some of the therapeutic solutions for chronic wounds (Kranke et al. 2004; Kavros et al. 2008; Pai and Madan 2013; Mulder et al. 2014). According to special characteristic features of stem cells, such as self-renewal, differential potential, using of stem cells has become a novel therapeutic approach for chronic wounds. Several studies have indicated various types of stem cells such as Bone marrow-derived mesenchymal stem cells (BM-MSCs), bone marrow progenitor cells, epidermal stem cells (ESCs), adult ASCs for accelerating the wound healing (Morasso and Tomic-Canic 2005; Maharlooei et al. 2011; Teng et al. 2014). BM-MSCs have been proved to play an important role in tissue repair by releasing growth factors. This type of stem cells have some limitations such as the need to large numbers of bone marrow cells and invasive procedures (Teng et al. 2014). To overcome these limitations, scientists have introduced other sources such as ASCs. Some properties of ASCs such as multipotent capacity, immune privilege, immunomodulatory properties, capability of harvesting with less invasive procedures and less ethical issues have granted privilege to these cells in comparison to other types of stem cells (Nambu et al. 2009; Arjmand et al. 2017).

5 Adipose Tissue-Derived Stromal Cells

5.1 Definition

Identification of different stem cells which can preserve the multi-lineage differentiation ability, is essentially important in developing valuable cell sources for regenerative medicine (Guilak et al. 2006). Among the multiple sources of stem cells, MSCs have attracted more attention during recent years, mainly because of their ability to differentiate into particular cell types and secretion of various biomolecules (Zuk et al. 2002; Desiderio et al. 2013). More than 50 years ago MSCs were isolated from bone marrow for the first time (Frieedenstein et al. 1968). More recently, the adipose tissue was introduced as a new source of MSCs (Zuk et al. 2001). ASCs are easily accessible in plentiful quantities and can be obtained from adipose tissues by less invasive methods. Also, they have a higher proliferation capacity and lower

ethical concerns compared with BM-MSCs. Moreover, ASCs are preferable to BM-MSCs in some biological characteristics, containing the immunosuppressive features (Kern et al. 2006; Schubert et al. 2011; Qomi and Sheykhhasan 2017). ASCs have a multi-lineage potential which can provide a capacity to recover and repair damaged tissues such as injured skin (Tsuji et al. 2014; Tobita et al. 2015). This capacity make them a worth-full solution for cell therapy (Gir et al. 2012; Dai et al. 2016). Additionally, regarded to the importance of using adult stem cells compared to embryonic stem cells in terms of ethical concerns, application of ASCs as adult stem cell sources is highly remarkable in regenerative medicine purposes (Johal et al. 2015; Qomi and Sheykhhasan 2017).

5.2 Isolation and Characterization of ASCs

By the increased incidence of obesity and liposuction surgeries in the world, ASCs can be isolated in large number from different adipose tissues containing subcutaneous and localized fat (Bunnell et al. 2008; Locke et al. 2009; Kolaparthy et al. 2015). The first method to isolate these cells were presented by Rodbell and colleagues in the 1960s (Rodbell 1966; Bunnell et al. 2008). Accordingly, there are two different methods for ASCs isolation including enzymatic and non-enzymatic digestion. Enzymatic digestion (collagenase, trypsin, etc.) as the most usual method, can produce larger number of cell specifically progenitor cells in comparison with other one (Oberbauer et al. 2015; Dai et al. 2016). While, non-enzymatic method involved washing and shaking lipoaspirate is the more considerable method because of it's easy, time and cost-effective procedure (Zimmerlin et al. 2010; Millan et al. 2014; Aronowitz et al. 2015; Oberbauer et al. 2015; Gimble and Wu 2016). In addition, the other source of adipose regenerative cells are stromal vascular fraction (SVF) which consist of heterogeneous cells and can be purified more, to access the ASCs (Han et al. 2015; Pak et al. 2017). ASCs have some characteristics such as the expression of multiple particular MSC surface markers such as CD73, CD90, and CD105, and the lack of expression of endothelial and hematopoietic cell markers (Camilleri et al. 2016; Wankhade et al. 2016; Mildmay-White and Khan 2017). In addition, they can be differentiate into mesodermal cell lineages including adipocytes, chondrocytes, and osteoblast (Dominici et al. 2006; Baer and Geiger 2012).

5.3 Differentiation Potential of ASCs

The potential of ASCs to differentiate into a multiple cell lineages was demonstrated in several studies (Zuk et al. 2001, 2002; Guilak et al. 2006; González-Cruz et al. 2012). According to this potential, ASCs can trans-differentiate into other tissue-specific cells based on various signaling pathways (Tsuji et al. 2014). For instance, implanted ASCs in wound sites are able to trans-differentiate into multiple skin cell types including keratinocytes and fibroblasts which can contribute to wound regeneration (Ebrahimian et al. 2009; Hong et al. 2013; Hassan et al. 2014; Hu et al. 2014; Tsuji et al. 2014; Tobita et al. 2015; Gaspar et al. 2016).

6 The Role of ASCs in Wound Healing Phases

The wound healing cascade is initiated following dermal injury by the release of different cytokines and growth factors (Schaffer and Nanney 1996; Park et al. 2017). Although these cytokines and growth factors are naturally secreted by the cells which involved in wound healing such as fibroblasts and keratinocyte. Furthermore, there are evidences that the paracrine function of stem cells is important in this process. Hence, the angiogenic, proliferative and anti-inflammatory factors that released by ASCs may accelerate wound treatment via different ways (Fig. 1). Some of these factors consist of VEGF, TGF-β1, IGF, HGF, bFGF, KGF, SDF-1, PDGF, and IL-6 (Rehman et al. 2004; Dittmer and Leyh 2014; Hassan et al. 2014; Tsuji et al. 2014; Cerqueira et al. 2016; Ma et al. 2017). These biomolecules play various role in each phase of wound healing process. In the

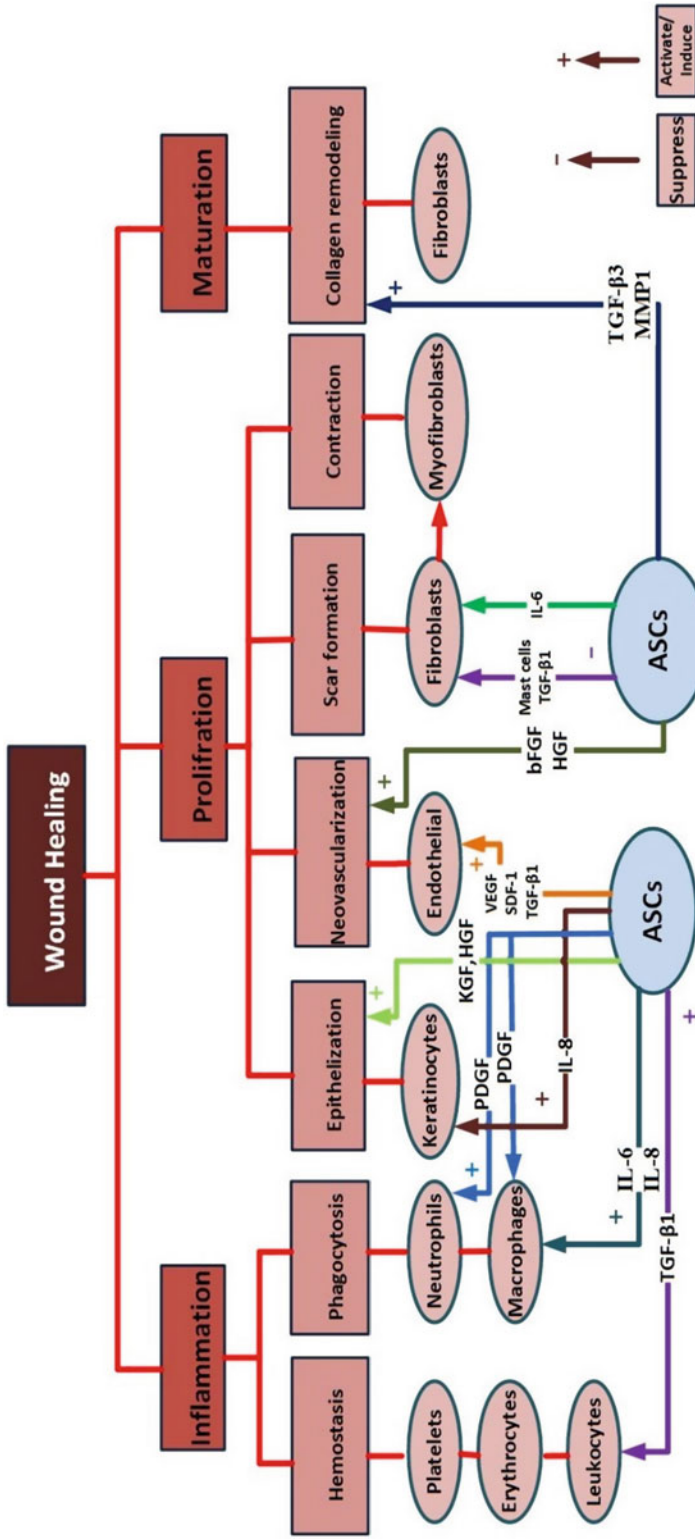

Fig. 1 Cellular and molecular mechanisms of ASCs in wound healing. ASCs affect different phases of wound healing like proliferation, re-epithelization, and angiogenesis by secretion of divers cytokines such as IL-6 and IL-8. IL-8 enhances proliferation and chemotaxis of keratinocytes, directly promote endothelial cell proliferation and survival. On the other hand, IL-8 and IL-6 could enhance activation of macrophages in the inflammation phase. Mast cells and TGF-β1 promote proliferation of fibroblasts that increase collagen synthesis and scar formation. ASCs are involve in fewer scar formation through suppressing the activation of Mast cells and TGF-β1. TGF-β1 can promote migration of leukocytes, erythrocytes and platelets in hemostasis process. TGF-β3 and MMP1 can be released by ASCs in scar remodeling phase and play a pivotal role in this phase of healing. ASCs also release multiple growth factors like PDGF, VEGF, bFGF, KGF, HGF that affect different phases of wound healing (Heo et al. 2011; Yun et al. 2012; Hassan et al. 2014; Yolanda et al. 2014)

inflammation phase, PDGF can stimulate the migration of neutrophils, macrophages, and fibroblasts, TGF-β1 can mediate the migration of leukocytes into the injured tissues, and the increased level of IGF may reduce the activity of proinflammatory cytokines, like TNFα and IL1β (Pierce et al. 1991; Werner and Grose 2003; Pakyari et al. 2013). ASCs can promote angiogenesis, re-epithelialization, neovascularization, collagen synthesizing, and skin cells proliferation by secretion of various growth factors including VEGF, TGF-β1, HGF, KGF, SDF-1, IL-8, and IL-6 during the proliferation phase. In the long time inflammatory conditions, enhance in TGF-β1 expression cause an increase in fibroblasts to myofibroblasts differentiation. Myofibroblasts could prompt contraction with secreting some factors like α-SMA (Crozier 1994; Jimenez and Rampy 1999; Hoeben et al. 2004; Conway et al. 2007; Bao et al. 2009; Matsumoto et al. 2013; Pakyari et al. 2013; Hassan et al. 2014; Johnson and Wilgus 2014; Leonov et al. 2015). Finally, at the last phase or in remodeling phase, ASCs can inhibit the fibroblast proliferation by decreasing the activity of mast cells and TGF-β1, this cause reducing scar formation. ASCs can inhibit the breakdown of collagen by MMP. On the other hand ASCs can lead to collagen remodeling by releasing TGF-β3 and increasing MMP (Yun et al. 2012; Pakyari et al. 2013). Generally, tissue recovery can be improved by ASCs through their differentiation into skin type cells or by their autocrine and paracrine secretions (Zarei and Soleimaninejad 2018).

