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Embryonic Stem Cells in Development and Regenerative Medicine

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Abstract

After progressive improvement in embryonic stem (ES) cell field, several studies have been conducted to explore the usage of ES cells in regenerative medicine. Unlimited self renewal and pluripoteny properties, combined with encouraging preclinical trials, remark that ES cell technology might be promising for clinical practice. ES cells, which can form three germ layers *in vitro*, are potential candidates to study development at the cellular and molecular level. Understanding the cell fate decision and differentiation processes during development might enable generating functional progenitor cells for tissue restoration. Progression in gene modifications and tissue engineering technology has facilitated the derivation of desired cells for therapy. Success in differentiation protocols and identification the regulatory pathways simplify the research for clinical applications. Although there are established protocols for cell differentiation *in vitro* and promising preclinical studies *in vivo*, many challenges need to be adressed before clinical translation. In this review, ES cells are discussed as a model of development *in vitro* and as a potential candidate for regenerative medicine. This review also dissusses current challenges for ES cell based therapy.

Keywords

Embryonic stem cells · Development · Differentiation · Regenerative medicine · Tissue engineering

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Abbreviations

ALS	Amyotrophic Lateral Sclerosis
ASCs	Adult Stem Cells
BDNF	Brain-Derived Neurotrophic Factor
BMP	Bone Morphogenic Protein
EB	Embryoid Body
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ES cells	Embryonic stem cells
FACS	Fluorescence-Activated Cell Sorting
FGF	Fibroblast Growth Factor
Flt3L	Fms-like tyrosine kinase 3 ligand
FoxO1	Forkhead box O1
G-CSF	Granulocyte Colony-Stimulating Factor
GDNF	Glial-Derived Neurotrophic Factor
HSCs	Hematopoietic Stem Cells
ICM	Inner Cell Mass
IL	Interleukins
IPS	Induced Pluripotent Stem Cells

LIF	Leukemia Inhibitory Factor
MACS	Magnetically Activated Cell Sorting
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
MSCs	Mesenchymal Stem Cells
NGF	Nerve Growth Factor
PODXL	Podocalyxin-like protein-1
RA	Retinoic Acid
SCF	Stem Cell Factor
SCNT	Somatic Cell Nuclear Transfer
SHH	Sonic Hedgehog
TSCs	Trophoblast Stem Cells
XENCs	Extraembryonic Endoderm Cells

1 Introduction

Different types of mature human cells, residing in the specific tissues and organs, have limited capacity of proliferation which restricts tissue regeneration process (Jopling et al. 2011). However, stem cells have an unlimited lifespan and division potential with a broad range of differentiation capacity. Human stem cells are classified into two major categories based on source and differential potential: embryonic stem (ES) cells and adult stem cells (ASCs) consisting hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). ES cells are capable of differentiation into all cell lineages which makes them remarkable tools for developmental processes and cell therapy studies. ES cells derived from the inner cell mass (ICM) of blastocysts are pluripotent, providing them unrestricted differentiation potential. In defined culture conditions, ES cells could be maintained in undifferentiated state and differentiated into other cell lineages (Nishikawa et al. 2007). Pluripotent ES cells normally produces compact colonies at undifferentiated state and differentiated colonies are likely to be more flattened at the edges where colony morphology loose spherical structure (Yabut and Bernstein 2011). Pluripotent ES cells are characterized by the expression of specific markers including OCT4, cMYC, KLF44, NANOG, SOX2 (Adewumi et al. 2007) which defines the dynamics of stemness and transformation potential. In addition to the mechanisms that regulates pluripotency, self-renewal of ES cells is controlled by sustained expression of proto-oncogenes that needs to be clarified with further studies (Nishikawa et al. 2007).

Strategies to test pluripotency *in vitro* involve embryoid body (EB) formation by inducing differentiation of ES cells in feeder free non-adherent culture systems followed by triggering the transformation of specific cell populations derived from three embryonic germ layers (Itskovitz-Eldor et al. 2000). The development of teratomas as disorganized structures when ES cells are grafted into immunodeficient mice is the most well established pluripotency analysis *in vivo* (Ritner and Bernstein 2010). Because ES cells have unlimited proliferation and transformation capacity *in vitro* and *in vivo*, they have become the aim of interest of many researches in recent years as a comprehensive cell source to study development and new therapeutic approaches for regenerative medicine. Moreover, advanced genetic modification of ES cells is an important step, allowing the generation of convenient cell lineages that are desired for regenerative medicine in cell-based therapies.

In this review, the current strategies to study ES cells as a model of human development and regenerative medicine and the improvements of cell based approaches will be described in detail and the challenges for experimental research and clinical applications will be briefly discussed.

