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Key Transcription Factors in the Differentiation of Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) are multipotent cells that represent a promising source for regenerative medicine. MSCs are capable of osteogenic, chondrogenic, adipogenic and myogenic differentiation. Efficacy of differentiated MSCs to regenerate cells in the injured tissues requires the ability to maintain the differentiation toward the desired cell fate. Since MSCs represent an attractive source for autologous transplantation, cellular and molecular signaling pathways and micro-environmental changes have been studied in order to understand the role of cytokines, chemokines, and transcription factors on the differentiation of MSCs. The differentiation of MSC into a mesenchymal lineage is genetically manipulated and promoted by specific transcription factors associated with a particular cell lineage. Recent studies have explored the integration of transcription factors, including Runx2, Sox9, PPARγ, MyoD, GATA4, and GATA6 in the differentiation of MSCs. Therefore, the overexpression of a single transcription factor in MSCs may promote trans-differentiation into specific cell lineage, which can be used for treatment of some diseases. In this review, we critically discussed and evaluated the role of transcription factors and related signaling pathways that affect the differentiation of MSCs toward adipocytes, chondrocytes, osteocytes, skeletal muscle cells, cardiomyocytes, and smooth muscle cells.

Keywords

Mesenchymal Stem Cells; Transcription factors; Multilineage Differentiation

Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that are capable of self-renewing and differentiating into functional cell types. The ease of isolation, the high migratory

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capacity, the relatively high expansion rates, and the ability to avoid the allogeneic responses after transplantation (Chen et al., 2004; Fouillard et al., 2007; Le Blanc and Ringden, 2005; Ripa et al., 2007; Sekiya et al., 2002) make them attractive candidates in regenerative medicine. Over the last few years, MSCs have been isolated from various tissues and organs including adipose tissue, bone marrow, placental tissue, umbilical cord blood, the testes, the liver, the pancreas, the spleen, amniotic fluid, menstrual blood, dental pulp, the dermis and the lung (De Coppi et al., 2007; Guan et al., 2006; in 't Anker et al., 2003; Kruse et al., 2006; Meng et al., 2007; Pierdomenico et al., 2005; Ringe et al., 2008; Sabatini et al., 2005; Sellheyer and Krahl, 2010). They are characterized by their spindle shaped morphology and their ability to differentiate *in vitro* into adipocytes, chondrocytes and osteocytes. Previous reports suggest that there is no single specific marker to distinguish MSCs from other cells that exhibit similar fibroblastic characteristics. Hence, these cells are immunophenotypically characterized by positive and negative expression of multiple surface antigens. MSCs express surface antigens such as CD44, CD73, CD29, CD90 and CD105 and lack hematopoietic and endothelial markers such as CD11, CD14, CD31, CD34 and CD45 (Haynesworth et al., 1992; Lodie et al., 2002; Suva et al., 2004). *In vitro*, MSCs usually grow as a monolayer culture in a medium containing 10% fetal bovine serum and L-glutamine.

The multi-lineage differentiation of MSCs has been extensively studied *in vitro* and *in vivo* since their first discovery. These studies have demonstrated that MSCs have the potential to differentiate into several mesoderm-type lineages, including myogenic, adipogenic, osteogenic and chondrogenic lineages (Figure 1).

During the differentiation of MSCs toward a specific cell type, there are a multitude of stimuli and inhibitors that play important roles in the initial commitment and later stages of differentiation. The differentiation of MSCs into specific mature cell types is controlled by various cytokines, growth factors, extracellular matrix molecules, and transcription factors (TFs). In vitro, the differentiation of MSCs requires certain inducers including some growth factors (Table 1).

To date, many studies have shown that transcription factors can affect the differentiation of MSCs toward different mature cell types. Through the activation of transcription factors, the effect may occur by upregulating the expression of genes responsible for induction and progression of specific-cell type differentiation. In the following sections, we will discuss the characteristics and function of various transcription factors that affect the differentiation of MSCs (isolated from different sources) toward adipocytes, chondrocytes, osteocytes, skeletal muscle cells, cardiomyocytes, and smooth muscle cells.

Osteogenic differentiation

Differentiation of MSCs toward osteocytes is induced *in vitro* by incubating a monolayer of MSCs with a differentiation medium containing β-glycerophosphate, dexamethasone, ascorbic acid-2-phosphate and combinations of transforming growth factor-beta (TGF-β), bone morphogenetic proteins (BMPs) and vitamin D3 (Friedenstein et al., 1987; Okamoto et al., 2002). At the molecular level, interactions between hormones and transcription factors control the differentiation of MSCs into osteocytes. The major transcription factors that have

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key roles in the differentiation of MSCs into osteocytes are CBFA-1/Runx2 and Osterix (Augello and De Bari, 2010).

The osteogenic differentiation is regulated by various transcription factors such as Runt-related transcription factor 2 (Runx2), osterix, and β-catenin. The Runx gene consists of a runt domain (DNA binding domain) and Runx protein forms heterodimers with core binding factor β (Cbfβ)/ polyma enhancer binding protein 2β (Pebp2β) *in vitro*. Various studies have shown that the Runt-related transcription factor (Runx2) is required for osteogenic differentiation. The Runx2 transcription factor is an essential regulator for bone formation and the osteogenic differentiation of MSCs. It directs MSCs to differentiate into pre-osteoblast and inhibits the adipogenic and chondrogenic differentiation (Komori, 2006). Zhao and his colleague transduced mice with Runx2 in order to examine the osteogenic differentiation of bone morrow-derived MSCs. The data showed that Runx2-induced MSCs were able to form more osteocytes than the control, which consisted of virus- transduced MSCs. These findings indicated that Runx2 plays an essential role in osteogenic differentiation and demonstrated the ability to use Runx2 gene transfer to enhance the differentiation of MSCs into osteocytes (Zhao et al., 2005). The post-mitotic symmetric segregation of Runx2 mRNA to progeny cells has been also found to support the maintenance of osteogenic lineage commitment and osteoblast phenotype (Varela et al., 2015). Homeobox protein Hox-B7 (HOXB7) over-expression affected the mRNA expression of the key transcription factor, Runx2, and promotes osteogenic differentiation. HOXB7 can enhance the osteogenic differentiation by up-regulating Runx2 (Gao et al., 2015). Hoxa2 was also found up-regulated during osteogenic differentiation, while Hoxa9 was down-regulated (Seifert et al., 2015). In addition, overexpression of Hoxa2 induced the osteogenic differentiation, which inhibits Sox9 and chondrogenic differentiation (Seifert et al., 2015).

