

Different roles of TGF- β in the multi-lineage differentiation of stem cells

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Abstract

Stem cells are a population of cells that has infinite or long-term self-renewal ability and can produce various kinds of descendent cells. Transforming growth factor β (TGF- β) family is a superfamily of growth factors, including TGF- β 1, TGF- β 2 and TGF- β 3, bone morphogenetic proteins, activin/inhibin, and some other cytokines such as nodal, which plays very important roles in regulating a wide variety of biological processes, such as cell growth, differentiation, cell death. TGF- β , a pleiotropic cytokine, has been proved to be differentially involved

in the regulation of multi-lineage differentiation of stem cells, through the Smad pathway, non-Smad pathways including mitogen-activated protein kinase pathways, phosphatidylinositol-3-kinase/AKT pathways and Rho-like GTPase signaling pathways, and their cross-talks. For instance, it is generally known that TGF- β promotes the differentiation of stem cells into smooth muscle cells, immature cardiomyocytes, chondrocytes, neurocytes, hepatic stellate cells, Th17 cells, and dendritic cells. However, TGF- β inhibits the differentiation of stem cells into myotubes, adipocytes, endothelial cells, and natural killer cells. Additionally, TGF- β can provide competence for early stages of osteoblastic differentiation, but at late stages TGF- β acts as an inhibitor. The three mammalian isoforms (TGF- β 1, 2 and 3) have distinct but overlapping effects on hematopoiesis. Understanding the mechanisms underlying the regulatory effect of TGF- β in the stem cell multi-lineage differentiation is of importance in stem cell biology, and will facilitate both basic research and clinical applications of stem cells. In this article, we discuss the current status and progress in our understanding of different mechanisms by which TGF- β controls multi-lineage differentiation of stem cells.

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Key words: Transforming growth factor β ; Stem cells; Cell differentiation; Cell signaling

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INTRODUCTION

Stem cell research originated in the middle of last century^[1,2], thereafter developing rapidly. The concept of stem cells is used to explain not only physiological development and regeneration, but also the clonal origination of tumor cells, such as the confirmation by Fialkow *et al.*^[3] that chronic myelocytic leukemia is derived from a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage lineages. Adult stem cell (ASC)-based therapies have been successful for several decades, with the first transplantation of hematopoietic stem cells (HSCs) occurring over 50 years ago. HSC transplantation has now become a standard clinical procedure, particularly as a treatment for leukemia and lymphoma^[4,5]. There have been countless research studies on stem cells published in scientific journals in recent years. A very recent leap forward is the successful generation by Takahashi *et al.*^[6,7] in 2007 of induced pluripotent stem cells from mouse somatic cells or adult human dermal fibroblasts by transduction of four defined transcription factors, which avoided the ethical dispute concerning the clinical application of embryonic stem cells (ESCs). In 2009, the US Food and Drug Administration allowed the second human clinical trials using ESCs in an inherited juvenile macular degeneration, Stargardt disease. In 2010, the application guideline of the Chinese National Key Basic Research and Development Plan gave preferential support to the following research fields: the maintenance of stemness and stem cell differentiation, stem cells and senescence, stem cells and regeneration of organs, structure and function of proteins involved in stem cell differentiation and cell reprogramming. Nowadays, stem cells research encompasses nearly all fields of life sciences and medicine, and has become one of the most popular topics in basic life science research and regenerative medicine. Several of the most interesting clinical trials in stem cells are currently in progress^[8,9].

The transforming growth factor β (TGF- β) family is a superfamily of growth factors, which includes TGF- β 1, TGF- β 2 and TGF- β 3, bone morphogenetic proteins (BMPs), activin/inhibin, and some other cytokines such as nodal. The TGF- β family plays very important roles in regulating a wide variety of biological processes, namely: cell growth, apoptosis, differentiation, migration, extracellular matrix (ECM) production, immunity, angiogenesis, tumor metastasis and invasion, and embryonic development^[10,11]. In this article, we mainly discuss the various roles of TGF- β signaling in multi-lineage differentiation of stem cells.