2 ES Cells in Development

Understanding the ES cells from an embryological viewpoint is required to identify ES cell biology, develop experimental model systems and establish clinically relevant protocols for therapeutic applications. In parallel, improvement of ES cell-based differentiation models could lead to overcome differences between mouse and primate embryologic development and to trace fate decision of human ES cells in *in vitro* culture conditions.

Most of the data for mammalian embryologic development are based on mouse studies. However, human and mouse embryonic development have significant differences in terms of gene expression and regulation. (Pera and Trounson 2004; Rossant 2015). Therefore, human and mouse ES cells obtained from ICM of the embryo have disparities. Both human and mouse ES cells could be kept in *in vitro* culture conditions at undifferentiated state by maintaining normal karyotype after several passages and hold great potential for regenerative medicine applications (Keller 2005). ES cells could be a suitable model for development and regenerative therapy research as they are pluripotent which allows generation of mature differentiated cells of all tissues in the adult body.

Recent advances in cell culture protocols to obtain various cell lineages could not only provide reprogramming strategies but also serve unique models for early development or even support multiple cell-based regenerative medicine approaches.

2.1 Differentiation in Culture

Under defined culture conditions that enable controlled exit from the pluripotent state, ES cells transform into differentiated cell types of the embryonic germ layers: mesoderm, endoderm, and ectoderm (Smith 2001). As developmental signals regulating the embryogenesis also contribute to the differentiation of pluripotent cells, mouse and human ES cells demonstrate disparities in culture systems. Although human ES cells could give rise to trophoectoderm derived lineages by bone morphogenic protein (BMP)-4 induction (Xu et al. 2002b), mouse ES cells do not have the ability to differentiate towards trophoblastic lineages. Cell surface glycolipids and proteoglycans including SSEA-1, -3, -4, TRA-1-60 and TRA-1-81 are differentially expressed in mouse and human ES cell lines (Pera and Trounson 2004). Phenotypic differences such as colony morphology or feeder cell and leukemia inhibitory factor (LIF) requirements are also distinct for both cell lines (Pera et al. 2000). Although mouse ES cells need LIF to maintain the pluripotency in the culture conditions as they require physiologically for mouse embryogenesis, human ES cells do not respond LIF because of differentially activated pathways including STAT3 and LIF pathways (Chen et al. 2015; Ginis et al. 2004; Sato et al. 2004). All these differences are organized by different regulatory mechanisms and precede distinct differentiation and development patterns in culture conditions.

Therefore, ES cells as attractive pluripotent cell sources represent a promising tool for development researches and cell products for therapies. Optimization of culture conditions for directed differentiation is a major challenge that should be overcome to utilize potential of ES cells to obtain functional tissue-specific cells.

2.2 Differentiated Cell Lineages from ES Cells

Embryo is a bulk of cells with the same progeny in the beginning but soon after developmental program is activated and cells switch to a differentiated state based on their position and induction signals during embryogenesis (Dvash et al. 2006). ES cell could differentiate into cell types originated from three embryonic germ layers when vital factors that keep them at undifferentiated state are removed and specific culture conditions are applied (Smith 2001).

There are three general well established approaches for ES cell differentiation: EB formation (Doetschman et al. 1985), feeder cell dependent methods (Nakano et al. 1994) and feeder free extracellular matrix protein-based techniques (Nishikawa et al. 1998). EBs as three-dimensional multicellular structures are created by ES cell aggregates in non-adherent culture conditions, and mimic embryo development and gem layer specification (Doetschman et al. 1985). Because of easy manipulation and a broad range of cell lineages generated from EB culture, the protocol is a classic method for mouse and human ES cell researches. Apart from EB culture method, stromal cell lines as LIF supplier are

used as feeder cell layers to induce cell differentiation of ES cells to hematopoietic (de Pooter et al. 2003) or mesodermal lineages (Nishikawa et al. 1998). Alternatively, ES cells are placed on extracellular proteins such as matrigel, collagen or fibronectin to induce defined cell types. These three methods and comparison are summarized in Fig. 1. Several growth factors including fibroblast growth factor- (FGF), activin A, nerve growth factor (NGF) and BMP are used to modulate ES cell differentiation and generation of cells displaying three germ layer characteristics (Dvash et al. 2006).

2.2.1 Mesodermal Cell Lineages

Mesodermal lineages including cardiomyocytes, endothelial cells and hematopoietic cells have been obtained from ES cells by using co-culture or growth factor approaches. Basically, in order to generate hematopoietic lineages such as erythroid, myeloid, and lymphoid cells (Wang et al. 2005), ES cells are induced with growth factors including stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (Flt3L), interleukins (IL-3, IL-6), BMP-4 and granulocyte colony-stimulating factor (G-CSF). Hematopoietic commitment has been well-characterized in ES cell differentiation models and expected to be useful for developing transplantable cells in therapy.