7 Evidence-Based Applications of ASCs for Healing

AS mentioned in pervious parts, using adipose tissue as an appropriate source of stem cells for cell therapy is extremely considered (Zuk et al. 2001, 2002; Gimble et al. 2012; Aghayan et al. 2014; Larijani et al. 2015). In this regard, several clinical and preclinical researches have exhibited the capability of ASCs to accelerate wound repair (Atala et al. 2010; Gimble et al. 2012; Shingyochi et al. 2015; Bertozzi et al. 2017). Nie, et al. in their preclinical study showed that wound closure in normal diabetic rats can be accelerated by ASCs, via increased epithelialization and granulation tissue deposition (Nie et al. 2011). In addition, according to another study, Rocco, et al. reported improved wound healing in diabetic mice by using genetically modified ASCs (Di Rocco et al. 2011). One of the clinical administration of ASCs was performed in 2007, Rigotti et al. reported that injection of ASCs can be effectively applied for treatment of patients with progressive wounds following radiation therapy (Rigotti et al. 2007). During 2003 to 2010 García-Olmo et al. and also Kim et al. performed invaluable investigations to introduce the therapeutic effects of ASCs on the treatment of enterocutaneous fistulas and facial scars (García-Olmo et al. 2003, 2005, 2008, 2009a, b, 2010; Kim et al. 2011). According to the body of literature, there are no reports of adverse events related to clinical applications of ASCs in wound regeneration. Moreover, previous studies have reported that ASCs can assist normal process of wound healing.

8 Conclusion

Due to the universal health system challenges because of chronic wounds, there is an essential need for designing safe and cost-effective methods for treatment of this type of wounds. Among different therapeutic approaches, cell-assisted wound healing is potentially a qualified curative method. The body of literature in this field has especially focused on effects of MSCs in wound management. ASCs as a member of MSCs family are one of the fascinating cell sources to improve wound healing outcomes. According to their various advantages, they have been considered as a valuable alternative for wound regeneration (Huang et al. 2013; Zuk 2013). As the niche of each cell provides appropriate temperature conditions and chemical signals which regulates the function of the cell (Wagers 2012), it seems that ASCs which extracted from subcutaneous adipose tissue (a promising and cost-effective source of autologous ASCs) are more useful for repairing dermal

injuries. Based on several clinical and preclinical studies there are various delivery approaches for ASC therapy including; local injection, and using different scaffolds such as collagen gel, fibrin, cell sheets and etc. (Nambu et al. 2009; Lee et al. 2011; Steinberg et al. 2012; Lin et al. 2013; Rodriguez et al. 2015; Hanson et al. 2016; Dash et al. 2018) therefore different application methods have provided various options to use ASCs for many clinical indications. Additionally, Cell-Assisted Lipotransfer (CAL) is one of the applications of ASCs which have become one of the novel stem cells transplantation strategies specifically in field of skin reconstruction (Yoshimura et al. 2008). In the CAL as an autologous tissue transfer method, fat derived ASCs are attached to the aspirating fat which acts as a living scaffolds to provide optimized condition for grafting. In this strategy, ASCs promote graft survival and angiogenesis because of their abilities for differentiate into endothelial and vascular cells (Matsumoto et al. 2006). Therefore, angiogenesis and vascularization play a vital role in the same methods of wound healing. Further, ASCs have major potential to release angiogenic, vasculogenic, and other factors. Hence, they can stimulate their surrounding cells through the paracrine angiogenic and vasculogenic effects and accelerate wound treatment.

Although, we described some fundamental roles of ASCs in wound regeneration by their multifactorial mechanisms, but further basic researches and efficacy clinical trials are needed to determine the optimal delivery methods of ASC therapy and developing the use of ASCs in wound healing.

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Selection of Suitable Reference Genes for Quantitative Real-Time PCR Normalization in Human Stem Cell Research

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Abstract

Quantitative real-time polymerase chain reaction (qRT-PCR) is a widely utilized method for evaluating the gene expressions in stem cell research. This method enables researchers to obtain fast and precise results, but the accuracy of the data depends on certain factors, such as those associated with biological sample preparation and PCR efficiency. In order to achieve accurate and reliable results, it is of utmost importance to designate the reference genes, the expressions of which are suitable to all kinds of experimental conditions. Hence it is vital to normalize the qRT-PCR data by using the reference genes. In recent years, it has been found that the expression levels of reference genes widely used in stem cell research present a substantial amount of variation and are not necessarily suitable for normalization. This chapter at hand stresses the significance of selecting suitable reference genes from the point view of human stem cell research.

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Keywords

Reference genes · qRT-PCR normalization · qRT-PCR · Stem cells · Human · Housekeeping genes

Abbreviations

18S rRNA	18S Ribosomal RNA
2-D	Two-dimensional
3-D	Three-dimensional
3′UTR	3′ untranslated region of the genes
ACTB	Actin beta
ALAS1	5′-Aminolevulinate synthase 1
ASCs	Adipose stem cells
B2M	Beta-2-microglobulin
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CAPN10	Calpain 10
CASC3	Cancer susceptibility 3
CCDC108	Cilia and flagella associated protein 65
CSCs	Cancer stem cells
EF1α	Eukaryotic translation elongation factor 1 alpha 1
EID2	EP300 interacting inhibitor of differentiation 2
ESCs	Embryonic stem cells
FBXL12	F-Box and leucine rich repeat protein 12

fMSCs	Fetal tissue-derived MSCs
GADD45A	Growth arrest and DNA damage inducible alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSCs	Gingival stem cells
GUSB	Glucuronidase beta
h	Human
HMBS	Hydroxymethylbilane synthase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HSCs	Hematopoietic stem cells
IPO8	Importin 8
iPSCs	Induced pluripotent stem cells
LTB4R2	Leukotriene B4 receptor 2
MIAMI	Marrow-isolated adult multilineage inducible
MSCs	Mesenchymal stem cells
NUBP1	Nucleotide binding protein 1
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PPIA	Peptidylprolyl isomerase A
PUM1	Pumilio RNA binding family member 1
qRT-PCR	Quantitative real-time PCR
RABEP2	Rabaptin, RAB GTPase binding effector protein 2
RNF7	Ring finger protein 7
RPL13A	Ribosomal protein L13a
RPL19	Ribosomal protein 19
RPLP0	Ribosomal protein lateral stalk subunit P0
RPS18	Ribosomal protein S18
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
SLC4A1AP	Solute carrier family 4 member 1 adaptor protein
SRP72	Signal recognition particle 72
TBP	TATA-box binding protein
TFRC	Transferrin receptor
TNFRSF13C	TNF receptor superfamily member 13C
UBC	Ubiquitin
VEGF	Vascular endothelial growth factor A-165
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
ZNF324B	Zinc finger protein 324B

1 Introduction

Stem cells are defined as the cells with the ability to self-renew and differentiate into various mature cell types (Lanza and Atala 2014). They can be simply classified depending on their source (*e.g.* embryonic or adult stem cells), or potency (*e.g.* pluripotent or multipotent stem cells). Even though embryonic stem cells (ESCs) possess a much higher capacity than adult stem cells to differentiate into all cells of the body, because of ethical restrictions and other problems their use in clinical applications is quite limited (Elçin 2004). Adult stem cells are multipotent cells found in a number of different organs of the human body, such as the bone marrow, adipose tissue, blood, skin and liver. Although their potency is limited, they are easier to obtain, and can differentiate into various lineages, such as the bone, cartilage and fat cells (Passier and Mummery 2003).

In order for stem cells to be better understood and used effectively in the treatment of diseases, their self-renewal, proliferation and differentiation properties need to be quantitatively evaluated (Ragni et al. 2013). The quantitative real-time polymerase chain reaction (qRT-PCR) is the method widely used to identify the gene expressions of stem cells. qRT-PCR is a user-friendly technique which can quite accurately identify the changes in the mRNA expression levels and can give reproducible and verifiable results. The accuracy of the results obtained is affected by certain internal and external factors, such as the amount of the sample, preparation of the RNA, cDNA synthesis, and productivity of PCR. Therefore, the gene expression levels should be

normalized through comparison with the reference genes (Li et al. 2015; Kang et al. 2018).

The reference genes to be used for normalization should not be affected by the cell passage number, cell cycle status, various experimental conditions, and must be expressed decisively by different samples (Radonic et al. 2004; Kang et al. 2018). Housekeeping genes which are used as reference genes are vital for cellular life; they exist in all nuclear cells, and are expressed constantly in a number of different tissues (Ragni et al. 2013). The first study on the possible variations in the expressions of housekeeping genes was published by Schmittgen and Zakrajsek (2000). However, in recent times scientists have come to the fore with some important findings on housekeeping genes widely used in research, among which are the facts about their expressions being variable, and that they are not common for different cell types and experimental conditions (Li et al. 2015, 2017; Zhang et al. 2016).

Currently, it is a known fact that housekeeping genes are far from being common, and may not be reliable as reference genes to be used for different cell types and experimental conditions (Amable et al. 2013; Li et al. 2017; Aggarwal et al. 2018). Hence it is of utmost importance to select a reference gene, which is suitable for the particular cell type and experimental condition in use, to attain accurate and reliable results. In order to improve the accuracy and reliability of the results, either the selected reference gene needs to be optimized to the working conditions, or at least two reference genes should to be used together (Vandesompele et al. 2002; Thellin et al. 1999).

In this chapter, the significance of identifying the reference genes suitable for various experimental conditions is stressed. Hence, certain stem-cell types and reference genes common for different experimental conditions are classified to guide those researchers who are dealing with the issues of gene expression and function in stem cell research.

2 Normalization Methods Used in Stem Cell qRT-PCR Studies

Studies on gene expression and function constitute the main field of study of cellular and molecular biology. Gene profiling studies have acquired prominence especially in recent years. These studies contain significant information required for identifying and better understanding the molecular mechanisms of diseases and developing new systems needed in bioengineering applications (Butte et al. 2001; Eisenberg and Levanon 2003).

Various methods, such as the northern blotting, in-situ hybridization, microarray analysis, and qRT-PCR are widely used in gene expression studies (Radonic et al. 2004; Zhang et al. 2016). However, qRT-PCR is the most frequently used method in the identification of stem-cell gene expressions (Li et al. 2015). In this method, a certain region of the DNA molecule is enzymatically multiplied under in-vitro conditions, and quantified in real-time.

