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A novel role for VEGF in endocardial cushion formation and its potential contribution to congenital heart defects

Yuval Dor[1], Todd D. Camenisch[2], Ahuva Itin[1], Glenn I. Fishman[3], John A. McDonald[2], Peter Carmeliet[4] and Eli Keshet[1,*]

[1]Department of Molecular Biology, Hebrew University – Hadassah Medical School, Jerusalem 91120, Israel
[2]Mayo Clinic, Scottsdale AZ, USA
[3]Section of Myocardial Biology, Departments of Medicine, Physiology & Biophysics and Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, USA
[4]The Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, KU Leuven, Leuven B-3000, Belgium
*Author for correspondence (e-mail: keshet@cc.huji.ac.il)

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SUMMARY

Normal cardiovascular development is exquisitely dependent on the correct dosage of the angiogenic growth factor and vascular morphogen vascular endothelial growth factor (VEGF). However, cardiac expression of VEGF is also robustly augmented during hypoxic insults, potentially mediating the well-established teratogenic effects of hypoxia on heart development. We report that during normal heart morphogenesis VEGF is specifically upregulated in the atrioventricular (AV) field of the heart tube soon after the onset of endocardial cushion formation (i.e. the endocardium-derived structures that build the heart septa and valves). To model hypoxia-dependent induction of VEGF in vivo, we conditionally induced VEGF expression in the myocardium using a tetracycline-regulated transgenic system. Premature induction of myocardial VEGF in E9.5 embryos to levels comparable with those induced by hypoxia prevented formation of endocardial cushions. When added to explanted embryonic AV tissue, VEGF fully inhibited endocardial-to-mesenchymal transformation. Transformation was also abrogated in AV explants subjected to experimental hypoxia but fully restored in the presence of an inhibitory soluble VEGF receptor 1 chimeric protein. Together, these results suggest a novel developmental role for VEGF as a negative regulator of endocardial-to-mesenchymal transformation that underlies the formation of endocardial cushions. Moreover, ischemia-induced VEGF may be the molecular link between hypoxia and congenital defects in heart septation.

Key words: VEGF, Hypoxia, Endocardial cushions, Mouse

INTRODUCTION

VEGF plays essential roles in the formation of blood vessels by both the vasculogenic and angiogenic modes. In addition, VEGF is required for proper heart morphogenesis at stages where the heart is still avascular. The roles of VEGF in heart morphogenesis are not fully understood but it is thought that VEGF induces the pre-endocardial mesenchyme to become endocardial epithelium (endocardial vasculogenesis) (Mjaatvedt et al., 1999). Several studies have shown that development of the cardiovascular system is exquisitely dependent on normal levels and appropriately timed expression of VEGF. For example, haploinsufficiency in mice carrying only one functional VEGF allele results in early embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996). Conversely, even a modest increase in VEGF levels during development is detrimental (Miquerol et al., 2000) and administration of exogenous VEGF during vasculogenesis in quail embryos results in severe perturbations of vascular patterning (Drake and Little, 1995; Feucht et al., 1997; Flamme et al., 1995). With regard to heart morphogenesis, the contention that myocardial cells and endocardial cells might have a common bipotential progenitor cell (Mjaatvedt et al., 1999) further argues for the need for a tightly regulated expression of VEGF during early stages of heart morphogenesis.

Independent of its tight developmental regulation, VEGF is also robustly induced by environmental stresses. In fact, VEGF has emerged as a key player in the molecular homeostatic response to ischemia. Specifically, hypoxia and hypoglycemia induce production of VEGF, which functions to recruit new blood vessels to the hypoxic tissue, thereby improving perfusion and restoring oxygen homeostasis (reviewed in Dor and Keshet, 1997)). Hypoxia-inducibility of VEGF is cell-autonomous and has been demonstrated in a large variety of cell types. Thus, hypoxic episodes during embryogenesis are likely to induce transiently high levels of VEGF, superimposed on the developmentally regulated program of expression. This duality in VEGF regulation led us to propose that VEGF might link episodes of gestational hypoxia and congenital heart defects.

