



## Minireview

# Directed Differentiation of Pluripotent Stem Cells by Transcription Factors

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Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been used as promising tools for regenerative medicine, disease modeling, and drug screening. Traditional and common strategies for pluripotent stem cell (PSC) differentiation toward disease-relevant cell types depend on sequential treatment of signaling molecules identified based on knowledge of developmental biology. However, these strategies suffer from low purity, inefficiency, and time-consuming culture conditions. A growing body of recent research has shown efficient cell fate reprogramming by forced expression of single or multiple transcription factors. Here, we review transcription factor-directed differentiation methods of PSCs toward neural, muscle, liver, and pancreatic endocrine cells. Potential applications and limitations are also discussed in order to establish future directions of this technique for therapeutic purposes.

**Keywords:** differentiation, embryonic stem cell, pluripotent stem cell, transcription factor

## INTRODUCTION

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), hold great promise in many biomedical fields. Indefinite proliferation of PSCs provides an unlimited cell source. Pluripotent differentiation potential enables PSCs to generate any desired cell

type *in vitro*. PSC-derived cells such as neurons are particularly valuable for human due to the lack of alternative sources. Therefore, generation of functional and fully differentiated cells from PSCs has been an active field of stem cell research. Common strategies rely on a vast knowledge of developmental biology learned from animal studies. Small molecules and recombinant signaling proteins have been applied to PSC differentiation in order to derive specific lineage cells. Despite the attempt to faithfully mimic *in vivo* development, these approaches suffer from several major limitations. The first limitation is that procedures are time-consuming. Neural differentiation, for example, usually takes 4~6 weeks and functional maturation requires a few more months depending on neuronal subtypes (Tao and Zhang, 2016; Xie et al., 2013). The second limitation is variability with low purity. Dramatic difference in differentiation potentials across particular PSC lines has been well documented in recent studies (Bock et al., 2011; Hu et al., 2010; Kajiwara et al., 2012; Kim et al., 2011; Koyanagi-Aoi et al., 2013; Osafune et al., 2008; Wu et al., 2007). This variability influences the response of PSCs to signaling molecules, resulting in different purity of desired cell types. Furthermore, difficulty in quality control of small molecules and recombinant proteins adds more variability to protocols. Given that large scale production of mature cells with high purity is necessary for clinical applications, alternative strategies might shed light on how to produce disease-relevant cell types from PSCs.

During embryonic development, cell fates are determined

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by a network of transcription factors. Consistently, successful reprogramming of cell fates by forced induction of single or a combination of transcription factors has been reported. Most strikingly, overexpression of four transcription factors (OCT4, SOX2, KLF4, c-MYC) can reprogram fibroblasts to PSCs (Takahashi et al., 2006). In some cases, single transcription factor is sufficient for direct reprogramming of fibroblasts to other cell types such as neurons and myoblasts (Chanda et al., 2014; Davis et al., 1987). These examples are very striking because cell fate acquisition relies on sequential requirement of multiple transcription factors over the course of development according to traditional views. To test the feasibility of transcription factor-directed PSC differentiation, systemic induction of transcription factors has been performed in mouse ESCs (mESCs) (Correa-Cerro et al., 2011; Nishiyama et al., 2009). Interestingly, 63 out of 137 transcription factors were able to initiate and direct specific differentiation programs when they were induced individually, suggesting that transcription factor induction could be an efficient strategy to direct PSC differentiation. In this review, we present PSC differentiation methods based on transcription factor induction. Potential limitations of this approach and possible solutions are also discussed.

## NEURONS

Producing functionally mature neurons from human PSCs (hPSCs) is important to understand normal physiology of human neurons and pathology of neurological diseases. Conventional methods follow the developmental path of neuronal derivation. First, PSCs are differentiated into neural progenitor cells (NPCs) by embryoid body formation or dual SMAD inhibition (Chambers et al., 2009). After that, NPCs are directed into specific neurons by a combination of signaling molecules (Tabar and Studer, 2014; Tao and Zhang, 2016). Based on the finding that forced expression of three transcription factors (BRN2, ASCL1, and MYT1L) can reprogram mouse fibroblasts into functional neurons (Vierbuchen et al., 2010), whether the same combination of transcription factors could bypass the long developmental path from hPSCs to neurons has been tested (Pang et al., 2011). Surprisingly, hPSCs expressing these three transcription factors showed bipolar neuron-like morphologies as early as day 3. They expressed neuronal markers such as  $\beta$ -III-tubulin (also known as Tuj1) and MAP2 by day 8. Electrophysiological analysis showed that these hPSC-derived neurons generated action potentials as early as day 6. The rapid induction of neuronal fate implicates that transcription factor induction can bypass sequential developmental stages and drive direct differentiation of hPSCs into neurons. More strikingly, a single transcription factor, ASCL1, was sufficient to drive direct neuronal differentiation of both human and mouse PSCs (Chanda et al., 2014; Pang et al., 2011; Yamamizu et al., 2013). BRN2 and MYT1L likely contribute to neuronal maturation by increasing morphological complexity. ASCL1 induction during mESC differentiation increased the percentage of neural cells (PSA-NCAM+) from less than 5% (no ASCL1 induction) to almost 50%, highlighting the robustness of transcription factor-directed differentiation (Yamamizu et al.,