The generation of beating cardiomyocyte loci after cardiac lineage differentiation in ES cell culture has been studied in spontaneous and co-culture conditions (Mummery et al. 2003; Xu et al. 2002a). *In vitro* derived contracting cardiomyocytes were similar to early cardiac tissue (Snir et al. 2003), indicating that ES cell might give rise to physiologically functional early cardiomyocytes.

Endothelial cell differentiation and marker (CD31) expression has been detected in ES cell using EB protocol (Levenberg et al. 2002). Moreover, ES cell derived endothelial cells have demonstrated morphological features such as tube-like and vascular network-like structures.

Although early mesodermal lineage commitment and underlying mechanisms are still unknown, various mesodermal cell types have been generated from ES cells in *in vitro* cultures and seems to be promising for future therapies.

2.2.2 Endodermal Cell Lineages

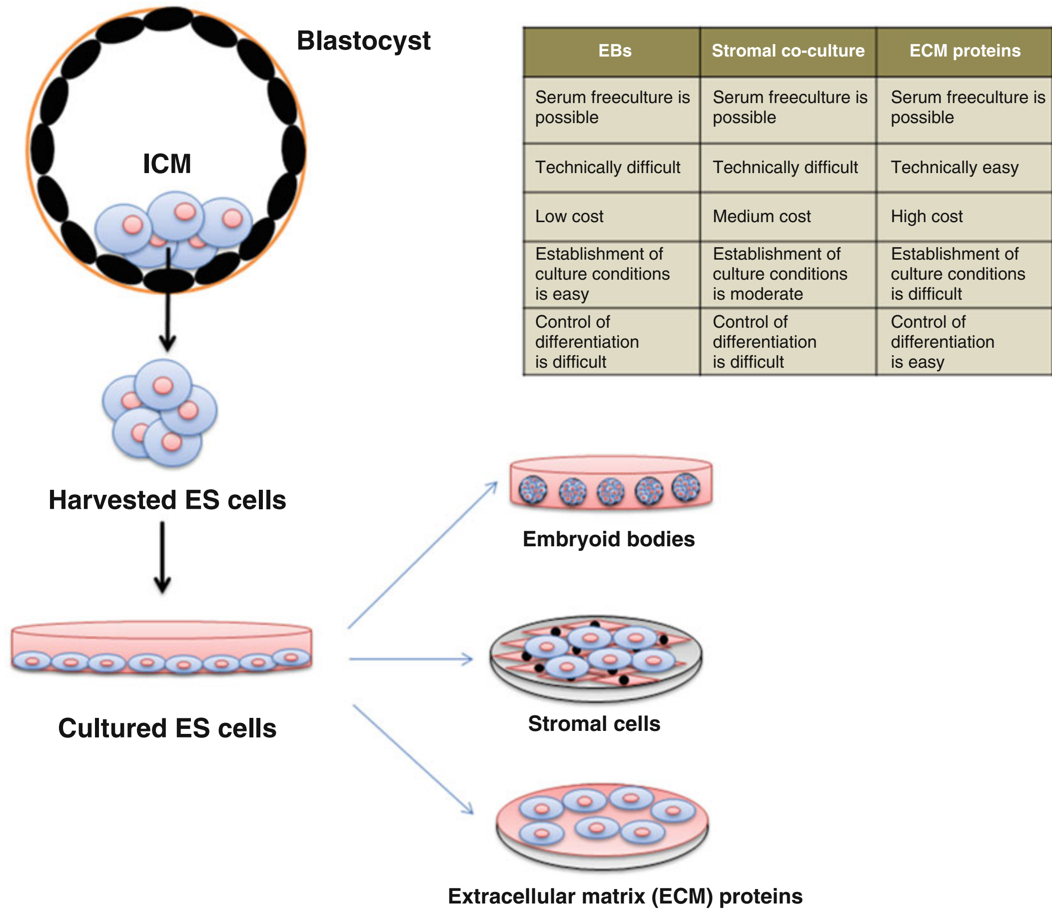
The differentiation and characterization of endodermal lineages from ES cells is crucial because they might be used as a therapeutic source for liver and pancreatic tissues. Despite the development of pancreatic β-cells and hepatocytes would be promising for the treatment of diabetes and liver disorders, derivation endoderm cell lineages is a slow process. Specific molecules to induce endoderm differentiation and identification of marker gene profiles for distinct endoderm populations should be addressed to obtain certain endodermal cells. Pancreatic and hepatic specific gene expression patterns have been observed in EB culture systems. Stimulation of human ES cells with sodium butyrate results in epithelial like cells with a hepatocyte marker profile. Similarly, insulin staining and switch to pancreatic lineage specific gene expression have been reported in EBs (Assady et al. 2001; Rambhatla et al. 2003).

