A null mutation of *Hhex* results in abnormal cardiac development, defective vasculogenesis and elevated Vegfa levels

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Summary

The homeobox gene *Hhex* has recently been shown to be essential for normal liver, thyroid and forebrain development. *Hhex*$^{-/-}$ mice die by mid-gestation (E14.5) and the cause of their early demise remains unclear. Because *Hhex* is expressed in the developing blood islands at E7.0 in the endothelium of the developing vasculature and heart at E9.0-9.5, and in the ventral foregut endoderm at E8.5-9.0, it has been postulated to play a critical role in heart and vascular development. We show here, for the first time, that a null mutation of *Hhex* results in striking abnormalities of cardiac and vascular development which include: (1) defective vasculogenesis, (2) hypoplasia of the right ventricle, (3) overabundant endocardial cushions accompanied by ventricular septal defects, outflow tract abnormalities and atrio-ventricular (AV) valve dysplasia and (4) aberrant development of the compact myocardium. The dramatic enlargement of the endocardial cushions in the absence of *Hhex* is due to decreased apoptosis and dysregulated epithelial-mesenchymal transformation (EMT). Interestingly, vascular endothelial growth factor A (Vegfa) levels in the hearts of *Hhex*$^{-/-}$ mice were elevated as much as three-fold between E9.5 and E11.5, and treatment of cultured *Hhex*$^{-/-}$ AV explants with truncated soluble Vegfa receptor 1, sFlt-1, an inhibitor of Vegf signaling, completely abolished the excessive epithelial-mesenchymal transformation seen in the absence of *Hhex*. Therefore, *Hhex* expression in the ventral foregut endoderm and/or the endothelium is necessary for normal cardiovascular development in vivo, and one function of *Hhex* is to repress Vegfa levels during development.

Key words: Homeobox, Transcription factor, Cardiac morphogenesis, Repressor, Vasculogenesis, Endocardial cushion, Epithelial-mesenchymal transformation, Vegf

Introduction

Hhex, as well as its highly conserved human and chick homologues, *HHEX* and *Prh*, was first cloned in 1992 (Bedford et al., 1993; Crompton et al., 1992; Hromas et al., 1993). *Hhex* is a proline-rich homeobox gene that functions as both a transcriptional repressor (Brickman et al., 2000; Guiral et al., 2000; Ho et al., 1999; Pellizzari et al., 2000; Tanaka et al., 1999) and activator (Denson et al., 2000a), and has been mapped to chromosome 10 in the human and 19 in the mouse (Ghosh et al., 1999).

Hhex is first expressed in cells that contribute to the murine cardiovascular system at E7.0, when it is detected in the extraembryonic mesoderm in a narrow band of cells within the nascent blood islands of the visceral yolk sac (Ghosh et al., 2000; Thomas et al., 1998). The blood islands are the regions of the embryo where primitive erythrocyte and blood vessel formation is initiated in a bipotential cell called the hemangioblast (Choi et al., 1998). Expression of *Hhex* in these cells is transient and is downregulated upon differentiation into endothelial cells. In the embryo proper, at the neural plate stage, *Hhex* is expressed in proximolateral mesoderm, a site where angioblasts are thought to arise (Coffin et al., 1991; Coffin and Poole, 1991) and a domain that includes tissue fated to form heart (Thomas et al., 1998). During headfold formation (E8.0), expression is seen in the ventral foregut endoderm adjacent to the heart and in endocardial cells of the developing cardiac tubes, but not in the intervening myocardial cell layer. Expression in the endocardium persists through E10.5. Additionally *Hhex* is expressed in regions where definitive vessels are known to form and in a pattern consistent with coalescing endothelial progenitors, such as the dorsal aortae, or the sprouting of new vessels (e.g. intersomitic vessels), suggesting that *Hhex* may participate in the initial phases of both vasculogenesis and angiogenesis. *Hhex* expression in the angioblasts of early blood

vessels is transient and downregulated as angioblasts differentiate into endothelial cells. Interestingly, *Hhex* expression in the endocardium persists longer than in endothelial cells of developing blood vessels, prompting the speculation that Hhex plays additional roles in cardiac development separate from its role in endothelial progenitors. This endothelial expression pattern is conserved across species as shown from studies in the frog (Newman et al., 1997), chick (Yatskievych et al., 1999) and zebrafish (Liao et al., 2000).

In addition to the expression data outlined above, there are functional data to suggest that *Hhex* plays a role in vascular development. Overexpression of *Xhex* in *Xenopus* results in an increased number of ectopic prevascular cells and a disorganization of vascular structure (Newman et al., 1997). Gain-of-function studies in which *Hhex* was ectopically expressed in zebrafish led to premature or ectopic expression of endothelial (and erythroid) genes, and *Hhex* could restore the expression of endothelial and blood genes in *cloche* mutants, a zebrafish mutation that affects early endothelial and blood cell differentiation (Liao et al., 2000). Interestingly, endothelial gene expression was not altered in *Hhex* loss-of-function mutants. *Hhex* and *scl*, another transcription factor important for both endothelial and hematopoietic development, can cross-regulate each other.

Gene targeting experiments have confirmed a critical role for *Hhex* in many developmental processes. Mice homozygous for a disruption of the *Hhex* gene die at mid-gestation (E13.5-E15.5) and have defects in forebrain, thyroid, monocyte and liver development (Keng et al., 2000; Martinez Barbera et al., 2000) (C. W. Bogue, unpublished). Recent data from our laboratory indicate that disruption of the *Hhex* gene also leads to a profound block in B-cell development (Bogue et al., 2003). In addition, investigators using in vitro $Hhex^{-/-}$ embryonic stem (ES) cell differentiation, in vivo yolk sac hematopoietic progenitor assays, and chimeric mouse analysis, found that *Hhex* is required for differentiation of the hemangioblast to definitive embryonic hematopoietic progenitors and, to a lesser extent, endothelial cells (Guo et al., 2003). Previous reports of *Hhex* mutant mice do not include any mention of cardiovascular abnormalities. In this report, we show that a null mutation of *Hhex* leads to profound abnormalities in vasculogenesis and cardiac morphogenesis. In addition, we show that a null mutation in *Hhex* results in elevated cardiac vascular endothelial growth factor A (Vegfa) levels that are responsible, in part, for the phenotype that we observe.

Materials and methods

Generation of $Hhex^{-/-}$ mice

The *Hhex* targeting construct and heterozygous ES cells were generated as previously described (Bogue et al., 2003). Two heterozygous clones were injected into C57BL/6J blastocysts to generate chimeric mice. Chimeric mice from one clone transmitted the mutant allele in the germ line, and these mice were interbred to produce $Hhex^{+/-}$ as well as $Hhex^{-/-}$ mice. The mice have not been fully backcrossed, thus all mice examined are on a mixed C57BL/6J and 129SVJ background. All $Hhex^{+/+}$ mice used for comparison were paired littermates of the $Hhex^{-/-}$ mice and therefore of this same mixed genetic background.

PCR genotyping

Newborn mice and embryos were genotyped by multiplex polymerase chain reaction (PCR) or Southern blot of DNA samples prepared from tails or yolk sacs using primers specific for the wild-type and targeted alleles (Hogan et al., 1994). Primer sequences are as follows: 5′ wild-type primer – agacgcaccaccatcaattt; 5′ targeted primer – ccacacgcgtcaccttaata; 3′ common primer – ccctgtagcggtgagaagag (all primer sequences are 5′-3′). Samples were amplified for 30 cycles (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute). PCR amplification of the *Hhex* wild-type allele gave a product 323 bp in size, and amplification of the targeted allele produced a band of 409 bp in size.

Morphological and histological analysis

Whole-mount embryos were photographed on a Nikon stereomicroscope. For histology, embryos were fixed overnight at 4°C in 4% paraformaldehyde, cryoprotected by incubation in 30% sucrose overnight, then frozen in Tissue-Tek in liquid nitrogen and sectioned (10 μm). Sections were stained with Hematoxylin and Eosin. For all analyses, at least 3-5 wildtype (littermates of $Hhex^{-/-}$ embryos) and $Hhex^{-/-}$ embryos were examined.

Immunohistochemistry

Immunohistochemistry of whole embryos and sections was performed using standard procedures (Hogan et al., 1994; Urness et al., 2000). Primary antibodies were used as follows: anti-αSM actin (α-SMA) (Sigma) at 1:500 dilution, anti-CD31 [platelet endothelial cell adhesion molecule (PECAM)] (Pinter et al., 1999) at 1:500 dilution, anti-Vegf (A-20, sc-152 goat, Santa Cruz) at 1:500 dilution, and anti-cytokeratin (Dako) at 1:500 dilution.