2013). Other transcription factors previously known to play important roles in neurogenesis were also tested. Ectopic expression of NEUROG2 can efficiently drive mouse and human PSCs into pure excitatory neurons in less than two weeks (Thoma et al., 2012; Zhang et al., 2013). These NEUROG2-directed neurons showed neuronal transcription profiles, formed synapses, and produced robust action potentials. Furthermore, when these human neurons were transplanted into mouse brain, functional integration and active electrophysiological properties were observed (Zhang et al., 2013), suggesting potential applications of these neurons for cell replacement therapy. Forced induction of another proneural transcription factor, NEUROD1, also drove rapid differentiation of hPSCs into excitatory neurons (Zhang et al., 2013).

GABAergic interneurons are inhibitory neurons that balance neuronal excitation in the brain. Given that the majority of cortical interneurons are originated from the medial ganglionic eminence (MGE) (Wonders and Anderson, 2005), four transcription factors (ASCL1, DLX2, NKX2.1, and LHX6) have been chosen based on their expression in the MGE (Sun et al., 2016). Through extensive comparison, a combination of three transcription factors (ASCL1, DLX2, and LHX6) has been found to be able to drive hPSCs into GABA and MAP2 double-positive neurons within 10 days (Sun et al., 2016). Co-expression of miR-9/9\* and miR-124 further increased the efficiency of interneuron derivation. These GABAergic interneurons exhibited electrophysiological properties within 6~8 weeks and functionally integrated into the host's neural circuit upon transplantation to mouse brain (Sun et al., 2016). In another study, co-expression of ASCL1 and DLX2 was sufficient to differentiate hPSCs into GABAergic interneurons with ~90% efficiency (Yang et al., 2017). These interneurons showed electrophysiological properties at five weeks after transcription factor induction and survived well in the transplanted mouse brain. Interestingly, ASCL1 also contributed to the conversion of hPSCs into dopaminergic neurons when it was co-expressed with NURR1 and LMX1A (Theka et al., 2013). This combination of transcription factors produced mature and functional dopaminergic neurons within 21 days.

Motor neurons are typically produced from PSC-derived NPCs that are caudalized and ventralized by retinoic acid and sonic hedgehog, respectively (Li et al., 2005; Wichterle et al., 2002). These methods suffer from a long and inefficient differentiation process. To overcome such problem, motor neuron-specifying transcription factors have been identified based on temporal gene expression analysis upon retinoic acid/sonic hedgehog treatment (Hester et al., 2011). Forced expression of three transcription factors, NEUROG2, ISL1, and LHX3, in hPSC-derived NPCs accelerated motor neuron differentiation (Hester et al., 2011). At day 11 post transcription factor induction, cells expressed motor neuron markers HB9 and CHAT, formed neuromuscular junctions, and exhibited electrophysiological properties. Given that conventional methods using retinoic acid and sonic hedgehog take ~45 days to acquire mature motor neurons, transcription factor-directed motor neuron derivation could be a faster and more efficient alternative method. ISL1-LHX3 fusion proteins can

also drive mESCs into mature motor neurons in the absence of sonic hedgehog by suppressing a spinal interneuron fate (Lee et al., 2012).

Many studies described above used a lentiviral vector for transcription factor delivery because this viral vector could infect PSCs in a highly efficient manner and drive stable expression of transgenes by host genome integration. However, host genome modifications could be a potential problem in terms of using transcription factor-directed differentiation methods in a clinical setting. To circumvent this issue, researchers have exploited alternative gene delivery tools such as protein transduction, synthetic mRNAs, and non-integrating viral vectors. For example, a cocktail of synthetic mRNAs encoding five transcription factors (NEUROG1, NEUROG2, NEUROG3, NEUROD1, and NEUROD2) induced

rapid differentiation of hPSCs toward neurons (Goparaju et al., 2017). Synthetic mRNA-directed neurons showed electrophysiological properties as early as 7 days after mRNA transfection. A combination of synthetic mRNAs encoding ATOH1 and NEUROG2 was also tested to differentiate hiPSCs into mature dopaminergic neurons (Xue et al., 2018). Delivery of three transcription factors, LHX3, NEUROG2, and ISL1, by non-integrating Sendai virus vectors efficiently drove hPSCs into HB9-positive and ChAT-positive motor neurons within two weeks after transduction (Goto et al., 2017). These examples suggest that transcription factor-directed differentiation methods can be further improved for clinical applications. The methods described above are summarized in Table 1.

