Chapter 2

Isolation of Arterial-specific Genes by Subtractive Hybridization Reveals Molecular Heterogeneity among Arterial Endothelial Cells

ABSTRACT

Arteries are distinguished from veins by differences in gene expression, as well as in their anatomy and physiology. The characterization of arterial- and venous-specific genes may improve our understanding of cardiovascular development and disease. Here we report the results of a subtractive hybridization screen for arterial-specific genes, and describe in detail the expression of a novel arterial-specific gene, *Depp* (**de**cidual **p**rotein induced by **p**rogesterone), using a GFP-Cre knock-in that permits a comparison of both instantaneous and cumulative expression patterns in situ. Several features of *Depp* expression are noteworthy. First, *Depp* is expressed in endothelial cells of peripheral tissues, but not in atrial or ventricular endocardial cells of the heart. Very few genes have been reported to discriminate between these two cell types, and therefore this specificity may be useful in generating conditional mutations in other genes implicated in cardiovascular development. Second, *Depp* reveals an unexpected degree of molecular heterogeneity among arterial endothelial cells. Third, *Depp* is up-regulated in subsets of endothelial cells, in settings of adult neo-vascularization, including tumor angiogenesis. Taken together, these data reveal unanticipated temporal and spatial heterogeneity among arterial endothelial cells of various tissues and organs, raising new questions regarding the functional significance of this diversity.

INTRODUCTION

Arteries are distinguished from veins by the direction of blood flow and by anatomical and functional differences. The recent observation that several genes, including *ephrin-B2* (Adams et al., 1999; Gale et al., 2001; Shin et al., 2001; Wang et al., 1998), *Dll4* (Krebs et al., 2000; Shutter et al., 2000), activin receptor-like kinase 1 (*Alk1*) (Seki et al., 2003), *EPAS-1*/hypoxia-inducible factor 2α (Tian et al., 1998; Tian et al., 1997), *Hey1*, and *Hey2* (Nakagawa et al., 1999) are specifically expressed in arteries but not in veins, while *EphB4* is enriched in veins (Gerety et al., 1999), indicates that there are multiple molecular differences between arteries and veins. These molecular differences may cause or reflect functional, physiological, and anatomical differences between arteries and veins.

Arterial- and venous-specific genes are also crucial for the proper development of the cardiovascular system. *Ephrin-B2* (Adams et al., 1999; Wang et al., 1998), *EphB4* (Gerety et al., 1999), *Alk1* (Oh et al., 2000; Urness et al., 2000), and *Dll4* homozygous mutants (Duarte et al., 2004; Gale et al., 2004) and *Hey1/Hey2* double homozygous mutants (Fischer et al., 2004) have severe angiogenesis defects accompanied by heart defects. *EPAS-1* homozygous mutants have angiogenesis defects (Peng et al., 2000), albeit less severe than defects in *ephrin-B2* or *EphB4* mutants. In addition to their role in development, recent data suggest that ephrin-B2 and EphB4 may play important roles in tumor angiogenesis (Martiny-Baron et al., 2004; Noren et al., 2004).

The identification of additional arterial- and venous-specific genes should contribute to our understanding of cardiovascular development and disease. However, most such genes discovered to date have been identified serendipitously; there have been relatively

few systematic attempts to identify novel arterial and venous-specific genes (Chi et al., 2003). Part of the reason for this is that it is very difficult to purify a sufficient number of arterial and venous endothelial cells for conventional subtractive hybridization or microarray screening, due to the absence of arterial- or venous-specific cell surface antibodies available for fluorescence-activated cell sorting (FACS). This technical limitation prompted us to employ differential screening of cDNA libraries constructed from small numbers of isolated arterial or venous endothelial cells. This screening method initially was developed to isolate pheromone receptor genes from single vomeronasal cells (Dulac and Axel, 1995; Pantages and Dulac, 2000), and has subsequently been applied to isolate novel genes from small numbers of cells from other tissues (Shimono and Behringer, 1999; Streit et al., 2000).

We have cloned several arterial-specific genes using this approach, and have characterized one novel arterial-specific gene, *Depp*, by generating a targeted mutation in the *Depp* locus. We introduced an *EGFP-Cre* fusion construct into this locus to visualize both the instantaneous expression of *Depp,* and to trace the lineage of $Depp^{+}$ cells. Although homozygous *Depp* mutant mice do not show any obvious cardiovascular defects during development or in adulthood, the expression of *Depp* reveals an unexpected spatial and temporal heterogeneity of arterial endothelial cells. Furthermore, *Depp* represents one of the few genes that differentiate peripheral vascular endothelial cells from endocardial cells. The availability of a mouse line expressing Cre recombinase in such a pattern may permit a genetic dissection of the relative roles in the heart and peripheral vasculature, of genes important in cardiovascular development.

RESULTS

Cloning of novel genes differentially expressed in arterial endothelial cells

To find genes specifically expressed in arterial or venous endothelial cells, we dissected umbilical cords from wild-type E11 embryos, and separated the cords into umbilical arteries and veins under a dissecting microscope, based on their different morphology. These pooled arteries or veins were dissociated into single cells and incubated with two pan-endothelial antibodies, anti-PECAM-1-FITC and anti-FLK1-PE, and PECAM-1^+/ $FLK1^+$ endothelial cells were isolated by FACS (Fig. 1B, C). All the isolated cells (2-3,000 cells) were re-suspended in lysis buffer. Duplicate aliquots of 10, 20, and 40 cell-equivalent volumes from the re-suspended cells were taken and subjected to PCR amplification as described (Dulac and Axel, 1995). The quality of the amplified cDNAs was evaluated by agarose gel electrophoresis (Fig. 1D). The gel pattern of the cDNAs ranged from 300 bp to 1 kb without any distinct bands, implying unbiased amplification. The cDNAs were further evaluated by virtual Northern blotting, using eight different genes as probes: a house keeping gene, *Tubulin*; three pan-endothelial markers, *Tie2*, *Flk1*, and *Flt1*; three arterial endothelial specific markers, *EPAS1*, *Dll4*, and *ephrin-B2*; and a venous endothelial marker, *EphB4* (Fig. 1E). *Tubulin* and the three pan-endothelial genes were expressed at a relatively similar level between the umbilical arterial (UA) and umbilical venous (UV) cDNAs. The arterial-specific genes were expressed at very low levels in UV, and strongly expressed in UA, as expected (Fig. 1E). Based on this evaluation, a cDNA pair representing 40 cell-equivalents each of UA or UV endothelial

cells was chosen for further subtraction and differential screening (for details see Experimental Procedures).

Virtual Northern blotting was performed for four candidate genes selected from the differential screen: *Depp*, connexin37 (*Cx37*), connexin40 (*Cx40*), and insulin-like growth factor binding protein-5 protease (*IGFBP-5P*). This experiment confirmed their specific expression in UA or UV cDNAs (Fig. 1F). These genes were then analyzed by in situ hybridization to verify their *in vivo* expression patterns. The in situ hybridization data confirmed the arterial-specific expression of all four candidate genes at E10.5 (Fig. 2G, H, I, J; arrows). *Depp* was expressed in the dorsal aorta at E8.75 (Fig. 2B) but down-regulated at E10.5 and E13.5. However, at E10.5 *Depp* expression was strongly detected in vessels branching from the dorsal aorta (Fig. 2G, white arrow) as well as in small vessels (Fig. 2G, black arrowheads). At E13.5, a subset of small vessels still expressed *Depp* (Fig. 2L, arrowheads). *Cx37* and *Cx40* expression in the dorsal aorta persisted from E8.75 to E13.5 (Fig. 2C-M, D-N), the latest stage examined. *IGFBP-5P* was not detectible in endothelial cells at E8.75 (Fig. 2E, arrow) but was expressed in the dorsal aorta at E10.5 and E13.5. This gene was also expressed in the notochord during the interval examined (E8.75 to E13.5) (Fig. 2E-O, black arrowheads).

Targeted mutagenesis of a novel arterial-specific gene, *Depp*

Depp, **de**cidual **p**rotein induced by **p**rogesterone, was cloned from human tissues and its sequence deposited into a public database in 1999, without further information. We chose *Depp* for further analysis due to its unique expression pattern and sequence novelty. Sequences of rat, porcine, and bovine *Depp* orthologs have been deposited in the public

databases, but no apparent orthologs in zebrafish, fugu, or chicken have been reported. In addition, there were no other *Depp*-related genes in the mouse or human genomes. The N-terminal (about 59 amino acids) and C-terminal regions (about 21 amino acids) are highly conserved among the different mammalian species examined (Fig. 3A). DEPP is predicted to be a soluble protein based on its amino acid sequence, and *in vitro* transfection experiment using a *Depp-V5* epitope-tagged expression construct suggested that DEPP may be present in the cytoplasm (data not shown). *Depp* has several putative consensus phosphorylation sites (Fig. 3A, circle, star, and square), but does not contain any conserved structural motifs.

To assess the function of *Depp* in vivo, and to characterize its expression in more detail, we replaced the entire predicted coding region of the gene with an *EGFP-Cre* fusion gene, by homologous recombination in embryonic stem cells (Fig. 3B). Germline chimeric mice were then generated from these ES cells by blastocyst injection. This design permitted the "immediate" or "instantaneous" expression of *Depp* to be monitored by EGFP expression, and its cumulative or "historical" expression pattern to be examined by ß-galactosidase expression in $Depp^{EGFP\text{-}Cre/+}$;*Rosa26R* mice.

Depp is expressed in arterial endothelial cells and down-regulated in mature vessels during development.