A number of strategies have been developed in order that the most suitable normalization method can be selected and applied in qRT-PCR (Rebouças et al. 2013; Huggett et al. 2005). One of the strategies is to use tissue samples having the same size and volume together. Although this approach is easy to implement, the fact that the samples can exist in different conditions and are not biologically identical to one another, it can lead to experimental errors (Rebouças et al. 2013). Another strategy is the usage of either reference or housekeeping genes for normalization. The measuring of the reference genes together with the target genes in a parallel way is regarded as the most reliable method in the normalization of samples (Vandesompele et al. 2002). This strategy can be applied to most of the variants introduced before or during the PCR (Spiegelaere et al. 2015).

Despite the fact that the reference gene strategy is regarded as mostly reliable, stability of the reference gene can vary in the same types of cells

or tissues under different experimental conditions (Ceelen et al. 2011; Neuvians et al. 2005). For this reason, a preliminary work has to be done before the actual study, so as to identify the most stable reference genes for every cell type and experimental condition. In this preliminary work, the calculation of a normalization factor based on the geometric average of the reference genes and the most stable reference genes are selected and used according to this factor (Pfaffl et al. 2004). It is surprising to observe from the literature that this strategy is still not prevalently applied. Most researchers tend to prefer to use a single reference gene in general (Bustin et al. 2013; Ceelen et al. 2014; Zhang et al. 2016). As for the stem-cell studies, this strategy appears to be used in a more wide-spread fashion than the other fields. Also, various software that compare the stability of the reference genes have been developed so as to ease the usage of more than one reference gene together (Spiegelaere et al. 2015; Zhang et al. 2016).

3 Reference Genes Used for Normalization in Stem-Cell qRT-PCR Studies

Identification of the suitable reference genes has become one of the main problematic areas in molecular biology (Stürzenbaum and Kille 2001). Thus, hundreds of new reference genes have been identified by microarray studies, and some of them have been used for normalization (Warrington et al. 2000). The traditional reference genes widely used in qRT-PCR are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), β-tubulin, phosphoglycerate kinase (PGK), ubiquitin (UBC), ribosomal protein 19 (RPL19), and 18S ribosomal RNA (18S rRNA) (Rebouças et al. 2013).

The selection of the suitable reference genes in studies on proliferation and differentiation of stem cells is far from being an easy and straightforward step. Differentiation of stem cells not only comprises various morphological changes, but also alters the expression level of many genes (Vossaert et al. 2013; Van De Moosdjik and Van Amerongen 2016; Mughal et al. 2018).

A number of studies performed in recent years with hESCs and with adult stem cells from different sources have demonstrated that widely used reference genes possess variable expression levels and therefore are not suitable for normalization (Synnergren et al. 2007; Willems et al. 2006; Fink et al. 2008; Quiroz et al. 2010; Curtis et al. 2010; Wang et al. 2010). It is assumed that the variation in expression levels is due to cellular changes during differentiation (Vossaert et al. 2013). Besides, it is possible that the applied differentiation protocols may also have an influence on reference gene expressions (Vossaert et al. 2013; Synnergren et al. 2007; Willems et al. 2006). Therefore, in the identification of the stable reference genes optimization related to different cell types and differentiation protocols is required (Vossaert et al. 2013).

3.1 Reference Genes Used in Pluripotent Stem Cell Studies

ESCs are obtained from the inner cell mass of the embryos in the blastocyte phase. They have the potential to proliferate in an unlimited fashion, and have the ability to differentiate into all cell types of the human body (Thomson et al. 1998; Amit et al. 2000). Because of this potential, ESCs have a high significance in research of fundamental mechanisms of cell differentiation and development.

The fundamental molecular mechanisms of the differentiation and development of the human ESCs are still not completely known (Han et al. 2013; Noaksson et al. 2005). The qRT-PCR is the most frequently used method both in the measurement of the expressions of, for example Oct4 and Nanog transcription factors, the main pluripotency genes in the characterization of ESCs, and the expressions of which decrease considerably during differentiation (Boyer et al. 2005; Noaksson et al. 2005; Draper et al. 2002). The qRT-PCR method and selection of the suitable reference genes are of utmost importance

for revealing the real potentials of the ESCs and explaining in detail the mechanisms which direct cellular differentiation (Abeyta et al. 2004; Bhattacharya et al. 2004; Synnergren et al. 2007; Vossaert et al. 2013; Holmgren et al. 2015).

Compared to somatic cells, studies on stem-cell reference genes are quite limited, and only a small number of these have been conducted by using hESCs or human induced pluripotent stem cells (hiPSCs) (Holmgren et al. 2015). The stability of the traditional reference genes used in the differentiation of the human ESCs was first investigated by Synnergren and co-workers (2007). Their study in which three different human ESC lines were used has shown that most of the traditional reference genes have quite variable expression levels, and therefore are not suitable for human ESCs. It has been observed that only a small number of genes have been expressed in a stable manner. It is assumed that this difference stems from the genetic variation in each of the cell lines and the difference in their initial cultures (Abeyta et al. 2004). It has been stated that the number of passages may have an effect on the forming of this difference (Enver et al. 2005; Maitra et al. 2005). Synnergren and co-workers have managed to identify six different reference genes which exhibit stable expressions (Synnergren et al. 2007) (Table 1).

In another study, Willems and co-workers (2006) have evaluated the stability of the reference genes in cells from the mouse embryo, mouse ESCs, and two different human ESC lines. Contrary to the previous study (Synnergren et al. 2007), the 18S rRNA, GAPDH, and UBC genes were identified as the most stable reference genes during the differentiation of human ESCs, and the ACTB, HPRT1 and B2M genes were assessed as low in the stability ranking. The expressions of the PGK1 and TBP genes have surprisingly exhibited significant differences on the two cell lines. While the TBP gene was identified as the most stable gene in one cell line, it was identified on the other hand as a gene with the lowest stability in another cell line. Similar results were obtained for the PGK1 gene as well. In the process of the mouse ESC differentiation, it was found that the GAPDH, ACTB, and PGK1 genes were the most stable ones (Willems et al. 2006). The fact that the ACTB gene has been identified as one of the most stable genes in the mouse ESCs, whereas it has been in a lower stability ranking in the human ESCs shows clearly the importance of selecting the right gene for different biological samples, species and cell types.

Vossaert et al. (2013) have stated that B2M, RPL13A genes, and the Alu repeats have been identified as the most stable genes in the differentiation of the human ESCs. The human ESCs were differentiated by retinoic acid and the stability of the reference genes was compared. RPL13A gene and the Alu repeats have been identified as the most stable genes. Nevertheless when B2M, RPL13A and Alu repeats and the normalization data obtained from the TBP, RPL13A, and Alu repeats were compared, the difference was found to be minute. While some suggest that the use of three reference genes together for normalizing the qRT-PCR data, some others state that two genes are sufficient (Vandesompele et al. 2002). What differs in the study by Vossaert et al. (2013) is that, a new normalization method based on the measurement of the expression levels of the Alu repeats has been developed. Alu insertions are repetitive DNA sequences, ~300 base pair long, possessing a high number of copies and are found in the introns, the 3′ untranslated region of the genes (3′UTR) and in the intergenic rich regions (Batzer and Deininger 2002). The expressions of the Alu repeats are not influenced by the changes in the expressions of other genes, since they exist in the 3′UTR regions of the genes coding the proteins, and are spread along the genome. Also, primary specificity has only a small effect on this reference gene. As it is expressed in high levels, it has low Cq values even in the cases where sample amount is low (Vandesompele et al. 2002; Batzer and Deininger 2002). Alu repeats, owing to these advantages are thought to be an interesting and

Table 1 Reference genes used in human pluripotent stem cell studies

Gene symbol	Gene name	Accession number	Function	Reference
For human embryonic stem cells				
18S rRNA	18S Ribosomal RNA	X03205	Translation	Willems et al. (2006)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Metabolism/glycolysis	
UBC	Ubiquitin C	NM_021009	Protein degradation	
RNF7	Ring finger protein 7	Hs.134623	Ring finger protein	Synnergren et al. (2007)
FBXL12	F-Box and leucine rich repeat protein 12	Hs.12439	Protein-ubiquitin ligases	
NUBP1	Nucleotide binding protein 1	Hs.81469	ATP-binding proteins	
SRP72	Signal recognition particle 72	Hs.237825	Targets of secretory proteins to the endoplasmic reticulum	
SLC4A1AP	Solute carrier family 4 member 1 adaptor protein	Hs.306000	Data not available for function	
B2M	Beta-2-microglobulin	NM_004048	Immune response	Vossaert et al. (2013)
RPL13A	Ribosomal protein L13a	NM_012423	Component of the 60S subunit	
Alu repeats	–	–	–	
Both for human embryonic stem cells and human induced pluripotent stem cells				
EID2	EP300 interacting inhibitor of differentiation 2	Hs.18949	Data not available for function	Holmgren et al. (2015)
TNFRSF13C	TNF receptor superfamily member 13C	Hs.344088	B-cell survival in vitro/Regulator of B-cell population	
ZNF324B	Zinc finger protein 324B	Hs.186970	Data not available for function	
CAPN10	Calpain 10	Hs.112218	Calcium-dependent cysteine proteases	
RABEP2	Rabaptin, RAB GTPase binding effector protein 2	Hs.555978	Data not available for function	
LTB4R2	Leukotriene B4 receptor 2	Hs.642693	Data not available for function	
CCDC108	Cilia and flagella associated protein 65	Hs.147762	May be a transmembrane protein	

reliable new focus for the normalization of the qRT-PCR data in studies with stem cells (Batzer and Deininger 2002; Vossaert et al. 2013). In spite of its advantages, it is ambiguous whether this new strategy can become prevalent in directed differentiation studies with hiPSCs (Holmgren et al. 2015).

Studies investigating the stability of reference genes during hiPSC differentiation are very limited. In a microarray study, stable reference genes were identified during differentiation of hESCs and hiPSCs into the ectodermal, mesodermal, and endodermal lineages (Synnergren et al. 2007). This study revealed interesting results. For example, ACTB gene was identified as a stable gene for iPSCs, apart from the ectodermal differentiation, while other studies had demonstrated that ACTB was not suitable for hESC differentiation (Synnergren et al. 2007; Holmgren et al. 2015). Similarly, while the usage of the B2M gene for normalization during hESC differentiation was indicated as a possibility by Vossaert et al. (2013), this study reports that it is not reliable for the normalization of hiPSCs (Holmgren et al. 2015). To conclude the study, seven different genes (Table 1), which display a stable expression and are common for all the hESCs and hiPSCs have been identified (Holmgren et al. 2015). When studies carried out by using human ESCs and iPSCs are considered together, it becomes clear that traditional reference genes are generally not suited for normalization in human pluripotent stem cell research and reference genes have to be optimized for each stem cell differentiation protocol (Holmgren et al. 2015).

3.2 Reference Genes Used in Mesenchymal Stem Cell Studies

MSCs are adult stem cells which have the ability to self-renew and basically differentiate into cells of the mesenchyme, such as the osteoblasts, chondrocytes, and adipocytes (Pittenger et al. 1999). MSCs have immunomodulatory properties which make them an appropriate cell source for cell-based therapeutic approaches and regenerative applications (Ucceli et al. 2008). The selection of the suitable MSC source in specific regenerative medicine application depends on the nature of the study (Ragni et al. 2013). In order to illuminate and better understand the molecular mechanisms that control these abilities of the MSCs, it is necessary to quantitatively identify the gene expression profiles. The qRT-PCR method is widely used for this purpose (Curtis et al. 2010; Su et al. 2016; Elçin et al. 2017). The identification of the suitable reference genes that can be used during expansion and differentiation is vital for the MSCs, in order to be effectively used in clinical applications and continuation of research in this field.