**Table 1.** Transcription factor-directed PSC differentiation toward neurons

Cell Line	Final Cell Type	Transcription Factor	Delivery Method	Time to Functional Cell	Differentiation Efficiency	Functional Test	Therapeutic Effect	Reference
mESC	Excitatory neuron	NEUROG2	Stable transfection	~10 days	40% (TuJ1 <sup>+</sup> )	Electrophysiology Coculture with primary neurons	ND	Thoma et al., 2012
mESC	Excitatory neuron	ASCL1	Lentiviral vector	~5 days	ND	Electrophysiology	ND	Chanda et al., 2014
hESC	Excitatory neuron	Either NEUROG2 or NEUROD1	Lentiviral vector	~2 weeks	~100% (MAP2 <sup>+</sup> ), ~100% (NeuN <sup>+</sup> )	Electrophysiology Calcium imaging Transplantation into mouse brain	ND	Zhang et al., 2013
hESC	Excitatory neuron	NEUROG2, SATB2, FEZF2	Lentiviral vector	~35 days	NEUROG2: 83% (MAP2 <sup>+</sup> ) NEUROG2+FEZF2: 64% (MAP2 <sup>+</sup> ) NEUROG2+SATB2: 49% (MAP2 <sup>+</sup> )	Electrophysiology Transplantation into organotypic slice cultures of adult human cortex.	ND	Miskinyte et al., 2018
hESC	GABAergic neuron	ASCL1 <sup>a</sup> , DLX2, LHX6, miR-9/9*, miR-124	Lentiviral vector	~6 weeks	81.3% (MAP2 <sup>+</sup> ) 84.5% (GABA <sup>+</sup> /MAP2 <sup>+</sup> )	Electrophysiology Transplantation into mouse brain	ND	Sun et al., 2016
hESC	GABAergic neuron	ASCL1, DLX2	Lentiviral vector	~5 weeks	89.1% (GABA <sup>+</sup> /MAP2 <sup>+</sup> )	Electrophysiology Transplantation into mouse brain	ND	Yang et al., 2017
hiPSC	Dopaminergic neuron	ASCL1, NURR1, LMX1A	Lentiviral vector	~21 days	51% (TuJ1 <sup>+</sup> )	Electrophysiology Dopamine release Transplantation into mouse brain	ND	Theka et al., 2013
hiPSC	Dopaminergic neuron	ATOH1, NEUROG2	Synthetic mRNA	36-49 days	>90% (TuJ1 <sup>+</sup> ) >97% (TH <sup>+</sup> /TuJ1 <sup>+</sup> )	Electrophysiology Dopamine release	ND	Xue et al., 2018
hESC, hiPSC	Motor neuron	NEUROG2, ISL1, LHX3	Adenoviral vector	~21 days	49-72% (HB9 <sup>+</sup> CHAT <sup>+</sup> )	Electrophysiology Coculture with C2C12-derived myotubes	ND	Hester et al., 2011
hESC, hiPSC	Motor neuron	NEUROG1, NEUROG2, NEUROG3, NEUROD1, NEUROD2	Synthetic mRNA	~7 days	98.2% (TuJ1 <sup>+</sup> ) 83.2% (MAP2 <sup>+</sup> /TuJ1 <sup>+</sup> ) 94.3% (HB9 <sup>+</sup> /TuJ1 <sup>+</sup> ) 97.7% (ChAT <sup>+</sup> /TuJ1 <sup>+</sup> ) 100% (ISL1 <sup>+</sup> /TuJ1 <sup>+</sup> )	Electrophysiology Calcium imaging	ND	Goparaju et al., 2017
hiPSC	Motor neuron	LHX3, NEUROG2, ISL1	Sendai virus vector	~14 days	>90% (TuJ1 <sup>+</sup> ), >90% (HB9 <sup>+</sup> )	Electrophysiology Coculture with human myocytes	ND	Goto et al., 2017
mESC	Motor neuron	ISL1-LHX3 fusion protein	Genetic recombination	~6 days	77% (HB9 <sup>+</sup> /TuJ1 <sup>+</sup> )	Coculture with C2C12-derived myotubes	ND	Lee et al., 2012
hESC, hiPSC	Neuron	BRN2, ASCL1, MYT1L	Lentiviral vector	~6 days	ND	Electrophysiology	ND	Pang et al., 2011
hESC	Neuron	Either NEUROG1, NEUROG2, NEUROG3, NEUROD1, or NEUROD2.	PiggyBac vector	~7 days	40-90% (HuC/D <sup>+</sup> )	Electrophysiology Calcium imaging	ND	Matsushita et al., 2017
hiPSC	Neuron	NEUROG1, NEUROG2	Lentiviral vector	~14 days	>90% (MAP2 <sup>+</sup> ), >90% (SYN1 <sup>+</sup> )	Electrophysiology	ND	Busskamp et al., 2014
mESC	Neuron	Ascl1	Genetic recombination	~11 days	8.3% (TH <sup>+</sup> /TuJ1 <sup>+</sup> ) 37.6% (ISL1 <sup>+</sup> /TuJ1 <sup>+</sup> ) 27.2% (GABA <sup>+</sup> /TuJ1 <sup>+</sup> )	Electrophysiology	ND	Yamamizu et al., 2013