Cell proliferation assay and apoptosis (TUNEL) assays

10 μm frozen sections were prepared as described above. Adjacent sections of each specimen were collected and used for assays of cell proliferation and apoptosis. Cell proliferation was assessed by performing immunohistochemistry with anti-phospho-histone H3 (Ser28) (Upstate Biotechnology) and apoptosis was assessed using the TUNEL technique (ApopTag, Molecular Probes). Serial sections through the entire AV cushion were examined and the section with the most positive cells was used for quantitation. The number of phosho-histone H3- or TUNEL-positive cells/AV cushion/section was counted from three $Hhex^{-/-}$ and three $Hhex^{+/+}$ hearts. The data were analyzed statistically using the Student's *t*-test and significance was set at $P=0.05$.

Vegfa ELISA assay

Whole hearts were isolated from E9.5-E13.5 $Hhex^{+/+}$ and $Hhex^{-/-}$ embryos, sonicated in PBS and Vegfa levels were assayed using the mouse Vegfa ELISA detection kit QuantikineM (R&D Systems, Minneapolis, MN, USA). Results are expressed as (pg Vegfa)/(μg protein), $n=5$ hearts for each age. Each value was determined in duplicate. Means were compared using Student's *t*-test.

Atrio-ventricular canal endocardial cushion explant culture

Atrio-ventricular (AV) explant cultures were performed as described (Enciso et al., 2003). Briefly, the AV canal and ventricle (AV explant) from E10.5 mice with >28 somites were placed on rat tail type I collagen (Fisher, Collaborative Biomedical) gels which were hydrated for a minimum of 1 hour with 100 μl of Medium 199 supplemented with 1% FBS, 100 u/ml penicillin, 100 μg/ml streptomycin, and 0.1% each of insulin, transferrin, and selenium (GIBCO BRL). The AV explants were incubated at 37°C in 5% CO_2, and allowed to adhere for 6-8 hours; 100 μl of Medium 199 was then added to the AV explants and changed once daily. After 72 hours, the cultures were stopped and the explants were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, 10 mM PIPES (pH 6.8), 50 mM NaCl, 300 mM sucrose and 3 mM $MgCl_2$, and blocked overnight at 4°C in 3% BSA and 0.05% Tween 20 in PBS. They were then

incubated overnight with a 1:400 dilution of anti-α-SMA and a 1:500 dilution of anti-CD31. Explants were washed ten times with 0.2% BSA and 0.05% Tween 20 in PBS, then incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes), and then washes were repeated as above. Images were acquired using an Olympus confocal microscope.

To assess the effect of blocking Vegfa signaling on epithelial-mesenchymal transformation (EMT) in AV cushion explants, 25 μg/ml of the soluble murine recombinant Vegf receptor 1/IgG-Fc chimeric protein sFlt-1 [mFlt-(1-3)-IgG, a truncated Flt 1-3 Fc fusion protein (gift from Dr N. Ferrara, Genentech, San Francisco, CA) (van Bruggen et al., 1999)] was added to the culture medium of both $Hhex^{+/+}$ and $Hhex^{-/-}$ AV explants. The explants were cultured as noted above for up to 72 hours and then photographed. Immunohistochemistry using α-SMA and Pecam was performed as described above and images were acquired using an Olympus confocal microscope as described (Enciso et al., 2003).

For analysis of the effects of the addition of exogenous Vegf on EMT, AV cushion explants from wild-type mice with >28 somites were cultured in the presence or absence of either 10 pg/ml or 10 ng/ml recombinant mouse Vegf-A_{165} (CHEMICON International) for 72 hours (n=3 for each condition).

In order to quantify the extent of EMT in the explants, the ratio of the number of mesenchymal versus epithelioid-like cells was determined in four separate high-power fields/explant, as previously described (Enciso et al., 2003) and the mean values were compared using the Student's t-test with significance set at $P<0.05$.

Results

Hhex-deficient mice have multiple developmental defects, including marked abnormalities of heart and vascular development

To determine the function of *Hhex* in vivo, we generated a null allele using a targeting construct designed to delete exons 1 and 2 of the *Hhex* genomic sequence, which removed the transcription start site, translational start site and most of the homeobox as previously described (Bogue et al., 2003). Homozygous deletion of *Hhex* results in embryonic lethality, as evidenced by the fact that we have detected no live born $Hhex^{-/-}$ mice and that the ratio of $Hhex^{+/+}$:$Hhex^{+/-}$ in live born mice is 1:2 (data not shown). Genotyping of embryos at various gestational ages showed the expected ratios of wild-type:heterozygous:homozygous at E9.5. By 11.5, the number of homozygous offspring was less than expected, and there were no live $Hhex^{-/-}$ fetuses seen by E14.5. Analysis of $Hhex^{-/-}$ embryos from E9.5 to E14.5 reveals striking abnormalities in the development of the head/forebrain, liver, and thyroid (data not shown). Anterior truncations of the brain/head are apparent in a subset of $Hhex^{-/-}$ embryos with variability in the severity of the forebrain defect. In some embryos, the thyroid gland initially forms at E9.5, but subsequently degenerates and by E13.5 there is no thyroid gland present. These results are consistent with the $Hhex^{-/-}$ phenotype reported by Martinez-Barbera (Martinez Barbera et al., 2000) but differ in some aspects from the findings reported by Keng (Keng et al., 2000).

The cardiovascular abnormalities, which have not been previously reported, are apparent after E9.5 and consist of defects in heart septation, myocardium formation, and vasculogenesis. In many embryos, it appears that the defects in the cardiovascular system are the cause of death – many embryos have significant edema of the body as well as expanded, fluid-filled pericardial sacs. At E13.5, $Hhex^{-/-}$ embryos are edematous and contain markedly dilated blood vessels (Fig. 1). Compared to the $Hhex^{+/+}$ and $Hhex^{+/-}$ mice, $Hhex^{-/-}$ mice have marked hypoplasia of the right ventricle (RV), with a normally sized left ventricle (Fig. 1C,D). Transverse sections of $Hhex^{-/-}$ embryos confirmed the presence of a hypoplastic RV (Fig. 1F). In addition, there were several defects present that are associated with abnormalities of endocardial cushion formation and remodeling. First, there was a dramatic overabundance of endocardial cushion cells (ECCs) and cardiac jelly in both the AV cushion (AVC) and the endocardial cushion that forms the RV outflow tract (Fig. 1F,I). In some embryos, the excessive accumulation of ECC resulted in severe narrowing of the RV outflow tract (Fig. 1I). Abnormalities of the remodeling of the outflow tract endocardial cushion were evidenced by the invariable presence of a double outlet right ventricle (DORV) (Fig. 1I,J). However, we never observed the presence of a persistent truncus arteriosus, indicating that septation of the aorta and pulmonary artery is not affected. The abnormally large AVCs failed to condense and thin normally, resulting in dysplastic mitral and tricuspid valves (Fig. 1L). In some severely affected embryos, there was no evidence of atrioventricular valve formation at all (data not shown). Ventricular septal defects were seen in all embryos, which is further evidence that the AVC is not developing normally (Fig. 1O). A high-power view of the AVC in $Hhex^{-/-}$ embryos shows that the cells are mesenchymal in appearance, suggesting that they have undergone epithelial-mesenchymal transformation (EMT) (Fig. 1G).

Finally, the ventricular wall of the mutants is abnormally thin. In the wild-type heart at E13.5, the compact layer of the myocardium is approximately 6-8 cells thick and the cells are closely packed and oriented longitudinally around the heart (Fig. 1N). The trabecular layer is oriented perpendicular to the compact layer and is much thinner, being only 1-2 cells in thickness. In contrast, in the $Hhex^{-/-}$ hearts, the compact layer is only 2-3 cells thick and the cells are much more loosely packed, while the trabecular layer seems thicker and more cellular (Fig. 1P). In the most severely affected mutants, the myocardium appears to be composed almost entirely of the trabecular layer and it is not clear whether there is a compact layer at all (Fig. 1Q). Additionally, we used immunostaining with anti-cytokeratin to determine if the abnormal development of the myocardium was due to the absence of epicardium at E11.5. Like the wild-type hearts, the outer layer of the $Hhex^{-/-}$ hearts stained positive for cytokeratin, indicating the presence of the epicardial layer (Fig. 2). Thus, there is a significant defect in the formation and development of the myocardium in the absence of *Hhex* that is not due to the absence of the epicardium.

