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Review

Murine embryonic stem cell *in vitro* differentiation: applications to the study of vascular development

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Summary. The present review summarizes knowledge accumulated during the last decade concerning *in vitro* endothelial differentiation from embryonic stem (ES) cells. There is now growing evidence that ES cells may provide a powerful model system to determine the cellular and molecular mechanisms of vascular development. ES cells differentiate into the endothelial lineage by successive maturation steps recapitulating *in vivo* events observed in the embryo. Further maturation of ES-derived embryoid bodies either in three dimensional gels or in confrontation cultures with tumor spheroids can also provide a model of physiological or tumoral angiogenesis. The data obtained from experimental *in vitro* differentiation of genetically modified mouse ES cells highlight the potential and the complementarity of this model system to *in vivo* gene knock out studies. We also consider and discuss some of the potential applications of ES cell technology in vascular biology for future directions in basic research and medicine, by manipulation of differentiation and the generation of cell populations for analysis and transplantation for therapeutic use.

Key words: Vasculogenesis, Angiogenesis, ES cells

Introduction

Blood vessels provide an essential nutritive function during growth and repair of tissues but can also contribute to pathology such as cancer and various ischaemic and inflammatory diseases. Angiogenesis is involved in the vascularization of tumors (Carmeliet and Jain, 2000). It is also observed in non-neoplastic conditions, including diabetic retinopathy, rheumatoid arthritis, and psoriasis (Folkman and Shing, 1992; Carmeliet and Jain, 2000). The ability to fully understand the biochemical mechanisms that govern and control blood vessel formation are then essential for the development of new therapies to help combat such disorders.

In the embryo, blood vessel formation occurs by differentiation of vascular endothelial cells from angioblastic precursors, that in turn give rise to a primitive vascular plexus. This process is termed vasculogenesis (Risau, 1997; Yancopoulos et al., 2000). Most subsequent vessel formation in the embryo takes place by splitting or sprouting from pre-existing vessels, a process known as angiogenesis (Risau, 1997; Carmeliet, 2000). Nascent vessels are stabilized by mural cells (pericytes in small vessels; smooth muscle cells in large vessels) completing the angiogenic programme (Carmeliet, 2000; Yancopoulos et al., 2000). Although initially independent of the circulation, the vascular system is later shaped and remodelled by forces generated by the blood flow, to suit the local tissue needs (Risau, 1997). Terminal endothelial differentiation arises under microenvironmental control leading to several types of functionally and morphologically endothelial cell phenotypes (Augustin et al., 1994; Risau, 1995; Garlanda and Dejana, 1997). New vessels in the adult predominantly grow by angiogenesis and until recently, vasculogenesis was appearing to be a mechanism unique to embryonic development. However, several recent studies reported that vasculogenesis may also occur in the adult and can contribute to the neovascularization response. Bone marrow-derived endothelial progenitor cells have been isolated from circulating peripheral blood and shown to be incorporated into newly-formed blood vessels, consistent with a postnatal vasculogenesis (Asahara et al., 1997, 1999; Takahashi et al., 1999; Kalka et al., 2000).

During the last decade, embryonic stem (ES) cells have concentrated a large body of interest for analysis of differentiation into the endothelial lineage. The recent isolation of human ES cells has attracted considerable attention because of the promise of using these cells or their derivatives for research in developmental biology and medical applications, including cellular transplantation (Rathjen and Rathjen, 2001). Here, we report and discuss the lessons obtained from

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experimental *in vitro* differentiation of mouse ES cells that highlight the potential of this model system for the understanding of the mechanisms of vascular development and some of their future applications.