<sup>a</sup>ASCL1: mutant form of ASCL1 in which five serine residues are substituted with alanine  
ND: Not Determined

## PANCREATIC $\beta$ -CELLS

$\beta$ -cells are a type of endocrine cells in the pancreas that can produce insulins to control blood glucose levels. Because transplantation of  $\beta$ -cells is a promising approach to treat diabetes patients, many researchers have been working on generating functional  $\beta$ -cells from PSCs. Common and conventional methods for  $\beta$ -cell derivation follow the developmental process of pancreas (Van et al., 2009). PSCs are first induced into definitive endoderm which is further specified into pancreatic lineage cells. To facilitate  $\beta$ -cell differentiation, PAX4, an essential transcription factor for  $\beta$ -cell development, is overexpressed in mESCs (Blyszczuk et al., 2003). Constitutive expression of PAX4 increased the formation of insulin-producing cells from 10~20% to 60~80%. Furthermore, transplantation of PAX4-directed insulin-producing cells maintained normal blood glucose levels in streptozotocin-induced diabetic mice. Other studies have also confirmed that forced induction of PAX4 could be an efficient method to derive insulin-producing  $\beta$ -cells from mouse and human PSCs (Liew et al., 2008; Lin et al., 2007).

Other transcription factors that play key roles in pancreatic development have also been tested in PSCs. Forced expression of PDX1 could facilitate mESC differentiation toward insulin-producing cells although these cells lacked mature properties such as high insulin secretion and glucose-responsiveness (Miyazaki et al., 2004). It has been reported that biphasic induction of PDX1 that mimic the normal de-

velopment of pancreatic  $\beta$ -cells had better effects on mouse and human ESC differentiation than constitutive expression (Bernardo et al., 2009). The ESC-derived  $\beta$ -cells secreted insulins and C-peptides. However, they failed to respond to glucose stimulation, suggesting that PDX1 alone might not be sufficient for mature  $\beta$ -cell derivation from PSCs. However, a later study reported that mESC-derived insulin-producing cells by PDX1 overexpression were glucose-responsive. They reduced hyperglycemia when they were transplanted into streptozotocin-induced diabetic mice (Raikwar and Zavazava, 2012). Such discrepancy could be due to different differentiation media conditions used to differentiate PDX1-expressing ESCs into insulin-producing cells. PDX1 induction has also been proven to be useful for  $\beta$ -cell derivation when it is combined with other transcription factors such as NKX6.1 and NEUROG3 (Ida et al., 2018; Kubo et al., 2011; Walczak et al., 2016). Moreover, it has been reported that induction of NKX2.2 alone can result in appearance of insulin-producing cells (stained by Dithizone) as early as 14 days in mESC-derived embryoid bodies although glucose-dependent secretion is not observed (Shiroy et al., 2005). Overall, forced induction of pancreatic transcription factors tested so far can drive a pancreatic  $\beta$ -cell fate from PSCs, although functional maturation might require other transcription factor networks.

Transcription factor-directed methods for pancreatic  $\beta$ -cell differentiation are summarized in Table 2.

