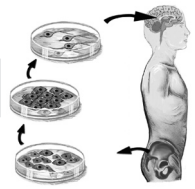

Human Embryonic Stem Cells for Vascular Development and Repair

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Abstract

Embryonic stem cells, derived from the inner cell mass of embryos in the blastocyst stage, are cells capable of perpetual self-renewal and long-term propagation and hold the potential to differentiate to progeny of the three embryonic germ layers. Since their derivation approximately two decades ago, exploration of mouse ES cells made major advances in ES cell differentiation research and in the successful development and propagation of various cell types. The subsequent derivation of ES cells from human embryos allows detailed study of early developmental events practically unreachable in early human embryos, and the potential derivation of a variety of adult cell types differentiated from the ES cells holds immense therapeutic promise. Recently, the study of ES cell-derived teratomas identified the partial presence of human ES cell-derived premature vessels within the teratoma, and a preliminary protocol for the *in vitro* derivation of a vascular progenitor was developed based on the study with the mouse ES cells. Furthermore, genetic profiling identified a pattern of expression of various endothelial and vascular smooth muscle cell genes that provide additional information on the degree of vascular development that ES cells undergo. Finally, the clinical application of ES cells in transplantation medicine is closer than ever following the affirmation that human ES cell-derived endothelial progenitors conferred increased neovascularization in transplanted engineered skeletal muscle. This review summarizes these recent advances in vascular development from human ES cells and their potential clinical applications.

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Since the advent of embryonic stem cell investigation, developmental biology has taken a tremendous leap toward understanding the processes that occur in the earliest stages of embryonic development. Advances in the research of mouse and human ES cell differentiation to the hematopoietic lineage enabled a description of lineage development that recapitulates the formation of blood cells in the early embryo and allowed the identification of a common progenitor cell to the hematopoietic and vascular lineages – namely, the hemangioblast [1,2].

Detection of contracting clusters within differentiating ES cells promoted investigation of ES cell-derived cardiomyocytes. Extensive research of the differentiating cardiomyocytes established that the differentiation pattern mimics that of the developing cardiomyocytes in the early embryo and facilitated the successful extraction and transplantation of ES cell-derived cardiomyocytes [3,4].

Spontaneously differentiating ES cells were also found to contain pancreatic island-like insulin-secreting cells, although the frequency of their appearance was too low to allow extensive characterization. Attempts are underway to produce enriched populations of ES cell-derived pancreatic island-like cells; this enrichment would permit a detailed characterization of these insulin-producing cells as well as the manufacture of an ample amount of cells that would answer the requirements for cell therapy [5].

Well-established studies of mouse ES cell commitment to the ectodermal lineage promoted attempts to drive human ES cells to neuronal differentiation, and several ectoderm derivatives have been successfully attained. Differentiation of ES cells in co-culture with stromal cells produced ES cell-derived neuronal cells with similar characteristics to dopaminergic neurons. Transplantation to newborn mice confirmed the ability of these ES-derived neurons to incorporate and function in the recipients' brain [6].

Concomitant with the acquired knowledge, utilization of the ES cells and the population of cells derived from them in cell therapy and transplantation is closer than ever.

Derivation, maintenance and differentiation of human ES cells

Embryonic stem cells are pluripotent cells derived from the inner cell mass of embryos in the blastocyst stage. They are unique by virtue of their immortality and their ability to maintain a developmental potential even with a prolonged culture, i.e., the ES cells remain in their undifferentiated state with a distinctive undifferentiated phenotype and a normal karyotype while continuously cultured [7].

The pluripotency of the ES cells is demonstrated when cells are injected to immunodeficient mice (nude mice). Shortly after injection, ES cells form a benign tumor, called a teratoma, which contains elaborate structures consisting of progeny of the three embryonic germ layers – the endoderm, mesoderm, and ectoderm [7].