The expression of Runx2 is regulated by many signaling pathways, including Wnt, BMP, and Notch signaling pathways. BMP binds to BMPR and activates intracellular Smad, which translocate to the nucleus and serve as a transcription factor (Fujii et al., 1999). Therefore, deletion of the BMP ligands disrupts osteogenic differentiation (Bandyopadhyay et al., 2006). Also, BMP9 promotes the activation of Smad1, 5, and 8 and osteogenic differentiation in MSCs (Xu et al., 2012). Smad-Runx2 interaction is required for osteogenic differentiation. Induced mutations in the C-terminal domain of Runx2 disrupt Runx2-Smad transcriptional activities, which suppress osteogenic differentiation (Javed et al., 2008). The TWIST transcription factor also acts as a downstream of Hypoxia-inducible factor-1α (HIF-α), and suppresses expression of Runx2 in MSCs. This suppression results in the regulation of the osteogenic differentiation (Yang et al., 2011b). Overexpression of HIF-1α was found to enhance proliferation, cell survival and expression of pro-angiogenic genes. However, the expression of the osteogenic markers, including BMP-2 and Runx2, were decreased, indicating no effect of HIF-1α overexpression on osteogenic differentiation of MSCs (Lampert et al., 2015).

Osterix belongs to the specificity protein 1 family (Sp1) of transcription factors. It has three zinc finger motifs. Nakashima et al. (2002) reported that the formation of cortical bone and bone trabeculae through either intramembranous or endochondral ossification did not occur in the absence of osterix. In the Runx2/Cbfa1 null mice, osterix was not expressed, which

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suggested that osterix acts as a downstream of Runx2. It was concluded that Osterix is required in order to direct MSCs to osteoblasts, and hence, necessary for bone formation (Nakashima et al., 2002). Additionally, activation of the Wnt signaling pathway in MSCs induces expression of Osterix and suppresses PPAR-γ (Kang et al., 2007).

β-catenin also plays a crucial role in the differentiation of MSCs into osteoblasts. The absence of β-catenin blocks osteogenic differentiation, and allows MSCs to potentially differentiate into chondrocytes (Day et al., 2005). β-catenin activity is regulated by Wnt signaling pathway. Wnt binds to the Frizzled receptor and LRP5 co-receptor, which leads to accumulation of β-catenin in the cytoplasm. From there, it translocates to the nucleus and interacts with the transcription factor LEF/TCF. The transcription factor β-catenin activates the transcription of downstream gene by binding LEF/TCF (Huelsken and Behrens, 2002). Indeed, the transduction of MSCs with LRP5 and treatment with Wnt3a increase the mineralized bone formation in mice (Qiu et al., 2007).

The transcription factor core binding factor-1 alpha (CBF-1α) plays an important role in osteogenic differentiation of MSCs. In bone marrow-derived MSCs, hypoxia gradually increases the expression of CBF-1α, which enhances the potential of MSCs to differentiate into osteocytes (Huang et al., 2011). CBF-1α is regulated by the Notch signaling pathway, which promotes the formation of Notch intracellular domain (NICD). This event leads to activation of CBF-1α in the nucleus. NICD-overexpression in transgenic mice results in osteosclerotic bone. Conversely, the loss of Notch signaling results in age-related osteoporosis (Engin et al., 2008). TNF-α has also been found to promote osteogenic differentiation of umbilical cord derived-MSCs through NF-kB signaling pathway (Marupanthorn et al., 2015). Several studies have shown that osteogenic differentiation of MSCs can be impaired or stimulated by other transcription factors. The DLX5 transcription factor belongs to the homeoprotein family and was expressed during the formation of bone. *In vitro*, overexpression of DLX5 prevents the terminal differentiation of MSCs into osteocytes, which is mediated by SOX2 (Muraglia et al., 2008). The Foxc2 transcription factor, which belongs to the winged helix/ forkhead family, stimulates osteogenic differentiation of MSCs, and the Wnt signaling pathway plays an essential role in this process (Kim et al., 2009). Yes-associated protein is a transcriptional co-activator driven by its association with the TEAD family of transcription factors, and it is involved in development, growth, repair, homeostasis, and progression of multiple cancers as a transcriptional regulator (Marupanthorn et al., 2015). It has been found that osteogenic differentiation was enhanced by the activity of YAP, which act as a mechanosensitive transcriptional regulator (Choi et al., 2015).

A range of transcription factors are known to have major regulatory roles in osteogenic differentiation of MSCs including the most widely studied transcription factors CBFA-1/ Runx2 and Osterix. There are some other transcription factors that have been also studied and reported to have functional roles in the differentiation of MSCs to osteocytes including HOXB7, CBF-1α, TNF-α, FOXC2, YAP, HOXA2, BMP9 and β-catenin. TWIST and HIF-1α have inhibitory effect on MSC differentiation into osteocytes through their direct or indirect interaction with Runx2.