Our in situ hybridization data indicated that *Depp* is expressed in arterial endothelial cells of the dorsal aorta, but without a direct comparison to other arterial markers we were unable to address the question of its arterial specificity in smaller-diameter vessels. To this end, we crossed $Depp^{EGFPCre/+}$ mice to $ephrinB2^{taulacZ/+}$ mice and examined

compound heterozygous embryos by double-labeling with antibodies to GFP and ß-galactosidase. At both E9.8 and E12.5, all GFP^+ endothelial cells were *ephrin-B2* $^+$ in the small vessels of limbs, as well as in dorsal aortic endothelial cells (Fig. 4C, F, I, L). (Note that the cytoplasmic tau-ß-galactosidase reporter used for *ephrin-B2* and the nuclear EGFP-Cre reporter used for *Depp* exhibit distinct subcellular localizations, therefore arterial endothelial cells often appear as green nuclei surrounded by red cytoplasm; Fig. 4I, L.) At no stage examined was *Depp* expression detected in veins (Fig. 4A, D, M; open arrowheads). These data confirm the pan-arterial specificity of *Depp* expression within the peripheral vasculature. The cellular resolution afforded by expression of the EGFP reporter revealed, unexpectedly, that at E9.8 *Depp* is expressed in only a subset of endothelial cells within the dorsal aorta (Fig. 4A, M, arrows). By E12.5, *Depp* was expressed in very few dorsal aortic endothelial cells, while it was expressed by a subset of endothelial cells branching from the dorsal aorta (Fig. 4D, arrow vs filled arrowhead). These data suggested that *Depp* is transiently expressed by subsets of developing arterial endothelial cells, in both the dorsal aorta and peripheral vasculature.

The heterogeneous expression of *Depp-EGFP* in dorsal aortic endothelial cells at E9.8 raised the question of whether the $EGFP^-$ cells represent a subpopulation that never expresses the gene, or rather cells that down-regulated expression prior to the stage analyzed. To distinguish these possibilities, we crossed $Depp^{EGFPCre/+}$ mice to *Rosa26R* Cre reporter mice. Double-labeling with antibodies to GFP and ß-galactosidase on sections of E9.8 and E12.5 $Depp^{EGFP\text{-}Cre/+}$*;Rosa26R* embryos indicated that most endothelial cells of E9.8 and E12.5 dorsal aorta expressed ß-galactosidase (Fig. 4N, O, Q,

R). These data suggest that most GFP$^-$ endothelial cells in E9.8 dorsal aorta had transiently expressed *Depp* earlier during development.

In the peripheral vasculature, *Depp* was maintained in limb vessels until at least E12.5, a time when *Depp* was barely expressed in dorsal aortic endothelial cells (Fig. 4D, arrow; G, J). By adulthood, however, *Depp* was down-regulated in most small diameter vessels of the limb (see below and data not shown). The transient nature of *Depp* expression raised the question of whether it was correlated with proliferation. Double-labeling with antibodies to GFP and phospho-histone3 (Fig. 8G, I; red vs green) or Ki67 (data not shown) indicated, however, that there was no correlation between proliferation and *Depp* expression.

Heterogeneous expression of *Depp* in adult vasculature

We next examined the expression of *Depp* in adult tissues, by performing double labeling with antibodies to GFP and PECAM-1. *Depp* was heterogeneously expressed in a subset of adult vessels, but this varied in different organs. For example, small-diameter vessels in the fat around the dorsal aorta, the heart, and the neocortex (Fig. 5A-F) contain abundant subsets of *Depp*$^+$ endothelial cells. The vasculature of these tissues contains more Depp-EGFP$^+$ endothelial cells than does that of some other tissues, such as kidney or liver (Fig. 5G, O); furthermore, the neocortex contains more *Depp*$^+$ endothelial cells than other regions of the brain (data not shown). *Depp* is also expressed in lung epithelial cells, pancreatic islet cells, and liver hepatocytes as well as in a subset of endothelial cells of these organs (Fig. 5K-P, arrowheads and arrows). *Depp* expression was restricted to arterial endothelial cells in P5 retina (Fig. 5Q, arrow); its expression in most other adult

organs was predominantly (but not absolutely) restricted to *ephrin-B2*$^{+}$ arterial endothelial cells (data not shown).

Within each tissue, *Depp* expression in the arterial vasculature was inhomogeneous. For example, in the kidney *Depp* is mostly restricted to the endothelial cells of the glomeruli (Fig. 5G, H; arrow). In the uterus of a pregnant female bearing E11 embryos, *Depp* was expressed in a small subset of endothelial cells (Fig. 5I, arrow); however, the placenta from the same female did not have any *Depp*$^{+}$ endothelial cells (data not shown). *Depp* was also expressed in a subset of uterine epithelial cells of pregnant females (Fig. 5I, arrowhead), but not in the uterus of a non-pregnant female (data not shown). This observation is consistent with the original identification of *Depp* as a decidual protein. We attempted to determine whether *Depp* expression could be induced in this tissue by progesterone administration, but no consistent results were obtained (data not shown).

We also examined expression of *Depp* in various settings of adult neovascularization. *Depp* was expressed in a subset of vessels in tumors and wounded skin (Fig. 5S-X). In some cases, the expression of *Depp* in such neo-vessels appeared greater than in neighboring, static vessels (Fig. 5W, X and data not shown). Examination of ß-galactosidase expression in such vessels in *Depp*$^{EGFPCre/+}$; *Rosa26R* mice indicated that many more cells were lacZ^{+} than were GFP^{+} (data not shown). Whether the high level of *Depp-Cre* mediated recombination in such neo-vessels represents a transient induction of the gene during neovascularization, or rather the recruitment of endothelial cells that once expressed *Depp* during development, cannot presently be distinguished.

Depp is not expressed in endocardial cells of atria or ventricles, but is expressed in a subset of outflow tract endocardial cells.

The fact that most endothelial-specific genes, including *ephrin-B2* (Wang et al., 1998), *EphB4* (Gerety et al., 1999), *Flk1* (Shalaby et al., 1995), *Flt1*(Fong et al., 1995) , *Tie1* and *Tie2* (Sato et al., 1993), VE-cadherin, CD34, PECAM-1, and TAL1 (Drake and Fleming, 2000), are also expressed in endocardial cells complicates the interpretation of cardiovascular phenotypes in mice bearing mutations in these genes. Therefore, we investigated whether *Depp* is, similarly, also expressed in endocardial cells.

Strikingly, *Depp-EGFP* was not expressed in endocardial cells of the atria or ventricles (Fig. 6A, B, D, E), whereas it was expressed in a subset of myocardial cells of the atrio-ventricular and bulbo-ventricular canals (Fig. 6A, C; arrow and arrowheads). Furthermore, *Depp-Cre* mediated lineage tracing experiments indicated that *Depp* is not expressed in progenitors of atrial or ventricular endocardial cells (Fig. 6K, N). Recombination (ß-gal expression) without GFP expression was detected in the trabeculated region of a ventricle (Fig. 6J, arrow and black arrowhead). The fact that myocardial precursors migrate from pharyngeal arches through outflow tracts to ventricles and further to atria (Cai et al., 2003; Kelly et al., 2001) may explain this observation; alternatively *Depp* may be transiently expressed in precursors of these myocardial cells. Further analysis of *Depp* expression in other cardiac regions revealed that *Depp* is also expressed in a subset of endocardial and myocardial cells of the outflow tracts (OFT; Fig. 6G, arrow vs arrowhead). This OFT expression was detected by both expression of GFP, and of the Cre-dependent reporter (Fig. 6P, arrow).

***Depp* is not required for vessel development or maintenance.**

The unique cardiovascular expression pattern of *Depp* prompted us to examine the cardiovascular development of *Depp* homozygous mutant embryos. Whole-mount staining with anti-PECAM-1 antibodies revealed that there were no obvious differences in vessel development or patterning between E9.5 wild-type and homozygous mutant embryos (Fig. 7A-D), or yolk sacs (Fig. 7E, F). Double-labeling with antibodies to α-SMA (Gothert et al.) and PECAM-1 (red) revealed no obvious differences between wild-type and homozygous mutant hearts in terms of ventricular trabeculation or atrial and ventricular morphology (Fig. 7G, H)

Although *Depp* is down-regulated in mature vessels by E10, its expression persists in small vessels in later stage embryos. Therefore, we examined the vascular morphology of wild-type and the homozygous mutant embryos at E15.5. Whole-mount staining with anti-PECAM-1 antibodies revealed no clear differences between E15.5 wild-type and homozygous mutant forelimb skin (Fig. 7I, J), stomach (Fig. 7K, L), intestine (Fig. 7M, N), and kidney (data not shown). Because retinal vessel development occurs after birth (Fruttiger, 2002) and is sensitive to angiogenic factors such as VEGF and angiopoietin-1 (Stalmans et al., 2002; Uemura et al., 2002), we also examined retina vasculature by double-labeling with antibodies to α-SMA and PECAM-1. However, there was no obvious difference between P5 wild-type and the homozygous mutant retinae (Fig. 7O, P). In addition, *Depp* homozygous mutant mice developed normally without any apparent physiological or developmental defects, and are fertile in both a 129/c57Bl6 mixed background and pure 129 background.

Depp is expressed in a subset of vessels in tumors and wounded skin; therefore, we asked whether pathological angiogenesis in tumors and wounded skin is affected in homozygous mutant mice, by implanting Lewis lung carcinoma cells subcutaneously or by making skin wounds in the back of the mutant mice. The tumor size and extent of vascularization, and the wound healing and skin vascularization, in the homozygous mutant mice were similar to those in heterozygous and wild-type mice (data not shown).

Arterial endothelial differentiation, proliferation, and survival are normal in *Depp* homozygous mutant embryos.

The arterial-specific expression of *Depp* prompted us to examine arterial differentiation in *Depp* homozygous mutant embryos. To address this, we intercrossed $Depp^{EGFP\text{-}Cre/+}$ with $ephrin\text{-}B2^{lacZ/+}$ mice. $Depp^{+/-};ephrin\text{-}B2^{lacZ/+}$ and $Depp^{-/-};ephrin\text{-}B2^{lacZ/+}$ embryos at E9.5 were processed for staining with anti-β-galactosidase antibodies to reveal *ephrin-B2* expression. *Ephrin-B2* was expressed in the homozygous embryos similarly as in the heterozygous embryos (Fig. 8A, B). Another arterial-endothelial marker, Cx40, was also expressed in the homozygous mutant (Fig. 8C, D); smooth muscle cells were normally recruited to the homozygous mutant dorsal aorta (Fig. 8E, F). We also examined proliferation (Fig. 8G-J) and apoptosis (Fig. 8K-L) in the mutant embryos, but there was no significant difference between the homozygous and heterozygous embryos at E9.5. These data suggest that arterial differentiation is normal in $Depp^{-/-}$ embryos, and that there are no genetic interactions between *ephrin-B2* and *Depp* affecting arterial differentiation.

