The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature

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Summary

Most internal organs are situated in a coelomic cavity and are covered by a mesothelium. During heart development, epicardial cells (a mesothelium) move to and over the heart, undergo epithelial-mesenchymal transition (EMT), and subsequently differentiate into endothelial and vascular smooth muscle cells. This is thought to be a unique process in blood vessel formation. Still, structural and developmental similarities between the heart and gut led us to test the hypothesis that a conserved or related mechanism may regulate blood vessel development to the gut, which, similar to the heart, is housed in a coelomic cavity. By using a combination of molecular genetics, vital dye fate mapping, organ culture and immunohistochemistry, we demonstrate that the serosal mesothelium is the major source of vasculogenic cells in developing mouse gut. Our studies show that the gut is initially devoid of a mesothelium but that serosal mesothelial cells expressing the Wilm's tumor protein (Wt1) move to and over the gut. Subsequently, a subset of these cells undergoes EMT and migrates throughout the gut. Using Wt1-Cre genetic lineage marking of serosal cells and their progeny, we demonstrate that these cells differentiate to smooth muscle of all major blood vessels in the mesenteries and gut. Our data reveal a conserved mechanism in blood vessel formation to coelomic organs, and have major implications for our understanding of vertebrate organogenesis and vascular deficiencies of the gut.

Key words: Blood vessel development, Gut, Mesothelia

Introduction

Our understanding of the molecular mechanisms regulating blood vessel formation has advanced greatly in recent years (Carmeliet et al., 1996; Cleaver and Melton, 2003; Ema and Rossant, 2003; Jain, 2003; Lammert et al., 2003). Still, many cellular mechanisms remain unresolved concerning the generation of blood vessels in the vertebrate embryo. One important question concerns the origin of cells that make up vessels. During blood vessel development in the embryonic trunk, body wall and limbs, as well as in extraembryonic tissues, endothelial tubes are thought to induce locally derived mesodermal mesenchyme to differentiate into smooth muscle and pericytes of the vessel wall (Cleaver and Krieg, 1999; Gerhardt and Betsholtz, 2003; Hirschi and Majesky, 2004; Majesky, 2003; Saint-Jeannet et al., 1992). This mechanism is generally accepted as the major form of blood vessel development in vertebrate embryos.

Studies of blood vessel development to coelomic organs, such as those encased in the pericardial and peritoneal cavities, are limited and have focused primarily on the heart. These studies have shown that the mesothelial covering of the embryonic heart [the proepicardium (PE) and its derivative the epicardium] is a major source of cells to the coronary system (Dettman et al., 1998; Manner, 1993; Manner et al., 2001; Mikawa and Fischman, 1992; Reese et al., 2002). Although debate remains about whether all coronary vasculogenic cells are derived from the PE/epicardium (Cox et al., 2000; Drake et al., 1997; Munoz-Chapuli et al., 2002), it is well established that cells of this mesothelium undergo EMT, migration and subsequent differentiation into coronary vessels (Dettman et al., 1998; Mikawa and Gourdie, 1996; Perez-Pomares et al., 2002; Vrancken Peeters et al., 1999). This form of blood vessel development is thought to be a unique mechanism, as its progenitors are derived from an epithelial mesothelium that subsequently produces vasculogenic mesenchyme (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Wada et al., 2003b).

The origin of vasculogenic cells to the alimentary canal, which is encased in the peritoneal coelom, is unknown. The structure of the gut is conserved among vertebrates and consists of the epithelial mucosa, submucosa, muscularis externa and serosa (serosal mesothelium and underlying mesenchyme) (Netter, 1997; Roberts et al., 1996). The gut is formed by simple embryonic structures: endoderm gives rise to the epithelial mucosa while the other layers are thought to arise from the lateral splanchnic mesoderm (Kiefer, 2003). Additionally, neural crest cells migrate into the gut and differentiate into neurons of the enteric plexus (Young et al.,

2000; Young and Newgreen, 2001). Still, the origin of the major vessels to the gut is not understood.

Vascular systems of the heart and gut have several striking similarities. Most significantly, the major vessels to heart and gut run on the surface of the organ and are intimately associated with their mesothelial covering (Netter, 1997). These characteristics have led us to test whether a conserved developmental mechanism, similar to coronary vasculogenesis, accounts for blood vessel formation in the gut. In the current study, we provide molecular genetic and experimental data demonstrating that the serosal mesothelium is the major source of vasculogenic cells of the gut. Our data show that the gut is initially devoid of its mesothelial covering and its surface blood vessels. Soon after formation of the tubular gut, non-resident cells migrate to and over the gut to form the serosal mesothelium. Subsequently, a sub-population of these cells undergoes epithelial-mesenchymal transition (EMT), migrates within the gut and gives rise to vascular smooth muscle cells that populate all major vessels of the gut. Our data indicate that the formation of coelomic mesothelium, whether it be epicardial or serosal, is coupled to vasculogenesis, and suggest that elements of a common developmental mechanism regulate the generation of blood vessels to the heart and gut.

Materials and methods

Generation of Wt1-Cre transgenic mice

The strategy we used to construct the WT280Cre YAC was similar to that described for the WT280LZ YAC (Moore et al., 1998), except that a nuclear localization signal (NLS)-tagged Cre recombinase open reading frame (ORF) was cloned into the *Sac*II site in the 5′ UTR of the human WT1 first exon instead of the β-Galactosidase ORF. The resultant WT280Cre YAC was isolated and microinjected into fertilized mouse oocytes by following published protocols (Schedl et al., 1993). Transgenic founders were identified by assaying tail DNA with Cre-specific PCR primers, and one such founder (denoted AG11) was found to direct mesothelial expression in embryos derived from matings with Rosa26R reporter mice (Soriano, 1999). An analysis of genomic DNA with three PCR primer pairs targeted to different regions of WT280Cre revealed that AG11 mice retained the short (SVA) arm of the YAC and the Cre gene, but had lost the long (LVA) arm of the YAC (see Moore et al., 1998). Embryos were used from timed matings between WT280Cre transgenic males (referred to as Wt1-Cre animals) and Rosa26R homozygous females, or ICR wild-type mice, where the day of plug is E0.5.

Immunohistochemistry and *lacZ* staining

Embryos were either embedded in OCT and snap frozen directly after dissection, or fixed in 4% paraformaldehyde (PFA), protected in 30% sucrose overnight, and then OCT embedded and snap frozen. Frozen sections were generated at 7 μm on a Leica Cryostat. PFA-fixed tissue was used for the detection of β-galactosidase (β-Gal) protein. Immunohistochemistry was performed according to standard protocols (Bader et al., 1982; Reese et al., 1999; Wada et al., 2003a). The following primary antibodies were used: polyclonal Wt1 (C-19) at a dilution of 1:200 to 1:500 (sc-192, Santa Cruz); monoclonal Wt1 (6F-H2) at 1:50 (M3561, Dako); polyclonal β-Gal at 1:5000 (55976, Cappel/ICN); polyclonal cytokeratin at 1:500 to 1:2000 (Z0622, Dako); monoclonal α-SMA (1A4) at 1:100 to 1:200 (A2547, Sigma); monoclonal Pecam at 1:50 (550274, Pharmingen). Mouse monoclonal antibodies to be incubated on PFA-fixed tissue were directly labeled using the Zenon labeling kit (Molecular Probes), according to manufacturer's instructions. Secondary antibodies were Alexa fluorophore-coupled (Molecular Probes) and were used at a dilution of 1:2000.

Whole-mount *lacZ* staining was performed according to standard protocols (Hogan et al., 1994). Staining was usually allowed to continue overnight, especially in younger embryonic stages. In larger embryos and mice, the gut and other tissues of interested were either dissected out, or the body wall was opened to fully expose the intestinal organs. For histological analysis, *lacZ*-stained specimens were dehydrated in Isopropanol and subsequently embedded in paraffin. Serial sections (7 μm) were collected and counterstained with Eosin. Hematoxylin and Eosin staining of sections from unstained tissues and embryos was performed following standard protocols.