ES cells as a model system for vasculogenesis and angiogenesis

Origin and properties of murine embryonic stem cells

Mouse embryonic stem cells are continuous cell lines derived directly from the inner cell mass of blastocyst-stage of early murine embryo (Abbondanzo et al., 1993). They retain the ability to differentiate into all cell types, a unique feature which is the basis of various applications of ES cell technology. ES cells are maintained *in vitro* as totipotent stem cells by culture on a feeder layer of embryonic fibroblasts or in the presence of the cytokine leukemia inhibitory factor (LIF). When LIF is removed, they are able to spontaneously undergo *in vitro* differentiation, either in monolayer or in aggregates (embryoid bodies), into all the derivatives of the inner cell mass cells. The embryoid bodies (EBs) can also give rise to highly differentiated cells when EBs formed in suspension are subsequently allowed to attach and to form outgrowths. Indeed, ES-derived hematopoietic cells, osteoblasts, neurons and neural precursors, adipocytes, hepatocytes, keratinocytes, chondrocytes, insulin-secreting cells, cardiomyocytes and muscle cells, and endothelial cells have been found (Risau et al., 1988; Bain et al., 1995; Keller, 1995; Bagutti et al., 1996; Dani et al., 1997; Wobus and Guan, 1998; Kramer et al., 2000; Soriat et al., 2000; Lumelsky et al., 2001; Phillips et al., 2001; Zhang et al., 2001; Jones et al., 2002), indicating that this model provides access to early cell populations that develop in a normal fashion.

ES cell differentiation into the endothelial lineage

Several ES differentiation protocols based on different culture systems described the presence of derivatives of the endothelial lineage in ES cell progeny. In all these studies, endothelial differentiation can be obtained regardless of the ES cell line strain used and independently of the cell culture procedure used, i.e. suspension culture, methylcellulose-supplemented semi-solid culture medium or the hanging drop method. This indicates that most of the endothelial differentiation program can be achieved during ES cell *in vitro* differentiation. The *in vitro* formation of blood island structures: immature hematopoietic cells surrounded by endothelial cells, was initially reported on the surface of cystic EBs (Doetschman et al., 1985). Further studies showed that ES-derived endothelial cells can form, within the wall of cystic EBs, vascular channels that connect cavernous areas often containing hematopoietic cells (Risau et al., 1988; Wang et al., 1992). Microscopic analysis has revealed that cells lining the lumen of these channels were connected by typical endothelial junctions (Wang et al., 1992). These experiments clearly established that both endothelial differentiation and development of a primitive vasculature, as illustrated in Fig. 1A, can reproducibly occur in EBs. The differentiation procedure in semi-solid medium seems to be preferable since EBs originate from single cells and exhibit a narrower distribution size than in suspension culture that may have effects on the differentiation synchrony (Wartenberg et al., 1998). Another procedure, based on the spinner flask technique, where ES differentiation is performed under continuous stirring, allows the cultivation of large amounts of EBs which represents an advantage for routine screening of potentially active agents (Wartenberg et al., 1998).

ES endothelial cell differentiation could also be observed in the absence of the complex embryoid structure formation either when small EBs (before day 4) were transferred into gelatinized plates (Bautch et al., 1996) or when ES cell differentiation was directly performed in monolayer culture on type IV collagen-coated plates (Hirashima et al., 1999; Yamashita et al., 2000), a major constituent of the endothelial basement

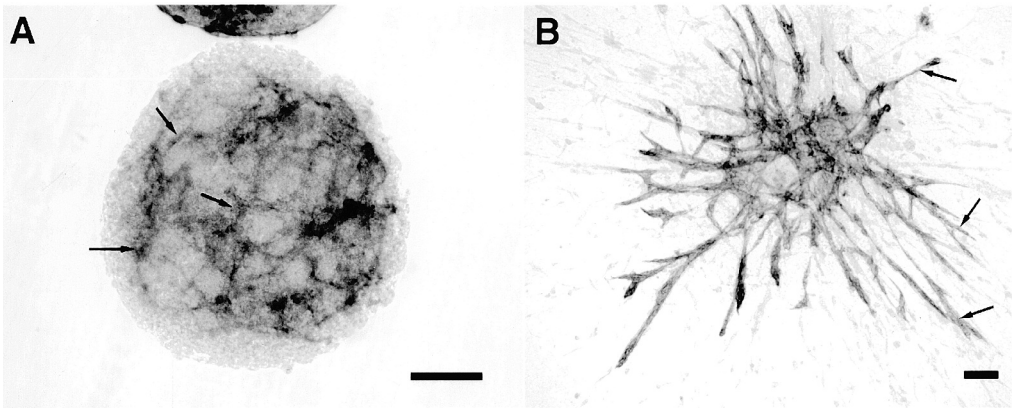

Fig. 1. Endothelial differentiation and vascular morphogenesis during *in vitro* ES cell differentiation. **A.** PECAM whole mount immunostaining of an EB after 11 days of differentiation. Endothelial differentiation and cord-like structure formation (black arrows) can be visualized. **B.** Endothelial sprouting from an EB embedded into type I collagen gel as a model of angiogenesis. Immunostaining of PECAM-positive sprouts, as indicated by arrows, were revealed 3 days after secondary culture in the presence of angiogenic growth factors. Scale bars: 100 μm.

