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flk-1, an *flt*-related receptor tyrosine kinase is an early marker for endothelial cell precursors

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SUMMARY

We have used RT-PCR to screen pluripotent murine embryonic stem cells to identify receptor tyrosine kinases (RTKs) potentially involved in the determination or differentiation of cell lineages during early mouse development. Fourteen different tyrosine kinase sequences were identified. The expression patterns of four RTKs have been examined and all are expressed in the mouse embryo during, or shortly after, gastrulation. We report here the detailed expression pattern of one such RTK, the *flt*-related gene *flk-1*. In situ hybridization analysis of the late primitive streak stage embryo revealed that *flk-1* was expressed in the proximal-lateral embryonic mesoderm; tissue fated to become heart. By headfold stages, staining was confined to the endocardial cells of the heart primordia as well as to the blood islands of the visceral yolk sac and the developing allantois. Patchy, speckled staining was detected in the endothelium of all the major embryonic and extraembryonic blood vessels as they formed. During early organogenesis, expression was detected in the blood vessels of highly vascularized tissues such as the brain, liver, lungs and placenta. Since *flk-1* was expressed in early mesodermal cells prior to any morphological evidence for endothelial cell differentiation (vasculogenesis), as well as in cells that form blood vessels from preexisting ones (angiogenesis), it appears to be a very early marker of endothelial cell precursors. We have previously reported that another novel RTK, designated *tek*, was expressed in differentiating endothelial cells. We show here that *flk-1* transcripts are expressed one full embryonic day earlier than the first *tek* transcripts. The expression of these two RTKs appear to correlate with the specification and early differentiation of the endothelial cell lineage respectively, and therefore may play important roles in the establishment of this lineage.

Key words: endothelial cell, angioblast, receptor tyrosine kinase, gastrulation, endocardium, embryonic stem cells, angiogenesis, mouse gene

INTRODUCTION

Embryonic stem (ES) cells provide an excellent system with which to study the molecular events involved in lineage determination and differentiation. ES cells are derived from the inner cell mass (ICM) of the early blastocyst and have the potential to form all embryonic cell lineages (Nagy et al., 1990) including those of the hematopoietic system (Forrester et al., 1991). ES cells are maintained in a proliferating, undifferentiated state by co-culture with feeder cells or in the presence of leukemia inhibitory factor (LIF) (Williams et al., 1988). When ES cells are grown in suspension culture in the absence of LIF, they aggregate to form embryoid bodies, which eventually differentiate in vitro into a number of cell types including visceral yolk sac, blood islands and beating hearts (Doetschman et al., 1985). These studies have been extended recently with the advent of well-defined culture conditions that promote the formation of early hematopoietic progenitors (Burkert et al., 1991; Schmitt et al., 1991; Wiles and Keller, 1991) and vascular endothelial cells (Risau et al., 1988; Wang et al., 1992). ES cells therefore provide a good in vitro model system for the study of the molecular and cellular processes involved in the formation of the embryonic circulatory system.

The heart is the first embryonic organ to develop in the mouse, becoming visibly obvious around embryonic day 8 (E8). Much of what is known about vertebrate heart development comes from studies performed in the amphibian and chick. The heart arises from paired anterior splanchnic mesodermal primordia which lie on either side of the midline and eventually give rise to epimyocardium and endocardium. The formation of the amphibian heart primordia appears to require interactions with the dorsal blastoporal lip (Sater and Jacobson, 1990). The nature of this inductive influence is not known, however, recent studies investigating the role of growth factors in the regulation of axolotl heart development suggest that TGF-β, PDGF and bFGF may participate (Muslin and Williams, 1991).

Blood cells originate in the extraembryonic mesenchymal cell aggregations of the yolk sac splanchnopleure known as the blood islands. Cells in the outer layer of the primordial blood islands flatten into vascular endothelium, while cells within become hematopoietic stem cells. Fusion of these blood islands eventually results in the formation of a continuous vascular channel system throughout the yolk sac. The vitelline (omphalomesenteric) vessels develop embryonically and eventually connect the embryonic vasculature with the yolk sac vasculature (Rugh, 1968). The major embryonic blood vessels are thought to arise due to a process of vasculogenesis which is defined as the formation of blood vessels by the in situ development of endothelial cells from angioblasts (Risau et al., 1988). Studies performed in quail-chick chimeras suggest that vasculogenesis mainly accounts for the formation of the heart, dorsal aorta, the cardinal and vitelline vessels as well as the extraembryonic vessels of the yolk sac (Coffin and Poole, 1988, 1991; Pardanaud et al., 1989). The formation of new blood vessels from pre-existing ones by vascular sprouting is known as angiogenesis and is thought to be responsible for the formation of vessels such as the intersomitic arteries (Coffin and Poole, 1988) and the vessels of the organs. It is not understood, however, how endothelial cell precursors assemble together to form vascular channels nor are the cues that direct the spatial organization of blood vessels within the embryo known.

We report here the isolation of several cDNAs encoding protein tyrosine kinases (PTKs) expressed in differentiated and undifferentiated ES cells. One of these cDNAs encoded *flk-1*, a putative growth factor receptor tyrosine kinase (RTK) that is closely related to the *c-kit/pdgfra/flt* family of RTKs (Matthews et al., 1991). Analysis of *flk-1* expression during early mouse postimplantation development revealed that *flk-1* mRNA was first detected during gastrulation in a spatially restricted subset of mesoderm cells in an area fated to become heart. Slightly later the gene is expressed in endothelial cell precursors and developing endothelial cells of the heart and embryonic and extraembryonic vasculature. Thus *flk-1* appears to be the earliest molecular marker of endothelial cell precursors. Furthermore, our results suggest that signalling pathways mediated by RTKs play important roles in regulating endothelial cell development and that flk-1 is probably a receptor for an angiogenic growth factor.