However, generation of functional hepatocytes and pancreatic β-cells have to be studied in detail and improved by identification of stimulant factors and underlying mechanisms that control endodermal germ layer specification.

2.2.3 Ectodermal Cell Lineages

Ectodermal differentiation from ES cells can be generated under appropriate culture conditions including spontaneous EB differentiation, serum free monolayer culture and retinoic acid exposure. Extensive research has been focused on neural differentiation as derivation of various neural cell types is easy to obtain when treated with fully defined culture conditions and might be a solution for neurodegenerative disorders.

Neurospheres in suspension culture systems express neuroectoderm markers including nestin, N-CAM and Pax6, and these progenitor populations could give rise to neurons, astrocytes and oligodendrocyte-like cells. These ES cell derived cells might be electrophysiologically



EBs	Stromal co-culture	ECM proteins
Serum freeculture is possible	Serum freeculture is possible	Serum freeculture is possible
Technically difficult	Technically difficult	Technically easy
Low cost	Medium cost	High cost
Establishment of culture conditions is easy	Establishment of culture conditions is moderate	Establishment of culture conditions is difficult
Control of differentiation is difficult	Control of differentiation is difficult	Control of differentiation is easy

Fig. 1 Comparison of for ES-cell differentiation protocols. There are three differentiation methods available for ES cell transformation: Embryoid bodies, stromal cell sources as feeders and extracellular matrix (ECM) protein coating. Pros and cons of each method in terms of technical difficulties and efficiency is summarized

active that responded neurotransmitters and survived when injected to mice brain (Carpenter et al. 2004; Pera et al. 2004). In addition to neural cell types, ES cell could give rise to other ectodermal lineages such as keratinocyte marker expressing epithelial cells. BMP-4 promotes keratinocyte development while inhibiting neural lineage development. ES cell derived keratinocytes form mouse skin-like structures in organ culture systems (Coraux et al. 2003; Kawasaki et al. 2000). Optimization of culture conditions for ectodermal derived cell populations could be a solution for various diseases such as neural system disorders in the future.

2.2.4 Extraembryonic Tissues

The outer cell layer of the embryo known as trophectoderm arises around blastocyst and is required for embryo implantation and placenta formation. Although mouse ES cells do not form the extraembryonic tissues, human ES cell are capable of differentiating into trophoblast cells *in vitro* (Thomson et al. 1998; Xu et al. 2002b). BMP-4 administration induces trophoblast marker expression, and chorionic gonadotrophin production in human ES cell cultures (Xu et al. 2002b). Although extensive research is required to evaluate the role of BMPs in trophectodermal layer appearance in the

mammalian embryogenesis, BMP treatment in the *in vitro* human ES culture system is useful to obtain different extraembryonic structures. BMP-2 treated human ES cells have been reported to produce the extraembryonic endoderm (Pera et al. 2004). OCT4 as one of the master transcription factor has regulated the trophoblast and endoderm differentiation patterns in mouse and human ES cells distinctly (Hay et al. 2004). Wei et al used CRISPR/Cas9 and upregulated Cdx2 and Gata6 for the derivation of two extraembryonic lineages such as typical trophoblast stem cells (TSCs) and extraembryonic endoderm cells (XENCs) which were similar to their counterparts *in vivo* (Wei et al. 2016). Identification of new factors and genes regulating the extraembryonic development might help understanding the pre-implantation stage during development.

3 ES Cell Originated Cell Types in Regenerative Medicine

Although some of the vertebrate species have broad regenerative potential, humans have limited tissue regeneration capacity that restricts restoration of damaged organs. Regenerative medicine using stem cells with extensive division, proliferation and differentiation potential has been an emerging area of research in recent years. ES cells are popular in regenerative medicine due to their self-renewal and pluripotent properties. Difficulties in access to adequate numbers of adult tissue specific stem cells make ES cells promising candidates for cell based therapy. With the ability of direct differentiation protocols and genetic modification tools, ES cell based regenerative medicine could be a solution for several diseases and tissue engineering applications. The pluripotency of ES cells regulates lineage commitment during differentiation which is required for regenerative therapeutic applications. This section of the review will discuss some of the cell lineages derived from ES cells for therapy and potential regenerative applications.