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Chondrogenic differentiation

In vitro, chondrogenic differentiation of MSCs is induced by a medium supplemented with ascorbic acid phosphate, dexamethasone, bovine serum albumin, linoleic acid, sodium pyruvate, transferrin, selenous acid, proline, L-glutamine, and TGF-β1 (Okamoto et al., 2002; Sottile et al., 2002; Suva et al., 2004). During differentiation, the morphology of MSCs changes from a fibroblast-like morphology into a round shape. Transcription factors play an important role in the regulation of the gene expression of collagen type 2, type 9, type 10, type 11, aggrecan and cartilage link protein, which are known as markers for chondrocytes (Bridgewater et al., 1998; Kou and Ikegawa, 2004; Lefebvre et al., 1997; Sekiya et al., 2000; Zhang et al., 2003). However, only a few genetic factors regulating chondrogenesis of MSCs have been identified.

The major transcription factor that have a key role in the differentiation of MSCs into chondrocytes is SRY-related high mobility group-box gene 9 (Sox9) (Augello and De Bari, 2010). Sox9 is an early transcription factor of chondrogenic differentiation and controls the expression of key genes in chondrogenesis. It controls the expression of collagen type 9 by binding to the promoter of this gene and forms trans-activating complexes with other proteins (Bridgewater et al., 1998; Wang et al., 2014). Targeting Sox9 directly by overexpression or inhibition of mRNA-145, has been shown to either decrease or increase the mRNA levels for the chondrogenic marker genes collagen type 2, type 9 type 11, and aggrecan (Yang et al., 2011a). The up-regulation miR-574-3p inhibits Sox9 and chondrogenic differentiation of MSCs (Guerit et al., 2013). Furthermore, a combination of Sox5, Sox6, and Sox9 was transfected into MSCs. The transfection resulted in a significant increase in the chondrogenic differentiation of MSCs (Park et al., 2011). TNF-α has been also found to upregulate Sox9 gene expression (Jagielski et al., 2014). Adenovirus-mediated BMP2 and Sox9 expression in mice embryonic MSCs have shown to effectively enhance chondrogenic differentiation *in vitro* (Liao et al., 2014).

The role of FOXO3A in chondrogenic differentiation of MSCs was investigated. Cells were transfected with miR-29a, whose direct target is FOXO3A. The overexpression of miR-29a has down-regulated the expression of FOXO3A and chondrocyte-specific markers during MSCs chondrogenic differentiation. The data revealed that down-regulation of miR-29a, and up-regulation of FOXO3A are important in the chondrogenic differentiation of MSCs (Guerit et al., 2014). Hoxa2 was found to be decreased during the chondrogenic differentiation of MSCs, and forced over-expression of Hoxa2 resulted in inhibition of MSCs differentiation toward chondrocytes lineage (Seifert et al., 2015). It was also reported that HOXD9 and HOXD13 were upregulated during chondrogenic differentiation of MSCs, and the inhibition of HOXD10, HOXD11 and HOXD13 inhibits MSCs differentiation into chondrocytes (Seifert et al., 2015).

Zinc-finger protein 145 (ZNF145) is a transcription factor that has been reported to play a role in the differentiation of MSCs into chondrocytes (Liu et al., 2007). Therefore, Liu and his research team examined the role of ZNF145 in chondrogenesis of MSCs. They found that the inhibition of ZNF145 decreased chondrogenic differentiation of MSCs, whereas overexpression of ZNF145 enhanced the expression of Sox9 and chondrogenesis (Liu et al., 2011). Smads have shown to function as regulators of chondrogenic differentiation of MSCs.

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Activation Smad 2 and 3 are dependent on the effect of TGF-β1 in the early stages of chondrogenesis (Zhang et al., 2015). Furumatsu et al. demonstrated that Smad3 binds the transcription factor Sox9, thereby impairing chondrogenic differentiation (Furumatsu et al., 2005). YAP, which was mentioned earlier in this paper as a regulator of osteogenic differentiation of MSCs, has been also found to have an inhibitory effect on the differentiation of MSCs to chondrocytes (Karystinou et al., 2015). Kondo and his colleagues have demonstrated that STAT3 plays a key role in the commitment of MSCs to chondrogenic lineage through the activation of STAT3 pathway by IL-6 (Kondo et al., 2015). Lui and colleagues (2014) investigated the role of Wnt11 in the chondrogenic differentiation of MSCs. Their data showed that Wnt11 overexpression stimulated the expression of chondrogenic gene regulators. In addition, the overexpression of Wnt11, in synergism with TGF-β, promoted MSCs chondrogenesis (Liu et al., 2014).

There are apparent master regulators of chondrogenic differentiation of MSCs that have been widely studied Including Sox9 and ZNF145. Other transcription factors have been also reported to have functional roles in the differentiation of MSCs to chondrocytes including FOXO3A, HOXD9, HOXD10, HOXD11, HOXD13, STAT3 and Wnt11. However, Smad3, YAP and Hoxa2 have inhibitory effect on MSC differentiation into chondrocytes through their direct or indirect interaction with Sox9. TNF-α was also reported to increase the expression of Sox9 leading to the chondrogenic differentiation of MSCs.

Adipogenic differentiation

Adipogenic differentiation of MSCs is stimulated by the incubation of MSCs in a medium containing 3-isobutyl-1-methyl-xanthine, insulin, indomethacin, triiodothyonine, Asc-2-P, basic FGF, and the glucocorticoid dexamethasone (Suva et al., 2004; Zhang et al., 2009). The differentiation of MSCs into adipocytes results in the accumulation of lipids in intracellular vacuoles (Prawitt et al., 2008). The inhibition or activation of some transcription factors is vital to the cellular commitment of MSCs to adipogenic differentiation.