MATERIALS AND METHODS

Embryos and ES cell cultures

Embryos were obtained from random bred CD-1 stocks (Charles River, Montreal). Embryos were staged as embryonic day 0.5 (E0.5) the morning that the vaginal plug was detected. D3 ES cells (Doetschman et al., 1985) were maintained in an undifferentiated state by growing on a layer of mitotically inactivated primary mouse embryo fibroblast feeder cells. The growth medium for ES cells was as described (Wurst and Joyner, 1992). ES cells were induced to differentiate into embryoid bodies in vitro by plating 5×10^4 cells/ml onto 10 cm Petri dishes and growing them in suspension culture for 4-8 days.

RNA isolation and analysis

Total RNA for RT-PCR was prepared from ES cells via the method of Chomczynski and Sacchi (1987). Total RNA used in RNase protection analyses was extracted from cell pellets with RNAzol (CINNA/BIOTECX Lab. Int.) according to manufacturers recommendations with the following modifications. Briefly, cell pellets (1×10^7 cells) were washed with ice-cold phosphate-buffered saline (PBS), homogenized in 2.5 ml of RNAzol, and phenol/chloroform extracted 3×. The RNA was isopropanol-precipitated and dissolved in 0.4 M sodium acetate, pH 5.2.

tek, *flk-1* and β-*actin* transcripts were detected by RNase protection analysis with a kit (Ambion) according to manufacturers recommendations. Antisense RNA probes were synthesized following manufacturers recommendations (Promega Biotec) in the presence of [^{32}P]CTP (3000 Ci/mmol; Dupont) following subcloning of cDNA fragments into either pGEM7zf(+) or pBluescriptII SK−. Probes corresponded to nucleotides (nt) 2416 to 2683 for *flk-1* (Matthews et al., 1991), 1257 to 1633 for *tek* (Dumont et al., 1993) and 883 to 970 for β-*actin* (provided by F. Shalaby). Digestion products were resolved in a 6% sequencing gel containing 8 M urea. Quantitation of digestion products was performed using a PhosphorImager system (Molecular Dynamics) and the supplied software (ImageQuant, Molecular Dynamics). All values were adjusted for C content, which was the nucleotide used to label the RNA strands, in addition to normalizing for loading as measured by the levels of β-*actin* message detected.

RT-PCR and sequencing

First-strand cDNA was generated by reverse-transcribing total RNA using oligo (dT) as primer. Forty cycles of PCR using PTKI and PTKII primers and the conditions described in Wilks (1989) generated a single band of the expected size (210 bp). Amplified products were digested with the appropriate enzymes (*Eco*RI and *Bam*HI), acrylamide gel-purified, electro-eluted and cloned into pGEM7zf(+) for double-stranded sequencing (Sequenase).

cDNA library screening

The ~210 bp BOD4-21 PCR clone was radiolabelled with [^{32}P]dCTP and used to probe ~10^6 plaques from an amplified, random-primed murine E13.5 λgt10 cDNA library (kindly provided by M. Hanks) to obtain larger cDNA clones. Hybridization was carried out overnight at 55°C in 50% formamide, 10% dextran sulfate (Pharmacia), 0.5% BLOTTO, 4× SSPE (1× SSPE= 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH7.4), 100 mg/ml sheared salmon sperm DNA, and 2×10^6 cts/minute/ml of probe. Filters were washed at 55°C twice in 2× SSC containing 0.1% SDS, dried and exposed overnight to Kodak XAR-5 film.

In situ hybridizations and probes

A partial *flk-1* cDNA subcloned from the above screen and corresponding to nt 1958 to 2682 according to the nomenclature of Matthews et al. (1991), was used as a template for in vitro RNA probe transcriptions. In situ hybridizations to sectioned or whole-mount embryos were performed as previously described (Yamaguchi et al., 1992; Conlon and Rossant, 1992).

RESULTS

Isolation of tyrosine kinase cDNAs from embryonic stem cells

We used RT-PCR and the degenerate oligonucleotide primers PTK1 and PTK2 (Wilks, 1989) to amplify tyrosine kinase sequences from ES cells. cDNA was generated from total RNA isolated from proliferating undifferentiated D3 ES cells or from D3 cells that had differentiated in vitro for 4, 6 or 8 days. The simple suspension culture conditions used generated a wide variety of differentiated cell types with visceral yolk sac, beating hearts and blood islands

Fig. 1. Amino acid sequence alignment of tyrosine kinases isolated from ES cells by RT-PCR. Black boxes indicate absolute sequence conservation, dark or light stippling highlight sequences identical in greater than 80% or 60% of the sequences respectively.

being the most obvious tissues formed (Doetschman et al., 1985; T. Yamaguchi, unpublished observations).

Fig. 1 illustrates the amino acid sequence alignment of tyrosine kinases isolated from ES cells by RT-PCR. Fourteen unique sequences including bmk/hck, c-yes, c-fyn, c-lyn, jak2 (Wilks et al., 1991), the insulin receptor, fgfr-1, pdgfra and c-kit were identified from various stages of ES cell in vitro differentiation. Note that internal blocks of conserved residues corresponding to conserved subdomains VII and VIII (Hanks et al., 1988) appear in all sequences and indicate that the PCR clones indeed represent tyrosine kinase-related sequences. Table 1 illustrates the identified clones and the stages of ES cell differentiation from which they were isolated. Three different tyrosine kinases, consisting of hck, fgfr-1 and ES79 were isolated from proliferating undifferentiated ES cells, of which hck was the most prevalent (Table 1). Both hck and fgfr-1 were also isolated from embryoid bodies. Ten PTK sequences, which were not identified in undifferentiated ES cells, were found in embryoid bodies. The fact that several PTKs were isolated at specific timepoints presumably reflects the diversity of embryonic cell types generated due to cell-cell interactions in vitro.