Increased proliferation and decreased apoptosis in endocardial cushions of $Hhex^{-/-}$ hearts

To elucidate the mechanism of the abnormal accumulation of ECCs in the hearts of $Hhex^{-/-}$ mice, we performed immunohistochemistry for PECAM and α-SMA and assessed the relative rates of both proliferation and apoptosis. In the endocardial cushions, there was no difference in the expression of either PECAM or α-SMA in the absence of *Hhex* (data not shown). The phenotype of the cells in the $Hhex^{-/-}$ AVC does not appear to be different from wild-type in that the cells are spindle-shaped and no longer express PECAM (Fig. 1Q and

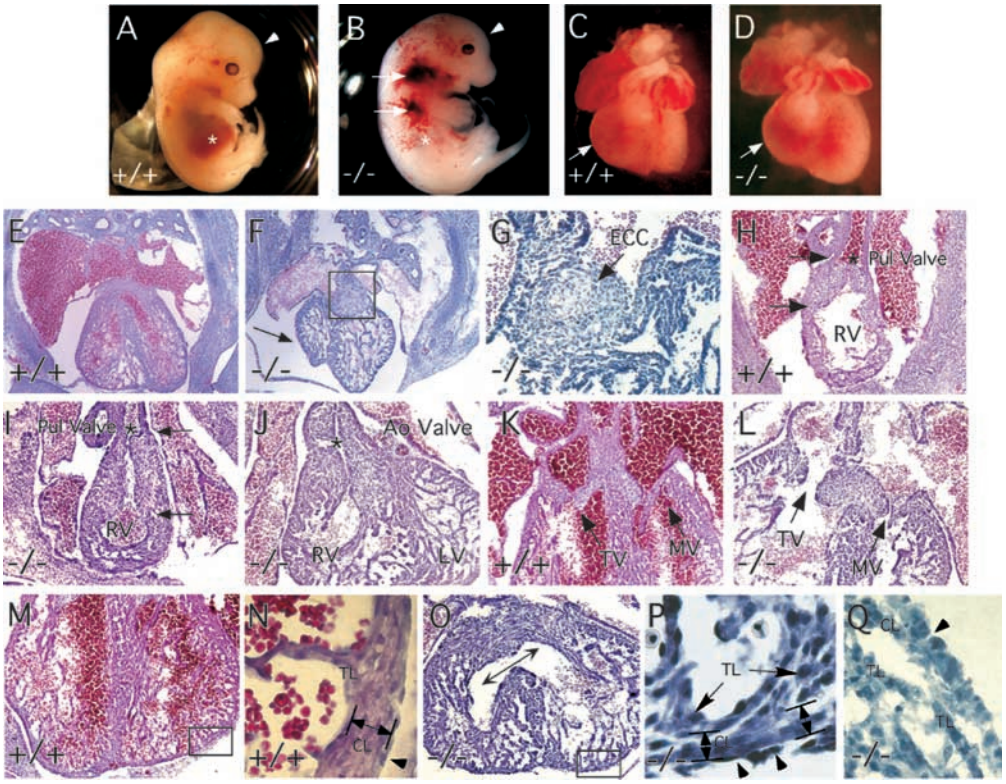

Fig. 1. *Hhex*$^{-/-}$ mice have multiple cardiovascular abnormalities. (A,B) 13.5 embryos and (C,D) whole hearts. (A,B) Compared to *Hhex*$^{+/+}$ embryos, *Hhex*$^{-/-}$ embryos have no livers (asterisks), forebrain truncations (arrowheads), and markedly dilated blood vessels (arrows in B). Hearts in *Hhex*$^{-/-}$ mice at E13.5 have a small right ventricle (arrows in C and D). (E-P) Transverse sections of E13.5 embryos. (E) Section of *Hhex*$^{+/+}$ heart inferior to outflow tracts showing normal morphology. (F) *Hhex*$^{-/-}$ heart with hypoplastic RV (arrow) and abnormal accumulation of ECCs (box). (G) Higher power view of boxed region in F reveals abnormal accumulation of cells in the atrio-ventricular cushion (AVC) region that are mesenchymal in morphology and represent ECCs. (H,I) RV outflow tract. (H) The *Hhex*$^{+/+}$ heart has a small region of ECCs just inferior to the pulmonic valve (asterisk). The arrows indicate the region of the RV outflow tract that is comprised of ECCs. (I) The *Hhex*$^{-/-}$ heart shows a marked increase in ECCs with subsequent narrowing of RV outflow tract. Arrows indicate the region of RVOT that is comprised of ECCs and the asterisk denotes the pulmonic valve. (J) A more caudal section of the same heart as in I showing the aortic valve (asterisk) communicating with RV, indicating the presence of DORV. (K,L) AV valve region showing abnormal accumulation of ECCs and dysplastic AV valves in *Hhex*$^{-/-}$ mice compared to wild-type mice. (M-Q) Ventricles of *Hhex*$^{+/+}$ and *Hhex*$^{-/-}$ hearts. In the *Hhex*$^{-/-}$ heart, a VSD (double arrow) is present and the myocardium is abnormally thin (O). High power view of boxed areas in (M) and (O) is shown in (N) and (P) and highlights the abnormally thin compact myocardial layer in *Hhex*$^{-/-}$ hearts. The thickness of the compact layer of the myocardium is indicated. Arrowheads point to epicardium. (Q) High power image through the myocardium of a severely affected embryo in which it is difficult to identify the presence any compact layer. RV, right ventricle; Ao, aorta; TV, tricuspid valve; MV, mitral valve; ECC, endocardial cushion cells; DORV, double outlet right ventricle; CL, compact layer; TL, trabecular layer.

data not shown), indicating they have undergone EMT. Interestingly, neither the *Hhex*$^{+/+}$ nor *Hhex*$^{-/-}$ ECCs continue to express α-SMA at E12.5 (data not shown). Thus, the abnormal accumulation of ECCs in *Hhex*$^{-/-}$ embryos is not due to an alteration in the temporal-spatial pattern of expression of either PECAM or α-SMA.

When assessed by phospho-histone H3 expression, the relative rate of cell proliferation in the *Hhex*$^{-/-}$ AV cushion cells at E13.5 was no different than in *Hhex*$^{+/+}$ embryos (Fig. 3C,D). In wild-type mice at E13.5, apoptosis occurs in AVCs as a part of the normal process of remodeling the AVC (Abdelwahid et al., 2002; Lakkis and Epstein, 1998). Using the TUNEL assay, we detected a 75% decrease in the number of apoptotic cells/cushion/section in *Hhex*$^{-/-}$ mice compared to wildtype (Fig. 3A,B). Thus, the large accumulation of ECC in *Hhex*$^{-/-}$ mice is accompanied by a marked decreased in the number of cells undergoing apoptosis while proliferation is unaffected.

Defective vascular development in *Hhex*$^{-/-}$ mice

We examined the development of the vasculature in *Hhex*$^{-/-}$ mice to determine if *Hhex* plays any role in vasculogenesis and/or angiogenesis. We found that, in *Hhex*$^{-/-}$ mice, blood vessel formation initiates normally, as there is no discernible difference in the vasculature between wild-type and mutant mice at E7.5-E8.5 when assayed by PECAM immunohistochemistry (data not shown). This indicates that vasculogenesis, the migration of angioblasts to discrete locations in the embryo, their differentiation into endothelial cells and coalescence into solid endothelial cords, and their formation into a primary capillary plexus (Conway et al., 2001; Risau, 1997) is not altered in the absence of *Hhex*.

The next stage of vasculogenesis involves extensive interaction of endothelial cells with themselves along with the recruitment and differentiation of mesenchymal cells into vascular smooth muscle cells (VSMCs) and pericytes (Conway

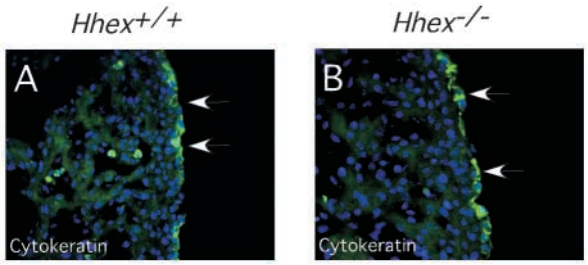

Fig. 2. The epicardium is present in *Hhex*$^{-/-}$ mice. Cross-sections through the ventricular region of E11.5 hearts from (A) *Hhex*$^{+/+}$ and (B) *Hhex*$^{-/-}$ mice. Immunohistochemistry for pan-cytokeratin was performed. Green staining and arrows indicates the cells that are cytokeratin-positive and therefore epicardial cells.

et al., 2001; Risau, 1997). At E9.5-E10.0, abnormalities of vascular development are first apparent in *Hhex*$^{-/-}$ mice and are manifested as disorganization of the developing cranial vasculature (data not shown). At this age, the abnormalities are subtle. By E11.5, abnormalities of vasculogenesis are quite dramatic (Fig. 4). The vessels of *Hhex*$^{-/-}$ mice appear larger, are disorganized, and there is a profusion of small finely branched vessels. This appears to be a generalized phenomenon and is not limited to any specific region of the embryo. By E13.5, the abnormal vascular phenotype is quite dramatic and is characterized by vascular structures with markedly dilated lumens. Examples include massive enlargement of the internal jugular vein (Fig. 5B), intercostal vessels (Fig. 5D), and vessels in the septum transversum mesenchyme, which normally form the hepatic and portal vessels and sinusoids (Fig. 5F). All of the enlarged vessels were lined with a layer of endothelial cells as assessed by histology and confirmed by staining with PECAM antibody (data not shown). This further demonstrates that *Hhex* is not required for differentiation of endothelial cells, as was previously demonstrated (Martinez Barbera et al., 2000), but is necessary for vessel remodeling and stabilization.