To date, several transcription factors have been identified to play crucial roles in the differentiation of MSCs into adipocytes. The adipogenic-specific peroxisome proliferation-activated receptor γ (PPARγ) is one of the transcription factors that regulates the expression of genes responsible for adipogenic differentiation (Nuttall and Gimble, 2004; Zhuang et al., 2015). PPARγ has been reported to be up-regulated during the adipogenic differentiation of MSCs, and the inhibition of this transcription factor suppresses adipogenesis (Bionaz et al., 2015; Morganstein et al., 2010; Yu et al., 2012; Zou et al., 2008). PPARγ2 and PPARγ1 isoforms were reported by Wei-Hua Yu and his colleagues to play a vital role in promoting the differentiation of MSCs into adipocytes. Interestingly, the knockdown of C/EBPα inhibited PPARγ2 but not PPARγ1, suggesting that PPARγ1 plays a lesser role in adipogenic differentiation (Yu et al., 2012). The binding of PPARγ to several ligands induces the activation and repression of PPARγ. TAZ was reported to function as a co-repressor of PPARγ, therefore blocking the adipogenic differentiation (Hong et al., 2005). A recent study also showed that up-regulated expression of PPARγ2 alone or combined with CEBPB or PRDM16 promoted the adipogenic differentiation with 90% efficiency.

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Furthermore, another study demonstrated that myocyte enhancer factor-2 interacting transcriptional repressor plays an important role in suppressing the adipogenic differentiation of MSCs through interaction with PPARγ2 and inhibiting the activity of this transcription factor (Chen et al., 2012).

The early B cell factor EBF-1 is a member of a cascade of transcriptions that play major roles in cellular function and differentiation. Also, EBF-1 plays a crucial role in promoting the differentiation of MSCs into adipocytes and osteocytes (Hesslein et al., 2009). Gene expression analysis also indicated that EBF-1 and PPARγ2 induced two different sets of genes and both are linked to adipogenic differentiation. The author concluded that EBF-1 and PPARγ2 induce the differentiation of MSCs into adipocytes with comparable efficiency (Akerblad et al., 2005).

GATA-2, a member of the GATA family of zinc finger transcription factors, is known to control the proliferation and differentiation of hematopoietic stem cells and various cell lineages. Interestingly, it was reported that GATA-2 maintains the hematopoietic differentiation by regulating adipogenic differentiation. The suppression of this transcription factor enhanced the differentiation of MSCs into adipocytes, whereas the activation of GATA-2 suppressed the adipogenic differentiation (Okitsu et al., 2007). Similarly, the knockdown of the forkhead transcription factor (Foxa1) increases adipogenic differentiation of MSCs and increases the expression of PPARγ, C/EBPα, which are key transcription factors in adipogenesis (Fujimori and Amano, 2011). These findings indicate that GATA-2 and Foxa1 play a suppressive role in the differentiation of MSCs into adipocytes. HOXC8 was also found downregulated during the differentiation of MSCs to adipocytes, and forced over-expression of HOXC8 in MSCs inhibited the adipogenic differentiation (Seifert et al., 2015).

The TWIST family of basic helix-loop-helix transcription factors has been reported to play a regulatory role in adipogenic differentiation. It has been reported that enforced high expression of Twist-1 and Dermo-1 in MSC cultures was associated with an increase in the gene expression of adipocyte-associated markers (Isenmann et al., 2009). These findings indicate that Twist-1 and Dermo-1 play mediatory roles in the differentiation of MSCs into adipocytes (Isenmann et al., 2009). Reduced expression of miR-194 was found with concomitant increases in COUP-TFII expression during adipogenic differentiation of MSCs suggesting that COUP-TFII has a key role in this process (Jeong et al., 2014). Sox2 and Oct4 were also found to have a key regulatory effect on the differentiation of MSCs to adipocytes. The overexpression of these two transcription factors showed higher adipogenic differentiation in comparison to control (Han et al., 2014).

A group of transcription factors have been described to have major regulatory roles in adipogenic differentiation of MSCs including the most widely studied transcription factors PPARγ1, PPARγ2 and EBF-1. There are some other transcription factors that have been also reported to have functional roles in the differentiation of MSCs to adipocytes including CEBPB, PRDM16, Twist-1, Dermo-1, COUP-II, Sox2 and Oct4. However, GATA2, Foxa1 and HOXC8 have inhibitory effect on the differentiation of MSCs into adipocytes.

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Myogenic differentiation

Skeletal muscle cells

In recent years, many studies have reported that MSCs possess the ability to differentiate into skeletal muscle cell lineage when treated with the demethylating agent 5-azacytidine (Jackson et al., 2007; Rowlands et al., 2008). Myogenic differentiation has also been promoted by co-culturing MSCs with skeletal myocytes, neonatal fibroblasts, and neonatal cardiomyocytes (Lee et al., 2005; Ramkisoensing et al., 2011). The inhibition or activation of some transcription factors is vital to the cellular commitment of MSCs to skeletal muscle cell differentiation. Myogenic differentiation of MSCs occurs via activation of some specific myogenic transcription factors, including paired box 3 (Pax3), MyoD, Myf-5, and myogenin (Braun and Arnold, 1996; Charytonowicz et al., 2011; Gang et al., 2008). Signals from these transcription factors lead to formation of sclerotome and dermo-myotome.

During the expression of Pax3, cells migrate through the dorsomedial lip of the dermomyotome to form the myotome and promote myogenic differentiation (Charytonowicz et al., 2011). Pax3 and Pax7 are members of the paired box family of transcription factors. They are known as master regulators of myogenic differentiation, as it contributes to early striated muscle development during skeletal muscle development and regeneration. The overexpression of Pax3 in MSCs after transduction promotes the myogenic differentiation and blocks the adipogenic, osteogenic, and chondrogenic differentiation of MSCs (Gang et al., 2008). In another study, MSCs were transfected with Pax3 and Pax7 genes and the resulting data implied that Pax3 and Pax7 transcription factors are required for commitment of MSCs to myocytes (Charytonowicz et al., 2011).