Table 1. Summary of tyrosine kinases isolated from different stages of in vitro ES cell differentiation

PTK	Undifferentiated ES cell	Embryoid body 4 day	Embryoid body 7 day	Embryoid body 8 day	
fyn				1	
yes		1	2		
lyn			1	1	
hck	29	10	14	5	
jak2		1	1		
InsulinR			1		
ES79	1				
PDGFRA		11	20	3	
kit			1		
flk-1 (BOD4-21)		2	2		
tek (BOD7-6)		1	6		
FGFR1	2	2	3		
FGFR4 (BOD8-7)			2	1	
BOD4-1		1			
totals	32	29	53	11	125

Values represent actual number of cDNAs identified by single-track sequencing.

Five of the fourteen sequences isolated were novel at the time of cloning, two of which have been isolated in other PCR screens but remain to be fully characterized: BOD4-1 (clone 1 from murine embryonic cerebellum (Stark et al., 1991); JTK5, a putative human homolog isolated from a chronic myelogenous leukemia cell line (Partanen et al., 1990)) and ES79 (clone 37 (Stark et al., 1991); tyro-3 from subtracted sciatic nerve cDNA (Lai and Lemke, 1991)). BOD8-7 corresponds to FGFR-4 (Stark et al., 1991) and we have recently reported the characterization of BOD7-6, a novel RTK, now designated *tek*, which was also isolated from embryonic heart (Dumont et al., 1992).

BOD4-21 initially showed sequence similarity with the *W/c-kit* subfamily of RTKs known to be potentially important developmental signalling molecules (Reith and Bernstein, 1991) and so we used it to screen a 13.5 day random-primed mouse embryonic cDNA library for larger cDNA clones. Sequence analysis of these clones revealed identity with the published sequence of *flk-1*, a cDNA isolated from mouse stem-cell-enriched fetal liver populations (Matthews et al., 1991). For the sake of clarity, we shall hereafter refer to BOD4-21 as *flk-1*.

RNA in situ hybridization analysis of *flk-1* expression during embryogenesis

We performed both standard and whole-mount RNA in situ hybridization analyses to determine the temporal and spatial expression patterns of *flk-1* during early mouse embryogenesis.

flk-1 expression during gastrulation

flk-1 transcripts were not detected in the E6.5 egg cylinder by whole-mount RNA in situ hybridization (data not shown). *flk-1* transripts were first detected by whole-mount analysis in yolk sac mesoderm of the streak-stage embryo (Fig. 2A) and in a wedge of proximal-lateral embryonic mesoderm that extended from the anteriormost part of the embryo back to the posterior primitive streak (Fig. 2A) around the time of amnion formation (~E7). Expression in these tissues appeared rather speckly, with small groups of cells being labelled. Lower levels of *flk-1* mRNA were

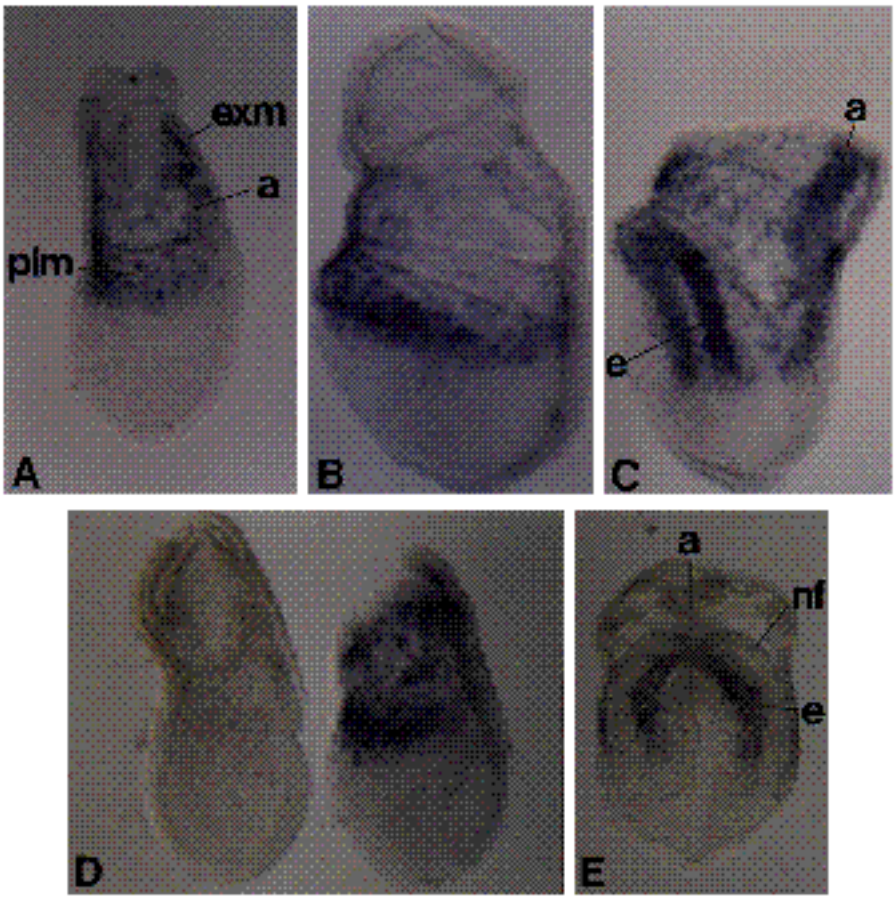

Fig. 2. Whole-mount in situ hybridization analysis of *flk-1* expression during gastrulation and neurulation. (A-D) Embryos are viewed laterally, with anterior to the left, primitive streak to the right. (A) Late-streak-stage embryo (~E7.25) displaying strong expression in proximolateral embryonic mesoderm and extraembryonic mesoderm. (B) Neural plate stage embryo. (C) Early headfold stage embryo (~E7.75). Note the strong expression of *flk-1* transcripts in the endocardial tubes and the allantois. (D) Comparison of *tek* (left embryo) and *flk-1* (right) expression in similarly staged E7.25 embryos. (E) Anterior view of *flk-1* expression in an embryo at a slightly earlier stage than that depicted in C. Abbreviations: proximolateral embryonic mesoderm (plm), extraembryonic mesoderm (exm), allantois (a), endocardial tubes (e), neural folds (nf).