**Table 2.** Transcription factor-directed PSC differentiation toward pancreatic  $\beta$ -cells

Cell Line	Final Cell Type	Transcription Factor	Delivery Method	Time to Functional Cell	Differentiation Efficiency	Functional Test	Therapeutic Effect	Reference
mESC	Insulin-producing cell	PAX4	Stable transfection	~36 days	80% (Insulin <sup>+</sup> )	Insulin release Transplantation to diabetic mice	Reduce blood glucose levels in diabetic mice	Blyszczuk et al., 2003
mESC	Insulin-producing cell	PAX4	Plasmid transfection	~18 days	55% (Insulin <sup>+</sup> )	Insulin release Transplantation to diabetic mice	Reduce blood glucose levels in diabetic mice	Lin et al., 2007
hESC	Pancreatic $\beta$ -cell	PAX4	Stable transfection	~28 days	ND	Proinsulin synthesis C-peptide release	ND	Liew et al., 2008
hESC mESC	Pancreatic $\beta$ -cell	PDX1-VP16 <sup>a</sup>	Stable transfection	~17 days ~28 days	ND 32% (C-peptide <sup>b</sup> )	Insulin expression Insulin release C-peptide release	ND	Bernardo et al., 2009
mESC	Insulin-producing cell	PDX1	Genetic recombination	16-24 days	ND	Insulin release C-peptide expression	No effect	Miyazaki et al., 2004
mESC	Insulin-producing cell	PDX1-AcGFP fusion protein	Stable transfection	~23 days	25~30% (Insulin <sup>+</sup> )	Insulin release Transplantation to diabetic mice	Reduce blood glucose levels in diabetic mice	Raikwar and Zavazava, 2012
hiPSC	Insulin-producing cell	PDX1-VP16 <sup>a</sup> , NKX6.1	Lentiviral vector	~17 days	ND	Insulin release C-peptide expression	ND	Walczak et al., 2016
hESC	Pancreatic endocrine cell	PDX1, NKX6.1, siPOU5F1	Synthetic mRNA	~13 days	5,6% (Insulin <sup>+</sup> )	Insulin, glucagon, and somatostatin expression	ND	Ida et al., 2018
mESC	Insulin-producing cell	PDX1, NEUROG3	Genetic recombination	~18 days	43% (Insulin <sup>+</sup> )	Insulin expression C-peptide, glucagon, and somatostatin release	ND	Kubo et al., 2011
mESC	Insulin-producing cell	NKX2.2	Stable transfection	~14 days	1% (Dithizone <sup>b</sup> )	Dithizone <sup>b</sup> staining Insulin release	ND	Shiroy et al., 2005

<sup>a</sup>PDX1 fused to the transactivator domain of the VP16 proteins from herpes simplex

<sup>b</sup>A zinc-chelating agent known to stain  $\beta$ -cell

ND: Not Determined

## SKELETAL AND CARDIAC MUSCLES

Muscular dystrophies including Duchenne muscular dystrophy and limb girdle muscular dystrophy are inherited disorders with skeletal muscle degeneration (Mercuri et al., 2013). PSC-derived skeletal muscles could be a promising source for transplantation therapy and disease modeling. Based on previous findings that MYOD1, a master transcription factor of myogenesis, can convert non-muscle cells into skeletal myocytes (Davis et al., 1987; Weintraub et al., 1989), whether MYOD1 induction could direct PSCs into skeletal muscles has been tested. MYOD1 overexpression facilitated myogenesis in mESC-derived embryoid bodies and generated skeletal muscle fibers (Dekel et al., 1992). However, only a subset of differentiating cells could be differentiated into skeletal muscles. Other studies have also confirmed that MYOD1 overexpression can induce myogenesis in mESCs

(Ozasa et al., 2007; Yamamizu et al., 2013). Interestingly, MYOD1 is unable to induce the myogenic program in hESCs, although extensive myogenic conversion has been observed in human fibroblasts (Albini et al., 2013). However, co-expression of SWI/SNF component BAF60C (encoded by SMARCD3) and MYOD1 is capable of driving hESCs into muscle cells, suggesting that the presence of epigenetic barriers can block MYOD1 functions in hESCs (Albini et al., 2013). BAF60C2/MYOD1-directed myospheres showed spontaneous contraction as well as typical skeletal muscle markers (myogenin, MyH3, and MyH8) at 20 days after differentiation. Ectopic expression of the catalytic domain of histone demethylase JMJD3 also enabled MYOD1 to activate the myogenic program in hPSCs (Akiyama et al., 2016). However, another study has been reported that MYOD1 alone is sufficient to drive hiPSCs into skeletal muscles (Tanaka et al., 2013). Such difference could be attributed to