3.2.1 Studies with Human Bone Marrow-Mesenchymal Stem Cells

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are a group of heterogenous cell population consisting of progenitors with the ability to self-renew and differentiate into cells of the connective tissue (*i.e.* bone, cartilage, adipose and muscle) (Dominici et al. 2006; Vater et al. 2011). In recent years, the use of autologous BM-MSCs has become quite wide-spread in cell-based treatments (Bianco et al. 2013). The osteogenic differentiation ability of BM-MSCs is especially utilized in bone tissue engineering studies involving scaffolds and bioreactor culture systems (Baykan et al. 2014). The in-vitro differentiation properties of BM-MSCs are generally evaluated using the histochemical and immunohistochemical techniques as a first step. However, various stages of cell differentiation need to be quantitatively identified so as to optimize the conditions, determine the kinetics of differentiation and compare the responses of different cell types to differentiation inducers (Amable et al. 2013). Thus, studies evaluating the differentiation properties of human BM-MSCs using qRT-PCR appear to be gradually on the rise (Quiroz et al. 2010). However, reference genes suitable and reliable to be used in human BM-MSC studies for different growth environments and differentiation conditions have not yet been fully identified. The research conducted so far shows that the reference genes widely used possess variable expression levels and are unsuited for BM-MSC studies (Curtis et al. 2010; Quiroz et al. 2010; Studer et al. 2012; Amable et al. 2013; Jacobi et al. 2013; Ragni et al. 2013; Schildberg et al. 2013; Rauh et al. 2014; Tratwal et al. 2014; Li et al. 2015).

The first study investigating the stability of the genes used in human BM-MSCs appeared in 2010 (Quiroz et al. 2010). This group investigated the expression stabilities of the two most frequently used reference genes, ACTB and GAPDH, and the RPL13A gene at two different stages of osteogenic differentiation (*i.e.* 14th and 20th days). This study concluded that the ACTB gene expression rises all through the differentiation process, leading to misinterpretation of the expression levels of osteogenic markers. Quaroz et al. state that the GAPDH expression shows variation between undifferentiated control cells and osteogenically-differentiated cells. The expressions of the osteogenic markers in the undifferentiated control cells are remarked to have risen as a result of the variation in the GAPDH expression. Thus, the ACTB and GAPDH reference genes are mentioned as unsuitable for the normalization of hBM-MSCs in osteogenic differentiation studies. On the other hand, the RPL13A gene has been presented as suitable for normalization, since the expression level was stable throughout the osteogenic differentiation. As for situations, in which GAPDH is used as the reference gene, it is emphasized that calibration is needed at various points in time of the study (Quiroz et al. 2010). Jacobi and co-workers have established in a similar study the GADD45A, PUM1 and RPLP0 genes as the most stable to

be used for normalization of hBM-MSCs. As for the ACTB, GAPDH, 18S rRNA and B2M genes, it was emphasized that they were not suited for these experimental conditions (Jacobi et al. 2013).

Another study conducted by Curtis et al., in which hBM-MSCs were used together with the marrow-isolated adult multilineage inducible (MIAMI) cells and RS-1 [Human leukemia] knockout cell lines, investigated the reference genes, whose expression was stable during neuronal and endothelial differentiation of these cells (Curtis et al. 2010). While the EF1α and RPL13A were identified as the most suitable two reference genes for proliferation, and differentiation of the cell lines and inter-species analyses, it was confirmed that expression of GAPDH showed a high rate of variation. This study has also shown that the YWHAZ and RPL13A genes other than the EF1α gene were the most suitable reference genes for normalization, in the inter-species study where human MIAMI cells were transplanted to rats to repair tissue damage (Curtis et al. 2010).

Studer and co-workers' 2012 study, which investigated the expression stability of the reference genes during osteogenic, chondrogenic and adipogenic differentiation was a first in its realm. They studied the expression levels of 7 reference genes widely-used in MSC research for the 9th, 16th and 22nd days of hBM-MSC differentiation. In this study, they used two different passages; *i.e.* passage 1 and 4 of hBM-MSCs and identified the RPL13A reference gene as the most suitable gene for normalization, reaching a conclusion similar to the previous researches (Quiroz et al. 2010; Curtis et al. 2010). Studer et al. (2012) have proposed the usage of the RPL13A as the single reference gene, since it has a high stability, rather than multiple reference genes recommended in Vandesompele and co-workers' study (2002). GAPDH and ACTB reference genes have been identified as genes, whose expressions vary the most and stated as unsuitable for differentiation studies by Studer et al. (2012).

Different results have been achieved in some other studies evaluating the expression stability of reference genes. Ragni and his team have found that the expression level of the TBP gene did not change; was expressed decisively and appeared to be one of the most stable genes during differentiation (Ragni et al. 2013). TBP gene has been established as one of the least stable reference genes in the differentiation of BM-MSCs and its usage has not been recommended in another study (Li et al. 2015). Several other studies have also reached the conclusion that expression levels of YWHAZ, PPIA, HPRT1 and RPL13A genes did not change and they could be used as the reference genes in research related to the differentiation of BM-MSCs (Amable et al. 2013; Ragni et al. 2013; Li et al. 2015). Despite the fact that the B2M gene, described as a gene whose stability is the lowest in differentiation studies (Amable et al. 2013; Ragni et al. 2013), it was only recommended to be used as a reference gene for hBM-MSC research in one study (Li et al. 2015).

In a study during which human BM-MSCs and adipose stem cells (ASCs) were differentiated into endothelial cells, human BM-MSCs were stimulated with vascular endothelial growth factor A-165 (VEGF) for 1 week and the stability of 9 reference genes which were widely used in differentiated cells and the unstimulated cells in the control group were evaluated. The conclusion of the study was that TBP was a gene with the highest stability in both the BM-MSCs and differentiation of ASCs into endothelial cells and could be used as a reliable reference gene. TBP and YWHAZ were presented as the most suitable double gene combination that could be used (Tratwal et al. 2014).

It is evident that the cultivation of cells in either two-dimensional (2-D) or three-dimensional (3-D) culture has significant impact on cellular behavior and function, such as intercellular communications controlled by gene expression (Dogan et al. 2016). BM-MSCs have been expanded and differentiated prevalently in 2-D cultures in most of the previous studies. However, it has been shown that hMSCs demonstrate biomimetic and more realistic properties when cultivated in 3-D (Caplan AI 2005; Inanc et al. 2008; Amini et al. 2012). Rauh and his team comparatively evaluated the expression of reference genes during the proliferation and

osteogenic differentiation of human BM-MSCs in both 2-D and 3-D environments for the first time (Rauh et al. 2015). They have observed that TBP, TFRC and HPRT1 were expressed decisively in 3-D culture environment and during differentiation, and were identified as the most stable reference genes. As for the ACTB and RPL37A genes, their expression levels were found to be quite variable and hence unsuitable for normalization as this would lead to false results (Rauh et al. 2015).

Another study which dwelled on the comparison of reference gene expressions, obtained from healthy and osteoarthritis patients has investigated the effect of the disease condition on reference gene expressions. This particular study has identified IPO8, TBP and CASC3, obtained from osteoarthritis patients as genes with the highest stability rating and recommended the usage of these in osteoarthritis studies. On the other hand, the ACTB and B2M were not found suitable with their low expression stabilities (Schildberg et al. 2013).

All these studies, conducted with BM-MSCs show that the RPL13A, YWHAZ, PPIA and HPRT1 are reliable reference genes for research on proliferation, differentiation into the three mesodermal lineages, and can be used in normalization (Table 2). The number of studies focusing on reference gene expressions, in which BM-MSCs are used for various diseases and environmental conditions is unfortunately inadequate. Research on reference gene stability in various diseases and environment conditions has to increase in number and reliable reference genes need to be identified so that the BM-MSCs can effectively be used in tissue engineering and regenerative medicine fields.

3.2.2 Studies with Human Adipose Stem Cells

Adipose stem cells are multipotent stem cells obtained from the stromal vascular fraction of the fat tissue, which have the ability to differentiate into a number of lineages, including osteogenic, chondrogenic, myogenic and neurogenic (Zuk et al. 2002; De Francesco et al. 2015). ASCs are suitable candidates for cell-based treatments, owing to certain features they possess. For instance, they can easily be isolated in high numbers; have high potential for proliferation, and exhibit low immunogenicity (Kim and Sung 2017; Palombella et al. 2017). ASCs regulate immune response by secreting various paracrine factors to repair the damaged tissues, stimulate angiogenesis, and contribute directly to tissue-repair (Park et al. 2008; Gimble et al. 2007, 2011; Palombella et al. 2017). In order to investigate these significant features of human ASCs and to benefit from them, first the variations occurring in the cells at the transcriptional level need to be identified. qRT-PCR method is usually employed for identifying these changes (Tratwal et al. 2014; Su et al. 2016; Palombella et al. 2017).

Reliable reference genes which can be used for normalization in ASC studies are not clear and definite as in BM-MSCs (Tratwal et al. 2014). The stability of the reference genes widely used during the expansion and differentiation of human ASCs have first been researched by Fink and his team of scientists (Fink et al. 2008). They have compared the expression levels of 12 reference genes of ASCs cultured under hypoxic conditions and the suitable reference genes that could be used for normalization during osteogenic and chondrogenic differentiation. As a result of their research, they have found that the expression levels of YWHAZ, TBP and GUSB were stable for all experimental conditions and suitable for normalization. On the other hand, expression levels of 18S rRNA, GAPDH and ACTB genes were found to be quite variable and not suitable to be used for normalization (Fink et al. 2008).