found in the extraembryonic mesoderm of the forming allantois. Transcripts were not detected in the ectoplacental cone, chorion, or embryonic ectoderm or endoderm. Expression in the yolk sac was concomitant with the beginning of blood island formation and the area of expression in the embryo included that fated to later form the heart (Tam and Beddington, 1992).

flk-1 expression during early vasculogenesis

As the neural plate formed (~E7.5), *flk-1*-expressing cells in the anterior half of the embryo appeared more coherently organized (Fig. 2B). Anteroventral views of an embryo displaying a small foregut pocket demonstrated that the labelled cells curved towards the midline to form the early endocardial tubes (Fig. 2E). By later headfold stages (~E7.75), labelled embryonic cells were divided into anterior and posterior expression domains by the appearance of the headfolds (Fig. 2C). Fig. 2C shows clearly that, in the anterior domain, the cells forming the paired endocardial tubes were intensely labelled. Posteriorly, *flk-1* mRNA was strongly expressed in a band of embryonic mesodermal cells lateral to the primitive streak. This band of labelled cells was continuous with the strongly labelled cells of the extraembryonic allantois (Fig. 2C). The observed posterior embryonic expression also appeared tube-like in structure and presumably corresponds to the forming allantoic arteries. High level expression persisted in the blood islands of the yolk sac.

At the late headfold stage (~E8), *flk-1* expression in the endocardium of the heart seemed to decline, as transcripts were not readily detectable by whole-mount in situ hybridization; however *flk-1* transcripts reappeared in the endocardium at low levels by the time 4-5 somites had developed (Fig. 3A). Strong expression continued in the yolk sac and close examination revealed the formation of short vascular channels (data not shown). The highest levels of *flk-1* expression were found in the allantoic stalk where the allantoic arteries arise from the dorsal aorta. Indeed, throughout somitogenesis the allantois and the allantoic stalk expressed the highest levels of *flk-1* (Fig. 3A-D). In the late headfold stage embryo, labelled cells were observed in the forming dorsal aorta, dorsal and posterior to the endocardial tubes (data not shown). *flk-1*-positive cells appeared in the early 3-5 somite embryo caudal and lateral to the heart in the forming vitelline veins. The vitelline veins join the vitelline vascular plexus of the yolk sac with the embryonic heart and thus serve to provide the embryo with blood cells. By the time 8-10 somites had developed, labelled cells were easily detected in the cephalic mesenchyme (Fig. 3B). These cells are presumably angioblasts which will give rise to the cephalic capillary plexus.

Standard RNA in situ hybridization analysis of E8.5 embryos demonstrated intense patches of *flk-1* expression in the cephalic mesenchyme (Fig. 4A,B) confirming our whole-mount analysis (Fig. 3B,C). Both approaches revealed that *flk-1* transcripts were easily detectable in the endocardium, omphalomesenteric (allantoic) artery and vitelline veins (Figs 3B, 4A,B). Analysis of sections