ES = embryonic stem

Embryonic stem cells were first derived from the inner cell mass of a mouse embryo in the early 1980s [8] and, with the establishment of their pluripotency and their successful propagation, attempts were made to isolate them from primate embryos and shortly after from human embryos [7]. Derivation of ES cells can be accomplished by three protocols – immunosurgery, mechanical scraping, and placing a whole blastocyst on an inactivated feeder layer [7,9,10]. In contrast to mouse ES cells that are able to effectively propagate in a feeder-free culture when supplemented with leukemia inhibitory factor [11], human ES cells when derived are unable to flourish in the presence of leukemia inhibitory factor alone and require plating on a feeder layer of inactivated mouse embryonic fibroblasts [12]. Future clinical applications of human ES cells require total independence of the cells from any non-human supportive cultures. Thus, attempts are underway to develop an animal-free culture system for the derivation and propagation of human ES cells, and recent reports indicate that a successful animal-free culture of human ES cells is indeed available. In these studies the human ES cells are derived and cultured either on feeders from a human origin such as foreskin fibroblasts or fetal and adult muscle and skin cells, or in a feeder-free culture on fibronectin-coated plates and with the addition of specific cytokines such as basic fibroblast growth factor [13].

Under the appropriate conditions, ES cells can be driven to differentiate. Differentiation can be induced via three pathways. The first is the formation of embryoid bodies, which are ES cell aggregates cultured in suspension, so that spontaneous differentiation of the cells to various cell types and lineages is imminent [14]. Second, ES cells can differentiate when cultured in monolayer, where cells are plated on a differentiation-inducing feeder, such as stromal cells. Third, directed differentiation of the ES cells can be induced by means of culture on extracellular matrix proteins such as collagen [6,15].

This paper will review the attempts to direct human ES cell differentiation to the vascular lineage and the progress made in the field of tissue engineering, which aims to construct a functioning vascular system *in vitro*.

Formation of blood vessels

Three distinct pathways enable the establishment of the vascular system in the developing embryo – vasculogenesis, angiogenesis, and arteriogenesis. Throughout the initial development of the embryo, the delivery of oxygen and nutrients and the disposal of metabolic waste products are carried out through diffusion. However, when the embryo increases in bulk, the ratio of its surface area to its volume becomes too low to facilitate efficient exchange of materials with diffusion alone. Therefore, the formation of a delivery apparatus is of great significance.

The *de novo* formation of a primitive vascular network from embryonic mesoderm in the process of vasculogenesis lays the foundations for the pending development of the mature vasculature. In the yolk sac, the precursor for both the hematopoietic and vascular lineages, termed the hemangioblast, forms aggregates in which the cells in the inner core will differentiate to cells of the hematopoietic system, whereas the cells in the periphery will undergo extensive migration during which they will differentiate into endothelial cells and assemble into a primary capillary plexus [16].

Vascular development then continues with angiogenesis. New blood vessels arise from the preexisting network of primitive vasculature by sprouting, differentiation and migration of endothelial cells [16]. Angiogenesis is crudely divided into three stages. It begins with vasodilatation and vascular endothelial growth factor-induced increased permeability of the vasculature, which allows extravasation of plasma proteins to the extracellular matrix. This process allows matrix-degrading proteins to enter the extracellular matrix and thus provide the path for the forthcoming migration of the differentiating endothelial cells [17]. Accordingly, this process is followed by the loosening of contacts between neighboring endothelial cells and between endothelial cells and their supporting cells and matrix. This enables destabilization of mature vessels, migration of endothelial cells to distant sites, and successive assembly of vessels [18].

Unlike vasculogenesis, which is mainly restricted to embryonic development, angiogenesis is common in adult life as well. It is responsible for occurrences of neovascularization in both physiologic processes (such as wound healing and pregnancy) and pathologic conditions (cancer and metastases) [19].

The third, less understood mechanism of blood vessel formation is arteriogenesis. Here, new arteries that contain fully developed tunica media appear either through the maturation of preexisting collaterals or through the *de novo* formation of mature blood vessels. Arteriogenesis involves all types of vascular cells, including smooth muscle cells and pericytes [20].