**Table 3.** Transcription factor-directed PSC differentiation toward skeletal and cardiac muscle cells

Cell Line	Final Cell Type	Transcription Factor	Delivery Method	Time to Functional Cell	Differentiation Efficiency	Functional Test	Therapeutic Effect	Reference
mESC	Skeletal muscle	MYOD1	Plasmid transfection	>13 days	20-50%	Skeletal muscle marker expression	ND	Dekel et al., 1992
mESC	Myocyte	MYOD1	Genetic recombination	~5 days	~60% (MHC <sup>+</sup> Myogenin <sup>+</sup> )	Skeletal muscle marker expression Cell fusion assay with C2C12 mouse myoblast cells	ND	Yamamizu et al., 2013
hiPSC	Skeletal myocyte	MYOD1	PiggyBac vector	~9 days	70-90% (MHC <sup>+</sup> )	Skeletal muscle marker expression Cell fusion assay with C2C12 mouse myoblast cells Transplantation to mice	ND	Tanaka et al., 2013
mESC	Skeletal muscle	MyoD	Genetic recombination	~6 days	66% (MHC <sup>+</sup> ) 23% (dystrophin <sup>+</sup> )	Skeletal muscle marker expression Transplantation to mice	ND	Ozasa et al., 2007
hESC	Skeletal muscle	BAF60C2, MYOD1	Lentiviral vector	~20 days	60.1% (MHC <sup>+</sup> )	Skeletal muscle marker expression Intracellular calcium recording Contractile properties	ND	Albini et al., 2013
hESC	Skeletal muscle	JMJD3, MYOD1	JMJD3: PiggyBac vector MYOD1: Synthetic mRNA	~5 days	57% (MHC <sup>+</sup> )	Skeletal muscle marker expression Contractile properties	ND	Akiyama et al., 2016
hESC, hiPSC			Synthetic mRNA		34~64% (MHC <sup>+</sup> )	Skeletal muscle marker expression Cell fusion assay with C2C12 mouse myoblast cells		
mESC	Skeletal myogenic progenitor	PAX3	Genetic recombination	~12 days	72.2% (Myogenin <sup>+</sup> ) 78.4% (MHC <sup>+</sup> )	Myogenic progenitor marker expression Transplantation to mice Contractile properties	Improved isometric tetanic force and rotarod performance in mdx mice <sup>a</sup>	Darabi et al., 2008
mESC	Skeletal myogenic progenitor	PAX7	Genetic recombination	~5 days	84.4% (MHC <sup>+</sup> )	Myogenic progenitor marker expression Transplantation to mice Contractile properties	Improved isometric tetanic force in cardiotoxin-injured mdx mice	Darabi et al., 2011
hESC, hiPSC	Skeletal myogenic progenitor	PAX7	Lentiviral vector	~14 days	87~91% (Myogenin <sup>+</sup> ) 93~96% (MHC <sup>+</sup> )	Myogenic progenitor marker expression Transplantation to mice Contractile properties	Improved isometric tetanic force in immunodeficient mdx mice	Darabi et al., 2012
mESC	Cardiomyocyte	ISL1	Plasmid transfection	~8 days	4.01% (Myh7 <sup>+</sup> )	Cardiomyocyte marker expression	ND	Kwon et al., 2009
hESC	Cardiomyocyte	ISL1	Protein transduction	~8 days	75% (beating area)	Cardiomyocyte marker expression Spontaneous contractile properties	ND	Fonoudi et al., 2013
mESC	Cardiomyocyte	GATA4, MEF2C, TBX5	Bacterial injection	~12 days	61% (MHC <sup>+</sup> )	Cardiomyocyte marker expression Spontaneous contractile properties	ND	Bai et al., 2015
hESC, hiPSC	Cardiomyocyte	GATA4, MEF2C, TBX5, ESRRG, MESP1	Bacterial injection	~14 days	55~62% (cTnT <sup>+</sup> )	Cardiomyocyte marker expression Spontaneous contractile properties	ND	Jin et al., 2018
mESC	Cardiac pacemaker cell	SHOX2	Adenoviral vector	~10 days	83% (beating embryoid body)	Electrophysiological properties Transplantation to the rat heart	Induced biological pacing in rat hearts	Ionta et al., 2015

<sup>a</sup>mdx mice: mouse model for Duchenne muscular dystrophy (DMD)  
ND: Not Determined

different media conditions used for muscle differentiation or variability related to hESC and hiPSC lines. More studies are warranted in order to make a clear answer to this issue.

PAX3 is a key transcription factor that initiates myogenesis in early paraxial mesoderm. Consistently, forced expression of PAX3 in mESC-derived embryoid bodies can greatly promote myogenic differentiation (Darabi et al., 2008). Myogenic progenitor cells can be further enriched from PAX3-directed cells by sorting out a PDGF $\alpha$ R<sup>+</sup>Flk1<sup>-</sup> population. Transplantation of PAX3-directed PDGF $\alpha$ R<sup>+</sup>Flk1<sup>-</sup> cells has shown potential for therapeutic engraftment in dystrophic mice (Darabi et al., 2008). Like PAX3, overexpression of PAX7 can also efficiently drive both human and mouse ESCs into muscle progenitor cells with muscle regeneration potential (Darabi et al., 2011, 2012).