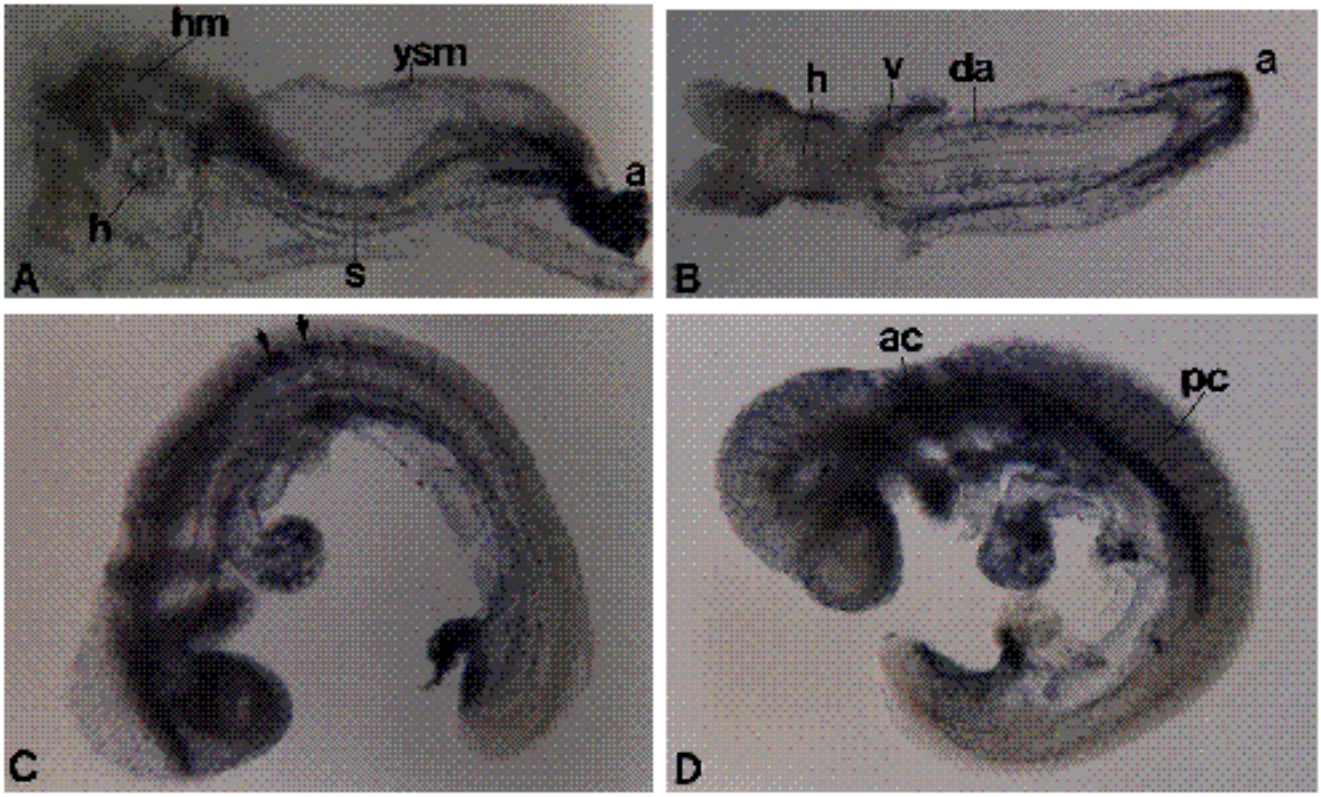

Fig. 3. Whole-mount in situ hybridization analysis of *flk-1* expression during somitogenesis. All embryos are depicted with anterior to the left, A, C, and D representing lateral views (dorsal is up), and B a ventral view. (A) A ~5-somite (E8.5) embryo displays particularly high levels of *flk-1* transcripts in the allantoic stalk. (B) A 10-somite embryo. Note that the dorsal aorta appears labelled throughout much of its length. (C) A 14-somite embryo reveals renewed intense expression in the heart. Arrows indicate foci of *flk-1*-labelled cells in the dorsal aorta adjacent to the somites. (D) An E9.5 embryo demonstrating *flk-1*-expressing endothelial cells forming a rich network of anastomosing blood vessels throughout the embryo. Abbreviations: head mesenchyme (hm), heart (h), somite (s), yolk sac mesoderm (ysm), allantois (a), vitelline veins (v), dorsal aorta (da), anterior and posterior cardinal veins (ac, pc).

through E8.5 yolk sac revealed expression in yolk sac mesoderm in patches of cells lining the perimeter of the blood islands (Fig. 4E,F). These cells form the vascular endothelium that surrounds the inner hematopoietic stem cells (Carlson, 1988). Interestingly, flat, elongated cells displaying the morphology characteristic of differentiated endothelial cells expressed low levels of *flk-1* transcripts suggesting that, at least in the yolk sac, *flk-1* expression is mainly confined to endothelial cell precursors. We did not detect concentrations of silver grains over the hematopoietic stem cells.

flk-1 expression during angiogenesis

While *flk-1* transcripts were easily detected by whole-mount RNA in situ analysis along the length of the dorsal aorta of 8-10 somite embryos (Fig. 3B), *flk-1* expression in the dorsal aorta changed significantly by the time ~14 somites had developed (Fig. 3C). Whole-mount analysis of 13-15 somite embryos revealed that *flk-1* transcripts were no longer expressed throughout the length of the dorsal aorta but rather that labelled cells were clustered in clumps adjacent to the anteriormost somites (see arrows, Fig. 3C). We suggest that the *flk-1*-expressing cells are clustered at sites where the intersegmental arteries bud out from the dorsal aorta by an angiogenic process.

Examination of *flk-1* expression in the head mesenchyme of 13-15 somite embryos showed small clumps of labelled cells which appeared to be anastamosing to form small blood vessels (Fig. 3C,D). High levels of *flk-1* mRNA continued to be expressed in the allantoic stalk and the endocardium. Transcripts could also be detected in the forming branchial arches and surrounding the otic vesicle (Fig. 3C,D). By the time 20-25 somites had formed (~E9.5), endothelial cells forming capillary plexi throughout the embryo were well-labelled (Fig. 3D). Long vessels within the head, vessels within the branchial arches, intersegmental vessels, posterior cardinal veins, vitelline veins and artery, the endocardium and the allantoic stalk were all clearly labelled. Expression in the vicinity of the future forelimb bud was also observed. This is likely the start of the formation of forelimb bud blood vessels.

Standard in situ hybridization analysis of E11 embryos demonstrated expression in the meningeal plexus surrounding the spinal cord, in the endothelium of intervertebral vessels, mesonephros, and liver primordia (Fig. 4G,H). Expression was also observed in the meninges of the developing brain, and in the umbilical artery and vein (data not shown). Analysis of E12.5 embryos revealed continued expression in these tissues as well as expression in craniofacial mesenchyme (Fig. 4C,D). Punctate staining of ves-