TGF- β SIGNALING

TGF- β was originally identified as a component of the "sarcoma growth factor" that was able to mediate transformation of non-neoplastic rat kidney NRK and murine fibroblasts^[12,13]. Since its discovery, the TGF- β family has been confirmed to regulate a wide variety of biological processes^[14]. TGF- β can promote or inhibit cell growth

depending on the cell type. For instance, TGF- β promotes the growth of fibroblasts, osteoblasts, and other mesenchymal cells, while it inhibits the growth of epithelial cells and neuroectodermal cells. TGF- β also has multiple immunomodulatory effects. In the initial stages of inflammation, TGF- β exhibits immune stimulation by recruiting inflammatory cells and producing inflammatory cytokines, while in the concluding stage of inflammation it shows immunosuppressive activity. Additionally, TGF- β can suppress or promote carcinogenesis depending on the cell type and stimulation context^[15]. Eight Smad proteins are encoded in the human and mouse genomes, including receptor-regulated Smads, (also known as RSmads - Smad1, Smad2, Smad3, Smad5, and Smad8), Co-Smad (Smad4, serves as a common partner for all RSmads), and inhibitory Smads (Smad6 and Smad7)^[16]. It is well-known that TGF- β exerts multiple biological effects *via* Smad^[17] and non-Smad^[18] pathways. In the Smad pathway, TGF- β binds to pairs of receptor serine/threonine kinases, known as the TGF- β type I (T β R-I) and type II (T β R-II) receptors, and forms a hetero-tetrameric receptor complex. In this complex, T β R-II phosphorylates a serine/threonine-rich region that is located N-terminal to the canonical kinase domain of T β R-I. Smad2 or Smad3 is then phosphorylated by T β R-I, and these receptor-activated Smads (R-Smads) later form a complex with a common Smad4. Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes, through physical interaction and functional cooperation with DNA-binding cofactors and transcriptional coactivators or corepressors^[16,19]. In non-Smad pathways TGF- β utilizes a multitude of intracellular signaling pathways including extracellular regulated kinase (ERK)^[20], p38 kinase^[21], c-Jun N-terminal kinase (JNK)^[22], phosphatidylinositol-3-kinase (PI3K)/AKT^[23], or Rho-like GTPase^[24] signaling pathways, to regulate cell function and sometimes coordinate with the Smad pathway.

STEM CELLS

Stem cells are a population of cells which has infinite or long-term self-renewal ability and can produce various kinds of descendent cells. Self-renewal and multi-lineage differentiation are two basic characteristics of stem cells^[25]. According to their differentiation abilities, stem cells are classified as totipotent stem cells, pluripotent stem cells, multipotent stem cells and monopotent stem cells. Based on their origins, stem cells are defined as ESCs and ASCs. ESCs have almost unlimited capacity for proliferation and the ability to form all cell types, but their clinical application is limited by ethical disputes, the formation of teratomas and the possibility of provoking immune reaction after their transplantation into a new host^[26,27]. Although ASCs can differentiate into only specified cell types, their easy isolation and amplification *in vitro*, low culture cost and the absence of ethical concerns have given ASCs great potential and a promising future in clinical applications. So far, ASCs have already

been found in a variety of tissues including HSCs, mesenchymal stem cells (MSCs), reproductive stem cells^[25].

DIFFERENT ROLES OF TGF- β IN THE MULTI-LINEAGE DIFFERENTIATION OF STEM CELLS

TGF- β is a pleiotropic cytokine and mainly regulates stem cell differentiation through the Smad pathway including Smad2, Smad3 and Smad4, which play crucial roles in mediating the intracellular responses to TGF- β , non-Smad pathways including ERK, p38 kinase, PI3K/AKT, and Rho-like GTPase signaling pathways, and their cross-talks.

TGF- β in smooth muscle differentiation

Both *in vivo* and *in vitro* experiments have proved that stem cells can differentiate into smooth muscle cells (SMCs)^[28-31], and stem cells have become the potential seed cells of SMCs in artificial blood vessels. TGF- β is a critical cytokine in the differentiation of stem cells into SMCs, but the downstream signaling mechanisms in this process have not yet been elucidated^[32-34]. In a model of smooth muscle differentiation, when neural crest stem cell line Monc-1 was induced into SMCs by TGF- β , a rapid induction of phosphorylation of Smad2 and Smad3 was observed. When the cells were transfected with small interfering RNA (siRNA) targeting either Smad2 or Smad3, α -smooth muscle actin (α -SMA) expression was decreased, which indicated that TGF- β -activated Smad2 and Smad3 were necessary for complete induction of at least one SMCs marker, α -SMA, and that Smad2 and Smad3 may cooperate to induce a smooth muscle phenotype in Monc-1 cells^[35]. When endogenous TGF- β was inhibited with an adenovirus expressing a soluble truncated TGF- β type II receptor, an antibody specific for TGF- β 1, or siRNA to knockdown expression of TGF- β 1, the increase in SMC-selective gene expression, α -SMA, myocardin, or smooth muscle myosin heavy chain (SMMHC), was also attenuated in the ESCs, and the decrease in α -SMA and myocardin expression could not be rescued by the addition of exogenous TGF- β 1. When using siRNA to knockdown expression of Smad2 or Smad3, it was found that α -SMA promoter activity was dependent on both Smad2 and Smad3 whereas SMMHC activity was Smad2 dependent, which suggested that different TGF- β signaling pathways may contribute to induction of early (α -SMA) *vs* late (SMMHC) SMCs markers^[36,37]. In sphingosylphosphorylcholine (SPC)-induced differentiation of human adipose tissue-derived MSCs to smooth muscle-like cells, G(i/o)-ERK-dependent autocrine secretion of TGF- β can activate a Smad2-SRF/myocardin-dependent pathway^[38]. Additionally, TGF- β 1-promoted smooth muscle differentiation of a murine embryonic mesenchymal progenitor cell (MPC) line, C3H10T1/2, is through the inhibition of Notch3 mediated by Smad activity and p38 kinase, activation of