In order to assess VSMC development, we stained embryos with an antibody to α-SMA. At E9.5 and E10.5, there were subtle abnormalities in the appearance of the vasculature characterized by decreased staining of the vasculature, especially the cranial vasculature, in *Hhex*$^{-/-}$ compared to controls (data not shown). However, by E11.5 there were striking differences in VSMC development in *Hhex*$^{-/-}$ embryos. In *Hhex*$^{+/+}$ embryos, VSMCs were present in the dorsal aorta, in intersomitic vessels, in branchial vessels and extending rostrally into the carotid vessels and smaller vessels of the head (Fig. 6A,D,G). In contrast, there was poor VSMC formation in *Hhex*$^{-/-}$ embryos at E11.5 (Fig. 6B,C,E,F,H,I). All *Hhex*$^{-/-}$ embryos showed decreased or delayed VSMC formation in the cranial vasculature, dorsal aorta, intersomitic and branchial vessels, although there was variability in the severity of the VSMC defects. In some embryos, α-SMA staining was present but decreased, and the pattern of expression was more irregular and punctate than in wild-type embryos. The investiture of cranial vessels with VSMC in some *Hhex*$^{-/-}$ embryos was markedly abnormal and correlated with the severity of the forebrain defects. In fact, embryos with the most severe forebrain phenotype had complete absence of VSMCs in the cranial vessels at E11.5 (Fig. 6F). The finding of variability in the severity of the forebrain defect is consistent with a previous report of a null mutation of *Hhex* (Martinez Barbera et al., 2000). However, since the *Hhex*$^{-/-}$ mice are on

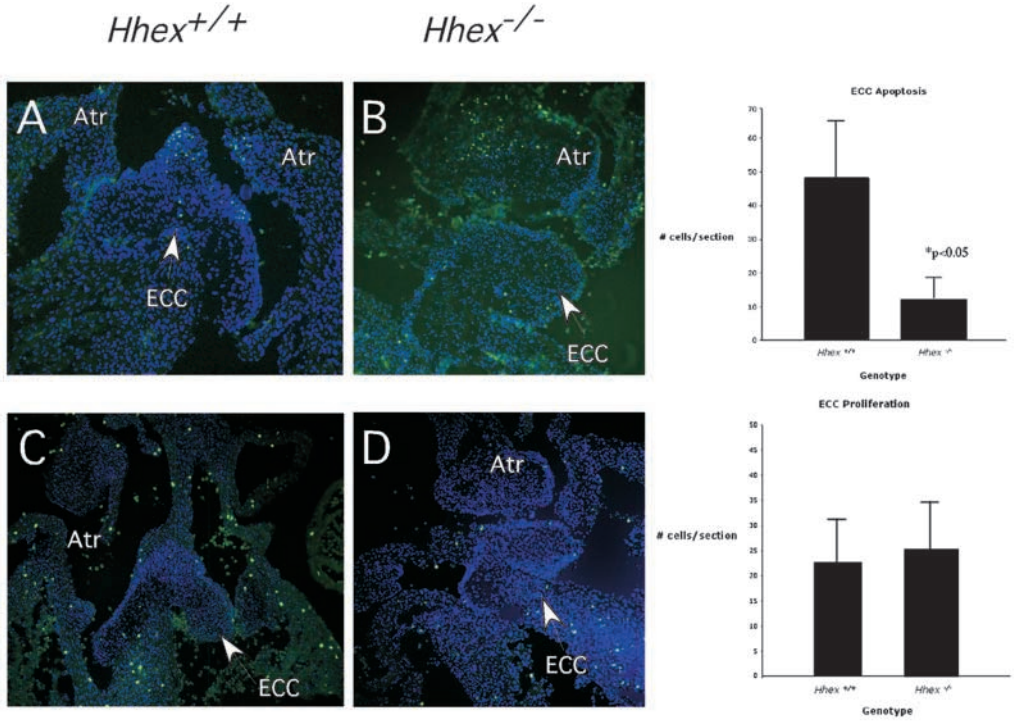

Fig. 3. AV cushion apoptosis is decreased in *Hhex*$^{-/-}$ mice. ECC apoptosis is decreased by 75% in the absence of *Hhex* (representative panels shown in A and B) while proliferation is unchanged (representative panels shown in C and D). Composite data are shown in the graphs on the far right (*n*=3 animals for each genotype). Bars show means+s.e.m. *n*=3 for each phenotype. ECC, endocardial cushion cells; Atr, atrium.

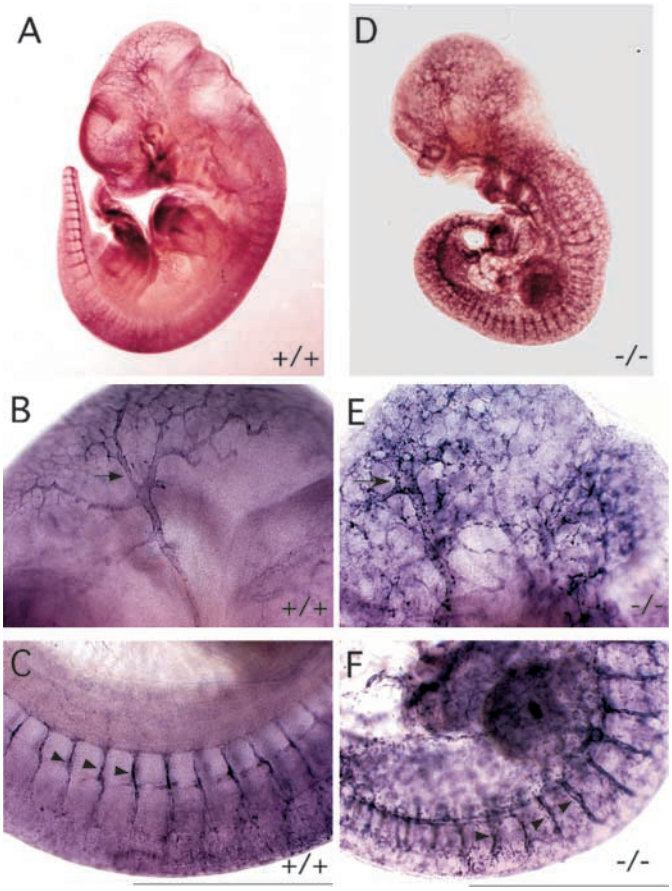

Fig. 4. *Hhex*$^{-/-}$ embryos have defective angiogenesis. (A,D) Low-power view of whole E11.5 *Hhex*$^{+/+}$ and *Hhex*$^{-/-}$ embryos stained with Pecam antibody. *Hhex*$^{-/-}$ embryos have a striking profusion of small vessels and disorganization of large vasculature throughout the whole embryo. (B,E) High-power view of cranial vascular detected with PECAM antibody. Note the disorganization of large vessels and the multiple ectopic small vessels that are present. Visualization of various focal planes indicates that the punctate staining represents small vessels branching off the larger vessels that are not in the plane of focus. Arrows indicate the same cranial vessel in each embryo for comparison. (C,F) The intersomitic vessels are also enlarged and have abnormal small branches (arrowheads).