In vivo labeling of embryonic guts and their culture

E12.5 embryos were freed from extraembryonic tissues, but remained attached to the placenta. CCFSE [5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate, succinimidyl ester 'mixed isomers', Molecular Probes] was diluted to 24 μM in sterile PBS, and, through a small opening in the ventral body wall covering the herniated intestines, injected with a mouth pipet into the cavity surrounding the guts. After CCFSE application, embryos were incubated for 1 hour at 37°C and 5% CO_2 in DMEM/10% FCS under sterile conditions. Subsequently, embryonic guts were isolated and cultured in 4-well dishes (Nunc) in Optimem (Life Technologies), supplemented with 1 mM L-Glutamine (Life Technologies) and a Penicillin/Streptomycin antibiotic mixture at 37°C and 5% CO_2 (Natarajan et al., 1999). Embryonic gut explants were fed every 2 days. At least two embryonic guts were fixed at the same time point, day 0 to day 3, with fresh 4% PFA for 30 minutes on ice, washed in PBS, protected overnight in 30% sucrose, OCT embedded and snap frozen. Control explants without CCFSE treatment showed no difference in tissue integrity and viability (not shown).

Quantification of labeled cells

Images of sections labeled with CCFSE and β-Gal, or β-Gal and Pecam/SMA were used for quantification. Images were viewed in Photoshop, where the color channels for blue (DAPI), red and green were separated for the counting of cell populations. Percentages were collected for each vessel, and an average percentage calculated for the number of vessels counted.

Results

Formation of the serosal mesothelium

In order to establish when serosal mesothelium is formed over the gut, and to follow its development, we employed immunohistochemical and histological analyses at selected stages of development. Both the Wilms tumor protein (Wt1) and cytokeratin have been reported as being expression markers for the epicardial mesothelium of the heart (Chan et al., 1988; Foley-Comer et al., 2002; Perez-Pomares et al., 1998; Watt et al., 2004), and also for the serosal mesothelium (Armstrong et al., 1993; Carmona et al., 2001; Foley-Comer et al., 2002; Moore et al., 1998). As seen in Fig. 1A,B, the epicardium of the heart was clearly identified by these two proteins. This pattern of Wt1 and cytokeratin expression served as a control for subsequent immunochemical and molecular genetic analyses of mesothelial formation in the gut.

At early stages of mouse embryogenesis [embryonic day (E) 8.5], the gut consists of two epithelial layers: endoderm and lateral splanchnic mesoderm. At E9.5-E10.5, the mesoderm of the gut has thickened into several cell layers by

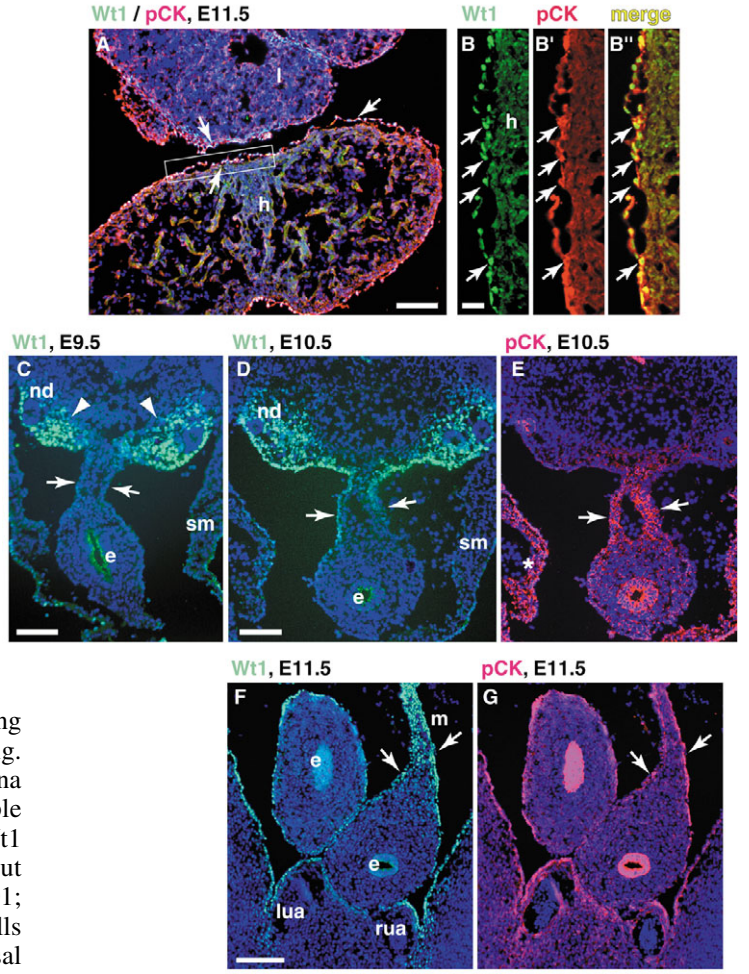

Fig. 1. Wt1 and cytokeratins as markers for mesothelial cells in the heart and gut. (A,B) Expression of Wt1 and cytokeratin mark epicardial mesothelium. (A) Merged image of Wt1 (green) and cytokeratin (pCK, red) colocalization in mesothelial cells (arrows) over the heart (h) and the adjacent liver (l) at E11.5. Boxed area in A is shown in B-B′′ to delineate colocalization. (C-G) Wt1 and cytokeratin also mark serosal mesothelium. (C) Expression of Wt1 is first observed in the urogenital ridge at E9.5 (arrowheads) but is absent from the mesentery (arrows) and gut tube. (D) At E10.5, Wt1 staining extends over the mesentery (arrows) but does not cover the entire gut. (E) Cytokeratin staining is absent from the gut surface, but residual expression is seen in splanchnic mesoderm of the mesentery (arrows). Note cytokeratin staining in somatic mesoderm (asterisk) of the body wall. (F,G) At E11.5, Wt1 (F, arrows) and cytokeratin (G, arrows) staining is evident on the entire surface of the mesentery and gut tube. Note that only cytokeratin is expressed in the endodermal linings of the gut (e). lua, left umbilical artery; m, mesentery; nd, nephric duct; rua, right umbilical artery; sm, somatic lateral mesoderm. Scale bars: 20 μm in B-B′′; and 100 μm for A,C-G.

cell proliferation. Antibodies for Wt1 detected strong reactivity in cells of the nephrogenic mesoderm at E9.5 (Fig. 1C), as previously reported (Armstrong et al., 1993; Carmona et al., 2001; Moore et al., 1998). Still, at E9.5, the simple squamous serosal mesothelium that also expresses Wt1 protein was completely absent from the mesenteries and gut tube (Fig. 1C) (Armstrong et al., 1993; Carmona et al., 2001; Moore et al., 1998). At E10.5, Wt1-positive mesothelial cells were first detectable at the proximal base of the dorsal mesentery, whereas the gut tube was still devoid of a mesothelium, as evidenced by the lack of Wt1 and cytokeratin surface staining (Fig. 1D,E). The absence of a mesothelium at this stage was also confirmed by histology, as cells on the embryonic gut surface were irregularly shaped and arranged, and did not have a squamous phenotype (see Fig. S1 in the supplementary material). Residual cytokeratin staining in splanchnic lateral mesoderm of the mesentery (Fig. 1E) was observed at E10.5, as it loses its epithelial nature. One day later (E11.5), Wt1-, cytokeratin-positive cells were present on the gut surface and the peritoneal wall along the entire anteroposterior axis (Fig. 1F,G). Staining of these two markers was confined to the mesothelial lining of the gut and to the mesentery, indicating that, by E11.5, the entire gut was enclosed by mesothelium (Fig. 1F,G). Thus, serosal mesothelial cells are the only cell type to express Wt1 in the gut at this stage, as has been previously reported (Armstrong et al., 1993; Carmona et al., 2001; Moore et al., 1998). Histological analysis at this stage revealed that cells on the gut surface were arranged in a regular manner, although not yet with a squamous phenotype (see Fig. S1 in the supplementary material). By E13.5, however, these cells comprise a thin layer of simple squamous epithelium that is typical for the serosal mesothelium (see Fig. S1). Thus, Wt1- and cytokeratin-positive serosal mesothelial cells appear in a proximal to distal manner from E10.5 onwards; by E11.5 they have completely enclosed the embryonic gut, and by E13.5 they have fully differentiated into a simple squamous epithelium. Genetic lineage marking (see below) confirmed this pattern of differentiation. Once established, the serosal mesothelium was present over the gut surface throughout prenatal and postnatal life (see Fig. S1).