Identification and characterization of an ES cell-derived vascular progenitor

The hemangioblast hypothesis

The concept of a common precursor for both the hematopoietic and vascular lineages emerged as early as 1920, based on the close proximity in time and place of hematopoietic and vascular differentiation in the yolk sac blood islands [21]. Shortly after, in his work with chick embryos, Murray [23] was able to isolate cells capable of differentiation to both blood and endothelial cells and thus strengthened the hypothesis of the existence of the hemangioblast. These observations facilitated the later studies that demonstrated the developmental pathways of the yolk sac blood islands. The yolk sac blood islands transiently appear as a uniform cell population, presumably hemangioblasts, and soon develop into a mass consisting of inner core cells that will differentiate to the hematopoietic lineage and an outer layer of cells committed to a vascular fate [22]. Advances in molecular genetics provided further corroboration with studies that found similarities in the pattern of gene expression of immature hematopoietic and endothelial cells and with gene mutation analysis, which identified a mutual dependence of the hematopoietic and vascular lineage development on the proper expression of several genes such as the

VEGF receptor *Flk*-1 [24]. The actual appearance of a hemangioblast-like cell population was available using a mouse ES cell differentiation model. ES cells, spontaneously differentiating as embryoid bodies, were analyzed at various early-stage time points. The analysis led to extraction of a hemangioblast-like cell called the blast colony-forming cell, which, when cultured further, gave rise to clusters containing early hematopoietic and vascular cells. The appearance of the blast colony-forming cells in the embryoid bodies is a transient one and it precedes the emergence of committed hematopoietic cells. These blast colony-forming cells were found to express *Flk*-1 and *Runx*1, the early genes of both vascular and hematopoietic development (although not expressing lineage-committed genes of either lineage), and the mesoderm-specific gene *brachyury*, and thus represent a mesodermal subpopulation of cells able to commit to either blood or vascular cells [25]. Following the identification of the blast colony-forming cell and based on its characterization, a possible candidate for the role of the hemangioblast was recently identified in the mouse embryo, also expressing *Flk*-1 and *brachyury*, strongly suggesting that the ES cells are a reliable model for embryonic development and hold the potential for future clinical application [26].