MyoD, Myf-5, and myogenin, which are members of the helix-loop-helix family transcription factors, play a key role in regulating myogenic differentiation. Interestingly, the expression of MyoD and Myf-5 does not occur within the same committed MSCs; therefore, MyoD and Myf-5 transcription factors determine different muscle cell lineages arising from different committed MSCs (Braun and Arnold, 1996). MyoD overexpression was found to inhibit Twist-1 through miR-206 induction, resulting in an increase in muscle cell differentiation (Koutalianos et al., 2015). Overexpression of MyoD1, using a vector to human-induced pluripotent stem cells, promotes these cells to undergo myogenic differentiation (Shoji et al., 2015). The overexpression of TAZ, which is known as a modulator of osteogenic and adipogenic differentiation of MSCs, was found to increase MyoD-mediated myogenic differentiation. This indicates that TAZ plays a regulatory role in the differentiation of MSCs toward myocytes (Jeong et al., 2010). Incubation of MSCs in myogenic medium significantly upregulates the expression of MyoD and myogenin, sugesting the regulatory role of the two transcription factors in the commitment of MSCs to myogenic lineages (Gang et al., 2004). Myogenic differentiation can be induced or inhibited by signaling factors. Insulin-like growth factor-II (IGF-II) induces the myogenic differentiation through the insulin-like growth factor receptor-1, which targets co-regulators of important cofactors for MyoD (Wilson and Rotwein, 2006). Tumor necrosis factor-α (TNF-α) also has a regulatory role in the differentiation of MSCs into myocytes. It down-regulates the expression of the MyoD transcription factor, which is required for myocyte

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development (Sitcheran et al., 2003). TNF-α inhibits myogenic differentiation through NF-κB activation and reduction of IGF-1 signaling pathway (Zhao et al., 2015). Similarly, Smad3, which belongs to receptor-regulated Smad family, suppresses myogenic differentiation through its association with myogenic transcription factors. TGF-β-activated Smad3 directly suppresses the transcriptional activity of MyoD and myogenin (Liu et al., 2001; Liu et al., 2004).

Several transcription factors have been reported to have key regulatory roles in skeletal muscle cell differentiation of MSCs including Pax3, Pax7, Myf-5 and MyoD. Few other transcription factors have been reported to have functional roles in the differentiation of MSCs to skeletal muscle cells including Myogenin, TAZ and IGF-II. However, TNF-α was found to have inhibitory effects on the differentiation of MSCs through the activation of NF-κB signaling pathway. Smad3 as well was reported to inhibit skeletal muscle differentiation of MSCs.

Cardiomyocytes

Myogenic differentiation has been promoted by co-culturing MSCs with skeletal myocytes, neonatal fibroblasts, and neonatal cardiomyocytes (Lee et al., 2005; Ramkisoensing et al., 2011). MSCs were reported to have the potential to differentiate into cardiomyocytes after two to three weeks of treatment with 5-azacytidine in a medium consisting of low-glucose DMEM supplemented with 10% FBS (Wei et al., 2011). At the molecular level, several transcription factors have been identified as regulators for cardiomyogenic differentiation of MSCs.

GATA4, which belongs to the GATA zinc finger transcription factor family, has been shown to regulate differentiation and growth of different cell types. GATA4-transduced MSCs were shown to have a higher expression of GATA4 than the control MSCs during cardiomyogenic differentiation, suggesting that the overexpression of GATA4 increases the potential of MSCs to differentiate into cardiomyocytes (Li et al., 2010). In the presence of myocytes, MSCs were able to differentiate into cardiomyocytes with a higher expression of GATA4 than the undifferentiated MSCs (Hatzistergos et al., 2010; Xu et al., 2004). Moreover, the overexpression of Nkx2.5 and GATA4 promotes differentiation of MSCs into cardiomyocytes, and Nkx2.5 and GATA4 are required for cardiomyogenic differentiation (Arminan et al., 2010; Yamada et al., 2007). During differentiation of MSCs into cardiomyocytes, the expression of Nkx2.5 and GATA4 was significantly enhanced by VEGF. Anti-VEGF antibodies also suppress the expression of cardiomyocyte markers (Song et al., 2007). The transduction of Wnt11 gene promotes differentiation of MSCs toward cardiomyocytes by up-regulating GATA4 (He et al., 2011). However, the mechanism behind the up-regulation of GATA4 by Wnt11 is still not clear.

Myocardin is a smooth muscle cell and cardiomyogenic transcription factor that contains two or more essential binding sites for serum response factor (SRF). *In vitro*, overexpression of myocardin gene in MSCs induced the expression of cardiomyogenic genes (van Tuyn et al., 2005). It was reported that forced myocardin expression in MSCs, after transduction of MSCs with human adenovirus vectors expressing myocardin, promotes the expression of several cardiomyogenic markers *in vitro*. However, myocardin-transduced MSCs did not

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result in complete cardiomyogenic differentiation suggesting that it only stimulates the expression of early developmental genes (Grauss et al., 2008). Thioredoxin-1 (Trx1), an antioxidant, transcription factor, and growth-factor regulator, was found to significantly enhance the differentiation of MSCs to cardiomyocytes. Genetically modified MSCs with an adenovector expressing Trx1, compared to control MSCs without adeno-vector expressing Trx1, showed significant increase in the proliferation and differentiation of MSCs to cardiomyocytes *in vitro* and *in vivo* after transplantation into rat heart tissue (Suresh et al., 2015). Moreover, Ding et al. demonstrated that genetically modified MSCs with an adenovector over-expressing Notch1 intracellular domain (NICD) showed increased differentiation capability of MSCs favoring differentiation into cardiomyocytes (Ding et al., 2015).

