

Delivery of growth factor-associated genes to mesenchymal stem cells for cartilage and bone tissue regeneration

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Abstract. Genetically-modified mesenchymal stem cells (GM-MSCs) have emerged as promising therapeutic tools for orthopedic degenerative diseases. GM-MSCs have been widely reported that they are able to increase bone and cartilage tissue regeneration not only by secreting transgene products such as growth factors in a long-term manner, also by inducing MSCs into tissue-specific cells. For example, MSCs modified with BMP-2 gene increased secretion of BMP-2 protein resulting in enhancement of bone regeneration, while MSCs with TGF- β gene did cartilage regeneration. In this review, we introduce several growth factors for gene delivery to MSCs and strategies for bone and cartilage tissue regeneration using GM-MSCs. Furthermore, we describe strategies for strengthening GM-MSCs to more intensively induce tissue regeneration by co-delivery system of multiple genes.

Keywords: growth factor; transcription factor; genetically-modified mesenchymal stem cells; cartilage; bone; tissue regeneration

1. Introduction

Critical-sized bone defects and articular cartilage injuries induced by various reasons including trauma, surgery, and diseases do not self-repair, which are a major challenge in the orthopedic field. The gold standard of clinical therapeutic strategies to enhance bone and cartilage regeneration is the use of tissue transplantation, since they possess the essential components such as progenitor cells as well as osteo/chondro inductive growth factors and extracellular matrix (ECM) (O'Driscoll 1998, Bauer and Muschler 2000, Kneser *et al.* 2006, Oryan *et al.* 2014). Autogenic, allogenic, and xenogenic tissue grafts have been applied to the treatment of bone and cartilage disease (Oryan *et al.* 2014). However, autogenic tissue grafts may lead to limited quantity and morbidity of the donor site, while allo- and xenogenic tissue grafts may give rise to serious

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immunogenic rejection (Silber *et al.* 2003, Clair *et al.* 2009).

Tissue engineering commonly involves the therapeutic cells, mechanically supportive scaffolds, and bioactive molecules and the combination of them generates functional engineered tissue *ex vivo* eventually to replace the damaged tissues (Lee and Shin 2007, Zhang *et al.* 2009, Santo *et al.* 2013, Santo *et al.* 2013). To realize ideal tissue engineering, firstly, selection of appropriate cell source is important. Recently, stem cells or tissue-specific progenitor cells have been emerged as promising cell sources for tissue engineering. They are capable of restoring the cellular component of damaged tissue through the release of trophic factors as well as differentiation into tissue-specific cells at the same time. It is also critical that bioactive molecules such as cytokine and growth factors provide signals at local injury site to stimulate cellular proliferation and differentiation. Furthermore, to induce effective cell survival and tissue formation, an engineered scaffold should support various features such as biocompatibility, porosity, mechanical functionality, bioactivity, and degradability. For instance, the scaffold system releasing growth factors would be a good approach to stimulate tissue regeneration continuously without loss of bioactive growth factors.