membrane that has been found to be optimal for the support of the *in vitro* mesoderm differentiation from ES cells. However, the formation of tubular structures, as compared with ES cell-derived embryoid bodies cannot be obtained when differentiation is performed in monolayer culture. Nevertheless, it represents a good alternative for an easy isolation of purified endothelial progenitors without the need of the enzymatic digestion of the embryoid body.

The ES differentiation system can then allow the dissection of the cellular events of vasculogenesis and gives accesss to endothelial cell progenitors. As stated above, this model system has allowed us to characterize several successive maturation stages during differentiation into endothelial cell lineage by the analysis of the expression of various endothelial cell markers (Vittet et al., 1996). In particular, these studies have indicated that PECAM (CD31) and Flk-1 may be markers of a prevascular stage in addition to being expressed on vascular and hematopoietic precursors (Kabrun et al., 1997; Redick et Bautch, 1999) and that the expression of VE-cadherin is a crucial step for the commitment into the endothelial lineage (Nishikawa et al., 1998). Although embryoid bodies are not structurally organized compared to the embryo, the emergence of cells belonging to the endothelial lineage was found to follow the sequence of events occuring *in vivo* (Vittet et al., 1996). In addition, the ES model has brought new insights into the cellular origin of cells in vascular development by the demonstration of the existence of "vascular progenitor cells" able to differentiate *in vitro* and also *in vivo* into both the endothelial and the mural compartment of the vasculature (Yamashita et al., 2000).

The embryoid body as an assay system for angiogenesis

The ES/EB system also appears to display many features of the angiogenic process. Experiments performed by plating EBs primarily grown in suspension culture onto gelatinized dishes or onto matrigel showed the formation of branching vascular structures indicative of extensive vascular morphogenesis from EBs (Bielinska et al., 1996; Bloch et al., 1997; Zhang et al., 1998; Goumans et al., 1999). A more complex vascular organization process can be obtained when performed in three dimensions (Yamashita et al., 2000; Féraud et al., 2001). We have recently demonstrated that sprouting angiogenesis can be recapitulated during ES-derived EB development into a type I collagen matrix (Fig. 1B). Endothelial sprouts exhibiting an angiogenic phenotype can arise from the pre-existing EB primitive vascular network when 11-day-old EBs are plated in type I collagen gels in the presence of angiogenic growth factors such as VEGF (Féraud et al., 2001). These endothelial sprouts, whose development can be inhibited by known angiostatic agents, were found to further organize into tube-like structures, in parallel with the differentiation of α-smooth muscle actin-positive cells. Three dimensional cultures in type I collagen gels of aggregates of sorted ES-derived Flk1-positive cells also showed the formation of tubular endothelial stuctures which are associated with α-smooth muscle actin-positive cells (Yamashita et al., 2000), a feature evoking pericyte recruitment during neovessel stabilization (Carmeliet, 2000). The ES/EB system then allows the study of vasculogenesis and angiogenesis, both processes being involved in new blood vessel formation in the adult, that represents a unique property conferring superiority to all other *in vitro* angiogenesis models.

In addition to normal physiological angiogenesis, the ES-derived EB system used in confrontation cultures with multicellular tumor spheroids is also suitable for studying tumor-induced angiogenesis. Invasion of endothelial cells into the tumor tissue can be observed in tumor spheroids cultivated in confrontation cultures with embryoid bodies which became efficiently vascularized within a few days (Wartenberg et al., 2001). This angiogenesis resulted in the growth stimulation of the tumor spheroid, the disappearance of the central necrosis, the improvement of oxygen supply and the downregulation of the expression of both HIF-1α and VEGF, features normally observed during the vascularization of a tumor tissue.