Hes1 by Smad2 but not Smad3, and Hes1 augmented transcription of smooth muscle differentiation gene, SM22 α , in collaboration with Smad3^[39]. However, the effects of TGF- β and Notch signaling on human MSCs and ESCs are different from those in C3H 10T1/2 cells. TGF- β can induce Notch ligand Jagged 1 (JAG1) and SMC markers, including α -SMA, calponin 1 (CNN1), and myocardin in human MSCs, which are dependent on the activation of Smad3 and Rho kinase. Knocking down JAG1 expression partially blocked α -SMA and CNN1 expression and completely blocked myocardin expression. Meanwhile, the activation of Notch signaling induced the differentiation of human MSCs and ESCs into SMCs, and resulted in an increase of neural markers and a decrease of endothelial markers in human ESCs (hESCs)^[34]. In other words, the effects of TGF- β and Notch on SMCs markers expression may depend on the cell types involved.

TGF- β in cardiac and skeletal muscle differentiation

Experimental evidence indicates that TGF- β can induce the differentiation of bone marrow stem cells (BMSCs) into immature cardiomyocytes. TGF- β 1 can increase the cellular expression of myosin, troponins, connexin-43, GATA-4, and NKx-2.5 in CD117+ BMSCs, and after the intramyocardial implantation of TGF- β -preprogrammed CD117+ cells in an acute myocardial infarction mouse model, injured myocardium was effectively regenerated and induced therapeutic angiogenesis, contributing to functional cardiac regeneration^[40]. Although TGF- β treatment increased the expression of the cardiac transcription factors, GATA-4 and NKx-2.5, in BMSCs in 1-3 d, and cardiac myosin, troponins and ANP in 3-14 d, the Ca²⁺ transient was relatively weak, the connexin-43 expression was irregular, and spontaneous beating was not detected within 28 d of observation. Furthermore, TGF- β stimulation up-regulated most of the TGF- β /BMP signaling pathway genes, including TGFBI, ACVR2B, and phosphorylated Smad2 and Smad3, within 24 h, which indicated that the TGF- β /BMP signaling pathway might play an important role in cardiac differentiation^[41].

However, TGF- β inhibits myoblast differentiation, apparently through two mechanisms acting in concert: a block in the expression of myogenic differentiation genes, such as myogenin, and TGF- β -induced changes in cell adhesion^[42]. Other studies showed that TGF- β intracellular effector Smad3, but not Smad2, mediated the inhibition of myogenic differentiation in MyoD-expressing C3H10T1/2 cells and C2C12 myoblasts, by interfering with the assembly of myogenic bHLH transcription factor heterodimers on E-box sequences in the regulatory regions of muscle-specific gene, MyoD, suppressing the transcription activity of a second class of essential myogenic factors, MEF2, and blocking the SRC family coactivator GRIP-1-induced redistribution of MEF2C to discrete nuclear subdomains in C3H10T1/2 cells, and the recruitment of GRIP-1 to the myogenin promoter in differentiating myoblasts^[43,44].