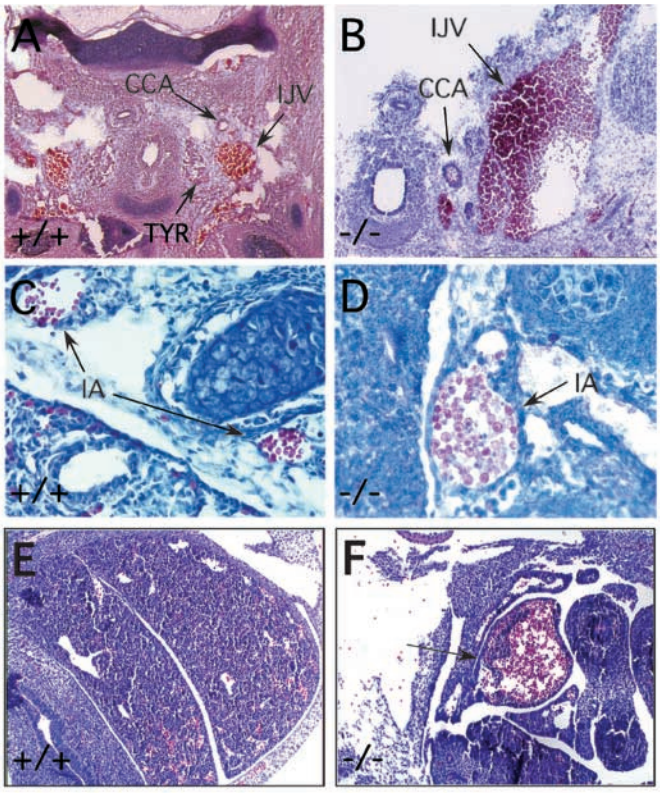

Fig. 5. Vascular abnormalities in *Hhex*$^{-/-}$ mice at E13.5. (A,B) Transverse sections through the neck region of E13.5 embryos show massive dilation of the internal jugular vein in *Hhex*$^{-/-}$ embryo compared to wild-type embryo. A and B are shown at the same magnification. Other examples of abnormal vascular structures include enlarged intercostal vessels (arrows) (C,D) and massive vascular lake (arrow) in the region of the septum transversum (F) in place of normal liver tissue and vascular structures (E). CCA, common carotid artery; IJV, internal jugular vein; TYR, thyroid; IA, intercostal artery.

a mixed genetic background (C57Bl/6J:129SVJ) we cannot exclude the possibility that the variability in phenotype is due to the mixed genetic background. It is unlikely that the phenotypic variability is due solely to mixed genetic background because Bort examined *Hhex*$^{-/-}$ mice with a similar phenotype after completely backcrossing them onto the C57Bl/6J strain, and found that the phenotypic variability of the forebrain defect persisted (Bort et al., 2004). Nonetheless, these data demonstrate that the absence of *Hhex* results in defective vasculogenesis.

Cardiac Vegfa levels are elevated in the absence of *Hhex*

The combination of dilated vascular structures, ventricular septal defects (VSD), defective outflow tract septation, thin compact myocardium, and abnormal endocardial cushion/AV valve development is reminiscent of mice in which Vegfa is constitutively overexpressed during embryogenesis (Miquerol et al., 2000). Interestingly, previous work from our lab and others has shown that Hhex and Vegfa are expressed in the same populations of cells during early heart development. This includes foregut endoderm at E8.0-8.5, endocardium at E8.5-9.5 and the endothelium of the cardiac outflow tract from E9.5-14.5 (Bogue et al., 2000; Miquerol et al., 1999; Thomas et al., 1998) and suggests the possibility of an interaction between these two genes. Therefore, we determined if Vegfa levels were elevated in *Hhex*$^{-/-}$ embryos. Using an ELISA to quantitate Vegfa levels, we found that between E9.5 and E12.5, Vegfa levels were significantly elevated in the hearts of *Hhex*$^{-/-}$ embryos compared to control embryo hearts (Fig. 7). During the early stages of cardiac morphogenesis and vascular development (E9.5-E10.5), the Vegfa levels were 2-3-fold higher in *Hhex*$^{-/-}$ embryos, levels that have previously been determined to be responsible for cardiovascular developmental defects (Miquerol et al., 2000). This indicates that *Hhex*, either directly or indirectly, normally represses Vegfa levels in the developing mouse.

To determine if the spatial domain, as well as the level, of Vegfa expression is altered in the absence of *Hhex*, we

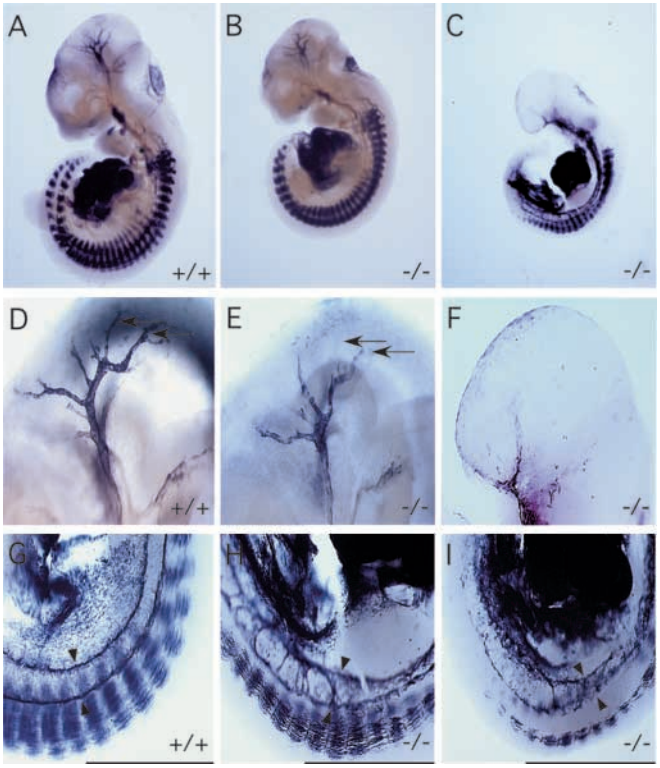

Fig. 6. The defect in angiogenesis in *Hhex*$^{-/-}$ mice includes abnormal VSMC development. Whole-mount α-SMA immunohistochemistry of E11.5 embryos. (A,D,G) Wild-type embryo. (B,E,H) *Hhex*$^{-/-}$ embryo with mild phenotype. (C,F,I) *Hhex*$^{-/-}$ with severe phenotype. In the mildly affected embryos, decreased α-SMA staining is seen in the cranial vasculature (E) and the dorsal aorta (H). Arrows in (D) and (E) highlight the same vascular branches in both wild-type and *Hhex*$^{-/-}$ embryos, showing the absence of VSMCs in some vessels. Arrowheads in (G) and (H) indicate the VSMCs in the dorsal aorta. Note the abnormal pattern of α-SMA staining in the *Hhex*$^{-/-}$ aorta. In severely affected embryos, there are no VSMCs present in the cranial vessels (F) and a dramatically decreased number are present in the dosal aorta (I), where the pattern of α-SMA staining is decreased and very irregular.

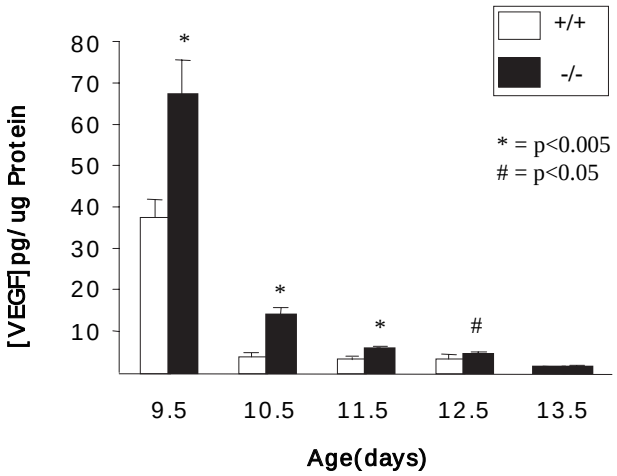

Fig. 7. Vegfa levels are increased in *Hhex*$^{-/-}$ hearts during cardiac morphogenesis. Vegfa levels were determined by ELISA at the gestational ages indicated. *n*=5 hearts for each age. Each value was determined in duplicate. Bars show means+s.e.m. Means were compared using Student's *t*-test.

performed immunohistochemistry for Vegfa expression in wild-type and *Hhex*$^{-/-}$ embryos at E9.5. Consistent with previous reports, Vegfa at E9.5 is expressed in the endoderm of the developing gut and in both the myocardium and endocardium of the heart (Fig. 8A,C,E). There is no difference in the localization of Vegfa expression in the heart and foregut of *Hhex*$^{-/-}$ embryos (Fig. 8B,D,F), nor is there any difference in expression in the area of the cranial vasculature (data not shown). As immunohistochemistry is not quantitative, we, as expected, did not detect a large difference in signal intensity between *Hhex*$^{-/-}$ and *Hhex*$^{+/+}$ embryos.