The gut surface is originally devoid of blood vessels

The major blood vessels to the gut run on its surface in association with the serosal mesothelium (Netter, 1997). If formation of these vessels is coupled to formation of the mesothelium, they should not be present before its arrival. At early stages of gut development (E8.5-E12.5), an endothelial plexus associated with intestinal endoderm was revealed using Pecam (platelet/endothelial cell adhesion molecule)-specific antibodies (Bogen et al., 1992). This endothelial gut plexus extends into the dorsal mesentery beneath the splanchnic lateral mesoderm. Importantly, at these stages there are no blood vessels detectable on the surface of the gut, as visualized by the absence of Pecam staining in this area (Fig. 2A). Beginning at E13.5, endothelial cells form tubes that extend from the gut plexus to the surface of the gut (Fig. 2B). Although these endothelial tubes penetrate the mesodermal wall of the gut, they are devoid of vascular smooth muscle, as demonstrated by the absence of anti-α-smooth muscle actin (SMA) staining (Fig. 2C). Note that anti-SMA staining visualized visceral smooth muscle in the gut wall within the same sections (Fig. 2C). At E16.5 and

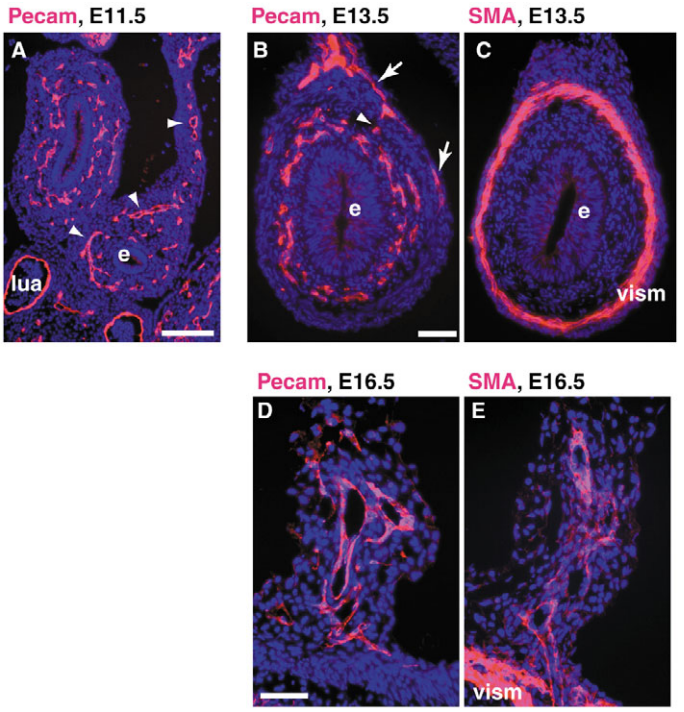

Fig. 2. Blood vessel formation in mesentery and gut. (A) Pecam is expressed in the endodermally associated plexus in the gut tube and mesentery (A, arrowheads) at E11.5. No surface staining is observed. (B) At E13.5, Pecam-positive cells are seen on the surface of the gut (arrows), connecting to the plexus via endothelial bridges (arrowhead). (C) At this time, no vascular smooth muscle cells are detected on the gut surface by anti-SMA staining; however, visceral muscle (vism) is detected. (D,E) In later stages, smooth muscle is associated with developing endothelial tubes in the mesentery and gut tube. e, endoderm; lua, left umbilical artery. Scale bars: 100 μm in A; 50 μm in B-E.

later stages, SMA is expressed in vascular smooth muscle cells that have been assembled by endothelial vessels in the mesentery and on the surface of the gut (Fig. 2D,E); the earliest SMA expression in vascular smooth muscle cells of the mesentery and gut was detected at E16.0, uniformly along the anteroposterior axis (B.W. and D.M.B., unpublished). These data demonstrate, first, that there are no surface blood vessels before the arrival of the serosal mesothelium, and, second, that the endothelial tubes first present on the gut surface extend from the endothelial plexus associated with the endoderm.

Lineage tracing of serosal mesothelium

To determine the fate of mesothelial cells, we have employed a Wt1-Cre recombinase/Rosa26R system (see Materials and methods) to irreversibly mark Wt1-positive mesothelial cells and their descendants. To trace the lineage of mesothelial cells, whole-mount and immunohistochemical analyses at selected times in development, as well as in neonates and adults, were performed. Whole-mount analysis of E11-E12 embryos revealed no *lacZ* staining over the herniated intestines, whereas a strong reaction was present along the dorsal body wall and at the root of the mesentery (Fig. 3A). Immunohistochemistry for β-Gal protein on sections confirmed the *lacZ*-staining pattern at this stage (not shown). From E12.5 forwards then, *lacZ*-positive cells extend from the base of the mesentery, progressively over the intestines in proximal to distal manner (Fig. 3B,C). Again, immunohistochemistry confirmed the presence of robust β-Gal expression on the surface of the gut, and its colocalization with Wt1 protein (Fig. 3E). Note that at E12.5, β-Gal expression is not continuous over the most distal portions of the gut tube (Fig. 3E), whereas Wt1 protein is expressed in all mesothelial cells (Fig. 3E′,E′′). Thus, the pattern of *lacZ*/β-Gal staining recapitulated the endogenous pattern of Wt1 expression in the developing serosal mesothelium, although it was delayed by at most one day