TGF- β in osteogenic and chondrogenic differentiation

TGF- β is abundant in bone and plays a critical role in bone remodeling^[45], which is a complex process and relies on the interplay between bone resorption and formation that involves osteoclasts, osteoblasts, and osteocytes^[46]. In the early stages of osteoblastic differentiation, TGF- β can provide competence, but at late stages of osteoblastic differentiation, TGF- β acts as an inhibitor^[47]. This maturation stage-dependent effect of TGF- β was also confirmed in highly pure CD14 osteoclast precursor cells. During the initial period (days 1-7), when exposed to TGF- β , TRAP activity and bone resorption were increased by 40%, whereas with the continuous exposure of TGF- β , TRAP activity, cathepsin K, and matrix metalloproteinase 9 expression as well as bone resorption were almost completely abrogated. The molecular mechanism may be that TGF- β promotes osteoclastogenesis through strongly stimulation of the p38 mitogen-activated protein kinase (MAPK) in the early stage, whereas continuous exposure to TGF- β abrogates osteoclastogenesis through down-regulation of RANK expression and therefore attenuation of RANK-RANK-L signaling^[46]. Additionally, one study showed that TGF- β inhibited osteoblast differentiation through inhibition of both Runx2 (Cbfa1) transcription and transcriptional activation of osteoblast differentiation genes by CBFA1, which was mainly mediated by Smad3, but not Smad2, and the class IIa histone deacetylases (HDAC) 4 and 5 acted as corepressors for TGF- β /Smad3-mediated transcriptional repression of Runx2 function in differentiating osteoblasts^[48,49].

TGF- β is known to be a potent inducer of stem cells chondrogenic differentiation, and continuous treatment with TGF- β is necessary for effective chondrogenesis^[50-52]. Chondrogenic gene expression and protein synthesis directly correlates with the length of stimulation time and the concentration of TGF- β . Pretreatment with TGF- β could prevent fully differentiated and MSCs encapsulated in alginate beads from transdifferentiating into osteoblasts^[53]. Although BMP-2 induces osteogenic and chondrogenic phenotypes in alginate-encapsulated adipose-derived ASCs following 14 d of stimulation, TGF- β 1 can inhibit BMP-2-induced differentiation of the osteogenic lineage, and combined growth factor treatment shows a synergistic effect on the expression of cartilage-specific genes and elevated release of cartilage-specific ECM proteins^[54]. Supplementation with TGF- β 1 could initiate and promote chondrogenesis of synovium-derived stem cell (SDSCs), but TGF- β 1 alone was insufficient to fully differentiate SDSCs into chondrocytes. However, HDAC4 overexpression can promote TGF- β 1-induced SDSC chondrogenesis but inhibit chondrogenically differentiated stem cell hypertrophy^[55]. The mechanism underlying this process is still unknown. Recent investigation suggested that C-type natriuretic peptide/NPR-B signaling pathway was activated during TGF- β 1 induced chondrogenic differentiation of human trabecular bone-derived MSCs and may be involved in glycosaminoglycan synthesis during this process in a

dose-dependent manner^[56]. The chondrogenesis of trabecular bone-derived MPCs was initiated and maintained by TGF- β 1 through the differential chondro-stimulatory activities of p38, ERK-1, and JNK. TGF- β 1-mediated MAPK activation also controlled wnt-7a gene expression and WNT-mediated signaling through the intracellular β -catenin-TCF pathway, which probably regulated the expression of cell adhesion protein, N-cadherin^[57]. However, it is reported that TGF- β inhibited early chondrogenic induction of human ESCs but was required at the later stages of the differentiation, and TGF- β can sustain an undifferentiated population of ESCs within the differentiation culture, suggesting that caution should be exercised to avoid possible teratoma formation *in vivo* when using TGF- β as a chondrogenic inducer of ESCs^[58].

TGF- β in adipogenic differentiation

Exogenous TGF- β is a potent inhibitor during the early stages of adipogenic differentiation induction. However, once morphologic differentiation begin, TGF- β is ineffective in blocking adipogenic differentiation^[59]. During the adipogenic differentiation of murine preadipocyte cell line 3T3-F442A, the cell-surface availability of TGF- β receptors, and mRNA levels for Smads 6 and 7 decreased strongly, but were unchanged for Smads 2, 3, and 4. Stably expressing a truncated type II TGF- β receptor enhanced differentiation and decreased growth. Stable overexpression of Smad2 or Smad3 inhibited differentiation. Inhibition of Smad3 function, but not Smad2 function, enhanced adipogenesis. Increased Smad6 and Smad7 levels blocked differentiation and enhanced TGF- β -induced responses. All of these indicated that in endogenous TGF- β signaling, Smad6 and Smad7 (even though known to inhibit TGF- β responses) were negative regulators of adipogenesis, and that Smad2 and Smad3 had distinct functions in the process of adipocyte differentiation^[60]. Others have also reported that TGF- β inhibited adipocyte differentiation by Smad3, but not Smad2^[61]. Smad3 and Smad4 can physically interact with adipogenic transcription factors, C/EBP β and C/EBP δ , to repress the transcriptional activity of C/EBPs. TGF- β /Smad3 signaling inhibited adipogenic differentiation primarily through functional repression of C/EBPs to decrease the expression of adipocyte marker genes such as adipisin and peroxisome proliferator-activated receptor γ ^[47,61]. In addition, a recent study also showed that the TGF- β /Smad3 signaling pathway played key roles in adipogenesis, and that TGF- β inhibited adipogenesis independent from the Wnt and β -catenin pathway^[62].