Hhex$^{-/-}$ AV explants have increased epithelial-mesenchymal transformation that is mediated by Vegfa signaling

To gain greater insight into the endocardial cushion abnormalities that occur in the absence of *Hhex*, we studied E10.5 AV explants cultured in collagen gels. This technique has been extensively validated and used to elucidate the factors involved in regulating EMT and morphogenesis of the AV canal (Brown et al., 1999; Camenisch et al., 2000; Lakkis and Epstein, 1998; Runyan and Markwald, 1983). AV endocardial cushion transformation occurs in vivo between 21-28 somites in the mouse. Cultured AV canal endocardial cushions isolated from mouse embryos that have already undergone EMT in vivo (>28 somites) show expansion of endothelial cells over the collagen gel and decreased mesenchymal cell formation and invasion (Camenisch et al., 2002). Thus, by the 28-somite stage in the mouse, AV cushion EMT is completed. As mentioned previously, one notable aspect of the phenotype of *Hhex*$^{-/-}$ mice was the accumulation of excess mesenchymal tissue in the regions of the AV cushion and the pulmonary outflow tract. We have shown that this excess accumulation of mesenchymal cells is not due to increased proliferation in the cushions over the normal developmental time course. However, it is possible that there is a normal rate of proliferation that extends over an abnormally prolonged time course. Our data indicate that the relative rate of proliferation is not increased in the absence of *Hhex*, while the normal rate of apoptosis, which is necessary for endocardial cushion remodeling, is decreased (see above). To address the question of whether EMT in the absence of *Hhex* is temporally prolonged, we cultured AV explants from E10.5 mice with >30 somites. Similar to previous reports, wild-type AV explants cultured for 72 hours predominately showed endothelial expansion over the surface of the collagen gel with little cell-cell separation (Fig. 9A) (Camenisch et al., 2002; Lakkis and Epstein, 1998). There were very few spindle-shaped mesenchymal cells and no migration of cells into the collagen gel. In striking contrast, explants from *Hhex*$^{-/-}$ mice had much fewer rounded endothelial cells and a dramatic increase in the number of elongated, spindle-shaped mesenchymal cells. In addition, there was much more invasion of the collagen gel by cells from the *Hhex*$^{-/-}$ explant (Fig. 9B). Quantitation of the extent of EMT in *Hhex*$^{+/+}$ and *Hhex*$^{-/-}$ explants confirmed this observation. The ratio of mesenchymal:epithelioid cells (transformed:untransformed cells) in *Hhex*$^{-/-}$ explants was 2.85-fold higher than in *Hhex*$^{+/+}$

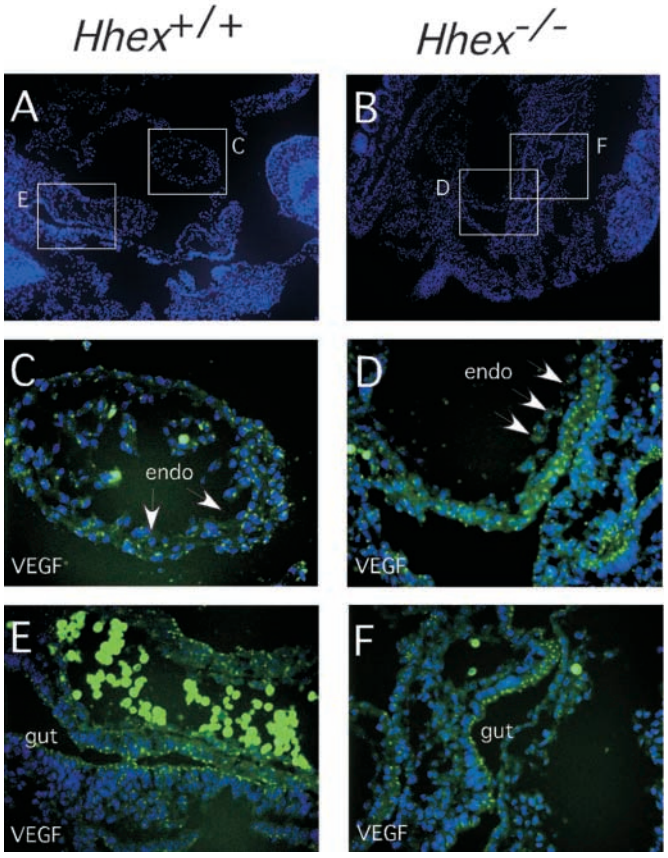

Fig. 8. The spatial expression pattern of Vegfa expression is not altered in *Hhex*$^{-/-}$ embryos. (A) and (B) Low-power views of sagittal sections of E9.5 embryos stained with Hoecsht (blue staining of cell nuclei). Higher-power views of the areas boxed in (A) and (B) are shown as indicated. Green staining represents presence of Vegfa. In the heart of both *Hhex*$^{+/+}$ (C) and *Hhex*$^{-/-}$ (D) embryos, Vegfa is expressed in both the myocardium and endocardium. Vegfa is also highly expressed in the developing gut endoderm of both *Hhex*$^{+/+}$ (E) and *Hhex*$^{-/-}$ (F) embryos.

explants (0.486 vs 0.170, $P<0.05$). This indicates that EMT and mesenchymal cell migration in *Hhex*$^{-/-}$ AV explants is increased and is ongoing at a time in development when EMT is usually complete.

To determine if elevated Vegfa levels mediate the increased EMT that occurs in the absence of Hhex, we treated AV explants from wild-type and *Hhex*$^{-/-}$ mice (>28 somites) in culture with sFlt-1. sFlt-1 is a soluble murine recombinant Vegf receptor 1/IgG-Fc chimeric protein that serves as a highly effective and specific inhibitor of Vegfa signaling by sequestering Vegfa, thereby preventing its interaction with its receptor Vegfr1 (Ferrara et al., 1998; Gerber et al., 1999). sFlt-1 was added to the culture medium of both *Hhex*$^{+/+}$ and *Hhex*$^{-/-}$ AV explants for 72 hours. Control explants were treated with culture medium alone. The addition of sFlt-1 to the culture medium of *Hhex*$^{-/-}$ AV explants, resulted in a marked decrease in the number of transformed mesenchymal cells in the explants and decreased migration of those mesenchymal cells into the collagen gel (Fig. 9B). There was no effect seen on the wild-type explants, and quantitation revealed that there was no difference in the ratio of mesenchymal:epithelioid cells in *Hhex*$^{-/-}$ AV explants treated with s-Flt compared to *Hhex*$^{+/+}$ explants. Thus, the increased EMT and mesenchymal cell migration present in *Hhex*$^{-/-}$ AV explants in vitro is mediated, at least in part, via the Vegf signaling pathway.

As further confirmation that elevated Vegf levels can increase EMT in AV explants from mice with >28 somites, we treated *Hhex*$^{+/+}$ explants with two doses of recombinant Vegf – 10 pg/ml and 10 ng/ml. At both doses, we observed a 16% increase in the ratio of mesenchymal:epithelioid cells in the treated explants compared to untreated explants ($P<0.05$ for both doses). While the magnitude of the effect on EMT of adding exogenous Vegf to wild-type AVexplants is much smaller than that seen in the *Hhex*$^{-/-}$ AV explants, this finding does support the conclusion that elevated Vegfa levels play an important role in the pathogenesis of the endocardial cushion defects present in *Hhex*$^{-/-}$ mice.

Discussion

We have disrupted *Hhex* and have found, in addition to previously reported defects in forebrain, thyroid and liver development, that *Hhex* is necessary for normal cardiac morphogenesis and vasculogenesis. Analysis of mutants revealed the presence of overabundant ECC and a number of cardiac structural defects dependent on normal ECC development including dysplastic AV valves, VSD, DORV and Right ventricular outflow tract (RVOT) obstruction. In addition, the formation of the myocardium was abnormal as evidenced by RV hypoplasia and thinning of the compact myocardium. Vasculogenesis was also severely perturbed – *Hhex*$^{-/-}$ embryos had a dramatic disorganization of sprouting endothelial tubes, altered formation of VSMC, and developed abnormally large vascular structures. In the mutant mice, cardiac Vegfa levels were elevated two- to three-fold and these elevated levels contributed, at least in part, to the abnormal EMT present in the AV cushions. These observations indicate that Vegfa lies downstream of Hhex and that Hhex normally acts to repress Vegfa levels in the developing heart.