Cultivation of the ES cell-derived vascular progenitor cell

Advances in the identification and characterization of the ES cell-derived hemangioblast from spontaneously differentiating embryoid bodies promoted intensified study in search of an induced differentiation procedure for the attainment of a vascular progenitor cell *in vitro*. In the mouse ES cell study, Nishikawa and colleagues [25,26] turned to isolation and subsequent culture of *Flk*-1-expressing cells with the aim of characterizing a vascular progenitor. The preference for the VEGF receptor *Flk*-1 as the target marker for isolation of vascular progenitors was based on several observations. As mentioned previously, both the ES cell-derived blast colony-forming cell and the embryonic hemangioblast candidate cell express *Flk*-1 – a recognized early marker for differentiating blood and endothelial cells. Furthermore, in an earlier study [27], Nishikawa himself reported that *Flk*-1 and VE-*cadherin*-expressing cells precede the appearance of both blood and endothelial cells and therefore represent a bipotential progenitor. The directed differentiation of endothelial progenitor cells was facilitated by the culture of undifferentiated, *Flk*-1-negative ES cells on type IV collagen-coated dishes followed by isolation of early differentiated *Flk*-1-positive cells and continuous culture on type IV collagen in the presence of VEGF. These *Flk*-1-positive cells were shown to produce blood vessels both *in vitro* and *in vivo* [28]. Interestingly, in the absence of VEGF, the *Flk*-1-expressing cells favored differentiation to smooth muscle actin-expressing mural cells, indicating that the vascular potential of these cells allowed the development of endothelial as well as of other vascular oriented cells. In a later study, Sone et al. [29] attempted to cultivate vascular progenitor cells that were the progeny of monkey ES cells; however, they encountered several obstacles. Unlike mouse ES cells, monkey ES cells were found to express *Flk*-1 even in their undifferentiated state, although undifferentiated monkey ES cells were unable to develop to endothelial-like cells in a similar manner to mouse ES cell-induced *Flk*-1-expressing cells. Still, when the *Flk*-1 expression pattern was investigated in a single cell co-culture of the ES cells with OP9 feeder layer, it was established that the cells lose *Flk*-1 expression during a 4 day differentiation procedure only to regain its expression at the end of 8 days of co-culture. *Flk*-1-expressing cells exposed to 8 days of co-culture with OP9 feeder layer demonstrated the ability to differentiate to either endothelial-like cells when cultured further on OP9 cells or type IV collagen in the presence of VEGF, or to mural-like cells when cultured on type IV collagen in the absence of VEGF supplementation [29]. Thus, it appears that a general protocol for the cultivation of vascular progenitor cells from various ES cell types is available provided that a preliminary ES cell type-specific culture protocol is developed for the proper acquirement of *Flk*-1 expression. In a different study, Kaufman and colleagues [30] described an alternative method for the development and propagation of endothelial-like cells from Rhesus monkey ES cells. This method included continuous culture of undifferentiated ES cells in the presence of several well-recognized angiogenic factors such as VEGF, *b*FGF, etc. Within 5 to 10 days of culture, ES cells acquired an elongated morphology reminiscent of endothelial cell morphology and were continuously cultured for approximately 20 population doublings while maintaining endothelial cell morphology and normal Rhesus monkey karyotype. ES cell-derived endothelial cells were able to assemble into capillary-like structures when placed on matrigel, were found to express endothelial specific markers, and demonstrated an *in vivo* angiogenic potential when injected into immunodeficient mice either in a matrigel plug assay or a tumor neovascularization assay.

With the successful induction of endothelial-like cells from mouse and monkey ES cells and the *in vivo* and *in vitro* affirmation of their endothelial characteristics, we tried to cultivate endothelial-like cells from human ES cells in our laboratory [15]. Based on the derivation of endothelial-like cells from ES cells seeded on type IV collagen, human ES cells were removed from their feeder layer, separated to a single cell suspension, and cultured on type IV collagen in the presence of endothelial differentiation-supporting medium for 6 days. The 6 day time interval was preferred since it is the documented time period that allows expression of endothelial and vascular smooth muscle cell markers in human ES cell-derived embryoid bodies [31].

At the end of the 6 day culture, two cell types were observed: small flat cells with large nuclei that were found to be in a proliferative state due to their ability to incorporate 5-bromo-2′-deoxyuridine, and a population of larger flat cells not able to incorporate BrdU. For continuous differentiation, the isolation of the proliferating smaller cells was required and was facilitated by filtration through a 40 µm strainer. Reculture of the filtered cells

VEGF = vascular endothelial growth factor

bFGF = basic fibroblast growth factor
BrdU = 5-bromo-2′-deoxyuridine

on type IV collagen in the presence of VEGF, reported previously to be a crucial factor in the directed differentiation of endothelial progenitor cells [28], resulted in endothelial-like cells. These cells presented several endothelial specific features, including extensive uptake of acetylated low density lipoprotein and partial expression of von Willebrand factor and CD31. Reculture in the presence of platelet-derived growth factor-BB, a factor effecting smooth muscle cell differentiation in mouse ES cells, resulted in a cell population expressing vSMC markers. Furthermore, reculture of the filtered cells on matrigel or collagen I gel in a three-dimensional system in the presence of VEGF resulted in cell sprouting and formation of network structures within the 3D culture, providing additional confirmation to the endothelial-like properties of the cells.

Taken together, it appears that ES cells are able to be induced to a vascular fate, generating cells considered to be vascular progenitors. More intense research is required to determine the nature of the vascular progenitors obtained and refine the differentiation protocols established.