TGF- β in endothelial differentiation

TGF- β signaling is a negative correlation factor in endothelial differentiation of stem cells. TGF- β inhibition can maintain the proliferation and vascular identity of purified endothelial cells derived from hESCs. The molecular mechanism is that TGF- β inhibition sustains Id1 expression in hESC-derived endothelial cells and that Id1 is required for increased proliferation and preser-

vation of endothelial cell commitment^[63]. It has been demonstrated that shear stress can induce endothelial differentiation from C3H10T1/2 cells. In this case, the molecular mechanism is that fluid shear stress significantly suppresses TGF- β 1 functions through down-regulation of TGF- β 1, TGF- β R1, TGF- β R2, positive signaling molecules Smad2, Smad3, Smad4, and up-regulation of negative signaling molecule Smad7, suggesting that the negative regulation of the TGF- β 1 system may be involved in shear-induced endothelial cell differentiation in C3H10T1/2 cells^[64,65].

TGF- β in other differentiations

Of course, TGF- β also regulates other kinds of cell differentiation. For instance, coculturing human MSCs (hMSCs) with rat neural stem cells (rNSCs) was found to stimulate astrocyte and oligodendrocyte differentiation of the rNSCs, driven by increased secretion of soluble factors such as TGF- β 1 by the hMSCs and probably through the Notch pathway^[66]. In addition, when using a mouse midbrain embryonic day (E) 12 neurospheres culture as an experimental model, TGF- β can cooperate with persephin for dopaminergic phenotype induction through receptor-mediated differentiation signaling, involving p38 kinase and PI3K/AKT pathways^[67]. Furthermore, after exposure of rat hepatic oval cells, progenitor cells in the liver, to TGF- β 1, expression of ECM genes were increased, and TGF- β 1 treatment induced an up-regulation of marker genes for hepatic stellate cells, such as desmin and glial fibrillary acidic protein, through an epithelial-mesenchymal transition process^[68]. Differentiation of T cells both *in vitro* and *in vivo* demonstrated that TGF- β 1 was highly expressed by Th17 cells and that T cell-produced TGF- β 1 acted on T cells to promote Th17 cell differentiation in a predominantly autocrine manner, with deletion of the TGF- β 1 gene from activated T cells abrogating Th17 cell differentiation^[69]. Induction of the nuclear receptor ROR γ may have a central function in the differentiation of human Th-17 cells from naive CD4+ T cells which requires TGF- β ^[70]. TGF- β 1 accelerated dendritic cell differentiation from common dendritic cell progenitors and directed subset specification toward conventional dendritic cells (cDCs) by inducing both cDC instructive factors such as IFN regulatory factor-4 and RelB, and the transcription factor inhibitor of differentiation/DNA binding 2 that suppressed plasmacytoid dendritic cells development^[71]. However, TGF- β repressed development of natural killer (NK) cells from CD34(+) progenitors, inhibited differentiation of CD16(+) NK cells, and also resulted in conversion of a minor fraction of CD56(bright)CD16(+) NK cells found in peripheral blood into CD56(bright)CD16(-) NK cells. This highlighted a possible role of the former as a developmental intermediate and of TGF- β in influencing the genesis of NK subsets found in blood^[72]. The three mammalian isoforms (TGF- β 1, 2 and 3) have distinct but overlapping effects on hematopoiesis. In addition, paracrine and autocrine actions of TGF- β also have re-

dundant but distinct regulatory effects on hematopoietic stem/progenitor cells. TGF- β can be pro- or anti-differentiative and can inhibit or increase terminally differentiated cell function depending on the differentiation stage, the local environment and the concentration and isoform of TGF- β , *in vivo* or *in vitro*^[73]. Further studies are needed to investigate how TGF- β regulates hematopoietic cell differentiation.

CONCLUSION

In summary, there are still lots of unsolved problems concerning TGF- β signaling, stem cells and especially their relationship, a current hot spot in life sciences. Although the Smad pathway is the core of TGF- β signaling in regulating stem cell multi-differentiation, the cross-talk of the canonical Smad pathway and non-Smad pathways should not be neglected. Further elucidation on the molecular mechanisms of TGF- β signaling in stem cell multi-lineage differentiation will be of great value in both basic research and the clinical application of stem cells.

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