3.1 Cartilage Tissue Engineering

Cartilage is an avascular supportive tissue which consists of chondrocytes and extracellular matrix (ECM) along with aggrecan and collagen fibrils. Due to its avascular nature, cartilage tissue has poor repair capacity leading to trauma induced osteoarthritis (Toh et al. 2011). Development of new cartilage tissue is challenging because of limited cell source for cell based therapies. Although some success has been achieved using MSCs and chondrocyte implantation; limited self-renawal, proliferation and differentiation abilities still remain to be solved. Therefore, understanding and controlling the ES cell differentiation to cartilage might be promising. ES cells should be cultured in defined culture conditions with appropriate cytokines, growth factors and stimulant chemicals to induce chondrocyte differentiation. Human ES cell derived EBs differentiated into chondrogenic pellets in serum free media followed by small molecules and chondrogenic medium (TGFβ3 and BMP4) treatment. (Yang et al. 2016). EB-based ES cell culture systems have led the formation of heterogeneous cartilaginous nodules and chondrocytes which de-differentiate easily to other cell types (Hegert et al. 2002; Jukes et al. 2008b). Obtaining MSCs from ES cells for subsequent chondrocyte differentiation is a suitable method as using progenitor cells instead of ES cells reduces the risk of teratoma formation and increases the success of direct differentiation to mesodermal lineages (Karlsson et al. 2009). These MSC cells derived from ES cells have been differentiated into chondrogenic cells in modified hydrogels (Hwang et al. 2006) and pellet culture systems (Nakagawa et al. 2009). Hyaline producing chondrocytes have been obtained from human ES cell derived MSCs by sequential BMP-2 and Wnt5a treatment *in vitro* and repaired rat chondral defects *in vivo* (Gibson et al. 2017).

Differentiated mouse ES cells under chondrogenic conditions have resulted in teratoma formation when injected to immunodeficient mice. However, when these differentiated cells were seeded on polycaprolactone scaffolds,

teratoma formation has not been observed (Fecek et al. 2008). Yamashita and colleagues used mouse ES cells in micromass culture system supplemented with TGF-β1 and BMP-2 to generate chondrogenic cells formed cartilage *in vivo* (Yamashita et al. 2009). In a different approach, the transformation of human ES cells into chondrocytes and cartilage-like tissue formation has been illustrated by a co-culture model and teratoma formation has not been observed following implantation (Hwang et al. 2008a). Similarly, human ES cell derived MSCs and bovine chondrocytes co-cultured and resulting cell pellets were implanted into the osteochondral defects of rat (Hwang et al. 2008b). Cartilage tissue was formed at the defect region (Takagi et al. 2007) and more hyaline containing tissue was observed when same model was used with hydrogel and growth factors such as BMP-7 and TGF-β1 (Toh et al. 2010). Consistently, human ES cells were implanted with PLLA and PEG scaffolds, and hydrogels that formed cartilaginous tissues after implantation into mice (Hwang et al. 2008a; Vats et al. 2006). Mechanical suppression induced by 3-D polydimethylsiloxane scaffolds triggered early cartilage marker gene expression and enhanced chondrogenic differentiation of ES cells (McKee et al. 2017). Cheng et al. used fibrin gel for implantation of chondrogenic cells derived from human ES cells into focal defects of nude rats and provided cartilage repair (Cheng et al. 2014). In addition to these differentiation conditions, incubation time in the culture media is also important to provide a cartilaginous structure. Chondrogenic induction of mES cells should be prolonged at least 14 days and differentiated mouse ES cells under chondrogenic condition created functional bone tissue and calcification only after 21 days (Jukes et al. 2008a). To sum up, while chondrogenic differentiation of ES cells in *in vitro* and *in vivo* culture conditions has been successful both for mouse and human ES cells, yet signaling pathways underlying the mature chondrocyte development from human ES cells, large scale production methods and appropriate delivery systems should be identified for long-term *in vivo* achievements and clinical benefits.

3.2 Neural Disease Therapies

Neurological diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and spinal cord disorders are the result of severe neuron damage and loss of function in the central and peripheral nerve systems (Kim and De Vellis 2009). Because nerve system has poor regeneration ability, cell replacement therapies have been the aim of interest as potential therapeutic options for the neurological disorders. ES cells with remarkable proliferation capacity and extensive potential to differentiate any desired cell type including dopamine neurons, glial cells, astrocytes and oligodendrocytes (Joannides et al. 2007) offer the advantage of new regenerative medicine strategies. Defined culture protocols have been established including retinoic acid treatment and three dimensional neurosphere cultures supplemented with FGF-2 to generate neural progenitors from ES cells. Engineered human ES cells which overexpressed FGF-2 exerted neuroprotective role in ventral root avulsion model indicating the activity of FGF signaling in neural dierentiation (Araújo et al. 2017). Although derivation of neural cells from ES cells is simple, certain identification criteria of neural subtypes for their different physiological functions are required for clinical application.