Based on therapeutic growth factors, genetically-modified mesenchymal stem cells (GM-MSCs), which are releasing bioactive growth factors, have been reported as an alternative means to enhance bone and cartilage tissue regeneration. Truly, it has been shown that transplantation of GM-MSCs which is expressing therapeutic growth factors generates new bone and cartilage formation much faster than exogenous treatment of growth factors (Lee *et al.* 2010). Thus, mesenchymal stem cells (MSCs) have received a great deal of attention as a delivery vehicle of growth factors following gene modification (Kofron and Laurencin 2006, Meyerrose *et al.* 2010, Porada and Almeida-Porada 2010, Qi *et al.* 2012) because they show sustained release of therapeutic growth factors as well as differentiation toward tissue specific cells at the same time. Therefore, these synergistic effects are able to distinctly enhance bone and cartilage tissue compared with individual treatment of MSCs and growth factors. In addition, GM-MSCs have unique immunosuppressive properties tolerating the immune response across human leukocyte antigen (HLA) mismatch between donor and recipient following transplantation. The gene modification of GM-MSCs for cartilage and bone generation has been mainly carried out using viral vectors, predominantly adenovirus. Considering clinical application, however, non-viral systems such as liposome carriers and electroporation would be more useful due to their safety and simple preparation. More recent studies have focused on appropriate combination of growth factors and transcription factors increasing tissue specific genes. The combination may have two advantages; 1) auto-stimulatory effects of growth factors 2) direct upregulation of target gene by transcription factors, followed by enhanced tissue regeneration. Furthermore, the 2A bicistronic vector allows the expression of two genes with a single promoter, which enables equivalent expression of two distinct genes simultaneously (Lee *et al.* 2010, Kang *et al.* 2012, Cha *et al.* 2013). Therefore, it could effectively release growth factors and increase MSC differentiation toward tissue specific cells followed by enhancement of tissue regeneration. In this review, we will discuss the advantages and future direction of GM-MSCs regarding growth factors for bone and cartilage tissue regeneration.

2. Bone tissue engineering

2.1 Growth factors in bone regeneration

Bone repair is accomplished by complex signal cascades controlled by numerous cytokine and growth factors that allow progenitor cells in the injury site to differentiate and trigger healing process (Kneser *et al.* 2006, Kimelman *et al.* 2007, Calori *et al.* 2009, Dimitriou *et al.* 2011). Current evidences based on *in vitro* and animal studies suggest that bone morphogenetic proteins (BMPs) have the highest osteoinductive potential among osteogenic growth factors (Dimitriou *et al.* 2011, Oryan *et al.* 2014). Especially, administration of BMP-2, -6, and -9 by gene delivery has been proposed as the most potent inducers of MSC differentiation into osteoblast *in vitro* and *in vivo* (Cheng *et al.* 2003, Kofron and Laurencin 2006, Calori *et al.* 2009, Evans 2012). The importance of angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) in bone regeneration has been newly recognized (Santo *et al.* 2013). More recent studies have focused on the combination of growth factors together with transcription factors and promoter binding proteins increasing osteoblast-specific genes (Lee *et al.* 2010). Great therapeutic potentials of GM-MSCs have been reported in preclinical animal models and it is summarized in Table 1.

2.2 Bone morphogenic proteins

BMPs, members of the transforming growth factor (TGF)- β superfamily, play an important role in the processes of bone formation and regeneration (Calori *et al.* 2009). It is synthesized and secreted by osteoblasts, then incorporated into the organic matrix during bone formation. Multiple BMPs including BMP-2, -4, -7, and -9 show the osteoinductive characteristics which lead to the differentiation of MSCs into osteoblasts (Kruyt *et al.* 2003, Kruyt *et al.* 2006). Currently, extensive preclinical investigation has demonstrated that GM-MSCs modified with BMPs promote the repair of critical sized bone defects and spine fusion, and induce ectopic bone formation. The groups of Peterson and Hsu determined the efficacy of GM-MSCs expressing BMP-2 (BMP-2-GM-MSCs) with collagen-ceramic carrier for bone regeneration of critical sized femoral defect (Peterson *et al.* 2005, Hsu *et al.* 2007). It revealed that BMP-2-GM-MSCs dramatically induced bone formation *in vivo*, but non-transfected MSCs did not. These results suggest that prolonged BMP-2 expression by MSCs may be essential process for the treatment of large bony defects. Zachos *et al.* (2007) also demonstrated new bone formation by implantation of bone marrow-derived MSCs (BMSCs) expressing BMP-2 (BMP2-BMSCs) in lateral intercondylar osteotomy model (Zachos *et al.* 2007). When cells were delivered in alginate (ALG) carrier or by direct injection in saline solution, successful bone repair was achieved using direct BMP-2-BMSCs injection. In ALG groups, bone healing was impeded by the development of a chondroid mass. In addition, the efficacy of genetically engineered bone implants, composed of β -TCP/HA biphasic calcined bone (BCB) and autologous BMP-2-BMSCs, was confirmed in tibial bone defects (Dai *et al.* 2005). The healing rate, trabecular bone, and biomechanical strength of the defects were significantly better in the BMP-2-BMSCs group when compared with non-transfected BMSCs groups. Xu *et al.* evaluated the ECM synthesized by implanted cells themselves (BMP-2-BMSCs) as the carrier for the repair of goat tibia bone, which showed the great osteoinductivity of BMP-2-BMSCs (Xu *et al.* 2005).