To more systematically examine the effect of the *Depp* mutation on endothelial cells, we analyzed the gene expression profiles of *Depp*$^+$ endothelial cells isolated by FACS from homozygous and heterozygous mutant embryos at E10. GFP$^+$/PECAM-1$^+$/FLK1$^+$ endothelial cells were isolated, and their cDNAs amplified and subjected to microarray analysis. There were no significant differences in the expression level of known endothelial genes between the homozygous and heterozygous *Depp*$^+$ endothelial cells (data not shown). A small subset of genes exhibited significant differences in their expression levels, but in situ hybridization for these genes failed to confirm these differences when comparing homozygous and heterozygous embryos. These data suggest that the differentiation of *Depp*$^+$ endothelial cells is relatively normal in *Depp* homozygous mutant embryos.

DISCUSSION

In this study, we undertook a systematic approach to identify arterial-specific genes, using primary endothelial cells from umbilical vessels of E11 embryos. We have described in detail the expression pattern of a novel arterial-specific gene, *Depp*, using a genetic approach that permits visualization of both instantaneous and cumulative expression in situ. The expression of *Depp* revealed several unexpected features of vascular endothelial cell heterogeneity. First, *Depp* is expressed in endothelial cells of peripheral tissues, but not in atrial or ventricular endocardial cells of the heart, identifying a molecular distinction between these two endothelial cell sub-types. Second, *Depp* revealed that arterial endothelial cells are molecularly heterogeneous. Finally, *Depp* is induced in subsets of endothelial cells, in some settings of adult neo-vascularization,

indicating heterogeneity within this population as well. The biological significance of the molecular heterogeneity revealed by *Depp* expression is not yet clear; nevertheless, it points to previously unsuspected diversity and complexity within arterial endothelial cell populations.

A systematic screen to identify novel arterial-specific genes

An important feature of our screen was the use of freshly isolated, rather than cultured, arterial or venous endothelial cells. While cultured cells may be a more convenient source of material, the arterial or venous identity of the endothelial cells may change in vitro. For example, cultured human umbilical vein endothelial cells (HUVEC) express an arterial marker, e*phrin-B2* (Kim et al., 2002). In addition, venous endothelial cells express *ephrin-B2* in response to VEGF in vitro (Y. Mukouyama and D. J. Anderson, personal communication), whereas they do not express *ephrin-B2* in vivo. These data suggest that arterio-venous identity is plastic in vitro, and underscore the importance of using freshly isolated, uncultured cells to screen for markers of such identity.

In a previous study, the expression profiles of 53 different populations of cultured endothelial cells were investigated using DNA microarrays. The comparisons performed included large vessel-derived endothelial cells vs. microvascular endothelial cells, and artery-derived endothelial cells vs. vein-derived endothelial cells (Chi et al., 2003). The study reported 59 putative artery-specific genes, and confirmed the arterial-specific expression of three of these genes (*Hey2*, *C17*, and CD44), using cultured endothelial cells, rather than in situ hybridization to embryonic tissue as we have done here. In contrast to the present work, this previous study did not identify *Cx37*, *Cx40*, *Depp*, or

IGFBP-5P as artery-specific genes. It is not clear whether these genes were not present on the 43K cDNA chips used in the earlier analysis, or whether the arterial-specific expression of these genes was lost under the culture conditions used to prepare the cells for microarray analysis.

Depp is expressed in peripheral endothelial cells, but not in atrial or ventricular endocardial cells.

We focused on *Depp* in part because our initial in situ hybridization analysis indicated that it is expressed in arterial endothelial cells of peripheral blood vessels, but not in atrial or ventricular endocardial cells of the heart. By contrast, most endothelial-specific genes are expressed in both endothelial and endocardial cells. This differential expression of *Depp* was confirmed using the GFP reporter. Furthermore, the cumulative expression pattern of *Depp*, visualized using the *Depp-Cre* line, revealed that transient expression of *Depp* does not occur in atrial or ventricular endocardial cells at any time up to the stages analyzed. This selectivity of *Depp-Cre* recombination may be useful in generating conditional mutations in other genes implicated in cardiovascular development, which are expressed in endothelial/endocardial but not in myocardial cells, to resolve the question of whether peripheral angiogenesis defects observed in conventional knockouts are primary, or secondary to cardiac defects. For example, we have used *Depp-Cre* mice to generate peripheral endothelial-specific knockouts of *ephrin-B2* (Gerety and Anderson, 2002; Wang et al., 1998), and observe vascular phenotypes in mutant embryos (D.S. and D.J.A., unpublished data). We note, however, that *Depp* is also expressed in endothelial cells of the cardiac outflow tract. Like *Depp*, *Connexin40*, a gap junction gene, is also

expressed in arterial endothelial cells and in OFT endocardial cells, but not in atrial or ventricular endocardial cell (Delorme et al., 1997). However, *Cx40-Cre* mice have not yet been described, so it is not clear whether this gene may be transiently expressed during endocardial development.

Molecular heterogeneity among arterial endothelial cells

The heterogeneous expression of *Depp* in the embryonic dorsal aorta indicates that neighboring endothelial cells within this vessel can be molecularly distinct. Definitive hematopoiesis takes place in the ventrally positioned endothelial cells of the dorsa aorta within the aorta-gonad-mesonephros region from E10 to E12 (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996). *AML1* is expressed in these ventrally positioned hemogenic endothelial cells, but not in dorsally positioned endothelial cells, and is required for the formation of intra-aortic hematopoietic clusters from the hemogenic endothelium (North et al., 1999). By contrast, *Depp* is not differentially expressed between dorsally and ventrally positioned, or between left and right, endothelial cells of the dorsal aorta. Rather, it is expressed in scattered endothelial cells distributed evenly throughout the dorso-ventral axis of the vessel wall, at E9.8 (Fig. 4A, M). A similarly scattered, heterogeneous expression pattern of *Alk1* in the dorsal aorta at E15.5 was recently demonstrated by X-gal staining in $Alk1^{LacZ/+}$ mice (Seki et al., 2003); however, *Alk1* seems expressed in most endothelial cells of the dorsal aorta at E11.5 (Seki et al., 2003). Therefore, *Depp* expression in the dorsal aorta at E9.8 uniquely reveals the heterogeneity of early embryonic arterial endothelial cells within a single vessel.

Heterogeneity within adult vessels has previously been reported using lectins and other markers (Aird, 2003; Caprioli et al., 2004).

The heterogeneous expression of *Depp* suggested that it might be correlated with proliferation. However, immunohistochemical analysis using anti-phospho-histone3 and anti-GFP revealed no correlation between *Depp* expression and proliferation. In addition, TUNEL assays showed no correlation between *Depp* expression and cell death. The functional significance of the heterogeneity revealed by *Depp* expression, therefore, remains to be elucidated.

Depp is induced in subsets of vessels in neo-angiogenic settings

Our analysis revealed that *Depp* is expressed in at least two settings of neo-angiogenesis: tumor vascularization and wound healing. *Depp* is also expressed in subsets of endothelial cells in a corneal micropocket assay (data not shown). Interestingly, a direct comparison of GFP with ß-galactosidase expression in such neo-vessels, in $Depp^{EGFP\text{-}Cre/+}$; *Rosa26R* mice, indicated that many more endothelial cells were $lacZ^{+}$ than were GFP^{+} (data not shown). On the one hand, this difference could reflect a transient induction of *Depp* expression during adult neo-angiogenesis, similar to its dynamic regulation during embryonic development. On the other hand, the difference could reflect the recruitment, to new adult vessels, of endothelial cells that transiently expressed *Depp* during embryonic development. Inducible control of Depp-Cre expression after tumor implantation or wounding will be required to distinguish between these possibilities. In both cases, however, the GFP reporter indicates that persistent expression or induction of *Depp* occurs in some endothelial cells of adult neo-vessels. If

Depp is indeed transiently expressed in such settings, then the *Depp* promoter could be useful to target other genetic modifications to sites of adult neo-angiogenesis.

Depp is not essential for vessel development or maintenance.

Despite its novel expression pattern, *Depp* homozygous mutants did not show any detectable phenotypic defects during development or in adulthood. This is surprising, as there are no other *Depp*-related genes in the mouse genome. Nevertheless, functionally redundant genes, whose amino acid sequences are not homologous to that of DEPP, might compensate for the lack of *Depp* in the homozygous mutants. The fact that DEPP is a cytoplasmic protein (data not shown), and has several putative consensus serine-, threonine-, and tyrosine-phosphorylation sites, suggests that it may function in a signal transduction pathway. If so, then this pathway might be compensated by other genes in the same pathway, or by other pathways, in *Depp* homozygous mutant mice. Alternatively, DEPP might be important only under special conditions of physiologic responses to stress, injury or infection, which we have not yet systematically investigated.

Recently, additional human and mouse *Depp* sequences have been deposited into the public sequence databases with new names: *Fig* (fasting induced gene) and *Fseg* (fat-specific expressed gene). In addition to its original name, *Depp* (decidual protein induced by progesterone), the new name, *Fig*, suggests that *Depp* expression might be dynamically regulated by extra-cellular signals, or physiological homeostatic mechanisms. The fact that *Depp* is expressed in uterine epithelia of pregnant females (Fig. 5I), but not of non-pregnant females, further supports the idea that *Depp* expression

is dynamically regulated by extra-cellular signals. The nature of these signals, and the significance of *Depp* regulation, remains to be determined.

Molecular logic of arterio-venous identity specification

Our screen was designed, in principle, to detect venous-specific as well as arterial-specific genes. However, despite analyzing the same number of *E. coli* colonies from the subtracted umbilical venous cDNA library as we did from the arterial cDNA library, we did not identify any venous-specific genes. Why did we fail to find a single venous-specific gene? *EphB4* is the only known venous endothelial-specific marker in mice (Gerety et al., 1999). Nevertheless, virtual Northern blots probed by *EphB4* (Fig. 1E) revealed a strong *EphB4* signal even in the arterial cDNA sample. Consistent with this, immunofluorescent staining of $EphB4^{LacZ/+}$ embryos with antibodies to ß-galactosidase revealed *EphB4* expression in arterial endothelial cells, albeit weaker than in venous endothelial cells (Shin et al., 2001). These data indicate that the differential expression of *EphB4* between arterial and venous endothelial cells is quantitative, rather than all-or-none. By contrast, expression of arterial markers such as *ephrinB2* is virtually undetectable in venous endothelial cells, by a number of methods. If this fundamental quantitative vs. qualitative difference in venous- vs. arterial-specific genes, respectively, is general, then the screening method used here may have systematically selected against the former category of genes, due to the PCR-based cDNA amplification step.