Gene expression pattern of ES cell-derived vasculature

A prominent feature of ES cell differentiation models is the accessibility to early embryonic events that are difficult if not impossible to study otherwise, i.e., with the embryo itself. A convincing example of the potential exploitation of ES cell differentiation models can be construed from the well-established data of the hematopoietic lineage differentiation derived from mouse ES cells and its uncanny similarity to the actual development of the early mouse embryo. In this model, assays – including genetic profiling, screening for specific cell surface markers, and the production of progenitor cells – demonstrated that ES cell-derived embryoid bodies remarkably reproduce the developmental stages that take place in the yolk sac of the mouse embryo [1,32].

A comparison of vascular developmental events of human ES cells and human embryos was carried out in our lab. In this study, Gerecht Nir et al. [33] followed the progress of differentiation of the vascular lineage in 4 to 8 week old human embryos and in ES cell-derived teratomas, with detection of cell surface markers by reverse transcriptase-polymerase chain reaction and immunohistochemical assays. In general, embryonic development was associated with protrusion of blood vessels into the generating tissues and organs. This was demonstrated with detailed immunolabeling of the developing vasculature with the endothelial specific markers *CD34, CD31 vWF* and Flk-1. Identification of the associated differentiation of vascular smooth muscle cells was facilitated solely by the staining of smooth muscle actin, particularly in the developing heart and connective tissues, with no detectable levels of other vSMC markers such as Calponin and smooth muscle-myosin heavy chain. In a parallel analysis of ES cell-derived teratomas, various small blood vessels were detected in the developing tissues. Identification of blood vessel origin was available with HLA staining, which only identified about 7% of the blood vessels formed as the derivatives of the implanted human ES cells; therefore, the bulk of the forming blood vessels in the teratoma belonged to the mouse host. Staining with *SMA* confirmed the presence of vSMCs in the vicinity of developing tissues within the teratoma, although only slight detection of human-specific *SM-MHC* was available. Developmental kinetics was ascertained with RT-PCR analysis of well-documented endothelial and smooth muscle cell genes. The 4 week embryo already expresses *SMA*, *calponin* and *caldesmon*, all vSMC-oriented genes, but acquires expression of SM-MHC only later at 8 weeks. As to endothelial specific expression, endothelial progenitor markers, such as Flk-1, Tal-1 and *CD133* and the endothelial markers *CD31*, *CD34* and *vWF* are all detectable in the 4 week embryo. In the 8 week embryo, a novel expression of genes involved in blood vessel remodeling, including vascular cell adhesion molecule and vascular endothelial cadherin, is also detectable.

A broader understanding of the genetic profile of spontaneously differentiating human ES cells and more specifically of vascular oriented gene expression of the ES cell-derived embryoid bodies was attained with micro-array analysis of human ES cells as compared to 1 week, 2 week and 4 week old embryoid bodies [34]. The micro-array analysis followed the expression pattern of 2308 genes expressed by the ES cells and the embryoid bodies described. The assay established a classification of the detected genes into two clusters according to changes in their pattern of expression. One of the clusters included genes associated with ES cell plupripotency and self-renewal, which were continuously down-regulated with the progressive differentiation, and the second contained genes that were increasingly up-regulated with the developmental process and were identified as a variety of genes associated with early developmental events and commitment to lineage. As to vascular progeny of the differentiating embryoid bodies, several genes that are detected in blood vessel formation were contained in cluster 2, and include *PECAM1*, *VCAM1*, *VEGF* and *ANG1* [Table 1]. Several transcription factors associated with the development of both blood and endothelial cells are also detected, including *TAL1* and *LMO1* [Table 1]. Temporal characterization of the expression pattern of the vascular associated genes indicated that a number of these genes are markedly increased in the differentiating embryoid bodies as compared to their expression level in the undifferentiated human ES cells. These genes include *PECAM1*, *VCAM1, VE-cad, CD34, CD41, CD45, TAL1, LMO2, GATA1, GATA2* and *GATA3.* Surprisingly, no up-regulation of the well-recognized marker for vascular differentiation, Flk1, was detected. However, several vascular related cytokines such as *VEGFA, VEGFC, ANG1, ANG2* and more, were identified as up-regulated with ES cell differentiation. Affirmation

vSMC = vascular smooth muscle cell
3D = three dimensional
vWF = von Willebrand factor
SMA = smooth muscle actin
SM-MHC = smooth muscle-myosin heavy chain
RT-PCR = reverse transcriptase-polymerase chain reaction
VCAM = vascular cell adhesion molecule
VE-cad = vascular endothelial cadherin