Applications of ES cell technology for cellular and molecular analysis of vascular development

Regulation of endothelial differentiation and angiogenesis

This system appears useful to help to characterize modulators of the earliest stages of differentiation and mitogenesis inside the endothelial lineage. These factors can be applied either directly on ES cells but also at different steps of the differentiation process. Up to date, all the examined factors were unable to exclusively direct the differentiation into one particular cell type but rather improved the differentiation into a cell lineage. Selection or sorting of cells are then necessary to obtain a homogeneous endothelial cell population. In the past years, several factors were shown to control endothelial differentiation of mouse ES cells. Endothelial cell differentiation and vascular structure formation were found to be promoted by a growth factor cocktail including VEGF-A, FGF2, IL6 and Erythropoietin (Vittet et al., 1996). Consistent with its pivotal role in the first steps of endothelial development *in vivo*, VEGF was found to be able to exert alone a significant activity in inducing the formation of PECAM-positive cells in ES-derived EBs (Vittet et al., 1996). Nevertheless, the other constituents of the growth factor cocktail have appeared efficient to optimize the endothelial differentiation response. Among them, FGF2, which has also been described in other studies to act as a potent morphogen during vasculogenesis (Doetschman et al., 1993). FGF2 and LIF together were characterized to contribute to the induction and to the support of embryonic vasculogenesis from an ES-derived

endothelial cell line (Gendron et al., 1996). The TGFß1 pathway also appeared to be an important regulator of vasculogenesis in ES-derived EBs. A functional role for the latent TGFß-binding protein-1 (LTBP1) in regulating endothelial cell development from *in vitro* ES cell differentiation has been observed by monitoring expression of endothelial cell markers and vascular cord formation (Gualandris et al., 2000). In this work, a modulating role of LTBP1 on TGFß availability was proposed. Differentiation of ES cells overexpressing TGFß1 was also found to result in an enhanced formation of cord-like tubular structures when EBs were plated on gelatin (Zhang et al., 1998). The functional role for both TGFß1 and FGF2 has recently been confirmed on the differentiation of human ES cells since these factors were observed to induce mesoderm differentiation from which endothelial precursor cells originate (Schuldiner et al., 2000). Nevertheless, future studies should be performed to analyze endothelial developmental processes in human models and to fully elucidate the regulatory elements of vasculogenesis.

Angiogenesis in ES-derived embryoid bodies was found to be sensitive to both angiogenic and antiangiogenic agents. Well-known angiogenesis activators (VEGF, FGF2) and inhibitors (PF4, Angiostatin, Endostatin, Thalidomide) were observed to significantly induce or inhibit the EB sprouting angiogenic response in three dimensional cultures (Sauer et al., 2000; Féraud et al., 2001). The ES/EB system then appears to be of primary importance to investigate the molecular mechanisms of angiogenesis, and in particular the mechanisms of action of angiogenesis inhibitors that remain largely unknown.

Analysis of the function of unknown genes: lessons from gene modification studies

ES cells have been used specifically to inactivate genes by homologous recombination. The *in vitro* differentiation of genetically modified ES cells was found complementary to *in vivo* studies on transgenic animals, and offers excellent alternatives to analyze the consequences of specific mutations, especially when these mutations are lethal for the embryos preventing analysis of further distal developmental events such as vascular system maturation and remodelling. Several gene knockouts have now been analyzed in this way (Table 1).

One of the most striking examples is constituted by the analysis of the VEGF-A signalling pathway which has been established to play a pivotal role in the formation of the embryonic vasculature. Impaired vessel formation is observed after targeted mutation of VEGF-A and its cellular receptors flt-1 and flk-1 (Ferrara, 2000). Targeted inactivation of the VEGF-A gene and even solely inactivation of a single VEGF-A allele in mice resulted in embryonic lethality because of severe defects in all examined steps of early vascular development including differentiation of blood islands, formation of large vessels, sprouting, and the spatial organization of embryonic vessels (Carmeliet et al., 1996; Ferrara et al., 1996). *In vitro* differentiation experiments performed with heterozygous or homozygous VEGF-A mutant ES cells allowed the characterization of a stage-specific differentiation step at which vasculogenesis is blocked because of VEGF-A deficiency. A population of Flk-1+, PECAM+ cells was found to accumulate which failed to organize into blood islands, thus suggesting that VEGF is required for the transition of these precursor cells to more differentiated endothelial cells (Bautch et al., 2000). These data are consistent with the requirement for the visceral endoderm where VEGF-A is primarily expressed for normal formation and organization of blood islands (Damert et al., 2002); a lack of blood islands and vascular channel formation also being observed in EBs deficient in Gata4, a transcription factor whose deficiency induces a specific block in visceral endoderm formation (Bielinska et al., 1996). Analysis of flt-1-/- ES cell differentiation has also brought additional insights into the mechanisms responsible for the flt-1 deficiency phenotype. Flt-1-deficient mice die at mid-gestation from severe defects in the organization of embryonic vasculature which display abnormal overgrowth of