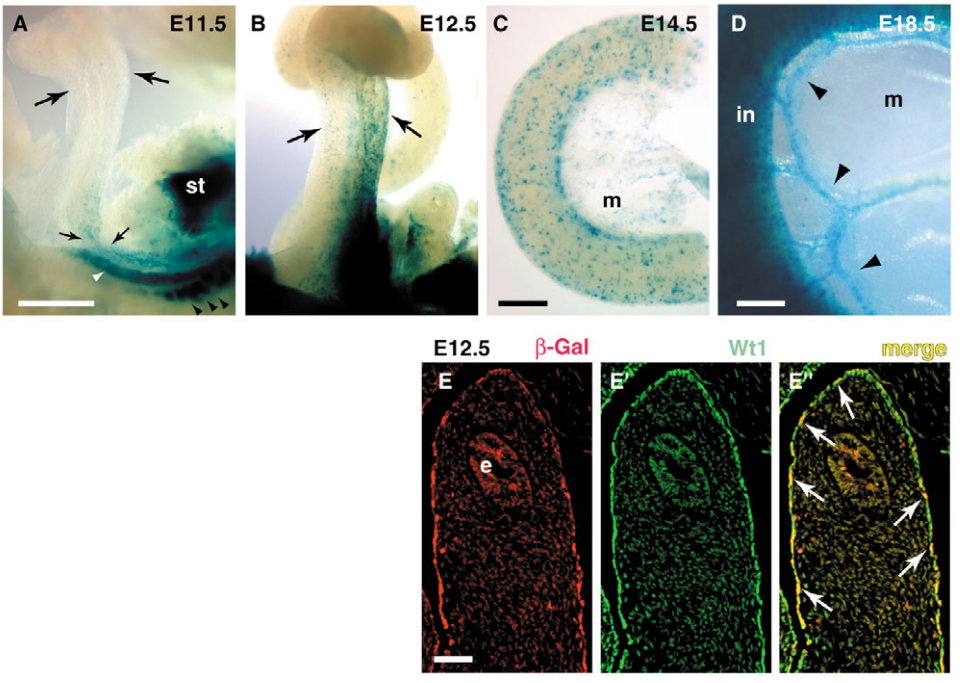

Fig. 3. Molecular genetic analysis of serosal mesothelial development. (A-D) Whole-mount analysis of *lacZ* expression marks the advance of the serosal mesothelium. (A) At E11.5, *lacZ* staining is prominent at the root of mesentery and intestine (small arrows), but is absent from the herniated gut (large arrows). Note the staining in nephric tubules (black arrowheads) and the urogenital ridge (white arrowhead). (B) At E12.5, *lacZ*-positive cells are seen covering more distal portions of the gut (arrows). (C) By E14.5, the intestine and mesentery (m) are positive for *lacZ* staining. (D) At E18.5, *lacZ* staining is strong over the entire gut tube and in the mesentery. (E-E′′) Immunohistochemistry for β-Gal (E, red) and Wt1 (E′, green) reveal that, at E12.5, most of the serosal mesothelial cells express β-Gal; β-Gal staining colocalizes with Wt1 staining (arrows; E′′). e, endoderm; in, intestine; st, septum transversum. Scale bars: 500 μm in A-C; 250 μm in D; 50 μm in E-E′′.

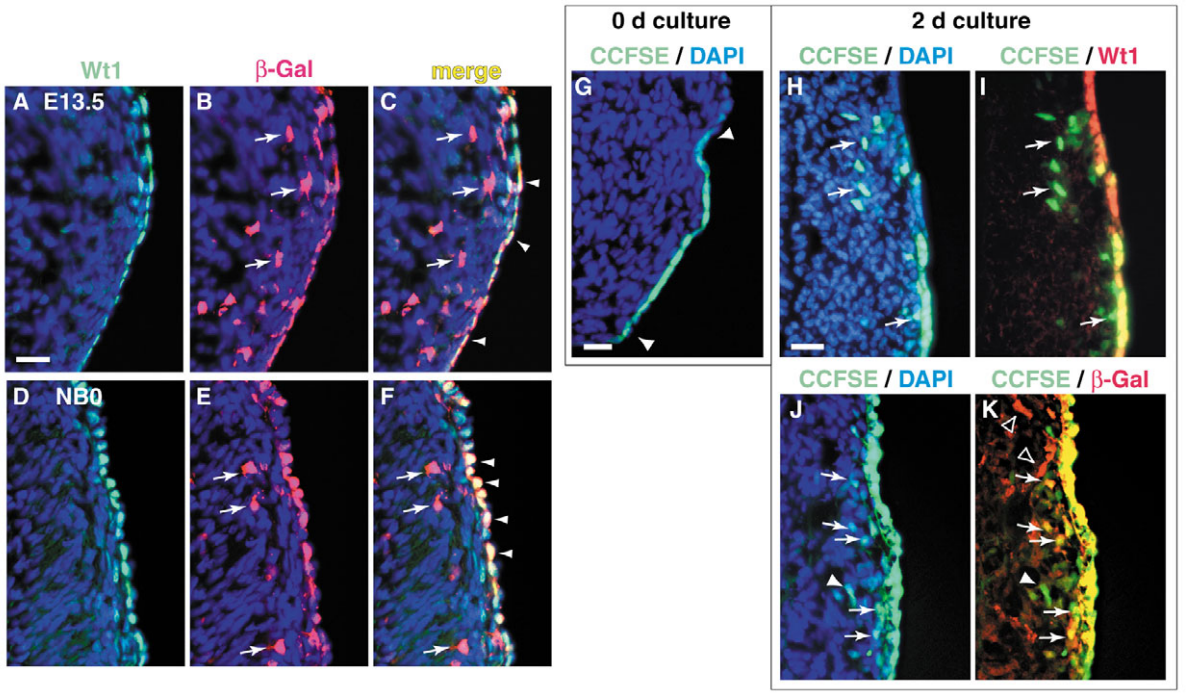

Fig. 4. Molecular genetic and vital dye lineage marking of serosal EMT. (A-F) Localization of Wt1 (green) and β-Gal (red) proteins during gut development. Wt1 expression is largely confined to the serosal mesothelium in the embryo (A-C) and newborn (NB0; D-F). The anti-β-Gal antibody marks cells in the subserosal space of the developing gut tube (arrows in B,C,E,F). Both Wt1 and β-Gal are expressed in most cells of the serosal mesothelium (C,F, arrowheads). (G-K) CCSFE (green) was used to vitally label surface cells of gut explants from E12.5 Wt1-Cre; Rosa26R embryos. (G) At day 0 of culture, surface cells were positive for CCFSE (area framed by arrowheads), but the subjacent mesenchyme is negative. (H,I) After two days of culture, CCFSE-positive cells (arrowheads) at the gut surface co-label with Wt1. CCFSE-marked cells in the subserosal space are not positive for anti-Wt1 antibody (arrows). Also, many mesenchymal cells label with both anti-β-Gal antibody and CCFSE (arrows, J,K). β-Gal-positive cells unmarked by CCFSE are also seen in the mesenchyme (open arrowheads). Note a small number of CCFSE-labeled cells without the β-Gal marker (filled arrowheads). Scale bars: 20 μm.

(compare with Fig. 1D-G) (Armstrong et al., 1993; Moore et al., 1998). At E18.5, *lacZ* expression was found covering the entire surface of the gut (Fig. 3D).

Serosal cells undergo EMT

Previous studies have suggested that Wt1 expression is linked to cells undergoing EMT (Armstrong et al., 1993; Carmona et al., 2001; Pritchard-Jones et al., 1990). To determine whether Wt1-expressing mesothelial cells and their descendants undergo EMT, we performed co-immunostaining for β-Gal and Wt1. In case of EMT, the progeny of Wt1-expressing serosal mesothelial cells were anticipated to react with the β-Gal antibody, but not the Wt1 antibody, in the submesothelial space. As expected, at E13.5, the gut mesothelium was positive for both β-Gal and Wt1 (Fig. 4A-C). Moreover, the submesothelial mesenchyme contained numerous cells positive for β-Gal but negative for Wt1. It should be noted that a very limited number of cells in the subserosal space was found to be positive for both Wt1 and β-Gal expression (Fig. 4A-C). Similar results were obtained at newborn stage, suggesting that at least a small portion of serosal mesothelial cells continuously undergo EMT (Fig. 4D-F).

The presence of β-Gal-positive cells in the submesothelial space of the gut suggests that these cells are the progeny of mesothelial cells that have undergone EMT. To experimentally confirm that surface serosal cells undergo EMT, we combined an explant culture system of the embryonic gut with genetic and in vivo labeling of serosal mesothelial cells (Natarajan et al., 1999). Embryonic guts from crosses between Wt1-Cre and Rosa26R were exposed to the lipophilic dye CCFSE, which has been well established to mark only surface cells (Morabito et al., 2001; Perez-Pomares et al., 2002; Perez-Pomares et al., 2004). CCFSE was placed locally on the herniated gut by injecting under the ventral body wall of E12.5 embryos, and, after one hour of incubation, the embryonic gut tubes, including the mesentery, were dissected out for subsequent culture in CCFSE-free medium. This approach should initially mark only surface serosal mesothelial cells (in localized areas), while cells undergoing EMT would be subsequently labeled in the delaminated underlying mesenchyme. In order to quantitatively delineate this process, we counted the number of surface and submesothelial CCFSE-labeled cells that were positive and negative for β-Gal protein (Table 1). As expected, at the time of CCFSE application, only mesothelial cells in labeled spots on the surface of the gut were positive for CCFSE (Fig. 4G and Table 1; arrowheads define the area of CCFSE labeling). Note that the overwhelming majority (96.1%) of these cells were also β-Gal positive (Table 1). No sub-serosal mesenchymal cells were labeled, indicating the effectiveness of surface labeling and the lack of immediate EMT. After 24 to 48 hours of culture, CCFSE-marked cells in labeled patches were still found in the serosal mesothelium, but were also present in significant numbers in the submesothelial mesenchyme of labeled areas (Fig. 4H,J).