Similar results have been found in other studies focusing on the stability of reference genes during differentiation of human ASCs into other lineages. Ragni and his team have observed that RPL13A, together with YWHAZ and TBP was a reference gene, having the best performance in differentiation studies and could be used for normalization. They have also found that 18S rRNA, B2M and ACTB were not suitable (Ragni et al. 2013). In a similar study, RPL13A has been identified as the most suitable gene for

Table 2 Reference genes used in human bone marrow-mesenchymal stem cell studies

Gene symbol	Gene name	Accession number	Function	References
Osteogenic differentiation				
GADD45A	Growth arrest and DNA damage inducible alpha	Hs00169255_m1	Stress response mechanism	Jacobi et al. (2013)
PUM1	Pumilio RNA binding family member 1	Hs00206469_m1	Translation, membrane organization	
RPLP0	Ribosomal protein lateral stalk subunit P0	Hs99999902_m1	Protein synthesis	
TFRC	Transferrin receptor	Hs99999911_m1	Vesicle transport	Rauh et al. (2015)
Osteoarthritis				
IPO8	Importin 8	Hs00183533_m1	Nuclear import of proteins	Schildberg et al. (2013)
CASC3	Cancer susceptibility 3	Hs00201226_m1	Core component of the exon junction complex	
Osteogenic and adipogenic differentiation				
PPIA	Peptidylprolyl isomerase A	NM_021130	Peptide binding	Li et al. (2015)
B2M	Beta-2-microglobulin	NM_004048	Immune response	
Osteogenic, adipogenic & chondrogenic differentiation				
GUSB	Glucuronidase beta	NM_000181.3	Metabolic pathways	Ragni et al. (2013), Amable et al. (2013), and Rauh et al. (2015)
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_000194.2	Nucleotide salvaging	
Osteogenic, adipogenic, chondrogenic, neural, endothelial, cerebral ischemia, interspecies analysis, cardiac differentiation				
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta	NM_003406	Signal transduction	Curtis et al. (2010), Quiroz et al. (2010), Studer et al. (2012), Amable et al. (2013), Ragni et al. (2013), Schildberg et al. (2013), Tratwal et al. (2014), Li et al. (2015), and Rauh et al. (2015)
TBP	TATA-box binding protein	NM_003194	Transcription, metabolic pathways	
RPL13A	Ribosomal protein L13a	NM_012423	Component of the 60S subunit	
Neural and endothelial differentiation				
EF1α	Eukaryotic translation elongation factor 1 alpha 1	NM_001402	Translation	Curtis et al. (2010)

normalization in the differentiation of ASCs. As for the ACTB, expression level was observed to be unstable during the ASC differentiation (Amable et al. 2013).

Almost in each other study, a different gene has been indicated for normalization of human ASCs (Table 3). For example, B2M gene has been found as suitable for normalization during trilineage differentiation in one study (Amable

Table 3 Reference genes used in human adipose stem cell studies

Gene symbol	Gene name	Accession number	Function	References
Proliferation				
PPIA	Peptidylprolyl isomerase A	NM_021130	Peptide binding	Su et al. (2016)
RPS18	Ribosomal protein S18	NM_022551.2	Component of the 40S subunit	Palombella et al. (2017)
Co-culture with glioma cell line				
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_000194.2	Nucleotide salvaging	Iser et al. (2015)
Osteogenic and chondrogenic differentiation				
GUSB	Glucuronidase beta	NM_000181.3	Metabolic pathways	Fink et al. (2008)
Osteogenic, adipogenic & chondrogenic differentiation				
B2M	Beta-2-microglobulin	NM_004048	Immune response	Amable et al. (2013), Ragni et al. (2013), and Palombella et al. (2017)
RPL13A	Ribosomal protein L13a	NM_012423	Component of the 60S subunit	
Osteogenic, adipogenic, chondrogenic and endothelial differentiation; proliferation in hypoxic culture				
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-Monooxygenase activation protein zeta	NM_003406	Signal transduction	Fink et al. (2008), Ragni et al. (2013), Tratwal et al. (2014), and Iser et al. (2015)
TBP	TATA-box binding protein	NM_003194	Transcription, metabolic pathways	

et al. 2013), it was found as unsuited in another one (Ragni et al. 2013). Although GUSB was identified as an ideal reference gene for hASCs in one study (Ragni et al. 2013), in another one it was placed in the middle of the reference genes ranking row (Fink et al. 2008). As for the GADPH, it was identified as a gene, having the lowest decisiveness in the proliferation and trilineage differentiation experiments in one study (Amable et al. 2013), but was placed in the middle of the reference genes row in other ones (Fink et al. 2008; Ragni et al. 2013). The fact that a common reference gene cannot be identified for human ASCs and various results are achieved for the same genes in each of the studies constitutes a substantial problem for researchers in the field. However, continuation of studies will enable identification of suitable genes, and elimination of incongruency among the results.

3.2.3 Studies with Human Fetal Mesenchymal Stem Cells

Studies carried out with human fetal tissue-derived MSCs (hfMSCs) are much lesser than the ones with hBM-MSCs and hASCs (Brady et al. 2014). Comparative studies conducted with hfMSCs and adult hMSCs have shown that the hfMSCs at early stages have a higher proliferation ability, differentiation potential and biological activity (Jo et al. 2013). To clarify the main mechanisms of self-renewal, differentiation into specialized cells and tissue-repair features of hfMSCs, their gene expression profiles need to be evaluated in detail (Li et al. 2015). Nevertheless, the number of reference gene studies with hfMSCs is quite limited (Table 4). The first study was on the identification of reference genes showing stable expression during osteogenic, adipogenic, and chondrogenic differentiation of hfMSCs (Ragni et al. 2013). GUSB, RPLP0 and TBP were found as genes with high level of consistency in the differentiation of umbilical cord-blood MSCs.

Table 4 Reference genes used in human fetal mesenchymal stem cell studies

Gene symbol	Gene name	Accession number	Function	References
Osteogenic and adipogenic differentiation				
PPIA	Peptidylprolyl isomerase A	NM_021130	Peptide binding	Li et al. (2015)
B2M	Beta-2-microglobulin	NM_004048	Immune response	
Osteogenic, adipogenic and chondrogenic differentiation				
GUSB	Glucuronidase beta	NM_000181.3	Metabolic pathways	Ragni et al. (2013)
RPLP0	Ribosomal protein lateral stalk subunit P0	Hs99999902_m1	Protein synthesis	
TBP	TATA-box binding protein	NM_003194	Transcription, metabolic pathways	
RPL13A	Ribosomal protein L13a	NM_012423	Component of the 60S subunit	Amable et al. (2013) and Li et al. (2015)

Besides, YWHAZ and B2M were identified as genes with least stability and were stated as unreliable for normalization (Ragni et al. 2013).

A similar study has evaluated the stability of reference genes during trilineage differentiation of Wharton's jelly MSCs. In this study, RPL13A was identified as a gene with the highest stability, and ACTB as the least constant gene for human Wharton's jelly MSCs (Amable et al. 2013). Another study has indicated RPL13A, PPIA and B2M as the most suitable genes for normalization during osteogenic and adipogenic differentiation. On the other hand, the 18S rRNA, ACTB and TBP were found to be the most variable genes, which would not yield reliable results (Li et al. 2015).

Identification of standard reference genes for normalization is vital for better investigating the self-renewal and differentiation properties of hfMSCs, and comparing the expression properties of MSCs (Li et al. 2015).

3.3 Reference Genes Used in Other Stem Cell Studies

Identification of the gene expression profiles of hematopoietic stem cells (HSCs) and cancer stem cells (CSCs) play an important part in understanding the molecular events that occur during hematopoiesis, and during the formation of benign and malignant tumors (Raaijmakers et al. 2002; Lemma et al. 2016; De Campos et al. 2018). The characterization of CSC species and cancer cells by comparison of their gene expression profiles is of utmost importance for understanding their biology, and for the development of new and more effective cancer treatment methods (Abbaszadegan et al. 2017). It has been observed that the variations originating from some properties of CSCs, such as adhesion, proliferation and metabolism alter the expressions of reference genes, widely-used for normalization, such as the ACTB gene (Lemma et al. 2016; De Campos et al. 2018).

Reference gene studies with human HSCs were first performed by Raaijmakers et al. in 2002. In this study, CD34+CD38- HSCs obtained from healthy donors and patients with acute myeloid leukemia (AML) were used; and the stability of traditional reference genes such as GAPDH and 18S rRNA was evaluated. Findings indicated that GAPDH, compared to 18S rRNA was expressed more constantly in HSCs obtained from both healthy donors and AML patients, and could be used as a reference gene (Table 5). As for the 18S rRNA expression, the expression was quite variable and indecisive for both HSC types (Raaijmakers et al. 2002).

In the first comprehensive study, the expression stabilities of 15 reference genes widely-used in benign cancer cells and CSCs obtained from human rhabdomyosarcoma, osteosarcoma, Ewing's sarcoma, breast cancer, and renal cancer tissues were compared (Lemma et al. 2016). As the result, while TBP, YWHAZ, PPIA and

Table 5 Reference genes used in other stem cell studies

Gene symbol	Gene name	Accession number	Function	References
For hematopoietic stem cells				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Metabolism/glycolysis	Raaijmakers et al. (2002)
For cancer stem cells				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Metabolism/glycolysis	de Campos et al. (2018)
TPB	TATA-box binding protein	NM_003194	Transcription, metabolic pathways	
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta	NM_003406	Signal transduction	Lemma et al. (2016)
PPIA	Peptidylprolyl isomerase A	NM_021130	Peptide binding	
HMBS	Hydroxymethylbilane synthase	NM_000190.3	Hydroxymethylbilane synthase	
For gingival stem cells				
TBP	TATA-box binding protein	NM_003194	Transcription, metabolic pathways	Taïhi et al. (2015)
SDHA	Succinate dehydrogenase complex flavoprotein subunit A	NM_004168.3	Major catalytic subunit of succinate-ubiquinone oxidoreductase	
ACTB	Actin beta	NM_001101.2	Cytoskeleton	
B2M	Beta-2-microglobulin	NM_004048	Immune response	
ALAS1	5′-Aminolevulinate synthase 1	NM_000688.5	Mitochondrial enzyme	

HMBS genes were identified as reference genes with the most constant expression stability, the tandem use of at least two genes was recommended. ACTB was identified as a gene with the lowest stability which could not be used for normalization. One of the important results of the study is the identification of specific reference genes, suitable for studying different tumor types (such as, sarcoma or carcinoma) (Lemma et al. 2016).

The gingival stem cells (GSCs), isolated from the gingiva have the ability for self-renewal and multipotent differentiation, is one of the interesting stem-cell types getting to be known in the recent years (Fournier et al. 2010). The fact that GSCs can easily be obtained from the gingival tissue with minor damage, has made this cell type one of the potential sources for use in prospective regenerative medicine applications (Fournier et al. 2013). GSCs share the same embryonic lineage with the maxillofacial bone, and constitute an alternative source for mesodermal stem cells. GSCs, with their osteogenic differentiation capability can especially be used to treat the upper jaw bone damages (Zhao et al. 2012). The only study for identifying the stable reference genes of human GSCs has been performed by Taïhi and co-workers (2015). In this study, the stability of 10 reference genes, widely-used during the in-vitro proliferation and also osteogenic differentiation of hGSCs has been investigated. SDHA, ACTB and B2M during the proliferation, and TBP, SDHA and ALAS1 during the osteogenic differentiation were identified as genes with the highest stability (Table 5), and were indicated as suitable for normalization (Taïhi et al. 2015). Identification of reliable reference genes is critical for the correct evaluation of hGSC properties; thus this may lead to better understanding of this cell source for future use in regenerative applications.

4 Discussion

Stem cells have significant potential in the emerging field of tissue engineering and regenerative medicine (Atala 2017). Thus, reliable quantitative methods are necessary for better understanding of the stem cell properties, such as self-renewal, proliferation, and differentiation (Taïhi et al. 2015; Amable et al. 2013). While qRT-PCR is a powerful method for evaluating gene expressions, the results have to be normalized with reliable reference genes. Findings show that the housekeeping genes may not be suitable for the normalization of the data (Synnergren et al. 2007; Vossaert et al. 2013; Ragni et al. 2013; Amable et al. 2013). Besides, some well-known reference genes are not suitable for common use in different cell types and experimental conditions (Li et al. 2015, 2017; Zhang et al. 2016). For this reason, identification of reference genes suitable for the cell types to be used and whose expression will not be affected by experimental conditions is vital for achieving accurate and reliable results.