Table 1. Vascular gene knockout analysis by *in vitro* ES cell differentiation.

GENE	*IN VITRO* PHENOTYPE	REFERENCE
ß1 integrins	Delayed endothelial cell differentiation in ß1-/- embryoid bodies	Bloch et al., 1997
α5 integrins	Delayed endothelial cell organization into vascular tubular structures	Taverna and Hynes, 2001
flk-1 (VEGFR2)	Impaired endothelial cell migration and vascular morphogenesis	Schuh et al., 1999
flt-1 (VEGFR1)	Deregulated mitogenesis in the endothelial lineage	Kearney et al., 2002
VE-cadherin	Defects in endothelial cell assembly and in vascular morphogenesis in VE-cadherin-deficient EBs	Vittet et al., 1997
TGFßRII	Induced aberrant TGFß signalling by overexpression of TGFßRII or of a dominant negative form of TGFßRII causes defects in vasculogenesis	Goumans et al., 1999
VEGF-A	Differentiation blockade of PECAM+/Flk1+ endothelial precursor cells	Bautch et al., 2000
Gata4	Gata4-/- ES cells retained the capacity to differentiate into primitive endothelial cells but failed to organize and form blood islands.	Bielinska et al., 1996
JunB	Inability of JunB-deficient endothelial cells to form vascular channels	Schorpp-Kistner et al., 2000

endothelial cells leading to many enlarged vascular structures within both the yolk sac and the embryo (Fong et al., 1995). Based on these observations it was suggested that the flt-1 signalling pathway may regulate normal cell adhesion between endothelial cells and endothelial cell-matrix interactions (Fong et al., 1995). A more recent study has established that the main factor leading to this phenotype corresponds to an increased commitment towards the hemangioblast (Fong et al., 1999), a common precursor for both hematopoietic and endothelial cell lineages. Analysis of the *in vitro* developmental potential of flt-1-deficient ES cells has revealed that the absence of flt-1, in addition, results in an aberrant increase in endothelial cell division throughout the stages of endothelial differentiation that supports a critical role for flt-1 for the fine tuning of VEGF-induced blood vessel growth (Kearney et al., 2002). Similarly, analysis of flk1-/- ES cell differentiation *in vitro* has been crucial to determine that flk1 deficiency does not affect the initial steps of endothelial commitment and differentiation but impairs subsequent endothelial cell migration and vascular morphogenesis (Schuh et al., 1999), issues that cannot be easily addressed in flk1-deficient embryos since early defects in blood island formation were found resulting from both hematopoietic and endothelial impairments (Shalaby et al., 1995).

Differentiation of ES cells with specific mutations for α5 integrin, ß1 integrin, jun-B, VE-cadherin and TGFßRII genes also revealed the expected defects in vascular development as they have been described *in vivo*. Indeed, consistent with the *in vivo* phenotype (Yang et al., 1993), α5-null EBs showed delayed and reduced formation of vascular structures (Taverna and Hynes, 2001). A delayed formation of cord-like structures, in accordance with a defect in the vascularization of ß1-/- ES cell-induced teratomas, could also be observed during differentiation of ß1-null EBs (Bloch et al., 1997). Vascular defects in jun-B-deficient embryos could be recapitulated during the *in vitro* differentiation of jun-B-deficient ES cells (Schorpp-Kistner et al., 2000). Although endothelial cells differentiated normally, VE-cadherin gene disruption impaired vasculogenesis in both embryo (Carmeliet et al., 1999; Gory-Fauré et al., 1999) and ES cell-derived EBs (Vittet et al., 1997). Defective or aberrant TGFß signalling in EBs led to the reduction of extracellular matrix deposition and failure of endothelial cells to organize into vessels (Goumans et al., 1999), as observed after targeted inactivation of the TGFß gene or the TGFßRII receptor which both resulted in defects in vasculogenesis (Dickson et al., 1995; Oshima et al., 1996).