To facilitate myocardial differentiation, transient overexpression of ISL1, a cardiac progenitor marker, has been tested in mESCs. Transfection of mESC-derived embryoid bodies with a ISL1-expressing construct increased the expression of cardiomyocyte markers (ACTC1, MLC2V, MYH7, SMA), suggesting an instructive role of ISL1 in myocardial differentiation (Kwon et al., 2009). The effect of ISL1 on myocardial differentiation has also been confirmed in hESCs by ISL1 protein transduction (Fonoudi et al., 2013). A combined delivery of three transcription factors, GATA4, MEF2C, and TBX5, to mESC-derived embryoid bodies activated the cardiac program and increased the efficiency of cardiomyocyte

derivation up to 60% (Bai et al., 2015). Another combination of five transcription factors, GATA4, MEF2C, TBX5, ESRRG and MESP1, has been tested in hPSCs. Delivery of these transcription factors to hPSC-derived mesoderm strongly promoted cardiomyocyte differentiation (Jin et al., 2018). Furthermore, a single transcription factor, SHOX2, greatly facilitated the derivation of cardiac pacemaker cells when it was delivered into mESC-derived embryoid bodies (Ionta et al., 2015). Most SHOX2-directed embryoid bodies exhibited spontaneous beating. When they were transplanted to the left ventricular apex of rat heart, SHOX2-directed embryoid bodies functioned as biopacemakers. Thus, many attempts with single or a combination of transcription factors have been made to produce functional muscle cells from PSCs. However, it seems that transcription factor-directed differentiation methods are not yet mature enough to produce highly pure muscle cells with functional maturity in a short culture period. New combinations of transcription factors together with small-molecule epigenetic inhibitors should be tested to further improve PSC-derived muscle derivation in the future. Transcription factor-directed methods for muscle cell differentiation are summarized in Table 3.

## HEPATOCYTES

Liver transplantation is a promising treatment for end-stage liver diseases. PSC-derived hepatocytes could not only be

**Table 4.** Transcription factor-directed PSC differentiation toward hepatocytes

Cell Line	Final Cell Type	Transcription Factor	Delivery Method	Time to Functional Cell	Differentiation Efficiency	Functional Test	Therapeutic Effect	Reference
mESC	Hepatocyte	HEX	Genetic recombination	~14 days	ND	Hepatocyte marker expression Liver metabolism-related gene expression	ND	Kubo et al., 2010
hESC, hiPSC	Hepatocyte	HEX	Adenoviral vector	~18 days	~84% (ALB <sup>+</sup> ) ~80% (CYP3A4 <sup>+</sup> ) ~88% (CYP7A1 <sup>+</sup> ) ~92% (Cytokeratin18 <sup>+</sup> )	Hepatocyte marker expression Liver metabolism-related gene expression Liver metabolite production	ND	Inamura et al., 2011
hESC, hiPSC	Hepatocyte	SOX17, HEX, HNF4A	Adenoviral vector	~20 days	~80% (ALB <sup>+</sup> ) ~80% (CYP3A4 <sup>+</sup> ) ~80% (CYP7A1 <sup>+</sup> ) ~80% (CYP2D6 <sup>+</sup> )	Hepatocyte marker expression LDL uptake Liver metabolism-related gene expression Liver metabolite production Glycogen storage Indocyanine green (ICG) uptake	ND	Takayama et al., 2012a
hESC, hiPSC	Hepatocyte	SOX17, HEX	Adenoviral vector	~18 days	ND	Hepatocyte marker expression Liver metabolism-related gene expression	ND	Takayama et al., 2011
hESC, hiPSC	Hepatocyte	FOXA2, HNF1A	Adenoviral vector	~20 days	~90% (ALB <sup>+</sup> )	Hepatocyte marker expression LDL uptake Liver metabolism-related gene expression Liver metabolite production Glycogen storage Indocyanine green (ICG) uptake Urea secretion Drug metabolism capacity	ND	Takayama et al., 2012b
hiPSC	Hepatocyte	CEBPA, CEBPB, FOXA1, FOXA3	Plasmid transfection	~7 days	ND	Hepatocyte marker expression	ND	Tomizawa et al., 2016
mESC	Hepatocyte	Either HNF4A, FOXA1, GATA2, or GATA3	Genetic recombination	7-14 days	ND	Hepatocyte marker expression Albumin secretion LDL uptake Glycogen storage	ND	Yamazumi et al., 2013
		HNF4A	Synthetic mRNA	~11 days		Hepatocyte marker expression Glycogen storage		