It is interesting to speculate why venous-enriched genes are still expressed at low levels in arterial endothelial cells, whereas arterial-specific genes are all-or-none. In zebrafish and mouse, VEGF and Notch signaling specifies arterial endothelial cells by

inducing arterial markers and repressing venous markers (Lawson et al., 2001; Lawson et al., 2002; Mukouyama et al., 2005). Mouse *Flt4*, a receptor tyrosine kinase, is initially expressed in both arterial and venous endothelial cells during early development, but becomes restricted to venous endothelial cells at E11.5, and then later to lymphatic endothelial cells (Dumont et al., 1998; Kaipainen et al., 1995). These and other data suggest that arterial specification might require the induction of a battery of arterial-specific genes and the repression of venous-specific genes, whereas venous specification might simply require the maintenance and/or up-regulation of venous-specific genes. Such a mechanism would be consistent with the view of the venous fate as a "default" fate for the artery vs. vein decision (Sato, 2003; Thurston and Yancopoulos, 2001). This view interprets the persistent expression of venous-enriched genes in arterial endothelial cells as a consequence of the developmental history of arterial fate-specification. Whether this persistent expression has any functional utility, for example with respect to phenotypic plasticity, is an interesting question for future investigation.

EXPERIMENTAL PROCEDURES

PCR-amplification, subtraction, and differential screening

Umbilical cords from wild-type E11 embryos were dissected in HBSS, and separated into umbilical arteries (UA) and veins (UV) under a dissecting microscope based on their different morphology: the arterial vessels are thinner than the venous vessels and bifurcate close to the embryo, while the venous vessels are single tubes. Each sample of pooled vessels as dissociated into single cells by digestion with type 1 collagenase (Worthington) and deoxyribonuclease type 1 (DNase 1; Sigma), labeled with FITC-

conjugated anti-PECAM-1 antibody (BD Pharmingen) and PE-conjugated anti-FLK1 antibody (BD Pharmingen), and PECAM-1^+/ $FLK1^+$ endothelial cells were isolated by FACS (Mukouyama et al., 2002). All sorts and analyses were performed on a FACS Vantage dual laser flow cytometer (BD Biosciences).

The isolated cells were re-suspended in 20 μl of lysis buffer, and aliquots containing 10-, 20-, or 40-cell equivalents were taken and subjected to single-cell based PCR amplification, essentially as described (Dulac and Axel, 1995). Each 5 μg of the amplified cDNAs were loaded on 1.5% agarose gels for electrophoresis to check the quality of the cDNAs, and transferred into a Hybond-N^+ membrane (Amersham) followed by virtual Northern blotting using 3'-biased cDNA probes against a house keeping gene, *Tubulin*; three pan-endothelial markers, *Tie2*, *Flk1*, and *Flt1*; three arterial endothelial specific markers, *EPAS1*, *Dll4*, and *ephrin-B2*; and a venous endothelial marker, *EphB4*. Based on these data, a single pair of 40-cell UA and UV cDNAs as selected for subtraction, which was performed essentially as described (Shimono and Behringer, 2000). Briefly, the cDNAs were digested with *EcoRI*, and the gaps created by *EcoRI* digestion were filled using the Klenow fragment of DNA polymerase I. Then, the cDNAs were loaded on 1.5% agarose gel to isolate cDNAs between 150 bp and 1.5 kb; each 150 ng of the gel-purified cDNAs as cut by *AluI*, *AluI* and *RsaI* restriction enzyme, or was left un-cut. These three cDNAs were pooled, ligated with phosphorylated linkers containing *EcoRI* sites, and amplified by PCR with a primer containing sequences complementary to the phosphorylated linker. For subtraction, the driver cDNAs were photobiotinylated, hybridized with unbiotinylated tester cDNAs, and removed by Streptavidin and phenol-chloroform extraction. This was followed by re-amplification of

the subtracted cDNAs using the same primer as used for phophorylated linker preparation. This subtraction step was repeated twice. The subtracted cDNAs were subjected to differential screening using the PCR-Select Differential Screening Kit (Clontech) with modifications. Briefly, the subtracted cDNAs were ligated with pGEM-T easy vector (Promega), about 800 *E. coli* colonies were picked and cultured, the cDNA in each *E. coli* was amplified by PCR with a primer (5'-TCCAGGCCGCTCTGGACAAAATATGA-3') and transferred into Hybond-N^+ membranes in quadruplicate, and Southern blotting was performed using 4 different probes, representing unsubtracted umbilical arterial and venous cDNAs, and subtracted umbilical arterial and venous cDNAs. cDNAs showing differential signal intensities were selected for virtual Northern blotting to check the expression levels of the selected genes in the unsubtracted umbilical arterial and venous cDNAs (Fig. 1F). Those genes exhibiting differential expression between the unsubtracted arterial and venous cDNAs were further examined for in situ hybridization, to check their in vivo expression patterns (Fig. 2).

Generation of *Depp* mutant allele

The arms of the *Depp* targeting construct were subcloned from a 129/SvJ genomic DNA BAC clone (Roswell Park Institute). An *EGFP-Cre* fusion construct containing a poly A signal from pBS592 (Le et al., 1999) was inserted into the start codon of *Depp*, and an FRT-flanked PGK-neo selection cassette (Meyers et al., 1998) was inserted into the stop codon of *Depp*. Then the 2.86 kb right arm fused with the FRT-flanked PGK-neo cassette was inserted downstream of *EGFP-Cre*, following the 2.8 kb left arm. In this

way, the entire coding region of *Depp* was deleted in this targeting construct. Homologous recombination was performed in mouse CJ7 embryonic stem (ES) cells following standard procedures. Correctly targeted ES cell clones were identified by Southern blot hybridization using a probe that flanked the 3′ arm of the targeting construct, and an internal probe in the 5' arm. Chimeric mice were produced by blastocyst injection and were mated to C57Bl/6 mice to establish lines. The *Depp* allele was deposited as **MGI:3526084**. The FRT- flanked PGK-neo selection cassette was removed by crossing with human ß-actin FLPe deleter mice (Jackson). The deletion of the selection cassette significantly decreased Cre activity without noticeably altering its specificity; therefore, mutant mice retaining the selection cassette were used in this paper.

Genotyping

The genotyping of *Depp* mutant mice was performed by PCR using two primer sets detecting the wild-type and the mutant allele: WT1 (5'-CTACGTCAGGTGTATCTGTCAGCT-3') and WT2 (5'-AAGAACCAGTCCTGTTGGGTCGAC-3') primers, 269 bp PCR products; Cre1 (5'-GTTCGCAAGAACCTGATGGACA-3') and Cre2 (5'-CTAGAGCCTGTTTTGCACGTTC-3') primers, 319 bp PCR products.

For *Depp;ephrinB2* and *Depp;Rosa26R* double mutant mice, the LacZ primers, LacZ1 (5'-CGCCCGTTGCACCACAGATG-3') and LacZ2 (5'-CCAGCTGGCGTAATAGCGAAG-3') producing 370 bp PCR products, were used with the above WT and Cre primers.

In situ hybridization

In situ hybridization was carried out essentially as described (Wang et al., 1998). Embryos were cryosectioned at 20 μm, and adjacent sections were hybridized with cRNA probes against *Flk1* (1035 bp), *Depp* (800 bp), *Cx37* (978 bp), *Cx40* (1000 bp), *and IGFBP- 5P* (897 bp).

Immunohistochemistry

Embryos younger than E10 were first fixed for 1-2 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, sunk in 15% sucrose/PBS, embedded in 15% sucrose and 7.5% gelatin in PBS, and 20 μm sections were collected on a cryostat. Older embryos were fixed for 2-4 hours, sunk in 30% sucrose/PBS, frozen in OCT medium, and 20 μm sections were collected on a cryostat. Adult organs were fixed for 6-8 hours, washed with PBS, cryoprotected in 30% sucrose/PBS overnight at 4°C, frozen in OCT medium, and 20 μm sections were collected on a cryostat. Staining was performed using anti-PECAM-1 antibody (Pharmingen, 1:300 overnight at 4°C), rabbit anti-GFP antibody (Molecular Probes, 1:1000, overnight at 4°C), chicken anti-GFP antibody (Aves Labs, 1:1000, overnight at 4°C), anti-ß-galactosidase antibody (5-prime 3-prime, 1:1000, overnight at 4°C), anti-CX40 antibody (Alpha Diagnostic International, 1:300, overnight at 4°C), Cy3-conjugated anti-α-SMA antibody (Sigma, 1:500, 40 minutes at room temperature), anti-NF-ATc1 antibody (gift from Dr. G. Crabtree, Standford U., 7A6, 1:300, 1 hour at room temperature), and anti-phospho-histone3 antibody (Sigma , 1:000, overnight at 4°C). For immunofluorescent detection; FITC-, Cy3-, Cy5-, Alexa-488-, or Alexa-568-conjugated secondary antibodies (Jackson, 1:300; Molecular Probes, 1:250, 40 minutes at room

temperature) were used. TUNEL labeling was performed according to the manufacturer's protocol (In Situ Cell Death Detection, Roche). All confocal microscopy was carried out on a Leica SP confocal (Leica).

For whole-mount staining, embryos and organs were first fixed for 1-2 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, and dehydrated in 100% methanol at -20°C. Staining was performed with anti-PECAM1 antibody (Pharmingen, 1:200 overnight at 4°C), rabbit anti-GFP antibody (Molecular Probes, 1:1000, overnight at 4°C), and Cy3-conjugated anti-α-SMA antibody (Sigma, 1:500, 1 hour at room temperature); either HRP-conjugated secondary antibodies (Jackson, 1:300, overnight at 4°C) or secondary antibodies conjugated to Cy5- or Alexa-488 (Jackson, 1:300, and Molecular Probes 1:250, 1 hour at room temperature) were used.