Generation of dopaminergic neurons from ES cells is of particular interest as they are involved in the pathogenesis of motor symptoms in the Parkinson's disease. ES cell derived dopamine neurons could be used in neuron replacement therapy for neurodegenerative diseases including Parkinson's disease (Tabar and Studer 2014). Both mouse and human ES cells can give rise to dopamine neurons that actively produce tyrosine hydroxylase, secrete dopamine and form *in vitro* synapses (Murry and Keller 2008). Over the years, different protocols have been used for direct differentiation to specify dopaminergic neurons from ES cells. Although stromal cell co-culture followed by FGF8 and sonic hedgehog (SHH) administration enhanced the dopaminergic neuron induction, addition of other growth factors

including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) and TGF-β3 increased tyrosine hydroxylase producing neurons (Joannides et al. 2007; Murry and Keller 2008). Identification of efficient protocols that could form fully defined dopamine neurons to be implantated is required to develop a promising strategy for Parkinson's disease.

Although there is not an available human clinical trial for ES cell derived neural progenitors for the Parkinson's disease, there have been several mice and rat *in vivo* researches. Neurospheres of human ES cells formed nestin positive neurons and remained in the brain of immunosuppressed rats; however, no benefit was observed due to the absence of differentiation signals required for direct cell specification (Ben-Hur et al. 2004). Similar to this report, *in vitro* differentiated ES cells expressing tyrosine hydroxylase were not successful after transplantation (Park et al. 2005).

Another challenging cell type for neural differentiation from ES cells is engraftable glial cells such as astrocytes and oligodendrocytes which are important for neurodegenerative diseases due to myelin sheath dysfunction. The differentiation protocol involves various steps such as neurosphere formation followed by treatment with FGF2 and epidermal growth factor (EGF) containing media. Approximately, 90% of the cells in this culture system have been detected as oligodendrocytes and remaining population were noted as astrocytes and neural cells (Nistor et al. 2005). These cells myelinated the axons when transplanted into the mouse, indicating the possibility of using *in vitro* derived oligodendrocytes for the treatment. There are some research for neuron recovery and remyelination by using neural progenitors derived from mouse and human cells in mice and rat spinal cord injury models (Kimura et al. 2005; McDonald et al. 1999). Keirstead and collegues used human ES cell derived oligodendrocytes in a rat spinal cord injury model and provided remylation to axons contributing to motor function (Keirstead et al. 2005). Konig et al showed the positive effect of mouse ES cell derived neural procursors for rat spinal cord avulsion injury model (Konig et al. 2017). Spinal cord injury restoration by using human ES cells have started in recent years and seems to be promising for patient therapy in clinal applications (Shroff 2016). Abbasi et al. demonstrated the successful neural differentiation of mouse ES cells in spesifically oriented polycaprolactone scaffolds which could be used in spinal cor injury (Abbasi et al. 2016).

Moreover, derivation of neural crest cells and functional peripheral neurons from human ES cells was shown in feeder free culture system indicating the potential promising regenerative application (Zhu et al. 2017). More research and trials need to be completed to move towards from basic research to clinical applications for neural regeneration.

3.3 Pancreatic Diseases Therapies

The development of potenatial cell based therapies for type I diabetes could be managed by generation of functional pancreatic β cells. ES cells as a source of insulin producing β cells might be used for transplantation in diabetic patients. Pancreas development starts from foregut endoderm and is regulated by retinoic acid (RA) and SHH signaling. Upregulation of transcription factor Pdx1 is the first indicator of pancreatic development. Pancreas development is controlled by FGF-10 and subsequently enhanced Notch signaling which suppresses transcription factor Ngn3 and induces β cells lineage. (Murry and Keller 2008). Although *in vitro* human ES cell diffentiation protocols were not very efficient for mature β cell development, insulin production and response to glucose have been observed (Jiang et al. 2007). In two previously published studies, human ES derived Pdx1 expressing cells transplanted into kidney capsule of diabetic mice did not form teratoma after transplantation and they were insulin positive *in vivo* (Shim et al. 2007). Fetal pancreatic tissue has been used to maturate human ES cell derived Pdx1 expressing progenitors as an alternative strategy and injected into kidney capsule of mice. Resulting cell population was insulin producing pancreatic β cells (Brolen et al. 2005). Saxena et al used synthetic lineage-control network engineering method as

an interesting approach to program transcription factors that are important for pancreatic differentiation including Pdx1. This network induced the differentiation of human ES cells into insulin producing pancreatic β cells (Saxena et al. 2017). In adition to cytokine and growth factor treatment modalities in *in vitro* culture, genetic modifications in pluripotent stem cells are promising to obtain functional pancreatic cells. mir-375, a key regulator of pancreatic development, enhanced insulin producing pancreatic islet differentiation of human ES cells in culture (Lahmy et al. 2016). Silencing of Forkhead box O1 (FoxO1) which is a regulator of pancreatic β cell differentiation increased pancreatic differentiation and insulin secretion upon glucose stimuli in human ES cells. Similar effects were observed in FoxO1 inhibitor treatment indicating the potential use of FoxO1 in ES cell based therapy applications (Yu et al. 2018). Additionaly, researchers have been trying to solve immunosuppression problems of transplanted pancreatic cells in preclinical studies. Human ES cell derived pancreatic progenitors were encapsulated with alginate and transplanted into streptozotocin treated mice. Transplanted implants control glycemic regulation and had viable cells after 174 day (Vegas et al. 2016). Although cell differentiation protocols are not enough to generate fully mature pancreatic cells *in vivo*, development of insulin producing cells from ES cells via established culture conditions could promote functional cell transformation.