BMP-2-GM-MSCs have been shown the therapeutic potential to enhance spinal fusion (Riew *et al.* 1998, Hsu *et al.* 2008, Miyazaki *et al.* 2008, Sheyn *et al.* 2011). Hsu *et al.* (2008) demonstrated successful spine fusion with the adipose tissue-derived MSCs (ADSCs) expressing BMP-2 (BMP-2-ADSCs) whilst none of the animals treated with ADSCs determined spine fusion after the surgery. Furthermore, direct delivery of BMP-2 protein without ADSCs illustrated that the

Table 1 Gene-modified MSCs-based therapy for bone regeneration and repair

Transgene	Cells*	Scaffold	Model	Animal	Reference
BMP-2	hADSC	Collagen-ceramic carrier	Femoral defect	Rat	(Peterson <i>et al.</i> 2005)
BMP-2	rBMSC	Collagen-ceramic carrier		Rat	(Hsu <i>et al.</i> 2007)
BMP-2	rBMSC	Alginate		Rat	(Zachos <i>et al.</i> 2007)
BMP-2	hBMSC	Collagen	Radial defects	Mouse	(Turgeman <i>et al.</i> 2001)
BMP-2	gBMSC	Biphasic calcined bone +collagen	Tibial defects	Goat	(Dai <i>et al.</i> 2005)
BMP-2	gBMSC	MSC-derived ECM		Goat	(Xu <i>et al.</i> 2005)
BMP-2 +VEGF	mBMSC	-		Mouse	(Kumar <i>et al.</i> 2010)
BMP-2	sBMSC	Collagen	Maxillary defects	Miniature swine	(Chang <i>et al.</i> 2003)
BMP-2	rBMSC	Collagen	Mandible defects	Rat	(Park <i>et al.</i> 2003)
BMP-2	raBMSC	Collagen	Spine fusion	Rabbit	(Riew <i>et al.</i> 1998)
BMP-2	hADSC	Collagen		Rabbit	(Hsu <i>et al.</i> 2008)
BMP-2	rBMSC	Collagen		Rat	(Miyazaki <i>et al.</i> 2008)
BMP-6	pADSC	Fibrin		Rat	(Sheyn <i>et al.</i> 2011)
BMP-2	rBMSC	Collagen	Intramuscular injection	Mouse	(Sugiyama <i>et al.</i> 2005)
BMP-2/-9	hBMSC	-		Mouse	(Aslan <i>et al.</i> 2006)
BMP-2	hBMSC	Collagen/Alginate/Hyaluronate/Agarose/Fibrin/Pluronic,	Intramuscular/subcutaneous injection	Mouse	(Xu <i>et al.</i> 2005)
BMP-2 +Runx2	hADSC	PLGA	Subcutaneous injection	Mouse	(Lee <i>et al.</i> 2010)
BMP-7	rADSC	Collagen		Rat	(Yang <i>et al.</i> 2005)
BMP-4	mBMSC	-	Femoral bone marrow cavity	Mouse	(Zhang <i>et al.</i> 2004)