Pathological studies: tumors, cutaneous wounding, and corneal micropocket assay

All procedures were carried out under protocols reviewed and approved by the IACUC (Institute Animal Care and Use Committee). Mice were implanted subcutaneously with 200 µl of 2 x 10^6 Lewis lung carcinoma cells (LLC), and sacrificed between 13 to 16 days postimplantation when the diameter of the tumors reached 1.5 cm. The tumors were dissected, weighed, fixed for 6-8 hours in 4% paraformaldehyde/PBS at 4°C, cryoprotected overnight in 30% sucrose/PBS at 4°C, frozen in OCT medium, and 20 µm sections were collected on a cryostat. Mice were also implanted intraperitoneally with 200 µl of 2 x 10^6 B16F10 melanoma cells, and sacrificed between 12 to 16 days postimplantation when they were lethargic. The tumors close to the pancreas and spleen were dissected, and processed for immunohistochemistry like LLC tumors.

Full-thickness cutaneous wounds were made on the back skin of mice using a sterile, disposable 6-mm dermal biopsy punch (Miltex). Animals were sacrificed and the tissue was examined 7 days afterward.

The mouse corneal micropocket assay was performed as described previously (Kenyon et al., 1996) using hydron-coated sucralfate pellets containing 100 ng of bFGF (PeproTech).

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FIGURE LEGENDS

Fig. 1. Screening procedure to identify arterial-specific genes. Umbilical vessels of wild-type E11 embryos were dissected (A, dotted lines), separated into arteries (A, red) and veins (A, blue), and incubated with anti-FLK1-PE and anti-PECAM-1-FITC antibodies. Modified from Carlson (Carlson, 1981). Double-positive endothelial cells from each sample (arterial or venous) were sorted by FACS (B, C). Electrophoresis of 5 μg of amplified cDNAs on 1.5% agarose gel reveals similar smear patterns among cDNAs amplified from 10-, 20-, and 40-cell equivalents (D). Amplified cDNAs representing 40-cell equivalents of umbilical arteries (UA, red) or veins (UV, blue) were selected for further analysis based on the amplification profiles of 8 marker genes: a house keeping gene, *Tubulin*; three pan-endothelial genes, *Tie2*, *Flk1*, and *Flt1*; three arterial-specific genes, *EPAS-1*, *Dll4*, and *ephrin-B2*; and a venous-restricted gene, *EphB4* (E). Note that arterial-specific genes are highly expressed in UA but not in UV, whereas *EphB4* expression is not restricted to UV. (F) Virtual Northern blotting with *Depp*, *Cx37*, *Cx40*, and *IGFBP-5P* probes confirms the specific expression of these genes in umbilical arteries but not in veins.

Fig. 2. Expression patterns of arterial-specific genes confirmed by in situ hybridization. Sections were taken from embryos at E8.75 (A-E), E10.5 (F-J), and E13.5 (K-O). In situ hybridization was performed with the anti-sense probes of *Flk1* (A, F, K), *Depp* (B, G, L), *Cx37* (C, H, M), *Cx40* (D, I, N), and *IGFBP-5P* (E, J, O). *Flk1* is a pan-endothelial marker; *Depp*, *Cx37*, *Cx40* and *IGFBP-5P* are genes isolated in the differential screen. These genes are specifically expressed in the dorsal aorta (arrows) but not in cardinal

veins (arrowheads). *IGFBP-5P* is also expressed in the notochord from E8.75 to E13.5 (E, J, O; black arrowheads). *Depp* is expressed in most enodothelial cells of the dorsa aorta at E8.75 (B), but is down-regulated at E10.5 (G) and is completely absent at E13.5 (L). However, small, branched vessels from the dorsal aorta still express *Depp* at E10.5 (G, black arrowheads and white arrow, respectively) and at E13.5 (L, arrows). Scale bars, 100 μm.

Fig. 3. Alignment of DEPP mammalian orthologs and targeting of the *Depp* locus. (A) Amino acid sequences of mouse, human, rat, porcine, and bovine DEPP homologues are aligned. Putative serine- (green circle), threonine- (blue star), and tyrosine-phosphorylation (red rectangle) sites are marked over the mouse sequence. (B) Restriction maps of the wild-type (WT) *Depp* locus, the targeting vector, and the targeted locus. The targeting vector contains an *EGFP-Cre* fusion construct fused with the initiator codon of *Depp*, which replaces the whole coding region of *Depp* (white box). H indicates a *HindIII* restriction enzyme site. The FRT-flanked PGK-Neo selection cassette can be removed by FLPe mediated recombination through mating with *FLPe* transgenic mice. (C) Confirmation of homologous recombination of the targeting vector by Southern blotting. Genomic DNAs of the ES cells were digested with *HindIII*, and hybridized with 5' internal probe A and 3' external probe B (B). WT, wild-type locus (14 kb); TG, targeted locus (8.7 kb) (D) Genotyping of E11.5 progeny of a *Depp* heterozygote intercross. *Cre* primers reveal a 319 bp TG band; *Depp* wild-type primers, a 269 bp WT band.

Fig. 4. Arterial-specific and dynamic expression of *Depp* in the dorsal aorta and forelimb at E9.8 and E12.5. E9.8 (A-C, G-I) and E12.5 (D-F, J-L) $Depp^{EGFP\text{-}Cre/+}$; $ephrin\text{-}B2^{LacZ/+}$ embryos were sectioned and processed for double-label immunoflouresence staining with antibodies to GFP (Gothert et al.) and β-galactosidase (red) to visualize *ephrin-B2* expression. Samples were analyzed by confocal microscopy. *Depp* expression in the dorsal aorta (A-F) is quite different from that in the forelimb (G-L). Note that *Depp* is down-regulated by E12.5 in the dorsal aorta (D-F), but is maintained in E12.5 forelimb (J-L). Note also that all $Depp^{+}$ endothelial cells are $ephrin\text{-}B2^{+}$. Arrows indicate dorsal aorta and black arrowheads indicate cardinal veins. E9.8 (M-O, S-U) and E12.5 (P-R, V-X) $Depp^{EGFP\text{-}Cre/+}$; *Rosa26R* embryos were processed for double-labeling with antibodies to GFP (Gothert et al.) and β-galactosidase (red) to visualize cells where *Depp-Cre* mediated recombination occured. Note that most endothelial cells in the dorsal aorta at E9.8 (M-O) and E12.5 (P-R) are β-galactosidase^{+}, indicating *Depp-Cre* mediated recombination. Scale bars, 100 μm.

Fig. 5. *Depp* expression in adult organs, P5 retina, tumors and wounded skin. (A-X) *Depp* expression in various organs and tissues from $Depp^{EGFP\text{-}Cre/EGFP\text{-}Cre}$ mice was revealed by double-labeling with antibodies to GFP (Gothert et al.) and PECAM-1 (red). The expression pattern from $Depp^{EGFP\text{-}Cre/+}$ mice is similar to, but weaker than the one from $Depp^{EGFP\text{-}Cre/EGFP\text{-}Cre}$ mice; therefore, expression data from $Depp^{EGFP\text{-}Cre/EGFP\text{-}Cre}$ mice were used. Lewis lung carcinoma cells (S-T) were subcutaneously injected, and B16F10 melanoma cells (U-V) were intraperitoneally injected. Two weeks later, the tumors were collected for immunohistochemistry. (W-X) Skin was removed using a

dermal 6 mm biopsy punch, and 7 days later, the wounded skin was excised for immunohistochemistry. Scale bars, 100 μm.

Fig. 6. *Depp* expression in E9.8 heart. (A-I) Triple-labeling with antibodies to GFP (A, D, G; green), PECAM-1 (B, E, H; red), and α-SMA (C, F, I; red) on the sections of E9.8 $Depp^{+/-}$ heart reveals that *Depp* is expressed in a subset of OFT endocardial cells (G, H; arrow) but not in atrial and ventricular endocardial cells (A, B, D, E). *Depp* is expressed in a subset of myocardial cells of both outflow tracts (G, I; arrowheads) and ventricles (A, C, D, F). (J-R) Triple-labeling with antibodies to β-galactosidase (J, M, P; green), PECAM-1 (K, N, Q; red), and α-SMA (L, O, R; red) on the sections of E9.8 $Depp^{EGFP\text{-}Cre/+}$*; Rosa26R* heart reveals that *Depp-Cre* mediated recombination is not observed in atrial and ventricular endocardial cells (J, K, M, N) but is observed in OFT endocardial cells (P, Q, arrows). Furthermore, *Depp-Cre* mediated recombination is observed in both OFT (R, arrow) and ventricular (O) myocardial cells. Unexpectedly, recombination occurs in the myocardial cells of trabeculated ventricles (J, L, M, O; arrowheads) and atria (J, L; arrows). Scale bars, 100 μm.

Fig. 7. *Depp* is not essential for blood vessel development. Whole-mount anti-PECAM-1 immunoperoxidase staining reveals no obvious differences between $Depp^{+/+}$ and $Depp^{-/-}$ embryos in E9.5 head (A, B), trunk (C, D), and yolk sac (E, F), or in E15.5 forelimb (L, J), stomach (K, L), and intestine (M, N). Double labeling with antibodies to α-SMA (Gothert et al.) and PECAM-1 (red) also reveals no evident difference between wild-type

and homozygous mutant embryos in E9.5 heart (G, H) and P5 retina (O, P). Scale bars, 300 μm.