In this study, the body of research that exists in scientific literature regarding the significance of identifying suitable reference genes for human stem-cells from different sources in varying experimental conditions has been compiled. The aim has been to classify the reference genes, common to different stem-cell types and conditions and thus assist to the stem cell researchers in their selection of the right reference genes. Evaluation of stem-cells studies showed that expression levels of the widely-used traditional reference genes, such as ACTB, GAPDH and 18S rRNA were quite variable and thus inappropriate for normalization. For hBM-MSCs in general, RPL13A, YWHAZ, PPIA and HPRT1 were expressed in a more stable manner during the proliferation, and tri-lineage differentiation, and could be used for normalization (Quiroz et al. 2010; Curtis et al. 2010; Studer et al. 2012; Ragni et al. 2013; Amable et al. 2013; Tratwal et al. 2014; Li et al. 2015; Rauh et al. 2014). Besides, RPL13A, YWHAZ and TBP genes were found to be stably expressed by the human ASCs as well (Fink et al. 2008; Ragni et al. 2013; Amable et al. 2013; Tratwal et al. 2014). As for other stem-cell types, such as hfMSCs, HSCs, CSCs and GSCs, we have refrained to specify a certain reference gene, as the number and content of these studies are inadequate (Li et al. 2015; Raaijmakers et al. 2002; De Campos et al. 2018; Lemma et al. 2016; Taïhi et al. 2015).

Since a common reference gene specific to different stem-cell types and experimental conditions could not be found, and different results are reached in each of the studies, it is envisaged that more reliable results can be achieved by using more than one reference gene together, rather than a single one.

5 Conclusion

In this article, the reference genes indicated as suitable for normalization of stem-cells having different potencies and properties, obtained from different sources and conditions have been presented as a whole. It was found that candidate reference genes needed to be optimized prior to each study, according to the specific conditions of the investigation. It became clear that the selection of the wrong reference genes could lead to false calculation and interpretation of the data. Thus, it is possible to say that one needs to be cautious when evaluating the results of the previous gene-expression studies on stem-cells, which do not contain the reference gene(s) optimization step. The use of optimized reference genes in studies can enable a proper understanding of the stem-cell features and expedite their translation into the clinical settings.

Competing Interests Y.M.E. is the founder and director of Biovalda Health Technologies, Inc. (Ankara, Turkey). The authors declare no competing interests in relation to this article.

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Induced Pluripotent Stem Cells and Induced Pluripotent Cancer Cells in Cancer Disease Modeling

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Abstract

In 2006, Noble Prize laureate Shinya Yamanaka discovered that a set of transcription factors can reprogram terminally differentiated somatic cells to a pluripotent stem cell state. Since then, induced pluripotent stem cells (iPSCs) have come into the public spotlight. Amidst a growing field of promising clinical uses of iPSCs in recent years, cancer disease modeling has emerged as a particularly promising and rapidly translatable application of iPSCs. Technological advances in genome editing over the past few years have facilitated increasingly rapid progress in generation of iPSCs with clearly defined genetic backgrounds to complement existing patient-derived models. Improved protocols for differentiation of iPSCs, engineered iPSCs and embryonic stem cells (ESCs) now permit the study of disease biology in the majority of somatic cell types. Here, we highlight current efforts to create patient-derived iPSC disease models to study various cancer types. We review the advantages and current challenges of using iPSCs in cancer disease modeling.

Keywords

Cancer disease model · Genome editing · Induced pluripotent cancer cells · Induced pluripotent stem cells · Reprogramming

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Abbreviations

AML	acute myeloid leukemia
APC	adenomatous polyposis cell
CML	chronic myeloid leukemia

COs	colorectal organoids
ER	estrogen receptor
ESCs	embryonic stem cells
FAP	familial adenomatous polyposis
HBOC	hereditary breast and ovarian cancer
iPCCs	induced pluripotent cancer cells
iPSCs	induced pluripotent stem cells
JMML	juvenile myelomonocytic leukemia
LFS	Li-Fraumeni syndrome
LSC	leukemic stem cells
MDS	Myelodysplastic syndrome
MSCs	mesenchymal stem cells
NS	Noonan syndrome
PDAC	pancreatic ductal adenocarcinoma
PR	progesterone receptor
sgRNA	single guide RNA
TALEN	transcription activator-like effector nuclease
ZFN	zinc finger nuclease

1 Introduction

In 2006, Kazutoshi Takahashi and Shinya Yamanaka pioneered the induction of pluripotent stem cells, termed induced pluripotent stem cells (iPSCs), from mouse embryonic or adult fibroblasts by inducing expression of four transcription factors, Oct4, Sox2, Klf4, c-Myc (referred to as the "four Yamanaka factors"), and growing the cells under mouse embryonic stem cell (ESC) culture conditions (Takahashi and Yamanaka 2006). Later, Shinya Yamanaka's and James A. Thomson's research groups successfully demonstrated the reprogramming of adult human fibroblasts as well as differentiated adult human somatic cells to human iPSCs (Takahashi et al. 2007; Yu et al. 2007). These iPSCs demonstrated gene expression, morphology, pluripotency gene epigenetic profiles and three germ-layer differentiation capacity that was comparable to ESCs. The technique of reprogramming differentiated adult cells back to pluripotent iPSCs has paved the way for the creation of patient-specific iPSC lines that has revitalized the field of both stem cell research as well as personalized medicine.

Soon after the first reports of iPSC creation by transcription factors, many groups confirmed these findings both in mice (Maherali et al. 2007; Wernig et al. 2007) and humans (Park et al. 2008b; Lowry et al. 2008). Early progress was limited by the low efficiency of iPSC generation, typically less than 0.1% of transfected fibroblasts (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008b). Initially, iPSCs were generated using either retroviruses or lentiviruses. Mouse iPSCs derived retrovirally are apparently normal, but retroviruses may cause iPSCs to be immunogenic (Zhao et al. 2011; Nakagawa et al. 2008; Aoi et al. 2008), limiting their application in animal models. Lentiviruses and some retroviruses can infect both nondividing and proliferating cells, limiting selectivity of the reprogramming process. Finally, because retroviruses or lentiviruses induce genomic integration of the targeted genetic material, it is impossible to fully guard against insertional mutagenesis. Thus, to reduce the risks associated with translational applications of iPSCs, many integration-free methods for iPSCs generation have been reported. These methods include adenovirus (Stadtfeld et al. 2008; Zhou and Freed 2009), Sendai virus (Fusaki et al. 2009; Seki et al. 2010; Ban et al. 2011), mRNA transfection (Warren et al. 2010), miRNA infection/transfection (Subramanyam et al. 2011; Anokye-Danso et al. 2011), Piggy Bac (Kaji et al. 2009; Woltjen et al. 2009; Mali et al. 2010), minicircle vectors (Narsinh et al. 2011), episomal plasmids (Okita et al. 2008; Hu et al. 2011), and direct protein insertion (Zhou et al. 2009; Kim et al. 2009). Among these, episomal plasmids and Sendai viruses are now the most commonly used tools for iPSC research.

The maturation of genome editing technologies over recent years has now facilitated making arbitrary genetic modifications to iPSCs, for example introducing a particular oncogenic mutation into patient-derived wild-type iPSCs or correcting a mutation in patient-derived mutant iPSCs (Hockemeyer and Jaenisch 2016). While numerous genome editing systems exist, CRISPR/Cas9 technology has been proven to be particularly useful in stem cell research and

human disease modeling (Cong et al. 2013; Matano et al. 2015; Schwank et al. 2013), as it affords that DNA-binding specificity that is encoded solely by the single guide RNA (sgRNA). Zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) platforms are also used to engineer hPSCs (Sexton et al. 2014; Soldner et al. 2011), though these approaches are often more costly and labor-intensive and less efficient than CRISPR/Cas9.

2 iPSCs in Cancer Disease Modeling

While the generation of iPSC lines (whether from affect patient fibroblasts or healthy donors) with unique genetic backgrounds represents an impressive scientific feat on its own, the full potential of this technology is only realized in conjunction with well-defined differentiation protocols. With appropriate such protocols, the effect of the discrete genetic alteration can be serially interrogated on a specific differentiated cell type and all of its progenitors, opening avenues for "disease modeling in a dish".

Many research groups have implemented iPSC modeling to better understand the underlying molecular mechanisms governing human diseases as well as to better study targeted therapies. iPSC lines have been produced to model various human diseases, including Huntington's Disease (An et al. 2012), Alzheimer's Disease(Israel et al. 2012; Doulatov et al. 2017; Kondo et al. 2013), Parkinson's disease (Kriks et al. 2011; Devine et al. 2011; Nguyen et al. 2011), Down syndrome (Briggs et al. 2013), familial dysautomania (Lee et al. 2009), cardiomyopathy (Carvajal-Vergara et al. 2010; Ang et al. 2016; Yazawa et al. 2011; Moretti et al. 2010; Itzhaki et al. 2011; Karakikes et al. 2014), liver metabolic disorders (Rashid et al. 2010; Yi et al. 2012), amyotrophic lateral sclerosis (Richard and Maragakis 2015), and urinary and prostate tract diseases (Moad et al. 2013).

One additional natural application of iPSCs that has only recently come to attention is cancer modeling. Because cancer is fundamentally a genetic disease, a select number of researchers have therefore begun to apply iPSC and reprogramming methods as well as induced pluripotent cancer cell (iPCC) technology to better understand the process of oncogenesis and offer novel treatment approaches (Fig. 1).

2.1 Li-Fraumeni Syndrome

Li-Fraumeni syndrome (LFS) is a genetically inherited autosomal dominant familial syndrome due to germline p53 mutations and characterized by a high incidence at a young age of a number of otherwise rare tumor types, including osteosarcoma, soft tissue sarcoma, breast cancer, gliomas, adrenocortical carcinoma and leukemia (Li and Fraumeni 1969; Zhou et al. 2017). Lee et al. generated LFS patient-derived iPSCs and explored the effect of the p53 mutation on osteoblastic lineages in order construct a disease modeling platform to explore the pathological mechanisms of mutant p53 in osteosarcomagenesis. Osteoblasts differentiated from LFS iPSC-derived mesenchymal stem cells (MSCs) recapitulated primary osteosarcoma-associated gene signatures and demonstrated impaired osteogenic differentiation ability (Lee et al. 2015). LFS iPSC-derived osteoblasts allow for the investigation of the role of mutant p53 in early osteosarcomagenesis prior to the acquisition of additional genomic mutations that are commonly observed in patient tumor samples. As p53 had been known to suppress H19 expression (Okita et al. 2008), Lee et al. investigated the influence of a p53 gain-of-function mutant on the expression of H19 in LFS iPSC-derived osteoblasts using transcriptomic analyses. The study indeed confirmed H19 downregulation in the p53 mutant and also demonstrated that H19 downregulation in part mediates the development of mutant p53-driven osteosarcoma.