*BMSC, bone marrow-derived MSC; ADSC, adipose-derived MSC; m, mouse; r, rat; ra, rabbit; h, human; s, swine

relatively high dose of BMP-2 delivery (10 microgram) could only lead to the higher rate of spine fusion compared with the low dose (1 microgram) (Hsu *et al.* 2008). Sheyn *et al.* (2011) investigated that BMP-6-ADSCs were capable of inducing spinal fusion *in vivo*. To evaluate the ectopic bone formation, GM-MSCs were transplanted into intra-muscular, subcutaneous and bone marrow cavity space (Sugiyama *et al.* 2005, Xu *et al.* 2005). Aslan *et al.* achieved *in vivo* bone formation by using BMSCs modified with the BMP-2 or -9 genes. Both cells were transplanted into intramuscular space in NOD/SCID mice, which showed new bone formation at 4 weeks postinjection (Aslan *et al.* 2006). The increasing of endosteal bone formation was examined by transplantation of BMP-4-BMSCs at the femoral bone marrow cavity. Interestingly, Xu *et al.* (2005) examined the efficacy of GM-MSCs expressing BMP-2 using various scaffold including alginate, collagen, agarose, hyaluronate, fibrin, and pluronic to augment bone formation (Xu *et al.*

2005). Following preparation of scaffold/BMP-2-GM-MSCs/BCB mixture, it was implanted into the subcutaneous site of athymic mice. Results showed that alginate, collagen, and agarose gel promoted new bone formation *in vivo*, but either fibrin or pluronic gel didn't.

In our recent studies, we reported that gene delivery of BMP-2 together with RUNX2, an essential transcription factor for control of osteoblast differentiation, dramatically enhanced the osteogenic differentiation of ADSCs and new bone formation *in vitro* and *in vivo*. To co-express both genes, a single bicistronic vector linked to the 'self-cleaving' 2A peptide sequence was constructed, which could permit the introduction of two distinct genes with a single transfection (Fig. 1) (Lee *et al.* 2010, Kang *et al.* 2012). These 2A non-viral vectors also supported higher safety and cost effectiveness compared with viral vectors systems that could cause host genome integration and induce internal toxicity. It revealed that the ADSCs modified with BMP2/Runx2 genes (BMP2/Runx2-ADSCs) showed a high expression of osteogenesis-related markers (osteopontin, osteocalcin and collagen type I) and increased mineralization compared to BMP2-ADSCs *in vitro*. Furthermore, a significant increase of mineralized bone formation was observed in the regenerated bone transplanted by BMP2/Runx2-ADSCs compared with results by treatment of BMP2-ADSCs (Fig. 2). These findings demonstrated that a specific combination of growth factors and lineage-determining transcription factors may be more effective for stimulating an osteogenic differentiation of ADSCs. Additional studies demonstrated that BMP-7-ADSCs could induce ectopic bone formation in athymic nude rats when they were transplanted into subcutaneous region. It has been reported that the improvement of *in vivo* new bone formation is remarkable in BMP-7-ADSCs group compared to non-transfected ADSCs at 4 weeks post-implantation (Yang *et al.* 2005).

2.3 Growth factors other than BMPs

Whilst the current landscape of growth factor use for bone regeneration is dominated by the BMPs, a number of other growth factors are being investigated for use as a potential treatment for bone tissue (Kneser *et al.* 2006). In particular, the establishment of a vessel formation has been shown to play a crucial role in an early and critical event for bone formation, thus administration of angiogenic growth factors such as VEGF, and fibroblast growth factor-2 (FGF-

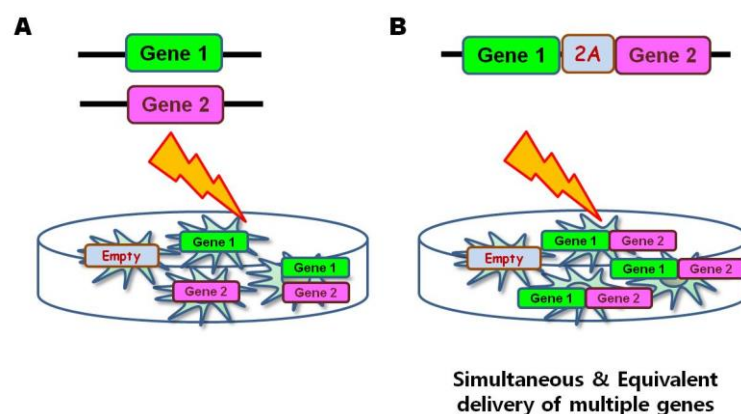


Fig. 1 Advantage of 2A vector in stem cell engineering. (a) Conventional method of gene transfection. (b) 2A bicistronic vector system ensuring equivalent expression of multiple genes simultaneously