Fig. 8. *Depp* is not essential for arterial differentiation, proliferation or apoptosis. (A, B) E9.5 *Depp*$^{+/-}$*;ephrin-B2*$^{LacZ/+}$ and *Depp*$^{-/-}$*;ephrin-B2*$^{LacZ/+}$ embryos stained with anti- β-galactosidase antibody to visualize *ephrin-B2* expression. *Ephrin-B2* is normally expressed in the dorsal aorta of a *Depp* mutant embryo (B, arrow). (C-F) E9.5 *Depp*$^{+/-}$ and *Depp*$^{-/-}$ embryos were processed for immunolabeling with antibodies to Cx40 (C, D) and α-SMA (E, F). Cx40 and α-SMA are normally expressed in the mutant embryo. Arrows indicate dorsal aorta and arrowheads indicate cardinal veins. (G-J) Double labeling with antibodies to GFP (Gothert et al.) and phospho-histone3 (PH3) (red) reveals no difference in proliferation between *Depp*$^{+/-}$ and *Depp*$^{-/-}$ embryos. Note that few GFP^{+} endothelial cells in limbs (G, H) are positive for PH3 and none in tails (I, J) are PH3^{+}. (K, L) Double-labeling with TUNEL (Gothert et al.) and anti-PECAM-1 antibody (red) reveals no difference in apoptosis between *Depp*$^{+/-}$ and *Depp*$^{-/-}$ embryos. Note that most endothelial cells are negative for TUNEL labeling. Scale bars, 100 μm.

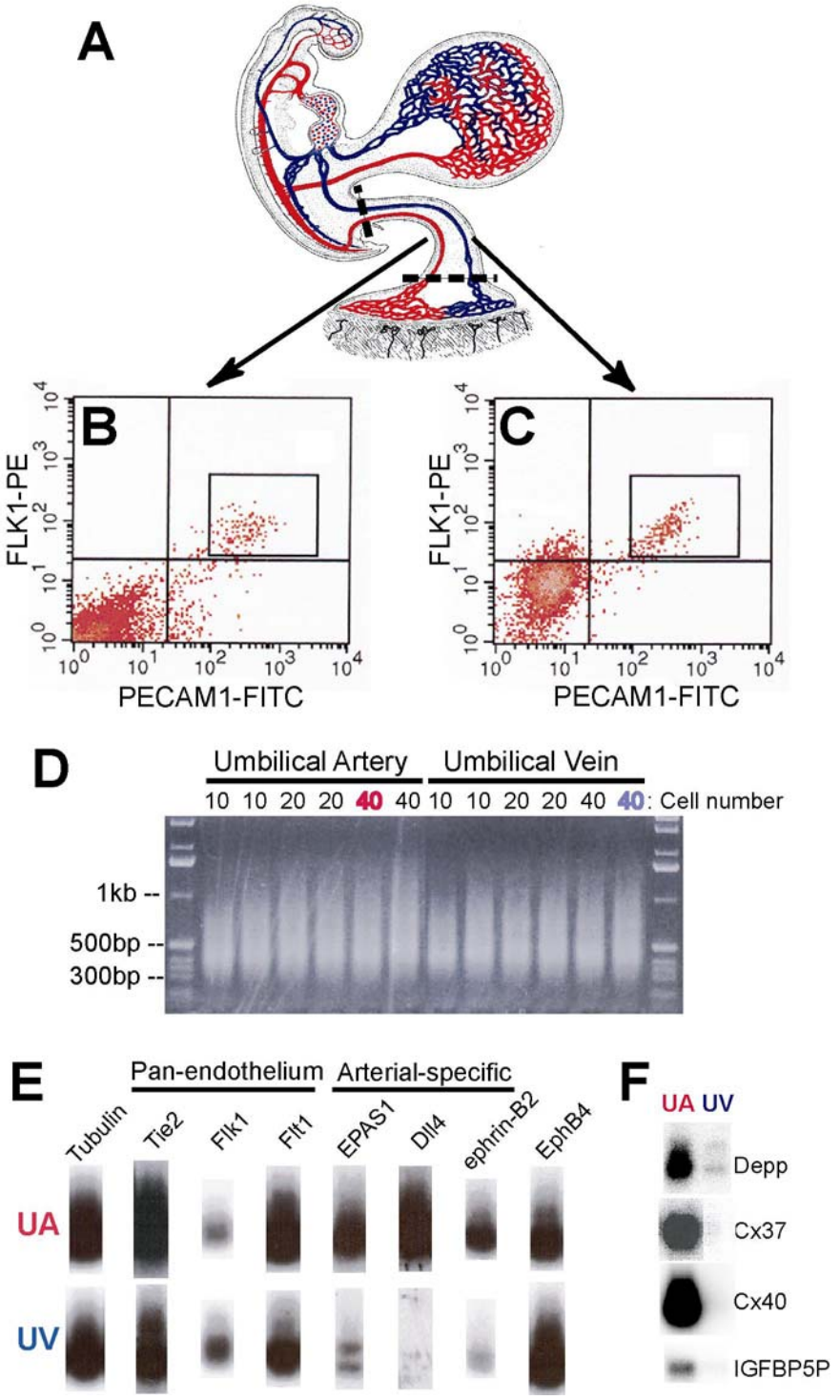

Fig. 1. Screening procedure to identify arterial-specific genes

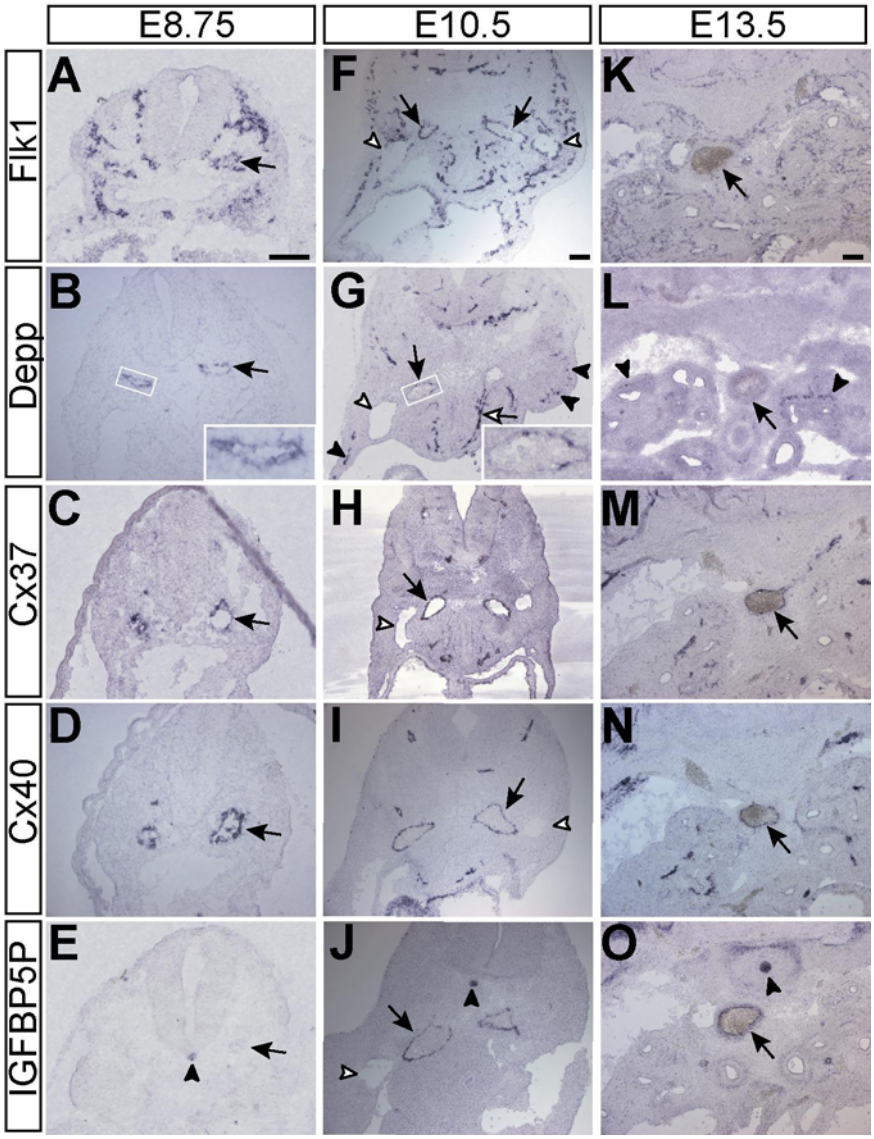

Fig. 2. Expression patterns of arterial-specific genes confirmed by in situ hybridization