Table 1. Genes associated with vascular differentiation, detected by micro-array analysis of developing embryoid bodies, at various stages of development

	Symbol	Title
v-SMC		
1	MYH11	Myosin, heavy polypeptide 11, smooth muscle
2	LMOD1	Leiomodin 1
3	PDGFB	Platelet-derived growth factor
4	PDGFRB	PDGF receptor,
5	TGFB3	Transforming growth factor
6	TGFBR2	TGF receptor II
7	TGFBR3	TGF receptor III
ECs		
1	PECAM1	CD31 antigen
2	VCAM1	Vascular cell adhesion molecule 1
3	PCDH12	Protocadherin 12
4	CDH5	VE-cadherin
5	VEGF	Vascular endothelial growth factor
6	VEGFC	Vascular endothelial growth factor C
7	FIGF	VEGFD
8	EPAS1	Endothelial PAS domain protein 1
9	FLT1	Vascular endothelial growth factor
10	FLT4	fms-related tyrosine kinase 4
11	ANGPT1	Angiopoietin 1
12	ANGPT2	Angtopoietin 2
13	GATA2	GATA binding protein 2
14	GATA3	GATA binding protein 3

The data are taken from Gerecht-Nir et al. [34].

of the data obtained by the micro-array was available with real-time quantitative PCR analysis. Results confirmed the micro-array analysis with regard to most genes tested, such as *PECAM1, CD34, ANG2* and others, all of which were up-regulated in the course of differentiation. However, the real-time PCR indicated an elevation in the expression of *TIE2* after 1 week of differentiation, an elevation not detected by the micro-array. This is associated with the documented decreased sensitivity of the micro-array analysis in comparison with the real-time PCR sensitivity [35]. Thus, the micro-array represents a powerful tool for documenting the shifts in gene expression from the onset of differentiation up to the resultant mature cell type, a tool that presumably can also be used to direct differentiation to a certain lineage and/or cell type.

A glance at the future

The potential application of ES cell-derived progenitor or fully differentiated cells for regenerative medicine was a declared goal at the advent of ES cell exploration. The progress in our understanding of the processes that occur during the initial stages of embryonic development has fostered the skill to produce cells for clinical purposes, even though it still requires refinement. The initial assembly of viable blood vessels from ES cell-derived vascular progeny has already been demonstrated in both mouse and human derived ES cells. In one mouse model, the derived endothelial progenitors were allowed to sprout within a 3D type I collagen gel [36]. Daily observations revealed a preliminary formation of vessel assembly before the end of 5 days of culture. The formed vessels increased in number and complexity, until eventually regressing to single cells after 2 weeks of culture.

Recently, Levenberg and team [37] described a method for the *in vitro* expansion of engineered skeletal muscle tissue developed by means of co-seeding the myoblasts with human ES cell-derived endothelial cells and embryonic fibroblasts on a porous biodegradable scaffold. The co-culture of the myoblasts in the presence of the human ES cell-derived endothelial cells allowed the construct to undergo a process of neovascularization prior to implantation, which contributed to the improved integration of the engineered muscle when transplanted to immunodeficient mice. The presence of endothelial cells apparently contributed to increased vascularization, to circulation and to improved survival of the implanted skeletal muscle.

This preliminary study provides a novel approach for ES cell manipulation in regenerative medicine and, combined with progress in establishing differentiation protocols and the improved availability of partially or fully differentiated cells, it seems that the study of embryonic stem cells has leaped forward not only in the field of vascular biology but in the overall understanding and use of ES cells.

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