Table 1. The majority of CCFSE-labeled mesothelial cells and their descendants co-label for β-Gal

A

	CCFSE surface			Submesothelial
0 days in culture	Total	β-Gal$^+$	β-Gal$^-$	Total
Number of cells	102	98	4	0
Average percentage		96.1%	3.9%	

B

		CCFSE surface			CCFSE submesothelial		
1-2 days in culture	CCFSE (total)	Total	β-Gal$^+$	β-Gal$^-$	Total	β-Gal$^+$	β-Gal$^-$
Number of cells	488	355	301	54	133	100	33
Average percentage			88.6%	11.4%		74.4%	25.6%
Average percentage		74.5%			25.5%		

At 0 days of culture (A), no CCFSE-labeled cells were detected in the submesothelial space.

After 1-2 days of culture (B), of a total of 488 CCFSE-marked cells counted, 133 were found in the submesothelial space (25.5%), and 355 were found in the surface mesothelium (74.5%). Of the 133 CCFSE-marked cells in the submesothelium, 100 cells were found to co-label with β-Gal (β-Gal$^+$, 74.4%) and 33 cells were without β-Gal co-label (β-Gal$^-$, 25.6%), whereas of the 355 CCFSE-positive cells in the serosal mesothelium, 301 cells were found to co-label with β-Gal (88.6%). Cell numbers were collected from five sections (0 days in culture), and 14 sections (1-2 days in culture), and average percentages calculated.

Indeed, when CCFSE-labeled patches were analyzed, on average 25.5% of all CCFSE-labeled cells were found in the submesothelial space (Table 1). Of this group of CCFSE-labeled submesothelial cells, on average 74.4% were also positive for the β-Gal marker (Fig. 4J,K and Table 1). Two important findings arise from these results. First, CCFSE-labeled cells in the submesothelium reveal that a subset of mesothelial cells undergoes EMT in this in vitro system (Fig. 4H,J). This result was similar to our observations with serosal EMT in vivo (Fig. 4A-F). Second, a large majority (74.4%) of CCFSE-labeled cells in the submesothelial space are co-labeled for β-Gal (Fig. 4J,K and Table 1), confirming that these marked cells are descendants of serosal mesothelial cells. By contrast, Wt1 expression was largely confined to the mesothelial surface of CCFSE-labeled cultured guts, whereas CCFSE-positive cells were readily apparent in the connective tissue space (Fig. 4H,I), reiterating the in vivo situation (Fig. 4A-C). This indicates that Wt1 expression is downregulated as cells become mesenchymal, as has been previously reported (Carmona et al., 2001; Moore et al., 1999), whereas β-Gal protein continues to be expressed. As expected, we also found β-Gal-positive cells that do not carry CCFSE, owing to the locally restricted uptake of the compound. In addition, we find a limited number of CCFSE-marked cells on the serosal surface (11.4%) and in the submesothelial space (25.6%) that are not reactive for β-Gal (Fig. 4J; Table 1). The implications of this finding are discussed below (see Discussion). Our analysis of CCFSE-labeled domains at random positions along the anteroposterior axis of the embryonic gut did not reveal any qualitative or quantitative differences in EMT. This indicates that EMT occurs in the same fashion independently from the position along the anteroposterior axis of the gut tube. Taken together, these data indicate that a subset of serosal mesothelial cells, like epicardial mesothelial cells, undergoes EMT.

Descendants of the serosa contribute to blood vessels in the mesentery and the gut

We next determined the fate of mesenchymal cells derived from the serosal mesothelium using the Wt1-Cre/Rosa26R system. Beginning at E16, and at all later stages, *lacZ*-stained cells were readily observed in association with differentiating blood vessels running in the mesentery and gut (Fig. 5 and data not shown). In the adult, *lacZ*-positive cells marked all blood vessels of the mesentery and their branches that dive into the gut (Fig. 5A,B). Although *lacZ* staining was strong in cells of the arteries, weaker staining was found in the accompanying veins (Fig. 5B). Close inspection of *lacZ*-positive cells in blood

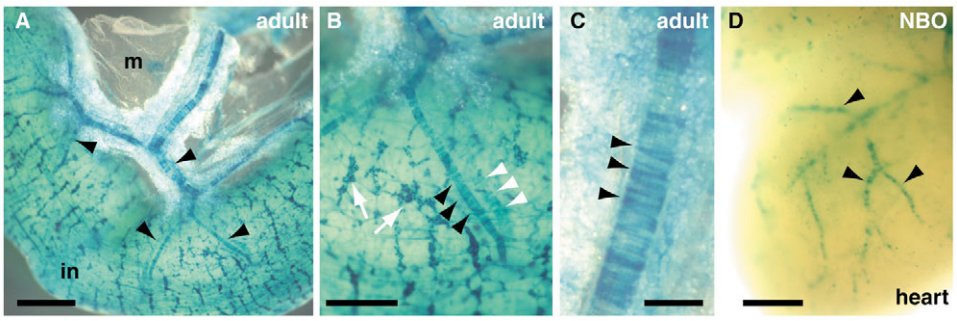

Fig. 5. Progeny of the serosal mesothelium contribute to blood vessels of the mesentery and gut. (A-D) Whole-mount *lacZ* staining characterizes Wt1-expressing cells and their descendents during postnatal stages in the gut (A-C) and heart (D). (A,B) After birth, major vessels in the mesentery and on the gut surface (black arrowheads) contain *lacZ*-positive cells. Veins are very lightly stained in whole mounts (B, white arrowheads). Note the blue staining outside of the vessel (B, white arrows). (C) At high magnification, *lacZ*-stained samples show the characteristic 'corkscrew' deposition of smooth muscle cells around a sample artery (arrowheads). (D) In the newborn (NB0) heart, coronary vessels contain *lacZ*-positive cells (arrowheads). in, intestine; m, mesentery. Scale bars: 1 mm in A; 500 μm in B,D; 200 μm in C.