GATA4 and NKX2.5 are the most widely studied transcription factors that are known to have major regulatory roles in cardiomyogenic differentiation of MSCs. There are some other transcription factors that have been studied and reported to have functional roles in the differentiation of MSCs to osteocytes including myocardin, Trx1, Wnt11 and Notch1.

Smooth Muscle cells

The most effective inducer of vascular smooth muscle cell differentiation of MSCs is TGF-β. It up-regulates the genes of smooth muscle cell markers alpha smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (SMMHC) and calponin (Deaton et al., 2005; Sinha et al., 2004). It has been also reported that TGF-β1 inhibits the proliferation of MSCs and promotes the avascular smooth muscle differentiation (Ross et al., 2006), and 5-azacytidine and amphotericin B treatments induce MSCs differentiation into myoblasts (Chamberlain et al., 2007). Previous studies have reported that MSCs possess the ability to differentiate into smooth muscle cell lineage (Jackson et al., 2007; Rowlands et al., 2008).

There are a few transcription factors, including myocardin, GATA6, and serum response factor (SRF), that are commonly known to assess smooth muscle cell differentiation (Ross et al., 2006). The treatment of MSCs with the MEK inhibitor up-regulated the expression of smooth muscle cell markers and induced the expression of myocardin, indicating that the inhibition of MEK signaling induces the differentiation of MSCs into smooth muscle cells (Tamama et al., 2008). Additionally, another study demonstrated that sphingosylphosphrylcholine (SPC) induces the differentiation MSCs into smooth muscle cells through a RhoA/Rho kinase-dependent mechanism. SPC was found to up-regulate the expression of the myocardin-related transcription factor (Jeon et al., 2008). It has recently been reported that GATA6, which is up-regulated by sphingosine 1-phosphate (S1P), is a novel player in smooth muscle cell differentiation (Donati et al., 2011). Moreover, TGF-β activates the transcription factors, GATA6 and SRF, during smooth muscle differentiation. This activation enhances the expression of smooth muscle marker genes α-SMA, SM22-α, SMMHC, and calponin in MSCs (Deaton et al., 2005). It was also reported that the expression of specific SMC markers were significantly increased with TGF-β supplementation when MSCs cultured on silk hydrogels compared to MSCs without TGF-β supplementation in media (Floren et al., 2016). PPAR γ was found to have an inhibitory effect on the differentiation of MSCs to myofibroblasts, which have some of the

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characteristics of smooth muscle cells. Transfection of MSCs with PPARγ-siRNA resulted in increased expression of alpha smooth muscle actin (α-SMA) with TGF-β treatment (Jia et al., 2015). The expression of the laminin isoform LM-521 during the differentiation process, in media containing TGF-β, was demonstrated to enhance the differentiation of MSCs to smooth muscle cells (Seeger et al., 2015). Moreover, olfactomedin 2 (Olfm2) was found to play an important role in TGF-β-induced differentiation of MSCs to smooth muscle cells, which was found to be upregulated during the differentiation. The knockdown of Olfm2 resulted in downregulation of smooth muscle cell markers expression, while the overexpression of Olfm2 increased the expression of the same markers (Shi and Chen, 2015). Olfm2 binds to serum response factor (SRF) and promotes SRF/CArG box interaction leading to increased expression of smooth muscle cell markers (Shi and Chen, 2015).

The major transcription factors that have been reported to have key roles in the differentiation of MSCs into smooth muscle cells are GATA6 and SRF. Forced expression of Olfm2 and treatment with TGF-β were found to increase the expression of smooth muscle cell markers during the differentiation of MSCs. However, PPARγ expression has inhibitory effect on the differentiation of MSCs into smooth muscle cells. There is still ongoing need for more studies to investigate the role of other transcription factors on the differentiation of MSCs to smooth muscle cells as well as their signaling pathways.

Endothelial cell differentiation

MSC therapy can be used for regeneration of the endothelial layer. Identification of cellular regulators that facilitate MSC differentiation to endothelial cells (ECs) is essential to the future of MSC therapy (Pankajakshan et al., 2013). Transcription factors could be transiently manipulated to induce differentiation. To date, there is limited published data that show the relationship between transcription factors and endothelial differentiation of MSCs. In our laboratory, we have been successful in differentiating MSCs into endothelial cells using gene based modifications. Using our protocol, we have identified Sox18 as a transcription factor that can be manipulated to enhance the differentiation of MSCs to ECs (Ikhapoh et al., 2015). We also found that the expression level of Sox18 was up regulated during the differentiation process through VEGFR-II. Additionally, we found that MSCs deficient in Sox18 maintain their undifferentiated phenotype (Ikhapoh et al., 2015). These results provided evidence that the overexpression of Sox18 in MSCs stimulates the translation of EC markers (Ikhapoh et al., 2015). Therefore, Sox18 is a critical regulator of MSC differentiation to ECs which can provide a new clinical application of MSC therapy in cardiovascular disease. The expression of HOXA7 and HOXB3 were also found to be upregulated during the differentiation of MSCs to endothelial cells, while the expression of HOXA3 and HOXB13 were significantly downregulated (Chung et al., 2009). Moreover, it was reported that HOXB5 increases the expression of vascular endothelial growth factor receptor-2 (VEGFR-II), which is a major player in the differentiation of MSCs to endothelial cells (Seifert et al., 2015). Bago et al. (2013) studied the effect of αNotch signaling on the differentiation of MSCs to endothelial cells and the formation of capillary-like structure *in vitro* and *in vivo*. Notch1 knock down by shRNA resulted in a lack of capillary-like structures formation. The expression of endothelial cell specific markers was also