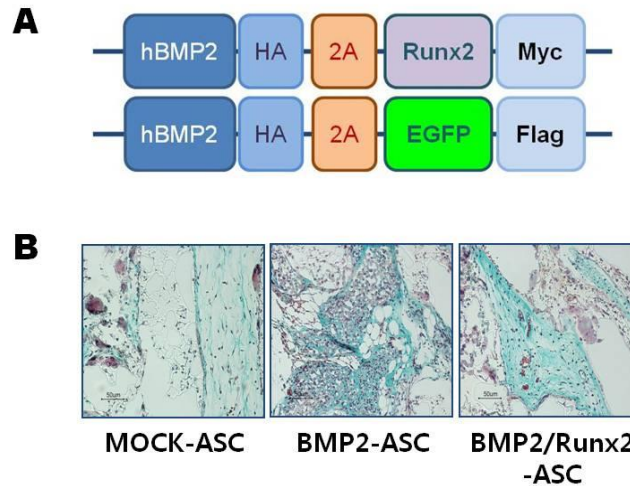


Fig. 2 Enhancement of bone regeneration by gene delivery of BMP2/Runx2 bicistronic vector into ADSCs. (a) Schematic of the 2A polycistronic vectors. (b) Bone formation in BMP2/Runx2 cotransfected ADSCs compared to controls (Modified from Lee *et al.* (2010) *Biomaterials* **31**(21), 5652-5659)

Table 2 Gene-modified MSCs-based therapy for cartilage regeneration and repair

Transgene	Cells	Scaffold	Model	Animal	Reference
TGF- β 1	raBMSC	PLS	Full-thickness articular cartilage defects in knee	Rabbit	(Guo <i>et al.</i> 2006)
TGF- β 1	raBMSC	Chitosan		Rabbit	(Guo <i>et al.</i> 2007)
TGF- β 1	raBMSC	Chitosan/poly (vinyl alcohol) hydrogel		Rabbit	(Qi <i>et al.</i> 2013)
GDF-5	raBMSC	Collagen		Rabbit	(Katayama <i>et al.</i> 2004)
BMP-2	rBMSC	Fibrin	Partial-thickness cartilage defects in knee	Rat	(Park <i>et al.</i> 2006)
TGF- β 1	hBMSC	-(cell pellet)	Osteochondral defects	Rat	(Pagnotto <i>et al.</i> 2007)
TGF- β 1	pBMSC	-	Subcutaneous injection	Mouse	(Xia <i>et al.</i> 2009)
TGF- β 1	hBMSC	-	-	-	(Ito <i>et al.</i> 2004)
TGF- β 1	hBMSC	-	-	-	(Kawamura <i>et al.</i> 2005)
IGF1 +(TGF- β 1 /BMP2)	bBMSC	-	-	-	(Steinert <i>et al.</i> 2009)

2), insulin growth factor-1 (IGF-1), and PDGF has been attempted to enhance bone regeneration (Chen *et al.* 2010, Santo *et al.* 2013). Currently, the importance of VEGF for bone repair has been recognized by Kumar colleagues. They found that MSCs transduced with both BMP-2 and VEGF genes enhanced bone formation in mouse model of segmental tibia bone defect and it was accompanied by increased vascularity and osteogenesis. Therefore, dual therapy with BMP2 and VEGF could synergistically influence on neoangiogenesis with even distribution of blood vessels around the healing area with uniform vascular diameter and size (Kumar *et al.* 2010).