Abnormal heart development in *Hhex*$^{-/-}$ embryos highlights the importance of the endoderm and/or endocardium in cardiac morphogenesis

Since *Hhex* is not expressed in the myocardium at any stage of cardiac development, the cardiac morphogenetic abnormalities seen in *Hhex*$^{-/-}$ mice are likely to be due to the absence of *Hhex* in the endoderm (definitive endoderm at E7.0. or the foregut endoderm at E8.0-8.5), the endocardium at E8.0-8.5, or both. It has long been appreciated that interaction of the cardiac mesoderm with adjacent endoderm is necessary for the normal development of the heart. Over the last 10 years, with the developmental of cardiac-specific molecular markers, much more specific information has been obtained regarding the role of the endoderm and the factors that mediate the interaction (reviewed by Fishman and Chien, 1997; Lough and Sugi, 2000; Nascone and Mercola, 1996). Exactly what stage of heart development is endoderm dependent is debated. However, an early inductive influence of endoderm is consistent with explant studies in both the frog (Nascone and Mercola, 1995) and chick (Schultheiss et al., 1995) which demonstrate the ability of anterior endoderm to induce cardiac-specific gene expression in cells fated normally to form other tissues. A

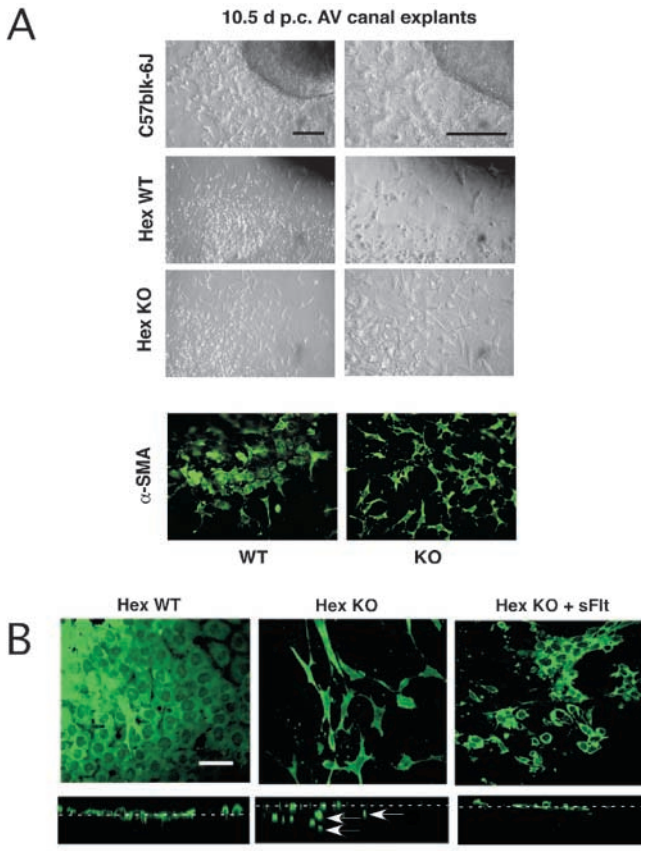

Fig. 9. E10.5 AVC explants from *Hhex*$^{-/-}$ hearts show increased EMT that is blocked by inhibiting Vegf signaling. (A) Top six panels show light microscopic images of E10.5 AVC explants from C57Bl/6J mice, wild-type mice from the *Hhex*$^{+/-}$ intercrosses, and *Hhex*$^{-/-}$ mice cultured for 72 hours showing increased numbers of transformed mesenchymal cells in the *Hhex*$^{-/-}$ explants. The bottom two panels are representative confocal images of endocardial cell outgrowths immunstained for α-SMA. In the wild-type explant (left panel), the cells are more epitheliod in morphology with relatively few mesenchymal cells. In the *Hhex*$^{-/-}$ explants, most of the cells are spindle-shaped mesenchymal cells that exhibit little cell-cell contact. Thus, there is ongoing EMT of AVC explants in the absence of *Hhex* at E10.5, a gestational age when EMT is usually complete. (B) Confocal images of E10.5 AVC explants immunostained for α-SMA in untreated wild-type explants (left panel), untreated *Hhex*$^{-/-}$ explants (middle panel), and in *Hhex*$^{-/-}$ explants treated with 25 μg/ml s-Flt (right panel). Below each panel is a corresponding *z*-plane image showing the distance cells have moved into the collagen gel, which is indicative of the invasive capacity of the cells. The broken line represents the top of the collagen gel. These panels show that wild-type cells at E10.5 are epithelioid in morphology, maintain close cell-cell contact, and do not invade the collagen gel. *Hhex*$^{-/-}$ cells undergo extensive transformation into spindle-shaped mesenchymal cells, maintain little cell-cell contact, and show extensive migration into the collage gel (arrows). Treatment of *Hhex*$^{-/-}$ explants with s-Flt reverts the cell morphology to the wild-type phenotype as evidenced by rounded cells with extensive cell-cell contact and no invasion of the collagen gel.

number of molecules expressed and/or secreted by the endoderm that affect cardiac myogenesis have recently been identified, including BMPs (signaling via SMAD proteins) (Galvin et al., 2000; Ladd et al., 1998; Lough et al., 1996), FGFs (especially FGF-2, 4) (Barron et al., 2000; Ladd et al., 1998; Lough et al., 1996; Zhu et al., 1999; Zhu et al., 1996) and Wnt proteins (Marvin et al., 2001). Most of these studies examined the role of endoderm or endoderm-derived molecules on cardiac mesoderm specification or cardiac myocyte development. Relatively little information is available on the role of the endoderm on cardiac morphogenesis. However, a recent study using vitamin A-deficient quail embryos suggests a role for anterior foregut endoderm in the regulation of heart tube morphogenesis (Ghatpande et al., 2000). These authors speculate that genes such as *Gata4* and *Hnf3b*, which are involved in foregut development may regulate other genes expressed in the foregut that are necessary for normal cardiac development. Interestingly, we have previously shown that *Hhex* is regulated, in vitro, by both *Gata4* and *Hnf3b* (Denson et al., 2000b).

In addition to affecting cardiac morphogenesis, the endoderm also appears to play an important role in directing endocardial development (Sugi and Markwald, 1996) (reviewed by Fishman and Chien, 1997; Lough and Sugi, 2000). One molecule that has been suggested as an endoderm-derived signal affecting endocardial development is Vegfa (Fishman and Chien, 1997; Lough and Sugi, 2000). Vegfa is highly expressed at E8.0-8.5 in the definitive gut endoderm in addition to being expressed in both the myocardium and endocardium of the developing heart (Miquerol et al., 1999). By E9.5, Vegfa expression in endocardial cells is restricted to the outflow tract and atrioventricular canal – cells that undergo EMT to form the endocardial cushions and are subsequently involved in the formation of cardiac cushions and valves. Thus deletion of *Hhex* expression in either the foregut endoderm or the endocardium (in particular the endocardium that gives rise to ECCs) could result in alterations of AV cushion development as well as abnormalities in ventricular myocardial development by altering Vegfa levels.

Excessive EMT in the endocardial cushions of *Hhex*$^{-/-}$ mice is associated with elevated cardiac Vegfa levels in the heart and is ameliorated by blocking Vegfa signaling in vitro

There is a growing body of evidence that Vegfa signaling plays an important role in cardiac morphogenesis in addition to its central role in vascular development. It is also now clear that Vegfa expression in vivo must be tightly regulated and perturbations of either Vegfa levels or the temporal-spatial pattern of Vegfa expression have profound effects on cardiac development. Haplo-insufficiency in mice carrying one functional *Vegfa* allele results in early embryonic lethality from abnormal cardiovascular development (Carmeliet et al., 1996; Ferrara et al., 1996) as does a mouse strain with a hypomorphic allele of *Vegfa* (Damert et al., 2002). In addition, even modest increases in Vegfa levels during early embryogenesis result in striking abnormalities of cardiac morphogenesis, including abnormal ventricular trabeculation, VSD, enlarged coronary and epicardial vessels, defective outflow tract remodeling and marked reduction in the compact layers of both ventricles (Miquerol et al., 2000). Exposure of developing embryos to hyperglycemia, which is associated with endocardial cushion defects in humans, has recently been shown to inhibit EMT in mouse embryos in culture, and this inhibition is mediated by a

hyperglycemia-induced decrease in Vegfa (Enciso et al., 2003). Interestingly, there is a report in which premature induction of myocardial Vegfa expression in E9.5 embryos inhibited endocardial cushion formation and treatment of E9.5 AV explants with $hVegf_{165}$ (100 ng/ml) inhibited EMT in vitro (Dor et al., 2001). Here we show that treatment of E10.5 AV explants with doses of Vegf in the 10 pg/ml-10ng/ml range results in a small but significant increase in EMT in vitro, consistent with our findings in vivo in *Hhex*$^{-/-}$ mice. Thus, during heart development, alterations in embryonic Vegfa levels appear to have pronounced effects on the endocardial cushions, and those effects critically depend on the timing, level and location of altered Vegfa expression. Our data indicate that cardiac Vegfa levels are elevated in the absence of *Hhex* and that the excessive EMT present is mediated by increased Vegfa signaling. Recently it has been shown that Hhex interacts with GATA transcription factors in endothelial cells, inhibiting signaling via the Vegf pathway by decreasing the expression of the Vegf receptor Flk1/KDR. This results in the attenuation of Vegf-mediated tube formation in primary endothelial cell cultures (Minami et al., 2004). Future experiments will focus on whether the effect on EMT we are seeing is solely due to elevated Vegfa levels or is also due to alterations in either the response to Vegf signaling (i.e. altered response by Vegf receptors), or in alterations in the levels of other Vegf isoforms.