Then, in addition to displaying, in large part, phenotypes consistent with the *in vivo* phenotypes, *in vitro* differentiation experiments with ES mutants have brought important additional informations that have allowed the identification of some of the mechanisms responsible for the observed mutant phenotypes. It can then be recommended to systematically analyze the *in vitro* differentiation potential of double mutant ES cells when performing an *in vivo* gene knockout. On the other hand, the ES/EB system may be of potential use for a pre-screening of ES cell clones before the production of transgenic lines (Gustafsson et al., 2001), avoiding the production and the screening of multiple transgenic lines, which constitutes an expensive and time-consuming labor.

Another potential use of the ES/EB system relies on the analysis of the consequences of a particular gene overexpression during the normal differentiation program. The experimental versatility of the ES differentiation system for the study of vascular development could then be further increased by the development of systems that allow precise control of gene expression. A conditional expression strategy has recently been achieved with an inducible expression system that relies on a tamoxifen-dependent Cre recombinase/loxP-based strategy and the use of bicistronic gene-trap expression vectors to turn on gene expression either in undifferentiated ES cells or in their differentiated derivatives (Vallier et al., 2001). Future use of such inducible expression systems under control of endothelial specific promoters would certainly bring new information about the timing of the effects of the gene tested and on their specific targets.

New trends and perspectives

Applications for the screening of active molecules, either angioactive or angiostatic

The sensitivity of the sprouting ES/EB assay to both positive and negative modulators of angiogenesis may be exploited for the optimisation and the development of performant angiogenesis assays. Combination of the ES angiogenesis model with gene reporter strategies may seriously improve such a model. This may be achieved by the generation of transgenic ES cells containing the green fluorescent protein (GFP) gene under control of an endothelial specific promoter that would allow the direct identification of endothelial cells. This strategy has recently been used with success by the generation of ES cell lines carrying EGFP as a reporter gene and an integrated puromycin resistance, both under the control of the vascular endothelial specific tie-1 promoter (Marchetti et al., 2002), thus providing a powerful means for endothelial lineage selection and/or identification. With the recent generation of human ES cell lines, the use of such reporter strategy would offer a powerful screening assay for both vasculogenesis and angiogenesis, which represent crucial steps for the identification and the development of new potent therapeutics.

Applications for the generation of immortalized endothelial cell lines

Since ES cells are relatively easy to transfect and are commonly used for targeted gene inactivation

experiments in mice, genetically manipulated endothelial cell lines carrying specific mutations can then be obtained. *in vitro* ES cell-derived endothelial progeny can be immortalized by using Polyoma middle T-containing vectors (Carmeliet et al., 1999; Balconi et al., 2000). Such immortalized cell lines may be particularly useful to analyze and characterize molecular mechanisms causing endothelial dysfunction (Carmeliet et al., 1999). However, ES-derived endothelial cells are not easily separated from EBs and maintained in culture, and immortalization might be preferably done when differentiation is performed in monolayer. Similarly, selective immortalization by a retroviral vector containing the SV40 Large T antigen of differentiated ES cell derivatives has allowed the derivation of an embryonic endothelial cell line that displays a vasculogenic potential (Gendron et al., 1996). The ES *in vitro* differentiation system may then be useful and represents a promising tool for the generation of immortalized endothelial cell lines at different endothelial maturation stages from either normal or mutated ES cells which could provide new endothelial models.

Differentiation towards lymphatics

During the last decade, enormous advances have been made towards elucidating the molecular and cellular events involved in vasculogenesis and angiogenesis but recent developments have identified the lymphatic endothelium as an important component for the process of tumor metastasis that could become one of the next foci for angiogenesis and metastasis research (Pepper, 2001; Plate, 2001; Karkkainen et al., 2002). The origin and the molecular mechanisms that govern differentiation towards lymphatics remain poorly understood. The development of the lymphatic system has mostly been thought to be derived by sprouting from embryonic veins through a process referred to as lymphangiogenesis. However, it has been assumed that besides the venous origin of lymphatics, there may be a lymphangioblastic cell lineage, at least in birds (Schneider et al., 1999). With the recent identification of markers, predominantly expressed in lymphatic vessels, such as the lymphatic endothelial hyaluronan receptor LYVE-1, the VEGF receptor VEGF-R3, the lymphoid chemokine SLC/CCL21 or the homeobox transcription factor Prox-1 (Karkkainen et al., 2002; Wigle et al., 2002), the ES *in vitro* differentiation system may be of great interest to solve these issues; and in particular to analyze whether lymphangioblasts can be identified in mammals.