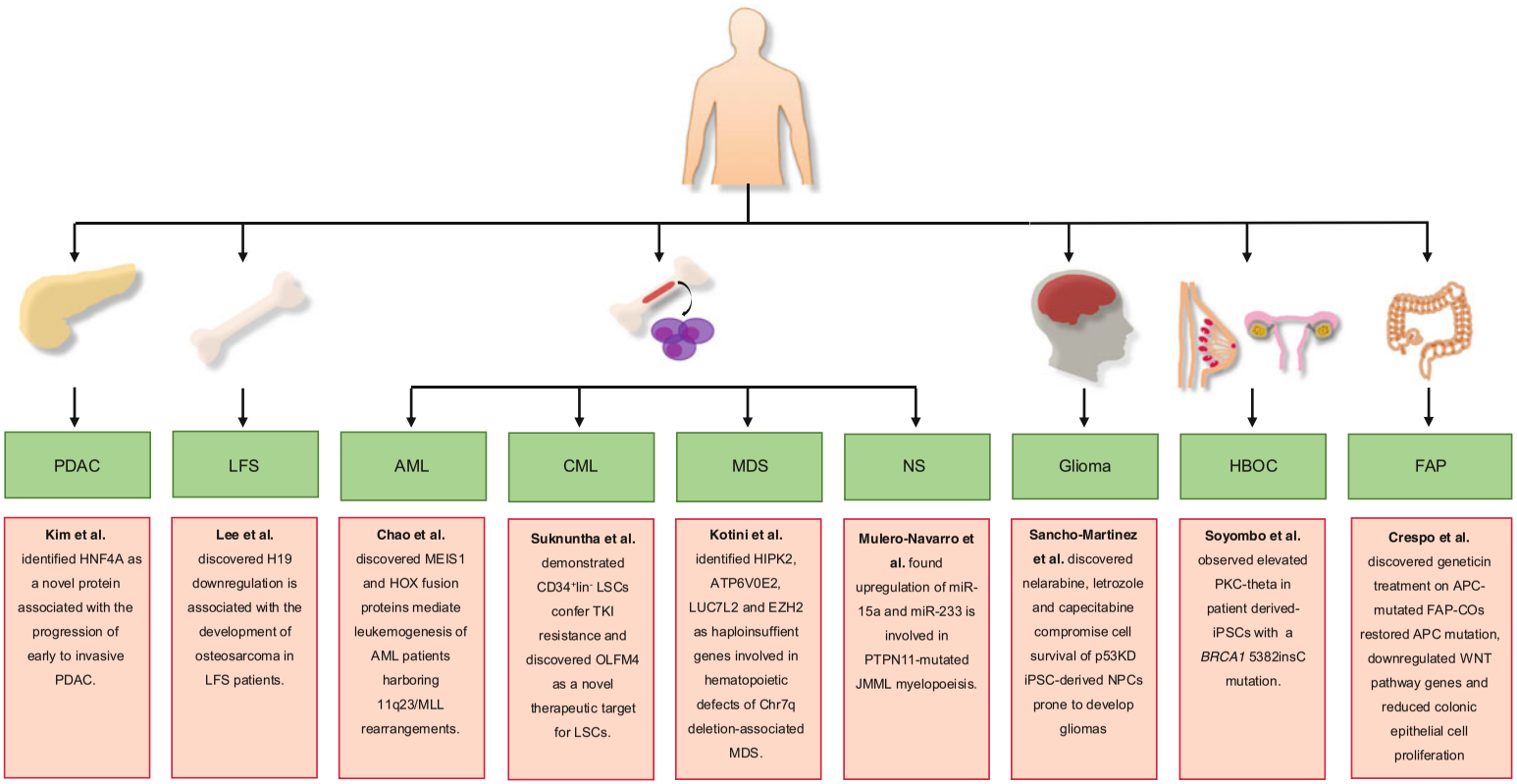

Fig. 1 Patient-derived iPSCs and iPCCs in cancer research. Taking advantage of their capability to differentiate into diverse cell types of the three germ layers, both iPSCs and iPCCs have been applied to model PDAC, LFS, AML, CML, MDS, NS, glioma, HBOC, and FAP

2.2 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a disease arising from transformation of hematopoietic cells harboring multiple genetic and epigenetic mutations as well as chromosomal rearrangements (Zwaan et al. 2015). Chao et al. established human AML iPSC lines carrying 11q23/MLL rearrangements by transducing myeloblasts with pluripotency reprogramming factors (OSKM) (Chao et al. 2017). These AML iPSC lines, when maintained in iPSC culture conditions, have reduced leukemic potential but reacquire their leukemic ability as well as genetic and epigenetic MLL signature expression patterns upon hematopoietic cell differentiation. Their findings show that the leukemogenesis can be driven by the reactivation of myeloid-specific MLL target genes within a background of expression of MEIS1 and HOX fusion proteins.

2.3 Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a disorder associated with the expansion and accumulation of myeloid progenitors in the peripheral blood and bone marrow (Rowley 1973). Expression of BCR-ABL in $CD34^+$ cells of CML patients has been linked to the pathogenesis of CML and BCR-ABL tyrosine kinase inhibitors (TKI) are now prescribed as first line treatment of CML (Druker et al. 2006). However, patients still have residual molecular evidence of CML post-treatment and leukemic stem cells (LSC) are thought to represent the reservoir of cells that permits persistence of CML post-treatment (Corbin et al. 2011). Suknuntha et al. established CML iPSCs from mononuclear cells of affected patients and subsequently generated LSC-like cells from differentiated CML iPSCs (Suknuntha et al. 2015). These LSC-like cells harbored primitive hematopoietic cell markers ($CD34^+$) but were negative for hematopoietic lineage markers (lin^-). Using the iPSC disease modeling platform, Suknunta et al. demonstrated resistance of this population to TKIs but was able to uncover olfactomedin 4 (OLFM4) as a novel agent with the potential to target the survival and proliferation of $CD34^+lin^-$ LSC-like cells.

2.4 Myelodysplastic Syndrome

Myelodysplastic syndrome (MDS) is a disease resulting from genetic mutations in hematopoietic stem cells. Some MDS patients can live with the disease for many years even with minimal clinical treatment, though others progress to develop AML (Sperling et al. 2017). However, the cellular mechanism by which MDS progresses to AML is not well understood. Kotiniet al. established patient-derived iPSCs that were able to recapitulate the entire progression spectrum of disease stages from MDS to transplantable leukemia (Kotini et al. 2015). Introducing a chr7q deletion into normal patient-derived iPSCs allowed for the modeling of pre-leukemia as well as transformed MDS. Using phenotype-rescue screening, they identified several distinct haploinsufficient genes (HIPK2, ATP6V0E2, LUC7L2 and EZH2) involved in producing the hematopoietic defects of chr7q deletion-associated MDS.

2.5 Noonan Syndrome

Noonan syndrome (NS) is an autosomal dominant disorder characterized by short stature, hypertelorism, webbed neck and exophthalmos (Noonan 1968; Roberts et al. 2013). Some patients with NS are also predisposed to developing malignant tumors including juvenile myelomonocytic leukemia (JMML). As both NS and JMML have been associated with gain-of-function PTPN11 mutations (Oishi et al. 2009), Mulero-Navarro et al. used hematopoietic cells differentiated from NS/JMML patient-derived iPSCs harboring PTPN11 mutations to investigate the role of PTPN11 mutations in NS-associated JMML (Mulero-Navarro et al. 2015). Hematopoeitic cells derived from NS/JMML patient iPSCs recapitulated aspects of the disease phenotype, including sensitivity to granulocyte-macrophage colony stimulating

factor as well as hyperproliferation of the myeloid population. Transcriptomic analysis comparing NS/JMML-derived $CD33^+$ myeloid cells with $C33^+$ control myeloid cells revealed that increased ERK activation and increased STAT5, an important component of JAK/STAT signaling pathway, was associated with development of JMML in NS patients. NS/JMML iPSC-derived $CD33^+$ myeloid cells also demonstrated increased proliferation and elevated expression of both miR-233 and miR-15a. By investigating the role of miRNAs in JMML pathogenesis, Mulero-Navarro et al. showed that upregulation of miR-233 alone is sufficient to induce PTPN11-mutated JMML myelopoiesis and that normal myelopoiesis can be restored through miRNA inhibition, a finding enabling novel therapeutic target for patients with JMML harboring PTPN11 mutations.

2.6 Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer-related death in the United States, with patient 5-year survival rates of less than 5% due to typically late-stage clinical presentation, local invasiveness adjacent to essential vasculature and biliary structures and the metastatic nature of the disease (Ying et al. 2016). Kim et al. reported successful reprogramming of one patient's late-stage PDAC cells harboring a typical KRAS mutation to iPSC-like cells (Kim et al. 2013). These PDAC iPSCs led to progression of invasive PDAC when transplanted into immunodeficient mice. Through proteomic analyses of the proteins secreted during progression of PDAC, Kim et al. were able to identify HNF4A as a novel protein associated with the progression of early to invasive PDAC.

2.7 Gliomas

Gliomas are one of the leading causes of CNS tumor-related deaths, with no current curative therapy available (Chen et al. 2012). Funato et al. discovered that neural progenitor cells (NPCs) could transform into glioma tumor-initiating cells (GTICs), leading to glioma development (Funato et al. 2014). As mutations affecting the p53 signaling pathway have been previously implicated in adult gliomas (Brennan et al. 2013), Sancho-Martinez et al. depleted p53 in wild-type iPSCs (so-called p53 "knockdown" or KD) and differentiated p53KD iPSCs to NPCs to investigate the mechanisms of gliomagenesis (Sancho-Martinez et al. 2016). These p53KD-NPCs were further transduced with mutant-active versions of SRC, EGFR and RAS to model activation of PI3K and MAPK pathways in adult gliomas (Guha et al. 2017). These genetically manipulated NPCs recapitulated GTIC properties *in vitro* and formed highly aggressive glioma-like tumors with the histopathological microstructure of clinical gliomas, namely undifferentiated stem cells and their differentiated derivatives. Sancho-Martinez applied this glioma iPSC disease modeling platform to discover three different chemical inhibitors (nelarabine, letrozole and capecitabine) whose exposure to GTIC-like cells compromised cell survival, highlighting the potential of this approach to generate potential glioma therapies.

2.8 Hereditary Breast and Ovarian Cancer Syndrome

Autosomal-dominant *BRCA1/2* mutations are the leading cause of hereditary breast and ovarian cancer (HBOC) syndromes (Futreal et al. 1994). Patients with inherited *BRCA1* mutations develop more aggressive breast cancers and at a younger age compared with patients with *BRCA2* mutations or sporadic breast cancers. The aggressiveness of tumors with *BRCA1* mutations could be due to *BRCA1*-deficient tumors commonly being estrogen receptor (ER) and progesterone receptor (PR) negative, suggesting that the tumors are driven by other oncogenes and precluding treatment with hormonal therapies (Turner et al. 2004). Soyombo et al. generated 24 iPSC lines (13 *BRCA1*-iPSCs and 11 wild-type iPSCs) from fibroblasts of patients carrying the Ashkenzaki

BRCA1 5382insC mutation to investigate the phenotype of patients with *BRCA1* mutation-associated tumors (Soyombo et al. 2013). All 24 iPSC lines showed embryonic stem cell-like morphology, expressed pluripotency markers and differentiation ability to all three germ layers. When comparing transcriptional profiles between *BRCA1* and wild-type iPSCs, Soyombo et al. discovered upregulation of *PRKCQ* expression, a gene that encodes for protein kinase C-theta (PKC-θ), in all 13 *BRCA1* iPSCs. They also detected elevated PKC-θ expression in more than half of primary tumor samples. As previous reports have linked PKC-θ activity to a subset of breast cancers (Gordge et al. 1996), results from Soyombo et al. support the potential of therapeutically targeting PKC-θ in patients with mutant BRCA1-associated cancers and possibly many other breast cancers.