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significantly decreased after Notch1 knock down (Bago et al., 2013). Other biomechanical and biochemical stimuli were found to induce the expression of endothelial cell markers (Ikhapoh et al., 2015b; Kim et al., 2016). Steady shear stress was reported to increase the differentiation of MSCs to endothelial cells. A fabrication of double-layered tubular scaffolds was used to mimic the structural microenvironment of blood vessels with a bioreactor system to apply fluid shear stress. MSCs that were cultured on the inner layer developed endothelial cell phenotype, and the expression of endothelial markers were significantly elevated (Kim et al., 2016). Furthermore, MSCs that were treated with angiotensin type II (ATII) and VEGF-A showed a higher expression of specific endothelial cell markers compared the MSCs that were treated with VEGF-A alone indicating the crucial role of ATII with VEGF-A in the differentiation of MSCs to endothelial cells (Ikhapoh et al., 2015b). However, the mechanism underlying the effect of ATII on the endothelial cell differentiation of MSCs is still not understood.

A group of few transcription factors have been reported to have functional effects on the differentiation of MSCs to endothelial cells including Sox18, HOXA7, HOXB3, HOXB5 and Notch1, while HOXA3 and HOXB13 were found to be downregulated during endothelial cell differentiation of MSCs. Moreover, treatment of MSCs with VEGF-A and ATII has been reported to stimulate the expression of endothelial cell markers during the differentiation of MSCs.

Conclusion

Among cell types, MSCs are a powerful candidate for regenerative medicine, and for the study of cellular differentiation. They represent an attractive cell source for transplantation because they can be isolated from different tissues. In addition to their ability to differentiate into adipocytes, chondrocytes, osteocytes, smooth muscle cells, endothelial cells, and cardiomyocytes, MSCs are capable of differentiating into additional cell lineage such as neurons and hepatocytes (Pacary et al., 2006; Talens-Visconti et al., 2006; Yuan et al., 2012). Each of these types of cells is associated with expression of a distinct set of proteins. However, there are many challenges to fully understand the mechanisms underlying the differentiation of MSCs in various lineages. These challenges include the identification of the signaling and transcription factors as well as the crosstalk between signaling pathways that promote the self-renewal and lineage differentiation in MSCs. The potential of MSCs to differentiate into a particular mesenchymal lineage relies upon up-regulation or suppression of genes specific to a lineage. During the differentiation, up-regulation or suppression of transcription factors occurs via specific signaling pathways or interaction with other transcription factors that act as co-regulators.

The differentiation of MSC into each of these cell lineages is promoted by specific transcription factors associated with a key cell lineage. In osteogenic differentiation, Runx2 is a key transcription factor that elevates the osteoblast differentiation and inhibits the adipogenic and chondrogenic differentiation. The expression of Runx2 is regulated by many signaling pathways such as the Wnt, BMP, and Notch signaling pathways. Chondrogenic differentiation is driven by Sox9. However, Nkx3.2 is required expression of Sox9 and suppression of osteogenic differentiation. Also, Sox9 can physically interact with and inhibit

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Runx2 (Augello and De Bari, 2010). This interaction can determine the commitment and fate of MSCs to differentiate into either osteocytes or chondrocytes. Adipogenic differentiation is mainly controlled by PPARγ, which cooperates with other transcription factors to promote the expression of adipogenic markers. The differentiation of MSCs into either adipocytes or osteocytes is also regulated by interactions of different transcription factors including the major players Runx2 and PPARγ, and the induction into one lineage blocks the commitment of MSCs to differentiate to the other (James, 2013). The commitment of MSCs to differentiate into either adipocytes or osteocytes is regulated through different signaling pathways including Wnt, Hedgehog, NELL-1, BMP, and IGF signaling (James, 2013). Finally, members of the helix-loop-helix transcription factor family form heterodimeric complexes with E proteins and promote myogenic differentiation. Their activities are dependent upon the interaction with MEF proteins. Other transcription factors, including GATA6, MyoD, and GATA4, function as drivers for smooth muscle cell, skeletal muscle cells, and cardiomyocytes, respectively (Figure 2).

There are few studies that have discussed and examined the differentiation of MSCs into fibroblasts, and the role of transcription factors in this process. Indeed, MSCs are capable of differentiating into fibroblasts-like cells in appropriate *in vitro* conditioned medium containing 100 ng/ml connective tissue growth factor (CTGF) and 50 mg/ml ascorbic acid (Hu et al., 2014; Tong et al., 2011). It has been also demonstrated that CTGF is sufficient to differentiate MSCs into fibroblasts, and the differentiated cells lost their ability to differentiate into other lineages (Lee et al., 2010). CTGF supplement decreases the expression of the MSC markers CD44 and STRO-1, and increases the expression of a fibroblast specific marker, fibroblast specific protein 1 (FSP-1) (Tong et al., 2011). The treated MSCs showed significant increases in the expression of type I collagen and tenascin-C (Lee et al., 2006). The fibroblast-like cells, differentiated from MSCs, were able to produce extracellular matrix proteins (Hu et al., 2014). Although the differentiation of MSCs into fibroblasts has many implications in the tissue engineering including tendons and ligaments repair (Lee et al., 2010), limited studies warrants further investigation. Additional studies are required to identify the signaling factors, master transcription factors, and signaling pathways that mediate fibroblastic differentiation of MSCs.