3. Cartilage tissue engineering

3.1 Growth factors in cartilage regeneration

Cartilage has a low regenerative capacity due to its lack of blood supply (Yuan *et al.* 2014). So, to repair the osteochondral defects, bone marrow stimulation techniques including osteochondral drilling, abrasion chondroplasty, and microfracture have been applied. However, these treatments have disadvantage to synthesize the fibrous cartilage showing inferior mechanical and biochemical characteristics compared to normal hyaline articular cartilage. In recent years, it has been showed that genetically engineered MSCs expressing chondroinductive factors leads to a stable chondrogenic differentiation and promotes new cartilage formation (Ito *et al.* 2004, Katayama *et al.* 2004, Kawamura *et al.* 2005, Guo *et al.* 2006, Park *et al.* 2006, Pagnotto *et al.* 2007, Steinert *et al.* 2009, Xia *et al.* 2009, Qi *et al.* 2013). In practice, gene delivery of growth factors including TGF- β 1, BMP-2, and IGF-1 have been shown to stimulate the synthesis of proteoglycans, aggrecan and type II collagen in MSCs and induce chondrogenic differentiation of MSCs (Fortier *et al.* 2011). Therefore, tissue regeneration by GM-MSCs secreting specific growth factors offers promising treatments for enhanced cartilage regeneration. GM-modified MSCs-based therapy for cartilage regeneration and repair is summarized in Table 2.

3.2 Transforming growth factor β

TGF β superfamily has been shown to play a central role in promotion of cartilage repair, thus has been chosen as promising candidates for gene therapy for articular cartilage defects. GM-MSCs, transduced to express TGF- β 1, enhanced chondrogenesis and significantly improved articular cartilage defects (Katayama *et al.* 2004, Guo *et al.* 2006, Guo *et al.* 2007, Qi *et al.* 2013).

In addition, TGF- β 1 transfected MSCs, seeded onto biodegradable poly-L-lysine coated polylactide (PLA) biomimetic scaffolds induced synthesis of hyaline cartilage specific ECM at the upper portion of the defect and the reconstitution of the subchondral bone at the lower layer of the defect (Guo *et al.* 2006). Furthermore, Guo *et al.* (2007) constructed the gene-modified cartilage composed of TGF β 1-engineered BMSCs and chitosan scaffolds, then implanted into the full-thickness articular cartilage defects in rabbit. The defects were filled with regenerated hyaline like cartilage tissue at 12 weeks after implantation (Guo *et al.* 2007). Qi *et al.* (2013) observed the therapeutic effect of Adv-TGF β 1-transfected BMSCs using thermo-sensitive chitosan/poly (vinyl alcohol) composite hydrogel as injectable material (Qi *et al.* 2013). The BMSCs expressing TGF- β 1 triggered the expression of specific markers of cartilage differentiation such as aggrecan and collagen II and generated functional hyaline cartilage. Similar studies have been performed to repair the osteochondral defects using adeno-associated virus (AAV)-TGF β 1-transduced MSCs pellet (Pagnotto *et al.* 2007). Although a progressive decline of TGF- β 1 occurred over the 12 weeks, improved cartilage repair were observed. This result suggests that AAV transgene expression persists long enough *in vivo* to positively influence cartilage repair. Furthermore, TGF β 1-expressing MSCs has been shown to enhance ectopic cartilage formation when transplanted subcutaneously into immunodeficient mice (Xia *et al.* 2009). Therefore, the genetic transfer of the TGF- β 1 gene into MSCs would be effective to enhance cartilage regeneration.