2.9 Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is a rare familial cancer syndrome characterized by multiple colonic polyps and a very strong predisposition to colorectal cancer (Aaltonen et al. 1993). Germ line mutations discovered in the adenomatous polyposis cell (*APC*) gene have been linked to the pathogenesis of FAP (Nagase et al. 1992). To further investigate genetic roles in the pathogenesis of colorectal cancer, Crespo et al. generated colorectal organoids (COs) from FAP patient-derived iPSCs and discovered upregulation of WNT pathway genes in FAP-COs (Crespo et al. 2017). They found enhanced proliferation abilities of colonic epithelial cells within FAP iPSC-derived COs, consistent with the early-onset FAP patient phenotype. Crespo et al. also attempted to use the CO system as a disease modeling and drug screening platform. After screening XYZ drugs, they found that treatment of FAP-COs with the aminoglycoside antibiotic G418 (Geneticin) restored colonic epithelial cell proliferation to normal and downregulated WNT pathway-associated gene expression. These findings validate the concept of applying organoids for iPSC-based cancer or pre-cancer drug screening.

3 Advantages of iPSC Over Other Patient-Derived Cancer Models

Starting with the seminal studies by George Daley's group (Park et al. 2008a), a growing number of scientists have employed iPSCs for disease modeling. Patient-derived iPSCs retain several advantages compared with other competing systems for use in disease modeling and drug screening. First, iPSC-derived cells are suitable for high throughput drug screening to predict toxicity/therapeutic responses. Previous widely used models of drug screening include immortalized cell lines, tumor-derived cell lines, and patient tumor samples, but the availability and capacity of expansion are limited by difficulty in acquiring certain samples, senescence and/or low-fidelity cellular replication. In contrast, iPSCs can be passaged and expanded indefinitely without evidence of genomic alterations prior to differentiation towards a lineage of interest. Second, ethical issues are eliminated by use of patient iPSCs rather than ESCs. Since iPSCs are derived directly from the somatic tissues of patients, no human embryonic tissue or oocytes are ever created or destroyed (Yamanaka 2010; Nsair and MacLellan 2011; Yoshida and Yamanaka 2010; Stadtfeld and Hochedlinger 2010). Third, preclinical testing on human cells bypasses the common predicament of identifying therapies with high efficacy in a non-human animal system and no efficacy in humans. Expensive preclinical testing on animals for drug toxicity can also be somewhat reduced, for example by using iPSCs in various cytotoxicity assays. Patient-derived iPSCs offer the greatest fidelity possible to their ultimate target, the patient. Fourth, current gene editing technologies (including TALEN, CRISPR/Cas9 and ZNF) are very well-adapted to iPSCs and clear protocols have already been established. Generation and/or correction of disease-associated mutations in other cell types may require more time-consuming experimentation and optimization. Lastly, iPSC models leave the door open for future cell-based therapies. The lower immunogenicity of modified patient-derived iPSCs compared with existing

iPSC or ESC lines offers at least theoretical benefits if those cells are ever to be reintroduced into patients. Mouse studies have found no evidence of increased T cell proliferation or an antigen-specific secondary immune response after transplantation of mouse iPSC-derived embryoid bodies or tissue-specific cells (Guha et al. 2017).

4 Challenges

Despite the advancements in the application of iPSCs for cancer disease modeling (Gingold et al. 2016; Lin et al. 2017; Zhou et al. 2017; Papapetrou 2016), obstacles surrounding this platform still exist. One of the main challenges of the iPSC cancer modeling system is the technical reprogramming of cancer predisposition syndrome-associated somatic cells to iPSCs. Genetic alterations associated with cancer-associated genes may affect the efficiency of iPSCs reprogramming, preventing or inhibiting the induction of pluripotency. For example, genetic mutations associated with Fanconi anemia have been shown to resist pluripotency induction, resulting in inefficient iPSC reprogramming (Raya et al. 2009). Also, reprogramming cancer cells to iPSCs, also known as iPCCs is challenging or impossible for certain cancer types. Cancer cells may possess as-yet-undefined epigenetic aberrations, defective DNA damage responses and genetic instability-induced reprogramming checkpoints. More developed and standardized protocols for the recovery of viable cells from tumor tissues are needed to improve the reprogramming efficiency to generate these iPCCs. Alternative reprogramming methods that substitute or add to the canonical "Yamanaka transcription factors" (e.g. a cocktail of NANOG, LIN28, p53 siRNA, UTF1 and hTERT) have been shown to provide higher iPSCs reprogramming efficiency (Yu et al. 2007; Zhao et al. 2008; Park et al. 2008b) but more progress is required to reliably reprogram specific cancer cells to iPSCs and/or iPCCs. Reliable and efficient differentiation of iPSCs to specific germ layers, progenitors and terminal lineages remains a persistent problem. As cancers arise from diverse progenitor cells or de-differentiated cells in distinct tissues (Visvader 2011), differentiation protocols with higher efficiency, defined reagents and scalability are still urgently required before the entire spectrum of cancers can be modeled using iPSCs.

Some within the stem cell community have also raised concerns about increased genetic instability of iPSCs compared to other pluripotent stem cells (PSCs) or somatic cells (Hussein et al. 2011). However, recent next-generation sequencing methods have provided evidence that gene expression in iPSCs is fundamentally stable. Young et al. showed that most of the genetic heterogeneity found in iPSCs is from background mutations in parental cells (Young et al. 2012). Supporting this, Abyzov et al. showed that 50% of copy number variants present in reprogrammed iPSCs are found in parental fibroblast cells and that iPSC clones manifest genetic variants from their specifically-derived fibroblast cells (Abyzov et al. 2012). Genetic heterogeneity found in iPSCs is often acquired during extended differentiation or expansion in culture, but at a rate consistent with normal adult somatic cells acquiring spontaneous mutations during cell division (Cheng et al. 2012; Mayshar et al. 2010; Laurent et al. 2011). These studies provide clear evidence that reprogrammed iPSCs are not genetically unstable. Nevertheless, the reprogramming of adult somatic cells or cancer cells to iPSCs or iPCCs does produce global epigenomic and transcriptomic changes (Apostolou and Hochedlinger 2013), resulting in the occasional generation of partially reprogrammed "iPSC-like" cells that could be dependent on endogenous transcription factor expression (Zhang et al. 2013; Stricker et al. 2013). Completely reprogrammed iPSCs should therefore be stringently selected based on strict criteria for pluripotency and transcription factor independence (De Los Angeles et al. 2015). Still, several studies have shown that iPSCs do not exhibit greater line-to-line variation, either phenotypically or transcriptionally, compared to human ESC lines, indicating that iPSCs are not inherently epigenetically unstable (De Los Angeles et al. 2015; Guenther et al.

2010). However, the epigenetic landscape of the source cancer cell might persist after induction of pluripotency and certainly has been shown to reoccur after iPSC/iPCC differentiation. Therefore, further research on characterizing the epigenetic landscape of iPSC/iPCC-derived cells needs to be conducted to better understand the limitations of applying iPSCs and iPCCs as a cancer-disease modeling platform as well as to investigate the relationship between genetic and epigenetic changes in specific cancer types.

5 Future Perspective

Although recent advances in iPSCs have confirmed the value of this system in disease modeling and improving treatments for numerous diseases, there are still substantial hurdles precluding fulfillment of this technology's potential. Ideally, we expect to be able to utilize iPSCs to model any genetic disease (monogenic, chromosomal or complex). This promise will require a combination of gene editing technologies such CRISPR/Cas9 or TALENs, isogenic cell lines with the induction or correction of relevant mutations, as well as the generation of different mutations in the same gene in the same patient-derived iPSC or engineered hESCs (Zhou et al. 2018; Xu et al. 2018; Tu et al. 2018).

Induction of mutations in genetically complex disorders is more than theoretically possible, though after a point such experimental constructs become impractically complex to engineer. The highly variable (but typically low) efficiency of iPSC differentiation across cell lineages indicates the need for optimize cell culture conditions and differentiation protocols. Experimentation on any cells derived from PSCs must be performed on a meticulously sorted population, as the inherent ability of PSCs to divide indefinitely in appropriate culture conditions and form teratomas *in vivo* can easily complicate interpretation of assays. iPSCs generated from retroviral/lentiviral systems carry additional limitations and risks related to the unpredictable integration of genetic information into various genomic loci.

Despite these challenges, iPSC disease modeling empowers multiple research areas in translational and basic science, such as the identification and validation of therapeutic targets, preclinical efficacy and safety studies and compound screening for drug discovery and drug repurposing (Kotini et al. 2017; Doulatov et al. 2017; Crespo et al. 2017). In addition, iPSC technology can also be extended from disease modeling to cancer immunotherapy. Several groups have paved the way for the application of iPSC-technology to improve and advance cancer immunotherapy. Serwold et al. utilized PSC technology to reprogram mature T cells to T-iPSCs and re-differentiated these cells back to T cells, resulting in the generation of antigen-specific cytotoxic iPSC-derived T cells (Serwold et al. 2007). The benefit of such an approach could potentially be expanded to clinically benefit cancer patients as the unlimited and antigen-specific cytotoxic T cells could be developed to target tumor-specific antigens for enhanced cancer immunotherapy effects. In lieu of potentially extending iPSC technology from disease modeling to cancer immunotherapy, Vizcardo et al. demonstrated the generation of iPSCs derived from mature cytotoxic T cells with specificity for melanoma epitope MART-1 (Vizcardo et al. 2013). When co-cultured with OP9/DLL1 cells, these iPSCs differentiated to $TCR\beta^{+}CD4^{+}CD8^{+}$ cells with a T cell receptor (TCR) specific for MART-1 epitope, paving the way for future research on the possibility of cloning functional iPSC-derived cytotoxic T cells for cell-based cancer immunotherapy. Most recently, Kooreman et al. showed irradiated iPSCs derived from mouse fibroblasts could reduce metastatic tumor load in murine models of breast, lung, and skin cancers (Kooreman et al. 2018). These irradiated iPSCs promoted a humoral and cancer-specific anti-tumor T cell response accompanied with increased $CD11b^{+}GR1^{hi}$ myeloid cells with no observed adverse effects. These data suggests that iPSC vaccine can be potentially used in clinical immunotherapy in the future. These advances in iPSC technology demonstrate the cutting-edge potential of applying iPSCs to future cancer therapies.

In conclusion, increasingly powerful and precise genome editing technologies are enabling the study of even unusual genetic combinations in cell types with otherwise highly limited source material. The extension of iPSC technology in the application of cancer immunotherapy also proves to be extremely promising thus, we anticipate the applications of these advances to cancer biology will only increase over the coming years and facilitate development of truly personalized cancer therapies.

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Conflicts of Interest Authors declare no conflicts of interest.

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