Heart development is particularly prone to malformations, with congenital heart defects (CHD) encountered in 1% of live births (Olson and Srivastava, 1996). The etiology of congenital heart defects is thought to be multifactorial, involving interplay between genetic and environmental factors. Recent studies have uncovered a variety of genes that, when mutated, might contribute to CHD. In contrast, the nature and mechanisms of action of the environmental insults have remained largely unknown (Chien, 1999). Among the environmental risk factors, the importance of embryonic hypoxia as a cardiac teratogen has long been recognized. Congenital cardiac anomalies are more prevalent at high altitude (Miao et al., 1988) and several studies have shown that experimental prenatal hypoxia in rats, mice and chick greatly increases the frequency of a host of cardiac malformations (Clemmer and Telford, 1966; Grabowski, 1970; Ingalls et al., 1952; Jaffee, 1974; Jaffee, 1977). To date, however, the mechanism by which prenatal hypoxia contributes to CHD has not been explored.

Among the congenital heart defects induced by hypoxia, most prevalent are valvuloseptal defects. A key process in the formation of heart septa and valves is the formation of endocardial cushion tissue, which partitions the initially uniform heart tube into an atrium and ventricle and the common outflow tract into a dorsal aorta and pulmonary artery. Endocardial cushion formation takes place by mesenchymal transformation of endocardial cells that invade the underlying expanded layer of extracellular matrix (cardiac jelly). Signals emanating from the myocardium, notably signaling by TGFβ family members, have been shown to play a role in endocardial-to mesenchymal transformation (Boyer et al., 1999; Brown et al., 1999). Mechanisms that regulate cushion placement within the heart tube, as well as mechanisms responsible for the restriction in the number of endocardial cells that eventually delaminates from the endocardial epithelium and undergoes mesenchymal transformation are poorly understood. Observations reported below that VEGF is upregulated specifically in the atrioventricular (AV) field of the heart tube shortly after the onset of endocardial cushion formation have suggested that VEGF might play a role in the regulation of the latter.

To further explore a novel role for VEGF in AV partitioning and to determine whether perturbation in the developmentally programmed dose of VEGF might induce septal defects, we have designed a binary transgenic system that allows conditional switching of VEGF production and secretion exclusively by the myocardium. Using this system, in conjunction with employing an explant system of AV tissue that recapitulates key steps in the formation of endocardial cushions (Bernanke and Markwald, 1982), we have indeed uncovered a novel developmental role for VEGF in keeping endocardial-to-mesenchymal transformation in check and a pathological role in mediating hypoxia-induced anomalies in heart septation.

MATERIALS AND METHODS

Transgenic mice

pTET-VEGF mice were obtained by zygote injection of the pTET-VEGF165 construct previously described (Benjamin and Keshet, 1997). MHCα-tTA mice were described before (Yu et al., 1996). Crosses were carried out between heterozygotes, so that most litters contained internal controls of MHCα-tTA$^+$, pTET-VEGF$^+$ and the wild-type genotype. To repress pTET-VEGF during early stages of heart development mothers were given 100 μg/ml tetracycline/3% sucrose in the drinking water from E3.5 to E9.5. To allow for development of double transgenics to term, mothers were implanted with a slow release tetracycline pellet (Innovative Research of America) before mating. Embryos and newborns were genotyped by Southern blotting (using coding regions of tTA and VEGF as probes) or by PCR. Primers for PCR genotyping of the pTET-VEGF (Benjamin and Keshet, 1997) and MHCα-tTA (Yu et al., 1996) transgenes were described before. Expression of both transgenes was verified by in situ hybridization, using tTA and VEGF probes.

Gene expression

In situ hybridization was performed on paraffin sections as previously described (Pe'er et al., 1995). Endogenous and transgenic VEGF was detected by a 1.8 kb cDNA fragment, containing the coding region and 3′UTR of the gene (Pe'er et al., 1995). Endogenous VEGF was specifically detected using a 3′UTR probe that is absent from the transgenic construct. tTA probe was prepared by subcloning of *Hin*dIII-*Spe*I fragment from the tTA coding region into pBluescript. Other probes were PCR amplified from cDNA and cloned into the TOPO-PCRII vector (invitrogen), using the following primers: VEGFR1, 5′-AACAGGCCGCGTCTTGCTCACCATG-3′ and 5′-GAACGAGGACCCACTCTTCACGCG-3′; VEGF-R2, 5′-GTGCA-GGATGGAGAGCAAGG-3′ and 5′-TGGACTCAATGGGCCTTCC-ATTTCTGTACC-3′.