Defective vasculogenesis in *Hhex*$^{-/-}$ mice

The development of the vertebrate vascular system involves a highly ordered series of molecular events that can be divided into two distinct processes: vasculogenesis and angiogenesis (reviewed in Carmeliet, 2000; Risau, 1997). Vasculogenesis is a process that involves the in situ differentiation of primitive precursor cells called angioblasts into endothelial cells that then assemble into the primitive primary capillary network. After this, the primitive capillary network grows and remodels into a complex network of mature blood vessels by the differential growth and sprouting of endothelial tubes and recruitment and differentiation of mesenchymal cells into VSMCs and pericytes. Communication between the endothelium and mesenchyme is critical for normal vasculogenesis and it has been shown that endothelial cells induce the differentiation of pericytes and VSMCs (Hellstrom et al., 1999; Hellstrom et al., 2001; Hirschi et al., 1999; Li et al., 1999). In fact, a recent study indicates that a common vascular progenitor cell can differentiate into both endothelial and smooth muscle progenitors when treated with Vegfa or Pdgf-BB, respectively (Yamashita et al., 2000).

It has been previously suggested by several groups that *Hhex* plays an important role in vascular development. This is based on several findings. First, *Hhex* mRNA and protein are transiently expressed in the developing blood islands of the mouse at E7.5 (where both vascular and hematopoietic precursors are found) (Ghosh et al., 2000; Thomas et al., 1998), and in the early vasculature of both mice and frogs (Newman et al., 1997; Thomas et al., 1998). In mice, *Hhex* expression in the developing vasculature is seen only in endothelial cells between E8.5 and E9.5 Second, in Xenopus, *Xhex* is transiently expressed in endothelial cells during vasculogenesis and overexpression of *XHex* sequences in the frog embryo causes disruption to developing vascular structures and an increase in the number of vascular endothelial cells (Newman et al., 1997). Third, in zebrafish, *hhex* was shown to act downstream of *cloche*, to induce premature and ectopic expression of endothelial and blood differentiation genes such as *fli1*, *flk1* and *gata1* when ectopically expressed, and to interact with the gene *scl* in a manner suggesting that *hhex* and *scl* can cross-regulate each other (Liao et al., 2000). However, analysis of a *hhex*-deficiency allele showed that *hhex* is not essential for early endothelial and blood differentiation. Fourth, Sekiguchi et al., made the interesting observation that Hhex is expressed in neointimal VSMCs of the rat aorta after balloon injury and in cultured VSMCs, whereas there is no *Hhex* expression in normal aorta or in mature endothelial cells, fibroblasts or cardiac myocytes (Sekiguchi et al., 2001). In that study, the authors showed that Hhex transactivated the promoter of SMemb/NHMC-B, a nonmuscle isoform of myosin heavy chain that has been shown to be a molecular marker of dedifferentiated VSMCs. The authors speculated that Hhex might play a role in the phenotypic modulation of VSMCs and in the response of the vasculature to balloon injury. Finally, when *Hhex* was overexpressed in endothelial cells in culture, the proliferation, migration, invasion and ability to form a vascular networks was completely abolished (Nakagawa et al., 2003). In addition, the overexpression of *Hhex* led to decreased expression of a number of vasculogenesis-related genes, including *Vegfr1*, *Vegfr2*, *neuropilin1*, *tie1* and *tie2*. This report suggests that Hhex acts as a negative regulator of vasculogenesis. However, in two separate *Hhex*-null mutations, abnormalities of vascular development are not reported (Keng et al., 2000; Martinez Barbera et al., 2000). Martinez Barbera reported that early vascular development (i.e. vasculogenesis), as assessed by *flk1* expression at E9.5, was normal (Martinez Barbera et al., 2000) while Keng did not examine vascular development in their targeted mutation of *Hhex* (Keng et al., 2000).

The findings presented here are the first to demonstrate that *Hhex* is necessary for normal vascular development in vivo. Interestingly, Pecam staining of our E9.5 *Hhex*$^{-/-}$ embryos shows that angioblasts coalesced into early vascular structures in a pattern similar to wild-type embryos (data not shown). Thus, our findings are consistent with those of Martinez-Barbera and show that vasculogenesis in *Hhex*$^{-/-}$ mice is not grossly disturbed. However, we found that at E11.5 the vasculature is disorganized with a profusion of small ectopic branches originating from dilated large vessels, and by E13.5 many vessels have large lumens and in some regions of the embryo, large sinusoidal structures form. The vascular defect in *Hhex*$^{-/-}$ embryos is also characterized by delayed or absent VSMC development suggesting that, in the absence of *Hhex*, there appears to be a defect in vasculogenesis. Additionally, these data are consistent with recently reported data suggesting that *Hhex* acts as a negative regulator of vasculogenesis and vasculogenesis-related genes (Nakagawa et al., 2003), and microarray data that show a three-fold increase in *vegf* RNA levels in *Hhex*$^{-/-}$ embryoid bodies (Guo et al., 2003). It is possible that the vascular phenotype we have observed is secondary to cardiac failure or AV valve insufficiency and is not primarily due to the absence of *Hhex* expression in the developing endothelium. However, we think this is unlikely given the fact that other groups have reported that both over- and under-expression of *Hhex* have effects on the expression of vasculogenesis-related genes and on vasculogenesis in vitro (Guo et al., 2003; Nakagawa et al., 2003).

Of perhaps even greater interest, relative to *Hhex* function, is the important role that the *Vegf* plays in vascular development. Overexpression of Vegfa is also pathologic and the link between overexpression of Vegfa and vascular malformations is well established. Exogenous administration of Vegfa during vasculogenesis in quail embryos results in severe perturbations of vascular patterning which includes abnormal vascular fusion and formation of vessels with abnormally large lumens (Drake and Little, 1995; Feucht et al., 1997; Flamme et al., 1995), and dysregulated expression of Vegfa in mice results in formation of abnormal vascular trees and irregularly shaped sac-like vessels (Benjamin and Keshet, 1997; Dor et al., 2002; Wong et al., 2001). In addition, recent evidence indicates that a common vascular precursor cell can develop into endothelial progenitors and smooth muscle progenitors, and that both Vegfa and Pdgf-BB affect to which lineage the cell will commit (Yamashita et al., 2000). In these cells, treatment with Vegfa in vitro promotes endothelial cell differentiation, resulting in decreased differentiation of the vascular progenitor cells into smooth muscle progenitors. The abnormalities of vasculogenesis in *Hhex*$^{-/-}$ mice are quite similar to the vascular abnormalities due to elevated Vegfa levels suggesting that the vascular defects in *Hhex*$^{-/-}$ mice may be due to Vegfa overexpression.

Our data demonstrate that *Hhex* is essential for normal cardiac morphogenesis and vascular development and that elevated levels of Vegfa are responsible, at least in part, for the developmental abnormalities seen in *Hhex*$^{-/-}$ mice. *Hhex* is one of the few genes identified that is not expressed in the myocardium yet, when mutated, has profound effects on cardiac morphogenesis. It is clear that both the foregut endoderm and the endocardium play important, yet relatively undefined, roles in heart development. Since *Hhex* is expressed in both of these sites during cardiac morphogenesis, studies of its function during development will yield important new mechanistic information on the roles of these two tissues in cardiac development. Additionally, our studies indicate that one function of *Hhex* is to control Vegfa levels in vivo. To our knowledge, this is the first example of a mutation in a homeobox transcription factor that results in elevated Vegfa levels. Relatively few genes have been identified as repressors of Vegfa and most are tumor-suppressor genes (e.g. *Smad4/DPC4*, *p53*, *p16* and the von Hippel-Lindau gene) (Haase et al., 2001; Harada et al., 1999; Schwarte-Waldhoff et al., 2000; Zhang et al., 2000). However, it is not yet clear how the absence of *Hhex* leads to elevated embryonic Vegfa levels. Interestingly, the mouse Vegfa promoter harbors a potential consensus *Hhex*-binding sequence that may allow *Hhex* to control Vegfa at the transcriptional level. However, indirect mechanisms are also certainly conceivable. Further studies of the interaction between *Hhex* and Vegfa will provide valuable insight into the control of cardiovascular development and is likely to have important implications for controlling therapeutic vasculogenesis as well.

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