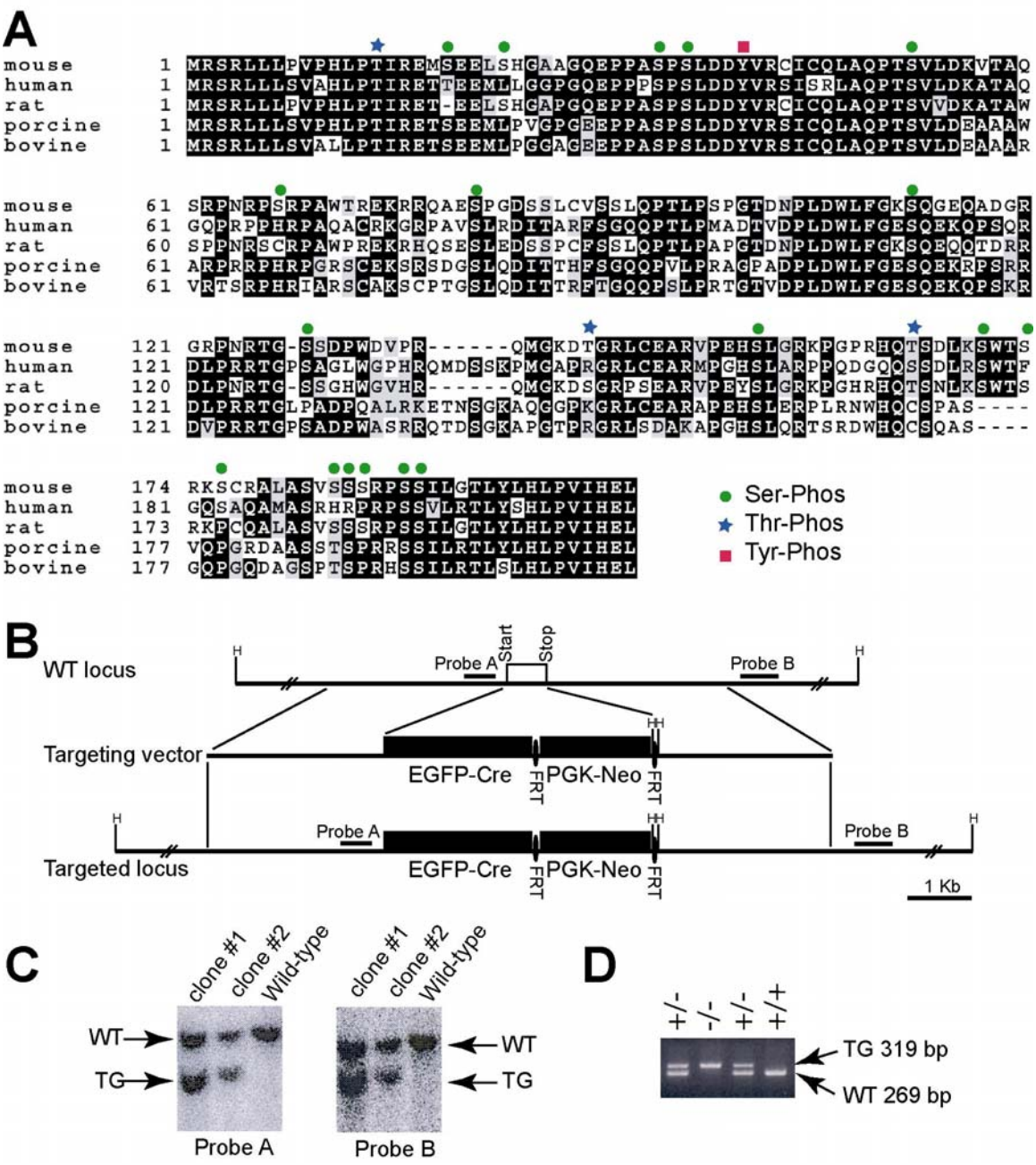

Fig. 3. Alignment of DEPP mammalian orthologs and targeting of the *Depp* locus

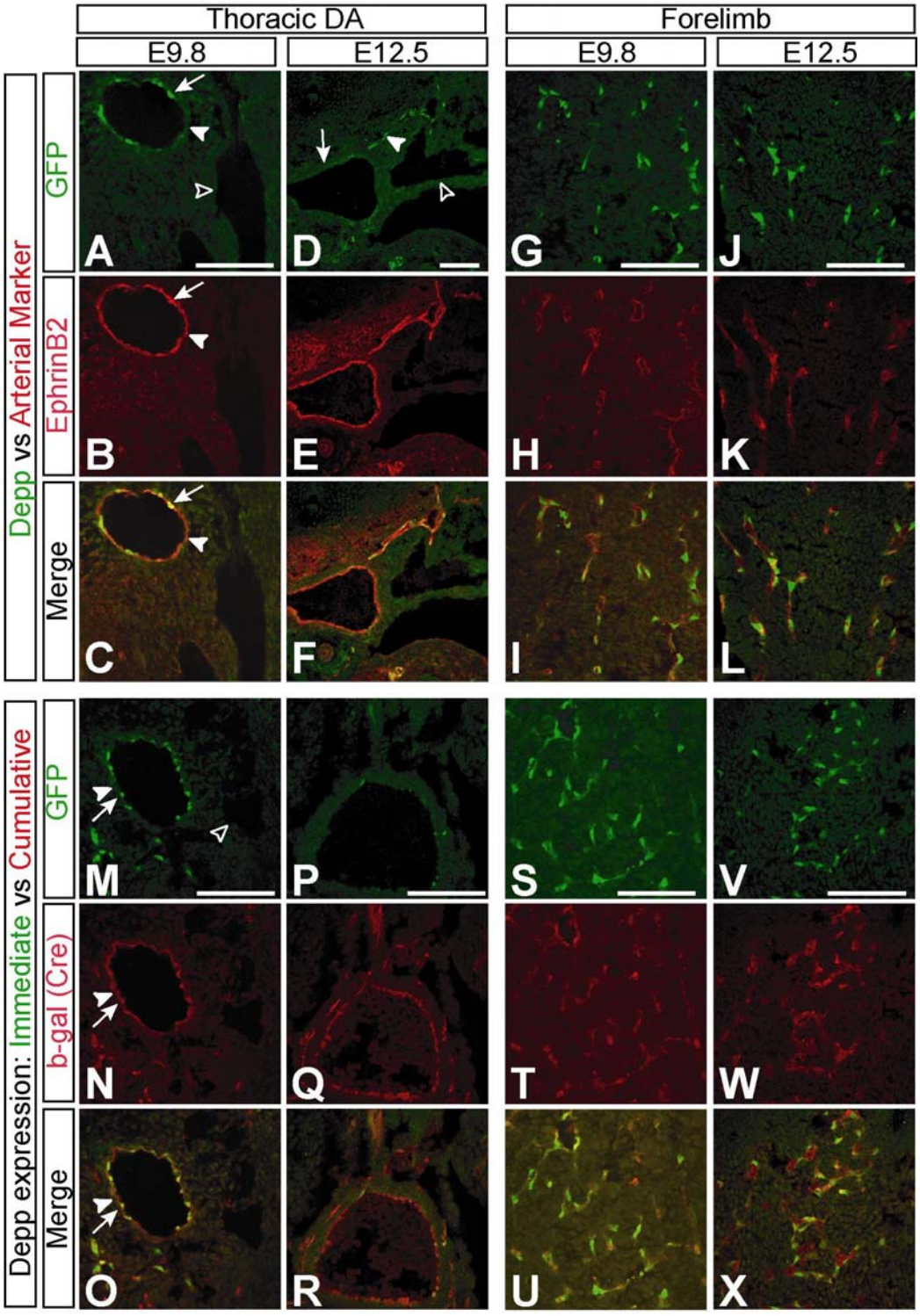

Fig. 4. Arterial-specific and dynamic expression of *Depp* in the dorsal aorta and forelimb at E9.8 and E12.5