Generation of cell populations for analysis and cell therapy

In the last few years, several studies have mentioned the existence of circulating angioblasts that can incorporate at sites of neovascularization (Asahara et al., 1997, 1999; Takahashi et al., 1999; Kalka et al., 2000; Kaushal et al., 2001). A potential and important source of these cells may be constituted by ES cells. Indeed, vascular progenitors can be obtained from differentiated ES cells (Yamashita et al., 2000) and were shown to give rise to the different blood vessel components: endothelial and smooth muscle cells. Recent studies have also mentioned that these ES-derived endothelial cells can incorporate into new blood vessels in mice (Yamashita et al., 2000; Marchetti et al., 2002). The ES/EB model, then, appears of particular interest for the study of the mechanisms of differentiation in these different lineages.

Moreover, the recent demonstration that human ES cells, similar to mice ES cells, can differentiate into the endothelial lineage and can organize *in vitro* into vessel-like structures when induced to form EBs (Levenberg et al., 2002) may represent an important advance for the treatment of pathological situations affecting the vascular system. These ES cells could then provide a source of endothelial cell progenitors and of mature endothelial cells that could be beneficial for potential applications in therapy such as cell transplantation, therapeutic agent delivery, or tissue engineering of grafts. However, because of the existence of potential differences in the behavior of mouse and human ES cells (Levenberg et al., 2002), further studies for the validation of the results obtained in mice appear necessary. In addition, more rigourous criteria are needed to establish how new cells form from transplanted ES cells, and whether they function normally. Indeed, very recent data reported that transplanted stem cells fused with the surrounding cells, which could make the cells genetically abnormal, precluding their medical use (Terada et al., 2002; Ying et al., 2002).

Signalling pathways and mechanisms controlling artery and vein specifications

Recent developments in the study of blood vessel formation have provided insights into the early molecular determinants that distinguish arteries and veins. The Eph family appears to be key molecules in this distinction. The receptor tyrosine kinase Eph-B4 was reported to mark future venous but not arterial endothelial cells, whereas its membrane-bound ligand ephrin-B2 reciprocally marks the arterial endothelium at the earliest stages of capillary plexus formation (Wang et al., 1998). These differences appear to result from an early specification at the angioblast stage (Zhong et al., 2001). However, how this distinction is established during vasculogenesis remains poorly understood. This arterial-venous cell fate decision was recently reported to be guided by the expression of gridlock (grl), an artery-restricted gene located downstream of the notch signalling pathway (Zhong et al., 2001).

Since the ES cell system can give access to different maturation steps in the vascular progenitors of the endothelial lineage, it might be suitable for the

elucidation of molecular mechanisms involved in the specification of angioblasts to artery or vein cell fate. In addition, the possibility of control of the arterio-venous cell fate appears important for the design and the expansion of vascular progenitors from ES cells for their introduction into selected areas of the body for specific vascular repair and endothelialization of tissue-engineered structures such as heart valves.

Conclusions

In addition to their use *in vivo* in transgenesis experiments, ES cells provide powerful *in vitro* tools for studies of gene expression during endothelial differentiation. ES-derived cells should indeed allow further characterization of mammalian vascular development with lineage markers as well as functional studies of purified cells. This *in vitro* system recapitulates most of the endothelial differentiation program as observed *in vivo* in the embryo. The ES/EB model appears also particularly useful for the identification of factors and genes potentially involved in the regulation of vasculogenesis, arterio-venous cell fate specification and of further angiogenesis in a three dimensional tissue context. The analysis of the consequences of genetic modifications, which can be easily introduced in these cells, offers excellent and complementary alternatives to *in vivo* studies on transgenic animals. Finally, with the isolation of their human counterparts, ES cells may provide a source of endothelial cells for transplantation and gene therapy.

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