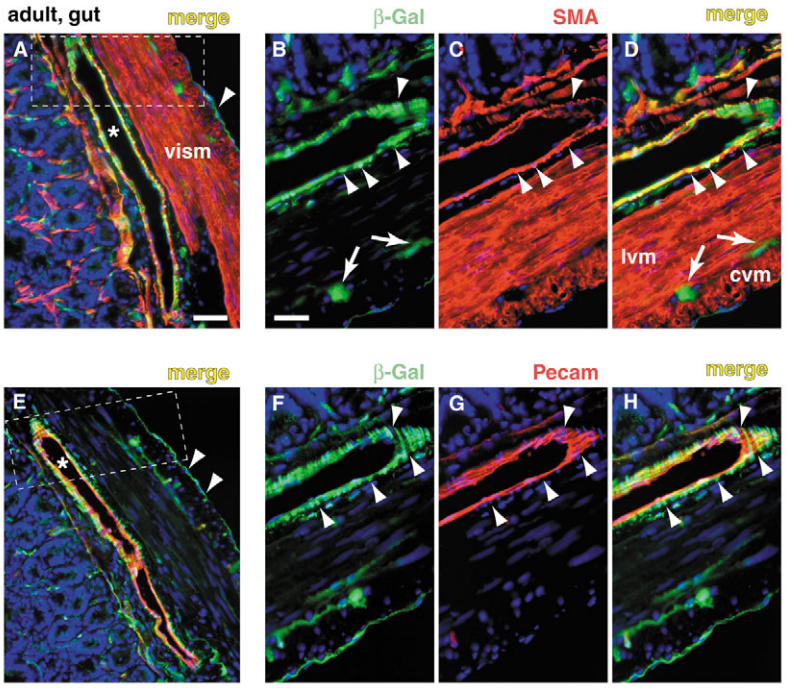

Fig. 6. Progeny of the serosal mesothelium differentiate into vascular smooth muscle cells.
(A-H) Immunohistochemistry for β-Gal and vessel markers in the adult gut. In co-labeling studies for β-Gal and SMA (A), or β-Gal and Pecam (E), the anti-β-Gal antibody marks blood vessels (asterisks) and the serosal mesothelium (arrowheads; A,E), but not the visceral smooth muscle (vism) of the intestine (A). High magnification (white box in A) of anti-SMA and anti-β-Gal staining reveals colocalization of β-Gal and SMA (arrowheads, B-D). Note the additional β-Gal-marked cells interspersed (arrows) between circular (cvm) and longitudinal visceral smooth muscle layers (lvm). High magnification (white box in E) of anti-Pecam and anti-β-Gal staining shows virtually no overlap of expression (arrowheads, F-H). Note that β-Gal-marked cells are arranged perpendicular to the endothelial Pecam-positive cells. Scale bars: in A, 50 μm for A,E; in B, 30 μm for B-D,F-H.

vessels revealed the characteristic perpendicular arrangement of smooth muscle in arteries (Fig. 5C) (Cleaver and Krieg, 1999). Note that *lacZ*-positive cells were also seen in non-vasculogenic regions in all organs of the gut (Fig. 5B).

Wt1 protein is also expressed in the epicardium (Fig. 1A,B). Thus, as a control, we analyzed the hearts of newborn mice that carry both the Wt1-Cre and Rosa26R alleles for descendants of the Wt1-Cre expressing cells. *lacZ* expression revealed that progeny of the epicardial mesothelium contribute to coronary vessels (Fig. 5D), as has been previously described (Dettman et al., 1998; Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Perez-Pomares et al., 2002; Vrancken Peeters et al., 1999).

Descendants of the serosa differentiate into vascular smooth muscle

To determine the identity of the *lacZ*-expressing cells of developing blood vessels in the gut, we performed immunohistochemistry for β-Gal, coupled with markers for vascular smooth muscle and endothelium. Immunostaining for β-Gal also serves to corroborate the *lacZ*-staining pattern, as recent reports have suggested that *lacZ* analysis of β-Gal activity in tissues does not always fully recapitulate the β-Gal expression pattern (Couffinhal et al., 1997; Mahony et al., 2002). Using standard fluorescence microscopy, β-Gal protein was found in smooth muscle cells that were co-stained for SMA and desmin adjacent to the vessel lumen (Fig. 6A-D, see also Fig. S2 in the supplementary material). However, colocalization of Pecam- with β-Gal-antibody was not observed to any significant degree (Fig. 6E-H). Of particular interest are three additional findings. First, Pecam-positive endothelial cells of the vessels are oriented perpendicularly with respect to the vascular smooth muscle cells (Fig. 6F-H), which in these images are labeled for β-Gal protein. Second, it should be noted that β-Gal protein is not expressed in the longitudinal and circumferential visceral smooth muscle layers, as seen in Fig. 6A-D. This indicates that the visceral smooth muscle is of different origin than the vascular smooth muscle of the gut. Third, we find cells that express β-Gal protein but are not associated with the vascular system of the gut (Fig. 6), suggesting that the serosal mesothelium contributes to other cell populations in the intestinal tract.

To further delineate the colocalization of β-Gal protein with endothelial and smooth muscle markers, we followed two approaches. First, we performed confocal microscopy on

Table 2. The majority of vascular smooth muscle cells of the gut and the heart are positive for β-Gal

	SMA$^+$ cells			Pecam$^+$ cells		
Adult gut	Total	β-Gal$^+$	β-Gal$^-$	Total	β-Gal$^+$	β-Gal$^-$
Number of cells	392	285	107	259	17	242
Average percentage		77.7%	22.3%		6.8%	93.2%
Adult heart	Total	β-Gal$^+$	β-Gal$^-$	Total	β-Gal$^+$	β-Gal$^-$
Number of cells	211	192	19	79	13	66
Average percentage		92.1%	7.9%		14%	86%

Cell numbers were collected from 18 (SMA) and 17 (Pecam) sections (gut), and eight (SMA) and five (Pecam) sections (heart), and average percentages calculated.

sections from adult intestines. Colocalization of β-Gal protein with SMA was readily apparent (Fig. 7A-C, see also Fig. S2 in the supplementary material), whereas overlap of β-Gal with the endothelial cell marker Pecam in blood vessels was not detected to any significant degree (Fig. 7D-F). Next, in order to quantify these results, we determined the percentage of β-Gal-positive cells in sections of SMA- or Pecam-stained blood vessels of the gut (Table 2). Our quantification revealed that, on average, 77.7% of SMA-positive cells of the gut vasculature co-label with β-Gal (from a total of 392 cells counted). By contrast, on average, 6.8% of Pecam-marked cells in the gut are also positive for β-Gal (from a total of 259 cells counted; Table 2). This result indicates that in the gut, a vast majority of vascular smooth muscle cells are descendants of the serosal mesothelium. The presence of β-Gal-negative vascular smooth muscle may indicate an additional source of this cell type.

Confocal analysis of sections from the hearts revealed that most vascular smooth muscle cells were also labeled by the β-Gal marker (Fig. 7G-I), whereas Pecam-labeled endothelium was mostly negative for the genetic lineage tracer (Fig. 7J-L). Quantitative analysis of sections from the heart resulted in a similar, although slightly different, outcome, as, on average, 92.1% of SMA-labeled cells of the coronary vasculature were positive for β-Gal, while, on average, 14% of Pecam-positive cells co-labeled with β-Gal (Table 2).

Once again, confocal experiments revealed that all major arteries and veins were populated by β-Gal-positive cells. Taken together, the results indicate that the smooth muscle cells of blood vessels in the gut are the progeny of serosal mesothelium.