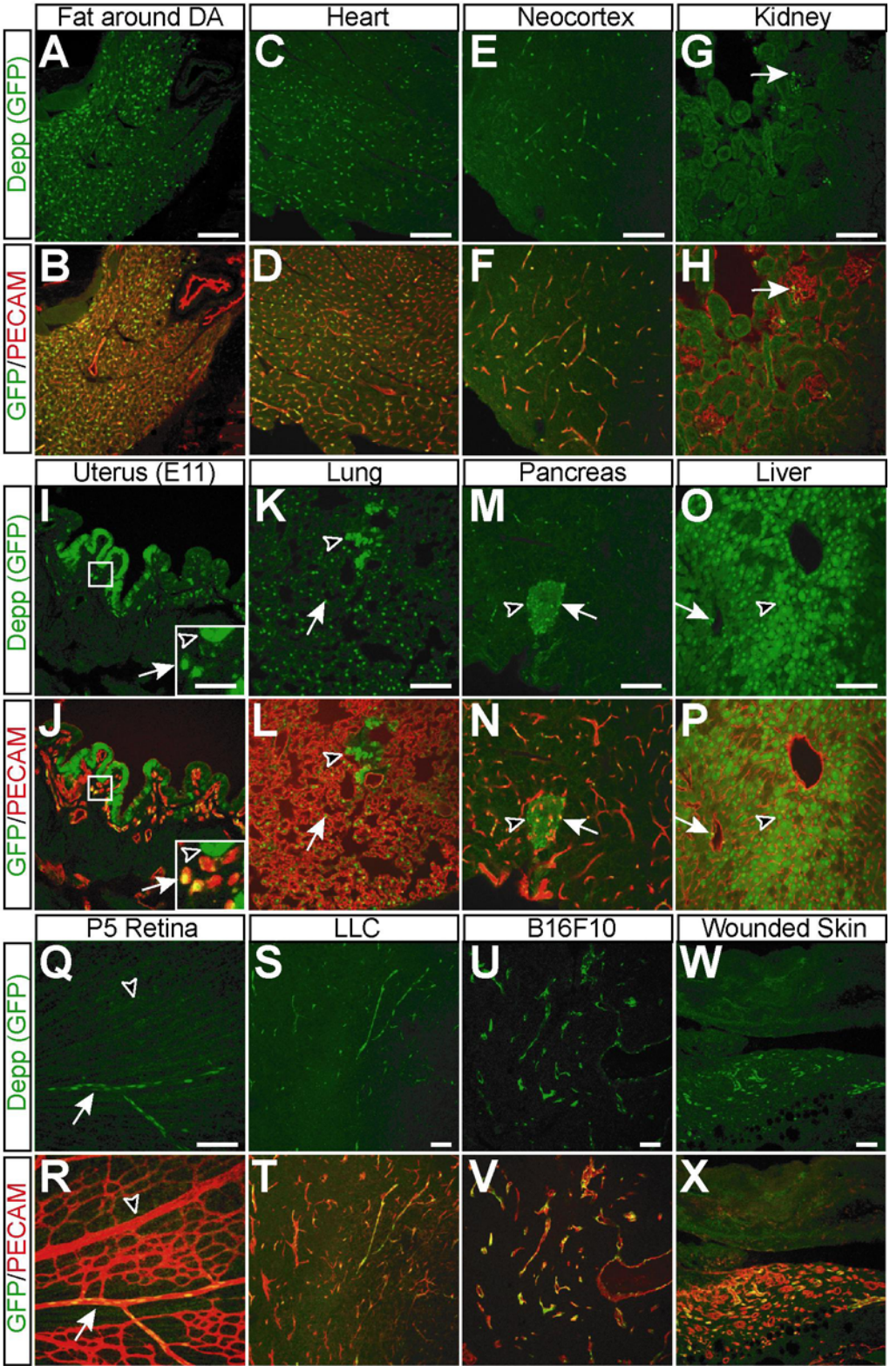

Fig. 5. *Depp* expression in adult organs, P5 retina, tumors and wounded skin

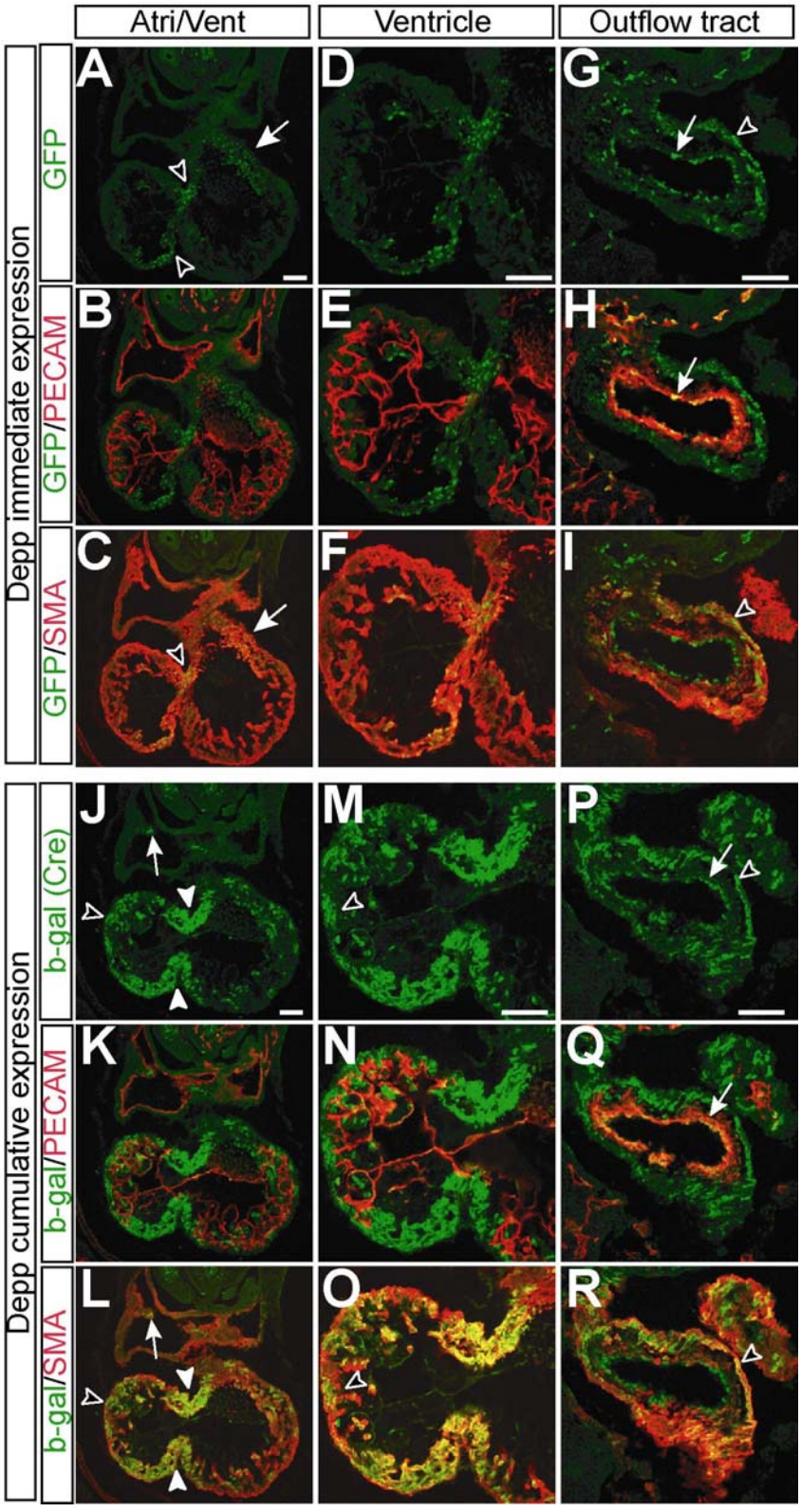

Fig. 6. *Depp* expression in E9.8 heart

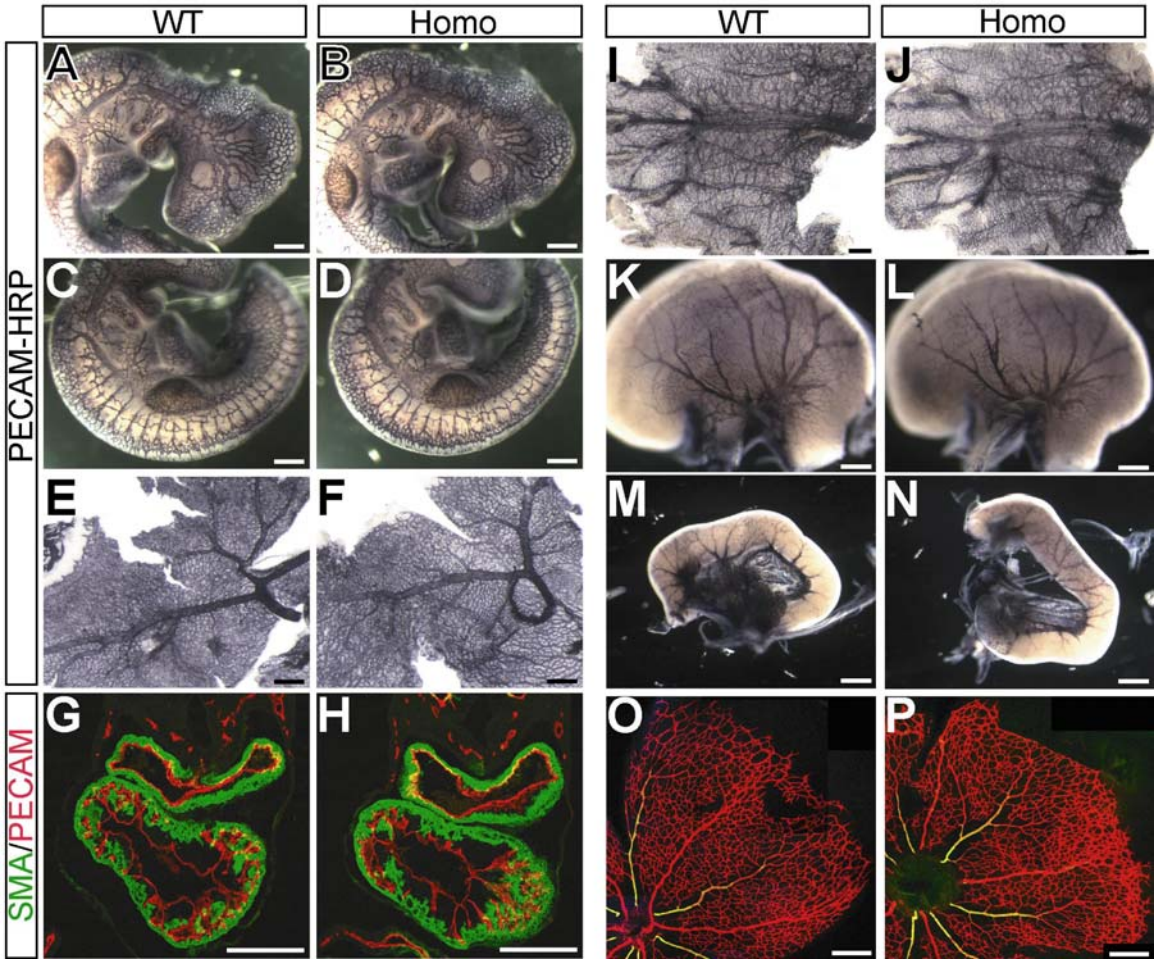

Fig. 7. *Depp* is not essential for blood vessel development

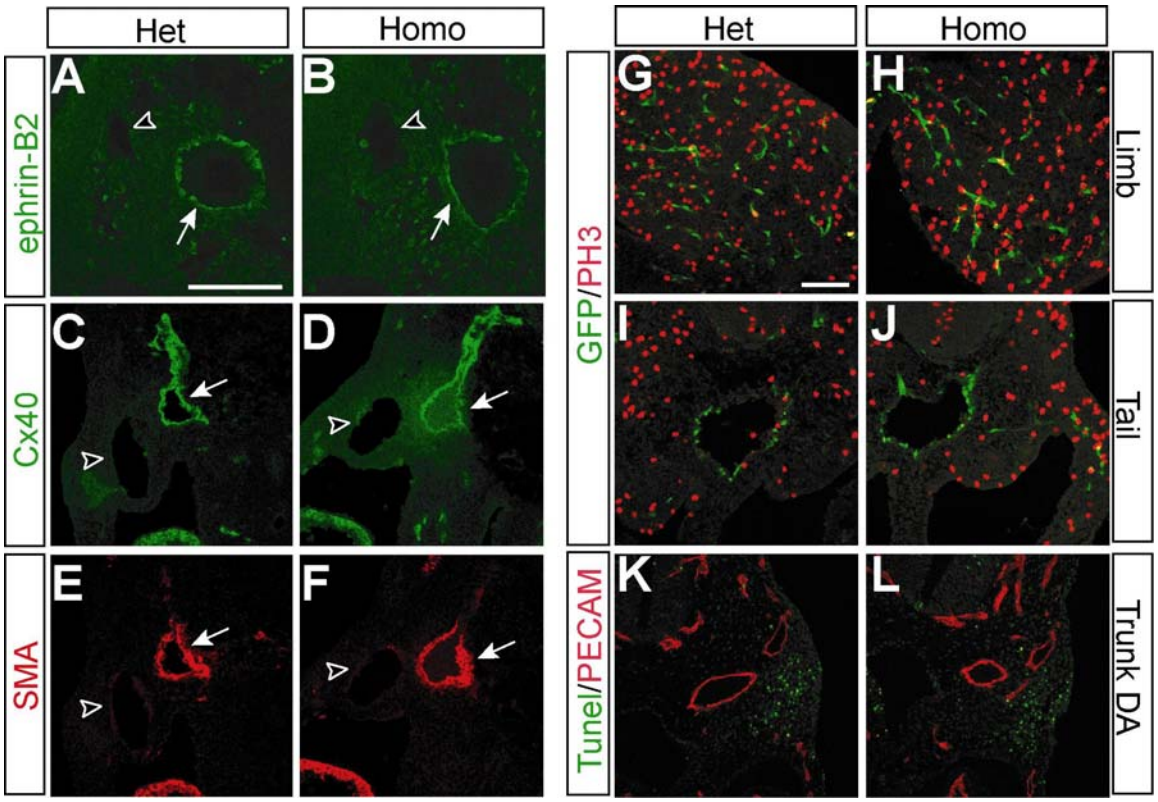

Fig. 8. *Depp* is not essential for arterial differentiation, proliferation or apoptosis