Several approaches have been tested and employed to introduce transcription factor genes into MSCs for use in regenerative therapy. Transfection methods have shown low efficiency in delivering DNA plasmid into MSCs and high mortality rates. Conversely, viral transduction methods have shown the ability to deliver DNA plasmid with high efficiency and low toxicity. However, the safety concern associated with viral transduction is a controversial topic. To date, numerous studies have shown that the delivery of transcription factors into MSCs increases and maintains the potential of these cells to differentiate into intended cell lineage. Therefore, forced expression of a single transcription factor in MSCs can promote trans-differentiation into several cell lineages, allowing for the treatment of many diseases. Further studies are required in order to avoid any activation of the innate tendency of these cells to differentiate into other (unintended) mesenchymal tissues.

The identification of specific transcription factors, receptors, and signaling molecules during differentiation is important in the understanding of the link between intracellular and

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extracellular signaling networks. Additional studies are warranted in order to deepen the understanding of the regulation of MSC differentiation by transcription factors at distinct levels during progression of a differentiation pathway. Since transcription factors naturally act as master regulators of cellular processes, they are expected to be excellent candidates for controlling differentiation of MSCs into any cell lineage, and transcription factor-based technologies are likely to be a prominent part of the next generation of MSC-based therapy.

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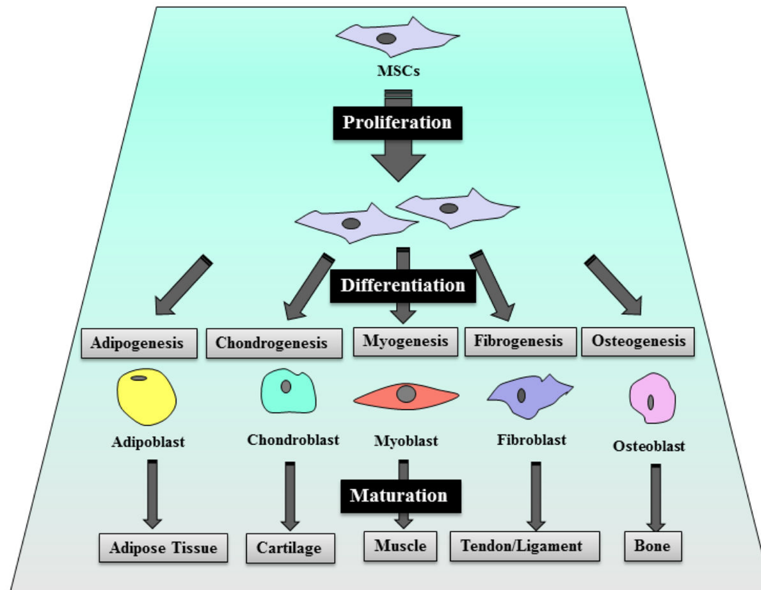

Figure 1.
Mesenchymal stem cells differentiate into osteoblast, chondrocyte, myoblast, fibroblast and adipocyte, depending on the stimuli in the differentiation-induction media (*in vitro*).

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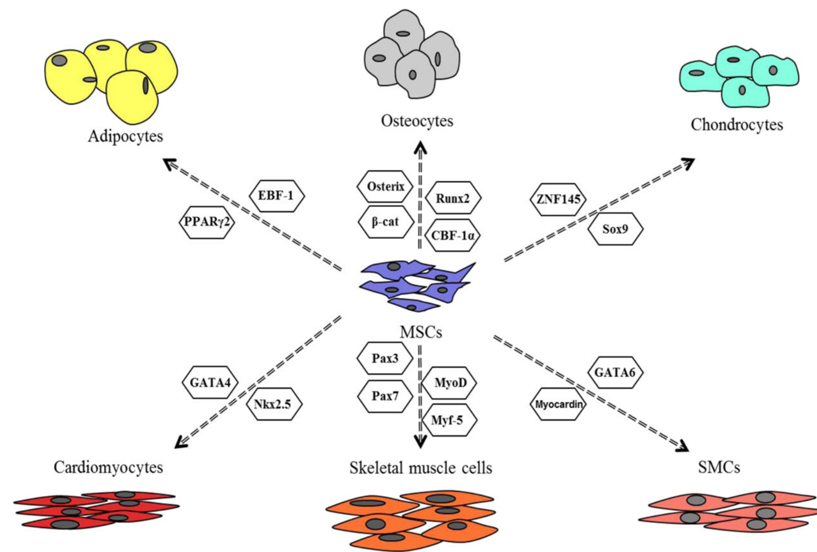

Figure 2.

Major transcription factors that promote differentiation of mesenchymal stem cells into osteocytes, chondrocytes, adipocytes, smooth muscle cells, skeletal muscle cells, and cardiomyocytes.

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Table 1

Factors in inducing differentiation of MSCs to specific lineage.

Inducer	Concentration	Effect	Direction of differentiation	Reference
Dexamethasone + 3-isobutyl-1-methylxanthine + insulin	1 μM 0.5 mM 0.01 mg/ml	Increase fat deposition in the cytoplasm	Adipocytes	(Yu et al., 2012)
Ascorbic acid + dexamethasone	50 μM 100 nM	Increases the activity of alkaline phosphatase	Osteocytes	(Yu et al., 2012)
TGF- β1 + ascorbic acid + dexamethasone	10 ng/ml 50 μg/ml 100 nM		Chondrocytes	(Vater et al., 2011)
TGF-β1	1, 2, 5 and 10 ng/ml	Stimulate SMC-specific genes expression	Smooth muscle cells	(Vater et al., 2011; Floren et al., 2016)
5-azacytidine	3 μM 10 μM	Inhibits DNA methyltransferase	Cardiomyocytes	(Yamada et al., 2007; Rowlands et al., 2008; Wei et al., 2011))
VEGF	10, 50 ng/ml	Phosphorylate VEGF-RII and increase its Activity	Endothelial	(Pankajakshan et al., 2013; Vater et al., 2011)