3.4 Heart Regeneration Therapies

Because post-natal cardiac regeneration is limited to some intrinsic cell repair processes, translation of cell based regeneration to efficient clinical trials is the focus of current research in this area. As ES cells are pluripotent, they could be able to differentiate into major heart cell types *in vitro* by using defined culture conditions and various growth factors inluding TGF- β, FGF-2 and BMP-2 (Singla 2009). The crucial point for ES cell-based cardiac regeneration is to generate mature cardiomyocytes that could achive electrical and mechanical interaction network successfuly. Mouse and human ES cell differentiation into cardiomyocytes in culture conditions could mimic the embryonic cardiac development in terms of cardiac marker gene expression. Pluripotency markers are downregulated and mesoendoderm marker Brachyury (T/Bra) and early cardiac markers such as Nkx2.5 and Tbx5 are upregulated during *in vitro* differentiation. Thereafter, late cardiac markers such as cardiac α-myosin heavy chain and cardiac β-myosin chain are observed. Although embryonic heart development is regulated by a complex series of events and gene regulatory networks, *in vitro* ES cell differentiation into cardiac lineages and marker expressions in a timely manner resembling embryogenesis is promising for fully elucidating the devolepmental process and presentation of future therapies (Zhu et al. 2009).

There are well-established protocols for both mouse and human ES cells differentiation *in vitro* including spontaneous EB and growth factor based defined culture conditons. Human ES cells maintained in monolayer culture systems in the presence of TGF-β, activin A and BMP-4 sequentially yielded at least 30% of cardiomyocytes with contracting foci and contributed to the regeneration of infarcted rat hearts (Laflamme et al. 2007). The positive contribution of human ES cell derived cardiomyocytes to myocardium regeneration has been shown in athymic nude rats. Implanted cardiomyocytes proliferated rapidly, expressed cardiac markers and produced a graft after transplantation without teratoma-like structure formation (Laflamme et al. 2005). Caspi et al. have transplanted beating human ES derived EBs to infarcted rat hearts and observed electrically active viable grafts 8 weeks after transplantation (Caspi et al. 2007). In another study, van Laake et al. have used co-culture method and transplanted human ES cell derived cardiomyocytes and endodermal cells together. Non-cardiac cells were eliminated after transplantation and grafts were viable at 12th weeks post-transplanatation (van Laake et al. 2007). Similarly, mouse ES cells derived cardiomyocytes were alive after transplantation into infarcted

animals and helped regeneration in the infarcted tissue (Kolossov et al. 2006; Ménard et al. 2005; Min et al. 2003). In another study, mouse ES cell derived cardiac progenitor cells were differentiated into cardiomyocytes when grafted into infarcted mouse hearts and did not cause teratoma. Moreover transplanted cells helped imroving heart function and differentiate into functional cells (Christoforou et al. 2010). Human ES cell derived cardiac progenitors has been used in some clinical trials for the recent years. Cardiac progenitors embedded in fibrin scaffolds were transplanted into patiend and situatons were improved without any teratoma or complication (Menasché et al. 2015). In a recent clinical trial, human ES cell derived cardiac cells were used for ischemic left ventricular dysfunction an improved for systolic function (Menasché et al. 2018). Although preclinical mouse and human ES cells studies are promising for future therapies, *in vitro* differentiation efficiency, cell purity before transplanataition, effective and stable grafting techniques should be improved and potential paracrine effets for graft integration to the host should be identified for successful clinical applications.

4 Challenges for Regenerative Therapy

There are many issues that need to be adressed for ES cell based clinical applications such as immunogenicity, tumorigenic properties, and mature and functional cell diferentiation with high purity. Potential challenges and strategies will be discussed in this part.