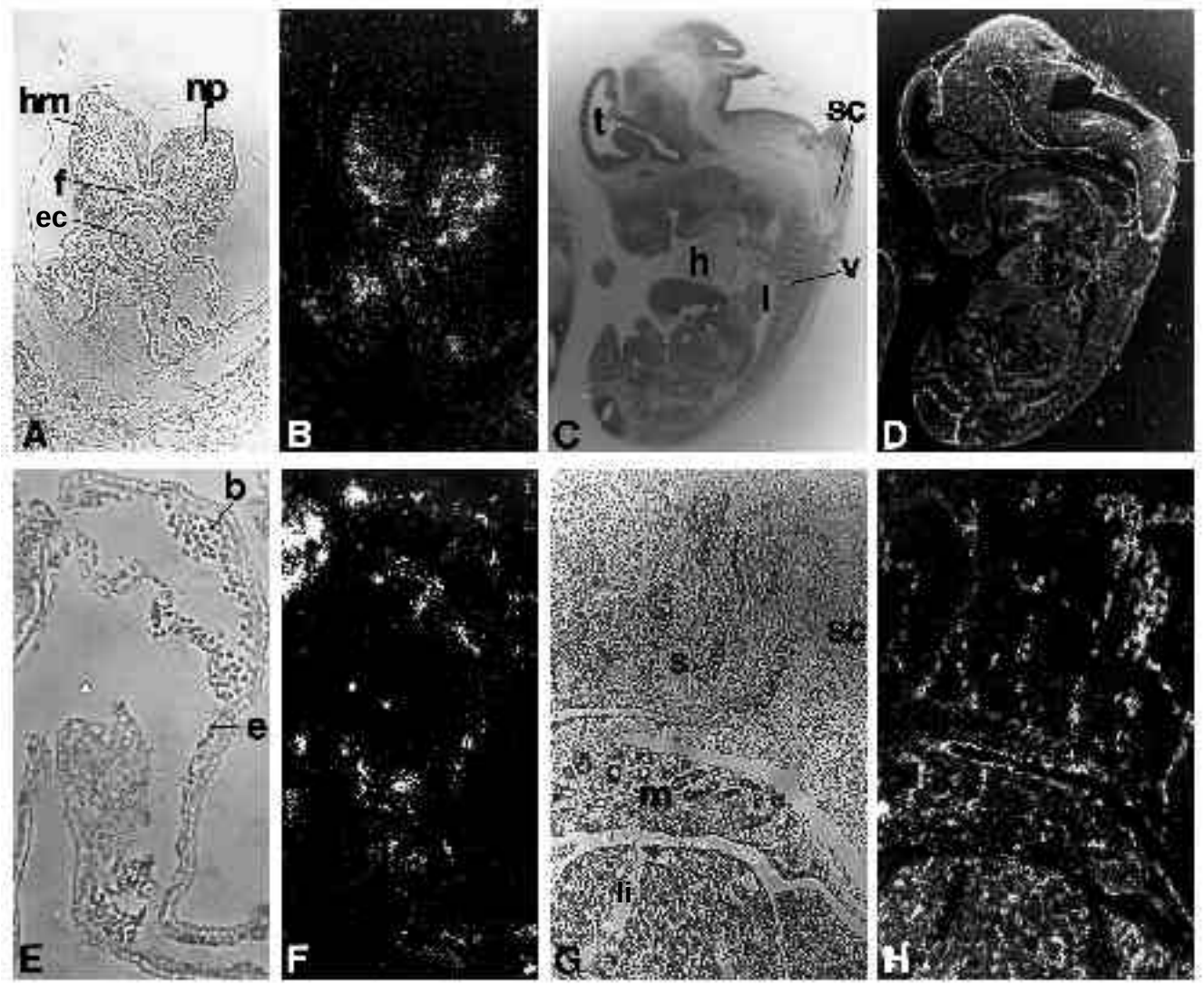

Fig. 4. Standard in situ hybridization analysis of *flk-1* expression in the mouse embryo. A, C, E, G are bright-field views; B, D, F, H are dark-field views displaying autoradiography signals. (A, B) Hybridization of *flk-1* antisense RNA probes to a transverse section of an E8.5 embryo at the level of the heart. Note strong signals in the endocardium and head mesenchyme. (C, D) A parasagittal section of an E12.5 embryo reveals widespread but specific expression of *flk-1* transcripts in highly vascularized tissues throughout the embryo. (E, F) High-power magnification of *flk-1* expression in E8.5 yolk sac blood islands. (G, H) High-power magnification of sagittal section through an E11 embryo demonstrating expression in embryonic liver, kidney, intersomitic vessels and the meninges. Abbreviations: endocardium (ec), head mesenchyme (hm), neural plate (np), spinal cord (sc), vertebrae (v), lung (l), blood island (b), sclerotome (s), liver (li), foregut pocket (f), heart (h), endothelial cell (e), mesonephros (m).

sels throughout the CNS was observed, as well as strong expression of *flk-1* in the leptomeninges. *flk-1* expression was observed in heart endothelium and the gut, and transcripts were easily detectable in the lung and the intervertebral vessels (Fig. 4C,D). Analysis of differentiated endothelial cells of the E12.5 endocardium revealed that they expressed easily detectable levels of *flk-1* transcripts. Sections through the E12.5 placenta show *flk-1* transcripts in the highly vascularized maternal decidual and labyrinthine layers (data not shown).

Comparison of *flk-1* and *tek* expression

Our in situ hybridization results demonstrated that *flk-1* transcripts were confined to cells of the endothelial cell lineage and their probable precursors in the embryo. We have previously shown that *tek*, an RTK that is closely related to *tie* (Partanen et al., 1992) and thus defines a new subfamily of receptors (Dumont et al., 1993), is also specifically expressed in endothelial cells (Dumont et al., 1992). Because the expression domains of *flk-1* and *tek* are similar, we directly compared their expression by both RNase protection and in situ hybridization analysis.

We attempted to stimulate the development of endothelial cell progenitors in vitro by differentiating ES cells in suspension culture (Risau et al., 1988; Wang et al., 1992). Vascular channels lined by differentiated endothelial cells were observed in roughly 15-30% of embryoid bodies examined. RNase protection analysis of total RNA isolated

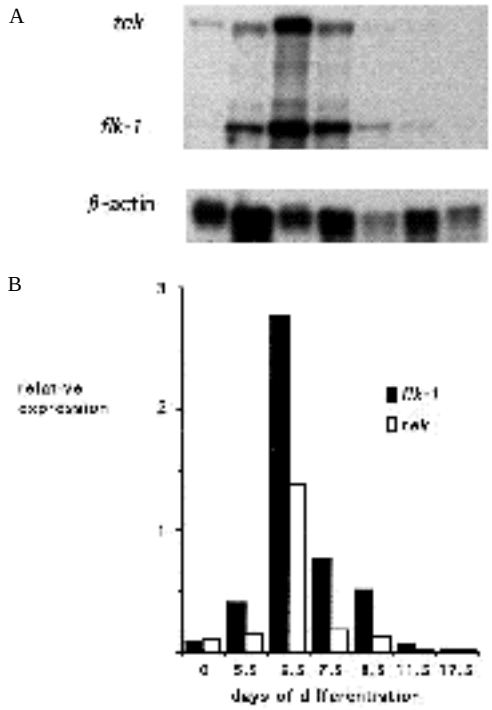

Fig. 5. Comparison of *flk-1* and *tek* expression in ES cells by an RNase protection assay. (A) Autoradiography of protected RNA probes hybridized with total RNA isolated from in vitro differentiated embryoid bodies. β-actin probes were used as loading controls. (B) Relative expression levels of *flk-1* and *tek* after normalizing with β-actin control signals.

from ES cells and embryoid bodies revealed that *tek* mRNA was expressed at low levels in the undifferentiated stem cells, while *flk-1* transcripts were not detectable (Fig. 5A,B). Both genes were up-regulated in a parallel fashion upon initiation of the in vitro differentiation protocol; however, *flk-1* transcripts were prevalent. Transcript levels for both genes peaked at ~6.5 days of suspension culture and diminished thereafter.