3.3 Growth factors other than TGF- β

Growth differentiation factor-5 (GDF-5) has been shown to enhance chondrocyte differentiation (Hotten *et al.* 1996, Tsumaki *et al.* 1999). Early experiments using GDF-5-transfected autologous BM-MSCs showed that the full-thickness articular cartilage defects were filled by hyaline cartilage and the efficacy of repair was superior to normal BMSCs transplantation (Katayama *et al.* 2004). BMP-2, belongs to the TGF- β superfamily, has been shown to increase ECM synthesis within cartilage lesion and ultimately lead to improved repair (Enomoto-Iwamoto *et al.* 1998). Park *et al.* examined the chondrogenic potential of BMP-2-activated MSCs from different origin, bone marrow-, perichondrium/periosteum- and fat- with respect to their suitability for articular cartilage repair (Park *et al.* 2006). Both BMSCs and perichondrium/periosteum-derived MSCs repaired the partial-thickness cartilage defects and appeared to be superior to ADSCs. Therefore, GM-MSCs are necessary to ensure regeneration effects constantly. On the other hand, encouraging results have been obtained by applying differentiated cells transduced to produce TGF- β 1, BMP-2 and IGF-1, individually, or in combination (Steinert *et al.* 2009). Notably, co-expression of growth factors (IGF-1 and TGF- β 1, or BMP2, or both at low doses) resulted in higher levels of glycosaminoglycan synthesis, stronger staining for proteoglycans and collagen type II compared with either transgene alone *in vitro*.

4. Conclusions

GM-MSCs will offer a novel approach for treating massive bone and cartilage defects. It

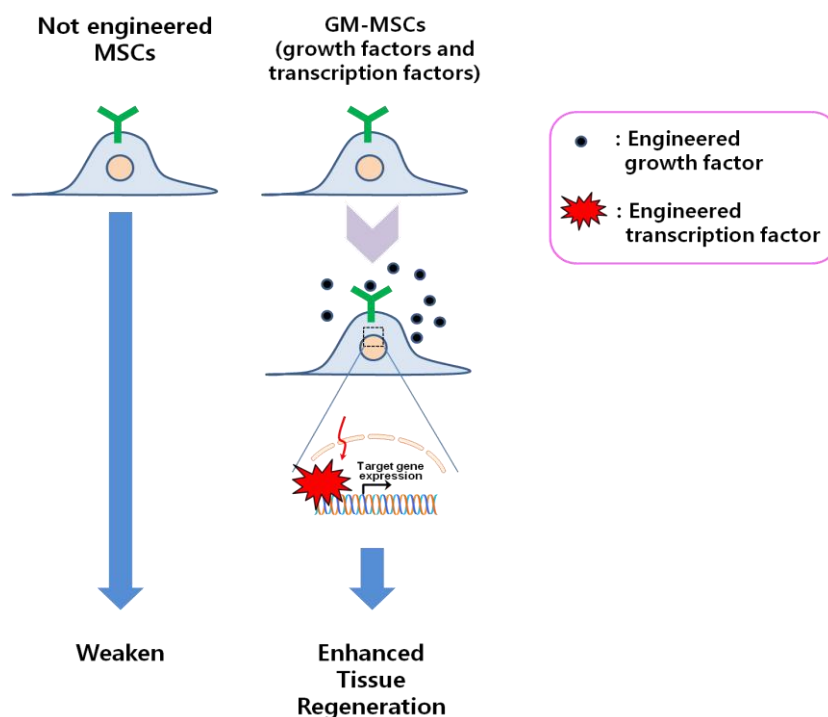


Fig. 3 Model of engineered stem cells. To make standardized MSCs, we should generate GM-MSCs which have growth factors and transcription factors to enhance bone and cartilage regeneration

provides the therapeutic growth factors secretion as well as the supply of MSCs at the same time. MSCs have several unique properties which make them ideal cell sources for delivering therapeutic genes: 1) relative ease of isolation; 2) the ability to differentiate into multiple cell types; 3) the ability to be extensively expanded in culture; 4) immunomodulatory functions; 5) anti-inflammatory properties; and 6) their ability to home to damaged tissues following *in vivo* administration. Thus, MSCs-based cell therapy, combined with genetic engineering, will provide a safe and effective means to regenerate osteochondral defects. Until now, we have tried to introduce the importance of GM-MSCs with growth factors and transcription factors to enhance bone and cartilage regeneration (Fig. 3). In conclusion, therapeutic application of GM-MSCs to regenerate bone and cartilage defects will stimulate and accelerate the development of cell therapy for the treatment of orthopedic diseases.

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