4.1 Teratoma Formation

Teratomas are complex tumors consisted of random tissues derived from three different germ layers and used to define pluripotency of human ES cells. Safety concerns regarding teratoma formation after transplantation is one of the major problems for therapeutic applications. In order to avoid teratoma formation, ES cells should be differentiated terminally into desired cell lineages before transplantation. Cell purification based on a visible identified phenotype, surface marker expression and genetic marker selection should be conducted carefully for most of the restoration applications. Fluorescence-activated cell sorting (FACS) and magnetically activated cell sorting (MACS) (Vodyanik et al. 2006) could be used to select cell populations based on surface marker expression profile. Although these techniques are highly efficient, lack of markers and requirements of cell expansion after sorting remain as techniqual problems. Moreover, fluorescence reporter genes could be inserted into the spesific places in the genome for selection of differentiated cell types. However; tagging these proteins in the genome and selection might cause tumorienicity problems after transplantation into host due to possible oncogenesis by chromosomal insertion. Another novel strategy to eliminate undifferentiated ES cells in culture is to use antibodies against undifferentiated cells. Choo et al have used a cytotoxic antibody against podocalyxin-like protein-1(PODXL) to remove undifferentiated cells (Choo et al. 2008). However expression of PODXL in multiple human tissues limits the usage in therapy. SSEA5, CD9, CD30, CD50 and CD200 have been used to select undifferentiated ES cells from culture as pluripotency markers. The problem with those antibodies is the unspesific expression in the differentiated tissues. SSEA5 is more spesific compared to other markers and has been utilized to remove pluripotent cells in culture (Tang et al. 2011). Similarly, targeting Claudin-6 which is a tight-junction protein and absent in differentiated cells has been proven as a succesfull strategy to sort undiferentiated cells (Ben-David et al. 2013). In addition to spesific antibodies, small molecules for certain differentiation culture systems have been developed. Targeting anti-apoptotic genes in pluripotent cells by using small molecules might block teratoma formation (Mohseni et al. 2014).

4.2 Immune Rejection

ES cells increased the expression of major histocompatibility complex (MHC) when they differentiated (English and Wood 2011). Therefore transplantation of terminally differentiated cells may cause immune rejection in tissue regeneration applications. Although immune rejection as a tough barrier for cell transplantation is adressed by several strategies including immunosupression and genetically engineered ES cells that secrete immunosuppressive cytokines, more efficient strategies should be identified. Blockage of leukocyte costimulatory molecules as a short-term immunosuppressive therapy has enabled xenogeneic engraftment of human ES cells (Pearl et al. 2011). Immune rejection might be avoided by engineering patient spesific ES cells via somatic cell nuclear transfer (SCNT) which is conducted by transfer of a somatic cell nucleus into an egg. Then ES cells could be isolated from blastocyts for further differentiaion and transplantation (Yeo and Lim 2011). This technique seems to be a solution for immune rejection; however, acquiring enough number of human eggs remains as a problem. Induced pluripotent stem cells (IPS) technology as an alternative technology is useful for eliminating immune rejection. Although IPS cells could be a sloution to immune reactions and easy in terms of ethical regulations; culture conditions, genetic manipulations for IPS formation and additional parameters that need control emerge new challenges. Moreover, expansion of IPS cells, preventing apoptosis and senesence are other obstacles for clinical research.

4.3 Patient Population and Preclinical Models

In addition, patient population and transplantation area should be selected appropriately for early clinical trials. Determination of right patient population before starting advanced clinical trials is critical. Patient populations with advanced disease stage should be selected to minimize the risk during ES cell based therapeutic applications (Murry and Keller 2008).

Identification of right preclinical models for ES cell based regenerative medicine applications is required before moving towards clinical applications. Although *in vitro* differentiation and cell purification still remain as major issues, preclinical success is another obstacle that need to be improved. Larger animal models such as non-human primates, dog or sheep could be used for preclinical studies, however; these models are expensive and diffucult to handle in practice. Murine models including the immunodefienct and genetically engineered mice are inexpensive and easy to work in the laboratory. Although small animals are usefull for preclinical studies, murine physiology is different from human and restricts the interpretation of preclinical results. Despite the limitations in ES cell research, differentiation protocols and models; ES cell based therapy still seems promising for future treatment options. Current researches and developed techniques might enable the efficient usage of ES cells for further regenerative medicine applications.

5 Future Perspectives

The main challenge for ES cell based therapy is the derivation of functional, mature and engraftable cells that are responsive to the signals provided by host tissue and differentiate into desired cell types easily. The major concern of ES cell based current clinical tirals for regenerative medicine is the unlimited differentiation capacity of ES cells (pluripotency) that likely results in teratoma formation. A strategy to bypass teratoma formation through uncontrolled ES cell proliferation and differentiaon is to establish appropriate culture conditions for *in vitro* transformation. Although several cell types have been obtained using well-established *in vitro* differentiaiton protocols, *in vivo* transplantation and disease models should be improved and optimized for successfull early stage clinical studies. Moreover, these differentiation strategies might enable scientists to understand

developmental basis of human embryologic development and tissue spesification. Identification of molecular elements that controls tissue spesification and cell fate desicion during development and cell differentiation may facilitate the derivation of various cell types that are relevant for clinical therapies.

The availability of desired cell lineages from ES cells in appropriate and efficient culture systems will provide an opportunity for the treatment of diseases in a safe and qualified way.

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