When the expression patterns of both genes were directly compared in vivo, however, it was clear that *flk-1* expression preceded the onset of *tek* expression. Whole-mount in situ hybridization of E7 embryos with specific probes revealed that, at the time when *flk-1* transcripts were first observed in the late streak-stage embryo, *tek* was not detectable (Fig. 2D). *tek* transcripts were not detected until after E8 (Dumont et al., 1992). Differences in *flk-1* and *tek* expression were also observed within specific vessels. For instance, *flk-1* was expressed in the dorsal aorta of the E8.75 embryo only in clusters of cells adjacent to the anterior-most somites (e.g. intersomitic arteries, see Fig. 3C), but *tek* was expressed throughout the entire length of the dorsal aorta of a similarly staged embryo (Dumont et al., 1992; T. Yamaguchi, unpublished observations).

DISCUSSION

Using RT-PCR, we isolated a number of different protein tyrosine kinases from ES cells and differentiating embryoid bodies. One of these, *flk-1*, was detected in embryoid bodies and was shown by in situ hybridization to be specifically expressed in vascular endothelial cells and their precursors in the developing mouse embryo. Its presence in embryoid bodies is consistent with this tissue distribution as ES cells have been shown to form endothelial cells in vitro (Risau et al., 1988; Wang et al., 1992). *flk-1* expression was first observed in the extraembryonic yolk sac mesoderm and the cells that populate the proximal-lateral embryonic mesoderm of the late primitive streak stage (E7) embryo. As development proceeded, *flk-1* expression became restricted to the endothelial cells of the yolk sac blood islands and, within the embryo itself, to the developing endocardial tubes and the allantois. Expression is later seen in presumptive angioblasts in the head mesenchyme and in all developing blood vessels during organogenesis.

This expression pattern is consistent with *flk-1* marking very early progenitors of the endothelial cell lineage as well as the differentiating endothelial cells themselves. In the extraembryonic mesoderm, blood islands arise at E7 when *flk-1* expression is first seen. The differentiating blood islands form both vascular endothelial cells and hematopoietic precursors. However, it is unclear whether these arise from the same precursor (Sabin, 1920), the putative hemangioblast, or whether they have separate precursors. Our studies do not allow us to say with precision that *flk-1*-expressing cells of the yolk sac include hemangioblasts as this cell type has not been well-defined (for discussion see Pardanaud et al., 1989) and would likely be rare. However, in later blood islands, only endothelial cell precursors are labelled suggesting that either *flk-1* is expressed in hemangioblasts, then becomes restricted to the angioblast lineage, or the angioblast lineage is separate and is defined by *flk-1* expression.

Within the embryo, the first expression in the proximolateral mesoderm is also suggestive of association with an early stage in vasculogenesis. Fate-mapping studies have shown that the heart arises from this region (Tam and Beddington, 1992) and analysis of the temporal progression of *flk-1* expression in this region suggests that *flk-1* is marking early progenitors of the endocardium. Our results indicate that murine endocardial tubes form by vasculogenesis in a fashion similar to that observed in the formation of the chick heart and the early major blood vessels (Coffin and Poole, 1991).

Other studies performed in the chick suggest that embryonic blood vessels are formed by both vasculogenesis and angiogenesis (for review see Noden, 1989, 1990). Our in situ studies show that *flk-1* is expressed in murine embryonic blood vessels that are forming by either process. *flk-1* is expressed in embryonic and extraembryonic angioblasts prior to the morphological appearance of blood vessels and continues to be expressed as these cells become endothelial cells and form tubes. *flk-1* is also expressed at sites where endothelial cells bud out from pre-existing vessels to form new blood vessels by angiogenesis (e.g. intersomitic vessels). Moreover, *flk-1* is expressed in the forming vessels of the central nervous system and neural crest-derived facial areas, both areas that do not give rise to endothelial cell precursors and therefore are vascularized by angiogenesis

(Noden, 1990; Stewart and Wiley, 1981). The common expression of *flk-1* in endothelial cell precursors of vasculogenic and angiogenic pathways suggests that the distinction between the two processes is one of descriptive convenience rather than a reflection of mechanism. Angiogenesis also occurs in the adult during wound healing and the vascularization of tumours (for review see Folkman and Klagsbrun, 1987). Preliminary results suggest that *flk-1* is expressed in the early capillaries that vascularize melanoma tumours (T. P. Yamaguchi and J. Rak, unpublished observations). Identification of *flk-1* as an endothelial-specific growth factor receptor may provide an avenue to develop reagents potentially useful in blocking tumour angiogenesis.

Although *flk-1* seemed to mark all early endothelial cell precursors, we did not detect *flk-1* transcripts in all mature endothelial cells. For example, *flk-1* expression was observed in angioblasts during the formation of the yolk sac blood islands, but was not easily detected in differentiated endothelial cells of more developed yolk sacs. In contrast, many of the endothelial cells of the E12.5 heart that displayed morphologies characteristic of fully differentiated endothelial cells expressed high levels of *flk-1*. Matthews et al. (1991) have independently cloned *flk-1* from fetal liver and shown by northern analysis that it is expressed in virtually all of the embryonic and adult tissues examined. We suggest that the *flk-1* transcripts detected by their northerns reflects the vascular component within each of the tissues examined. *flk-1* was also reported by these authors to be expressed in a population of fetal liver cells enriched for primitive hematopoietic stem cells. Our analysis cannot eliminate the possibility that *flk-1* also marks early hematopoietic progenitors, since primitive stem cells would be a very small component of either yolk sac or fetal liver. *flk-1* was not obviously expressed in the hematopoietic cells of the yolk sac blood islands that we examined, but this may be due to the difference in sensitivity between in situ hybridization and the PCR analysis Matthews et al. (1991) employed.

flk-1 and *tek* are markers for different developmental stages of the endothelial cell lineage