ND: Not Determined

unlimited sources for cell therapy, but also serve as an experimental model for metabolic diseases. Several key transcription factors for liver development have been tested to develop directed PSC differentiation methods. HEX (also known as HHEX) is an essential transcription factor that functions at the early stage of hepatic development. Forced expression of HEX in mESC-derived endoderm can dramatically increase the expression of genes related to hepatocyte maturation and function (Kubo et al., 2010). These findings have also been confirmed in hPSCs (Inamura et al., 2011). hPSCs were differentiated into definitive endoderm by Activin A followed by adenoviral delivery of HEX. Transient overexpression of HEX was sufficient to promote hepatocyte derivation. HEX-directed cells expressed both  $\alpha$ -fetoprotein (AFP) and albumin (ALB) on day 12. Furthermore, P450 cytochrome enzymes known to play a critical role in liver metabolism were also expressed in HEX-directed hepatoblasts (Inamura et al., 2011). Further maturation of hepatoblasts into functional hepatocytes was achieved by overexpression of HNF4 $\alpha$ , a key regulator of liver-specific genes (Takayama et al., 2012a). Interestingly, forced induction of HNF4 $\alpha$  in mESCs was able to bypass the natural order of hepatic differentiation and activate the hepatic program directly from mESCs (Yamamizu et al., 2013). ALB secretion and the uptake of low-density lipoproteins were observed in HNF4 $\alpha$ -directed cells as early as 7 days after differentiation. In their study, other transcription factors, such as FOXA1, GATA2, and GATA3, could also directly drive mESCs into hepatocytes (Yamamizu et al., 2013).

Sequential delivery of transcription factors has also been tested for efficient PSC differentiation toward hepatocytes (Takayama et al., 2011; 2012b). Forced induction of either SOX17 or FOXA2 was used to differentiate hPSCs into definitive endoderm. SOX17-directed endoderm cells were driven to hepatocytes by HEX overexpression (Takayama et al., 2011). In FOXA2-directed endoderm cells, HNF1 $\alpha$  transduction efficiently generated hepatocytes (Takayama et al., 2012b). Furthermore, a single-step protocol with a combination of transcription factors for hepatocyte derivation has been reported (Tomizawa et al., 2016). Forced induction of four transcription factors, CEBPA, CEBPB, FOXA1, and FOXA3, directly differentiated hiPSCs into hepatocytes when they were cultured in a medium that could promote hepatic differentiation. Transcription factor-directed methods for hepatocyte differentiation are summarized in Table 4.

## CONCLUSIONS

During development, fully differentiated cells emerge through several intermediate stages. For example, ESCs first produce early neuroectoderm that gives rise to region-specific neural progenitor cells. Neural progenitor cells further differentiate into post-mitotic neurons, a type of fully differentiated cells in the brain. Even in this simplified description, the end-stage cell fate is derived through three sequential cell fate transitions. An outstanding question in developmental biology is if multiple transitions are necessary for the acquisition of fully differentiated cell fates. A growing body of recent evidence shows that cell fate acquisition/conversion can occur directly by forced expression of transcription factors, suggesting that multiple developmental steps are unnecessary for generating fully differentiated cells. In this review, we also described examples of direct conversion of PSCs into mature cell types by transcription factors. Overexpression of transcription factors related to late developmental stages could bypass early developmental processes and rapidly produce functionally mature cells from PSCs. This is especially prominent in neuronal differentiation because many studies have shown that single proneural transcription factor is sufficient to convert PSCs into functional neurons with almost 100% efficiency. However, transcription factor-directed PSC differentiation seems to be less effective in other lineages. Although transcription factor induction can promote lineage differentiation, protocols are not yet mature in terms of efficiency or functional maturity. In some cases, transcription factors failed to initiate the differentiation program in PSCs likely due to epigenetic barriers. Small-molecule epigenetic inhibitors could be helpful to reset PSCs to be responsive to late-stage transcription factors.

Despite major advantages of transcription factor-directed differentiation over conventional methods, current methods are not ready for clinical applications yet. Besides a few tests for functionality, extensive systemic analyses including transcriptome and epigenome should be performed to validate if transcription factor-directed cells are safe and mature as their *in vivo* counterparts. Furthermore, virus-mediated transcription factor delivery might be inappropriate for clinical settings. Alternative delivery methods such as protein transduction and synthetic RNA transfection are under active investigation. Given that simple and scalable differentiation procedures with high purity are major bottlenecks for therapeutic applications of PSC-derived cells, transcription factor-directed PSC differentiation methods could be used as a promising strategy for future cell therapy.

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