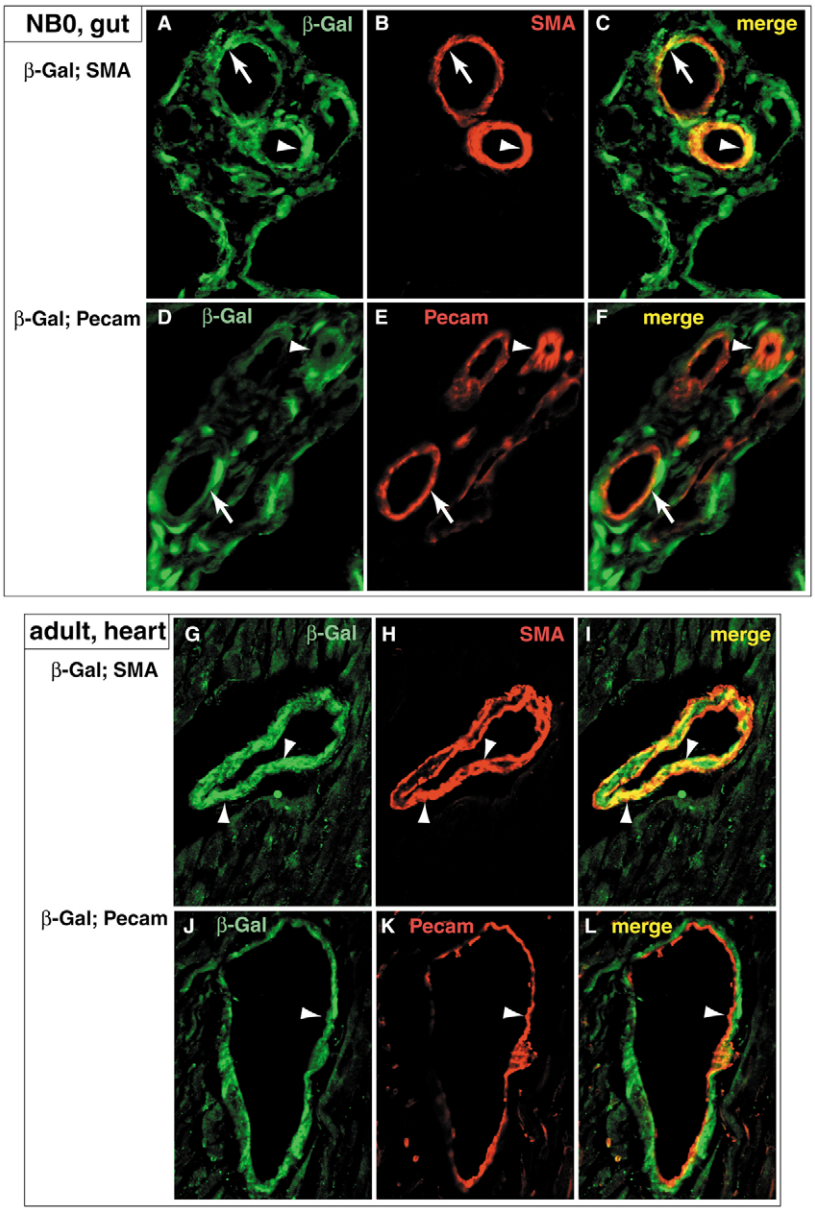

Fig. 7. β-Gal colocalizes with SMA, but not Pecam. Confocal images of immunohistochemistry in the newborn (NB0) gut (A-F) and the (adult) heart (G-L) of β-Gal with SMA (A-C,G-I), and with Pecam (D-F,J-L). Significant co-labeling is seen with anti-SMA and anti-β-Gal in vascular smooth muscle of the mesentery (A-C), and of the heart (G-I). By contrast, anti-Pecam and anti-β-Gal antibodies reveal essentially no overlap in endothelial cells of the mesentery (D-F), and the heart (J-L).

Discussion

We present data that for the first time link the presence of the serosal mesothelium, the outer lining of the intestinal tube, with the formation of blood vessels to the gut. Our findings show clearly that the Wt1 protein is expressed in the developing gut only in the surface serosal mesothelium, as has been previously observed by others (Armstrong et al., 1993; Carmona et al., 2001; Moore et al., 1998), but not in other cells within the intestinal wall. Based on the expression of Wt1 in the gut exclusively in the serosal mesothelium, we lineage trace the fate of mesothelial cells using a two-component genetic system consisting of a transgenic Wt1-Cre recombinase mouse line and the Rosa26R reporter mouse. We show that serosal mesothelial cells undergo EMT, as in our labeling study the majority of CCFSE-labeled, delaminated cells express β-Gal protein. Furthermore, our data reveal that the majority, but not all, of vascular smooth muscle cells of the gut express β-Gal, which indicates that they are derivatives of the Wt1-expressing serosal mesothelium. A similar mechanism has been reported for the formation of the coronary vessels from the epicardium during heart development (Dettman et al., 1998; Mikawa and Gourdie, 1996; Perez-Pomares et al., 2002; Vrancken Peeters et al., 1999).

On the basis of these data, we propose the following mechanism concerning blood vessel development to the gut (Fig. 8). At early embryonic stages (E9.5-E10.5), the gut tube consists of endoderm and splanchnic mesoderm without mesothelial covering. A vascular plexus associated with the endoderm is present before arrival of the mesothelium, whereas no blood

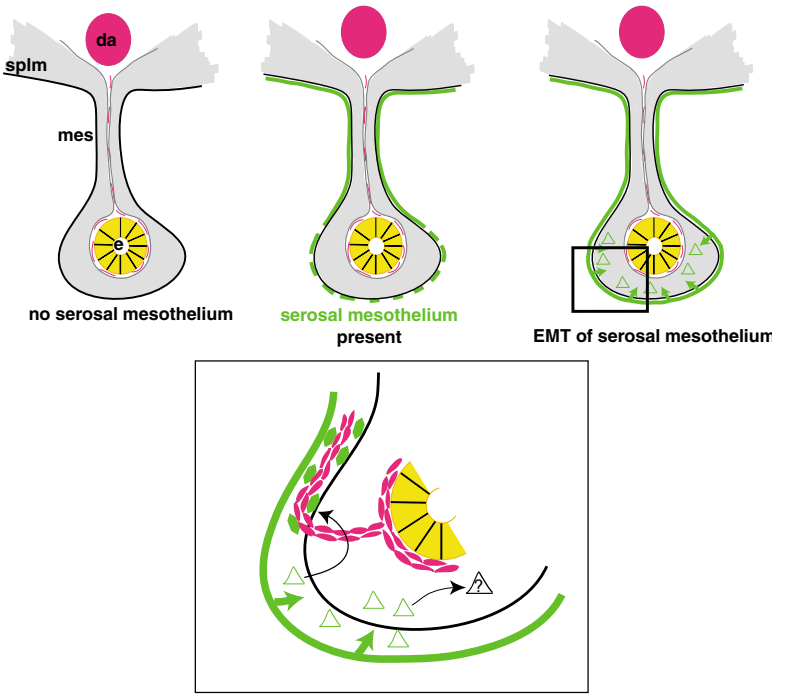

Fig. 8. The serosal mesothelium gives rise to mural cells of gut vasculature. (Left) At E9.5, the embryonic gut is not covered by a mesothelium. The gut consists only of endoderm (e) and splanchnic mesoderm (splm). A vascular plexus (red) runs between endoderm and splanchnic mesoderm, and within the mesentery. (Center) At E10.5, the Wt1/Cytokeratin-positive serosal mesothelium (green) starts to cover the mesentery and, subsequently, by E11.5, coats the entire gut. (Right) Serosal mesothelial cells undergo EMT into the subserosal space, where these mesenchymal cells discontinue Wt1 expression (open green triangles). (Box) Progeny of the serosal mesothelial cells differentiate into smooth muscle cells surrounding the blood vessels (red) that form in the subserosal space. A subset of these progeny differentiates into, as yet unidentified, non-vessel cells (triangle with question mark). da, dorsal aorta.

vessels are found on the surface of the gut. From E11, the serosal mesothelium can be visualized on the surface of the gut and the mesenteries, as well as covering the peritoneal cavity. At E12.5, mesothelial cells on the surface of the gut tube undergo EMT and are seen in the submesothelial space of the gut. From E13.5, endothelial tubes extending from the vascular plexus are seen at the surface of the gut. From E16.5, a subset of the descendants of mesothelial cells has differentiated into vascular smooth muscle cells that are found in all arteries and veins of the mesentery and gut. Therefore, we show that progeny of the serosal mesothelium differentiate predominantly into vascular smooth muscle and other non-vascular cells of the gut.

Wt1 expression as an indicator of the serosal mesothelium

Both epicardial and serosal mesothelia share expression of marker proteins, such as Wt1, cytokeratins and Bves (Armstrong et al., 1993; Carmona et al., 2001; Foley-Comer et al., 2002; Moore et al., 1998; Osler and Bader, 2004). Here, we have repeated earlier experiments that showed that both Wt1 and cytokeratin expression are indicators of the presence of the serosal mesothelium. At stages before E10.5, the surface of the embryonic gut lacks Wt1 and cytokeratin expression, and a simple squamous epithelium cannot be detected. Soon afterwards, robust expression of Wt1 and cytokeratin is detectable in a single layer on the surface of the embryonic gut, indicating the presence of the mesothelium. Futhermore, throughout embryogenesis and adulthood, Wt1 protein is found in the serosal mesothelium. Our analysis did not provide evidence that this protein is present in other cell populations in the embryonic and postnatal gut. However, we cannot exclude the possibility that Wt1 is transiently expressed in non-mesothelial cells in the gut. Still, given that both previously published reports and the current study demonstrate restricted expression of Wt1 to the serosal mesothelium, we conclude that Wt1 is a suitable marker for this epithelial structure.