The highly localized expression pattern of *flk-1* bears comparison with the expression pattern of *tek*, a second RTK that is expressed specifically in the endothelial lineage (Dumont et al., 1992) and which, by virtue of its structural similarity with *tie* (Partanen et al., 1992), defines a new endothelial-specific subfamily of RTKs (Dumont et al., 1993). Interestingly, a partial cDNA isolated from a human endothelial cell cDNA library appears to be the human homolog of *flk-1* as it displays high sequence similarity to *flk-1* and is expressed in endothelial cells (Terman et al., 1991). Several differences in the temporal and spatial expression patterns of *flk-1* and *tek* should be noted. First, *flk-1* was expressed one embryonic day earlier in the tissues in which *tek* eventually becomes expressed. Second, *tek* is not expressed at high levels in the allantoic stalk, site of intense blood vessel formation, whereas *flk-1* transcripts are extremely abundant. Third, at least in some tissues, *flk-1* appears to be somewhat down-regulated in endothelial cells as they differentiate, whereas *tek* is strongly expressed. Expression studies in differentiating ES cells, however, did not show the same temporal order of expression of the two genes. *tek* was expressed at low levels in undifferentiated ES cells, whereas *flk-1* was not detectable. Both genes were up-regulated in parallel during embryoid body differentiation although *flk-1* transcripts were more prevalent. Clearly a more detailed analysis of the correlation between expression of these genes and the events of vasculogenesis observed in embryoid bodies (Risau et al., 1988; Wang et al., 1992) is required to see how closely these events parallel the in vivo pathways. Within the embryo itself, our results suggest that the onset of *flk-1* expression correlates with the specification of the endothelial cell lineage while *tek* expression marks a more differentiated endothelial cell progenitor. Alternatively, the expression of these genes could reflect endothelial cell heterogeneity.

Candidate ligands for *flk-1*?

The identification of a ligand for the orphan RTK, *flk-1*, will help clarify the role of the *flk-1* signalling system in the endothelial cell lineage. A number of polypeptide factors with angiogenic or mitogenic activity for endothelial cells have been characterized (for review see Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). Both aFGF and bFGF have well-documented effects on endothelial cell proliferation in vitro and in vivo (for review, see Klagsbrun, 1989). However, neither factor appears to play a role in endothelial cell development in vivo because neither aFGF or bFGF are expressed in blood vessels during brain angiogenesis (Emoto et al., 1989; Schnurch and Risau, 1991). Furthermore, virtually none of the FGFRs are expressed by endothelial cells during early embryogenesis, making it unlikely that they play a role in the normal embryonic development of endothelial cells (Yamaguchi et al., 1992; Stark et al., 1991; Wanaka et al., 1991; Orr-Urtreger et al., 1991); however, low levels of *fgfr-1* and *fgfr-2* are detected in the endocardial cushions of relatively late (E12.5 and E16.5) embryonic hearts (Peters et al., 1992).

Besides the FGFs, other factors that modulate endothelial cell activity have been identified. Both the PDGF-B ligand and PDGFB receptor are thought to play a role in angiogenesis as they are expressed in endothelial cells of the developing human placenta (Holmgren et al., 1991). VEGF/VPF, which may be a ligand for flt-1 (De Vries et al., 1992), stimulates endothelial cell proliferation and blood vessel permeability and is structurally related to members of the PDGF family as all eight cysteines are conserved (Leung et al., 1989; Keck et al., 1989; Tischer et al., 1989; Conn et al., 1990; Breier et al., 1992). Interestingly, the *flk-1* sequence reveals similarity with the class III subfamily of RTKs (e.g. *pdgfr, kit, fms*); however, it is most closely related to *flt-1* (Shibuya et al., 1990). Matthews et al. (1991) have suggested that *flk-1* and *flt-1* may define a new subfamily of RTK as both are structurally similar, each displaying seven Ig-like repeats in their respective extracellular domains while other members of the class III family have five. The recent identification of a novel RTK, *flt-4*, which also contains seven Ig-like repeats and is closely related to *flk-1* and *flt-1*, supports this classification (Galland et al., 1992; Aprelikova et al., 1992). Given the sim-

ilarities between *flk-1*, *flt1*, *flt-4* and *pdgfr* and between VEGF/VPF and PDGF ligands, it seems likely that the ligand for flk-1 will also be structurally related to VEGF/VPF.

Mechanisms of induction of endothelial cells

The availability of two early markers of the endothelial cell lineage, *flk-1* and *tek*, opens up potential avenues for studying the mechanisms of induction of this lineage in the embryo using either embryo explants directly or ES cell embryoid body differentiation. Blood island formation in the chick yolk sac is dependent upon interactions between the yolk sac endoderm and the overlying splanchnic mesoderm (Wilt, 1965) but the inductive signals are not known. Recent studies in *Xenopus* suggest that the induction of myocardium is dependent upon interactions with the dorsal blastoporal lip, presumably via the Organizer activity (Sater and Jacobson, 1990). TGF-β1, and PDGFBB, can enhance myocardium formation in mesodermal explants of axolotl tissue, while bFGF inhibits (Muslin and Williams, 1991). With the availability of early endothelial markers, it is now possible to ask whether the same or different inductive interactions are involved in the formation of the endocardium. The later formation of endothelial precursors and their organization into vascular channels located in precise positions within the embryo also presumably involves inductive interactions with surrounding tissues. Chick/quail transplantation experiments suggest blood vessel assembly by endothelial cells is controlled by environmental cues residing within the mesenchyme (Noden, 1990). Biochemical and genetic studies should determine whether *flk-1* and *tek* and their respective ligands are required for these initial inductive interactions or for maintaining the differentiated properties of the cells.

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Note added in proof

Shortly after this manuscript was accepted for publication, a report of *flk-1* cloning and expression in endothelial cells was published (Oerlichs et al., 1993, *Oncogene* **8**). In addition, Rosnet et al. (1993) have proposed that *flk-1*, *flt-1* and *flt-4* collectively be designated as class V in the mammalian RTKs superfamily (*Oncogene* **8**).