The Wt1-driven Cre recombinase marks descendants of serosal mesothelium

The intriguing similarities between the epicardium and the serosal mesothelium, and recent findings that the epicardium is the source of the coronary vessels, led us to analyze the fate of the serosal mesothelium. We used a two-component genetic system in the mouse consisting of a Cre recombinase driven by the Wt1 promoter and the Rosa26R reporter mouse line, and have shown that serosal mesothelial cells are specifically and faithfully marked by the reporter (i.e. β-Gal expression). Also, we have combined this genetic system with the use of CCFSE as a surface marker (Morabito et al., 2001; Perez-Pomares et al., 2002; Perez-Pomares et al., 2004) and have revealed that mesothelial cells undergo EMT, as a large majority of CCFSE-positive submesothelial cells co-label for the β-Gal protein. Finally, our quantitative data indicate that the majority of vascular smooth muscle cells in the mesentery and the gut are β-Gal-positive and thus, we postulate, are descendants of the serosal mesothelium. Although the origin of the small, but significant, number of non-β-Gal-labeled vascular smooth muscle cells is unclear, we have no way to determine their origin at present. Possibly these non-labeled vascular smooth muscle cells originate directly from the splanchnic lateral plate mesoderm that overlies the endodermal epithelium. Alternatively, a small number of serosal mesothelial cells may not express the β-Gal protein, or may express at levels below detection. Overall, our current data indicate a faithful and specific expression of this protein in the serosal mesothelium and its descendants. However, the current methods, which have been previously used in related studies (Cai et al., 2003; de Lange et al., 2004; Jiang et al., 2002; Kawaguchi et al., 2002),

follow populations of Wt1-expressing cells and are not intended to address matters of clonal differentiation. Those analyses must await methods to mark individual progenitors.

Origin of endothelial cells during gut vessel development

Earlier studies had demonstrated that endothelial cells are produced from the PE in heart development (Mikawa and Gourdie, 1996; Perez-Pomares et al., 2002). Although we detect endothelial cells in small, but consistent numbers in our lineage studies in the gut and heart, the majority of endothelial cells in these vessels are not marked by our genetic labeling system. The original lineage studies of Mikawa and Gourdie (Mikawa and Gourdie, 1996) suggest that angioblasts are a minor population within the PE of the heart. A similar situation could be true for the serosal mesothelium. One explanation for this result is that angioblasts are associated with coelomic mesothelia but are not truly part of the epithelium. In this case, angioblasts may never express mesothelial markers such as Wt1 and, thus, may be largely undetected in our experimental model. Several authors have suggested that migratory angioblasts originating from the liver primordium are intermingled with the PE, and that the PE is composed of both epithelial and mesenchymal cells (Nahirney et al., 2003; Perez-Pomares et al., 1997; Perez-Pomares et al., 1998). These cells would be marked by direct retroviral or vital dye labeling, as employed in previous PE experiments (Mikawa and Gourdie, 1996; Perez-Pomares et al., 2002). Alternatively, if angioblasts are truly part of the advancing mesothelium, it is possible that they never express Wt1 or do not express Wt1-Cre at sufficient levels to be detected in our system.

Coupling vasculogenesis with the formation of coelomic mesothelium

Previous studies and our current data suggest a recurring relationship between the formation of coelomic mesothelia, either epicardial or serosal, and the delivery of vasculogenic cells to developing organs (Dettman et al., 1998; Kaufman and Bard, 1999; Manasek, 1969; Manner, 1993; Mikawa and Gourdie, 1996; Perez-Pomares et al., 2002; Perez-Pomares et al., 1998; Viragh and Challice, 1981; Vrancken Peeters et al., 1999). Both the heart and the alimentary canal are initially devoid of mesothelia (Manasek, 1969; Manner, 1993; Meier, 1980), but are housed within a common coelom. This coelom is later subdivided into pericardial and peritoneal cavities by the downward growth of the septum transversum (Kaufman and Bard, 1999). Mesothelial precursors of the epicardium and pericardial coelom arise in association with the septum transversum as it bisects the common coelom (Viragh and Challice, 1981). We can only speculate that a similar population of mesothelial precursors is delivered to the developing gut and peritoneal cavity, as its origins are unclear. The mesothelial precursors of the pericardial cavity have been shown to arise from the dorsal aspect of the coelom (Nahirney et al., 2003), and appear to migrate over the developing organs. Interestingly, our data suggest that the serosal mesothelium also forms dorsally in the peritoneal cavity, at the mesentery close to the urogenital ridges. Nevertheless, we would like to stress that, at present, we have no data providing evidence towards this mechanism. In the heart and the gut, EMT from the mesothelium produces cells that migrate to developing vessels, where described molecular mechanisms of vasculogenesis may regulate cell differentiation (Carmeliet et al., 1996; Cleaver and Melton, 2003; Hellstrom et al., 1999; Jain, 2003; Lammert et al., 2003). In addition, it is important to note that both epicardial and serosal mesothelia produce non-vasculogenic progeny that reside within the heart and the gut (Figs 5, 6) (Mikawa and Fischman, 1992; Munoz-Chapuli et al., 2002; Perez-Pomares et al., 1997; Perez-Pomares et al., 1998). Thus, although potential variation may exist, it appears that the vertebrate embryo employs elements of a common or conserved program for the generation of vessels to coelomic organs rather than any wholly variant mechanism in their development.

Coupling vascular development to the formation of mesothelia varies from blood vessel formation in other regions of the embryo. In the limbs, body wall and extraembryonic tissues, vasculogenic mesenchyme is thought to arise from locally derived mesenchyme (Cleaver and Krieg, 1999; Gerhardt and Betsholtz, 2003; Hirschi and Majesky, 2004; Majesky, 2003; Saint-Jeannet et al., 1992). The present data, along with previous studies on coronary development, suggest that vasculogenic mesenchyme is 'delivered' to organs within the coelom via its encapsulating epithelium later in development. Although known molecular signaling mechanisms are likely to regulate the angioblast/mesenchyme interaction, we propose that coupling the production of vasculogenic cells to the formation of coelomic mesothelium constitutes a distinct yet conserved cellular mechanism in blood vessel development.

Clinical relevance of the serosal mesothelium and its contribution to blood vessel formation

Surgeons have long used the omentum, the serosal mesothelium and its connective tissue companion, to repair injured blood vessels and intestines with good success (Bertram et al., 1999; Matoba et al., 1996; Roa et al., 1999; Sterpetti et al., 1992). Although the cellular basis of this reparative function is unknown, it is interesting to speculate that serosal mesothelium may serve as a source of diverse cell types in injury repair. A previous study from our group has shown that mesothelial cell lines can produce vasculogenic cells after stimulation with specific growth factors, suggesting a retention of embryonic potential (Wada et al., 2003a). Thus, it is possible that the serosa may provide a natural source of divergent cells to be used in the repair of damaged adult structures. Finally, a relatively uncommon developmental syndrome, called 'Apple Peel Bowel', producing intestinal atresia has been reported (Federici et al., 2003; Pumberger et al., 2002; Waldhausen and Sawin, 1997). Interestingly, this atresia or intestinal wasting is associated with regional loss of the serosa and its associated blood vessels, and is suggestive of a mechanistic relationship between the generation of coelomic mesothelia and vascular development.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/23/5317/DC1

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