Relative quantification of endogenous VEGF mRNA levels in different regions of the myocardium was carried out as follows: 3 μm sections were hybridized with a VEGF-specific probe encompassing the entire coding sequence. Brightfield and darkfield images (×100) were captured using a digital camera (SV-micro) and stored as Adobe Photoshop files. Darkfield images were converted in a way that the signal autoradiographic grains are visualized as discrete black dots. NIH-IMAGE software was used to count grains overlying cells in the ventricle, atrium and AV-canal regions (regions were defined and cells enumerated using the brightfield image). Relative grain densities in the respective regions of the heart tube were determined in 3 thin sections of each of 7 E10.5 hearts examined.

To determine the extent of VEGF upregulation following a tetracycline switch, a similar relative quantification of signal intensities was carried out using E9.5 embryos, a developmental time that precedes the formation of apparent heart defects. Here, control and double transgenic littermate embryos were co-processed and hybridized on the same slide.

In addition, the extent of VEGF upregulation was determined by quantitative RT-PCR as follows: E9.5 embryos from MHC-tTA+/– X pTET-VEGF+/– crosses were prepared. Total RNA was extracted from individually dissected embryonic hearts using TRI reagent (Sigma) and 10 μg glycogen as a carrier. DNA was extracted from the remainder of the embryo and was used for genotyping. Half of each RNA sample was reverse-transcribed in a volume of 20 μl using random hexamers as reverse transcription primers. 1 μl of each cDNA was amplified using either VEGF primers that yield an identical amplified fragment from both wild-type and transgenic cDNA, or ribosomal L19 primers serving as a standard. Primers used for both VEGF and L19 were described before (Benjamin and Keshet, 1997). PCR products were resolved by agarose gel electrophoresis and detected by blot hybridization with ^{32}P-labeled L19 or VEGF cDNA probes. Initial experiments were carried out to determine the range of PCR cycles required to obtain detectable bands with exponentially increasing intensities. Accordingly, 18 and 20 amplification cycles (94°C 1 minute, 55°C 1 minute, 72°C 1 minute each) were used for L19 and VEGF, respectively. Films were scanned and relative band intensities determined using NIH image. Results shown are the

average and standard deviation of the VEGF/L19 ratio from four wild-type and six transgenic hearts.

Explant system for in vitro mesenchymal transformation

All atrioventricular tissue was harvested from timed matings at E9.5 of development. The AV canal region was dissected from embryos in sterile Tyrodes buffer and placed on top of drained Rat type I collagen (Collaborative Biomedical Products, Bedford, MA) gels as described (Runyan and Markwald, 1983). The AV explants were maintained in M199 medium supplemented with 1% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1% insulin, transferrin and selenium (ITS, Gibco-BRL, Rockville, MD) in four-well microculture dishes (Nalge-Nunc, Naperville, IL). AV explants were allowed to firmly attach overnight (~14 hours) prior to addition of 100 μl of M199 explant media. Normal AV explant (normoxia) cultures were grown at 37°C with 5%CO_2, and hypoxic treatment created with BBL GasPak anaerobic system (Becton-Dickinson, Sparks, MD) at 37°C for 24 hours. Those tissues treated with either VEGF or sFlt (25 ng/μL) were placed in explant media containing the indicated reagent for 30 minutes prior to explantation and cultured in the presence of the same concentration. Explant cultures were fixed with 2% paraformaldehyde, rinsed twice with cold 1× PBS and stained with fluorescein Phalloidin and DAPI (Molecular Bioprobes, Eugene, OR) to observe cellular and nuclear morphology. Images were acquired using a Nikon microscope and Spot image software (Diagnostic Instruments, Sterling Heights, MI).

RESULTS

The pattern of myocardial VEGF expression suggests a role in heart partitioning

In situ hybridization analysis with a VEGF-specific probe revealed that by day E9.5, when the cardiac jelly within the AV canal is already swollen and its cellularization with mesenchymal cells has begun, VEGF is still uniformly expressed throughout the myocardial tube (Fig. 1B,C).

Shortly thereafter, however, when cellularization of the cushions has further progressed and the common chamber has been partitioned into a distinct ventricle and atrium (E10.5), expression of VEGF was upregulated locally in two diametrically opposed regions of the myocardium, which precisely mark the sites of atrioventricular canal formation (Fig. 1D,E). Relative levels of VEGF expressed in the ventricle, atrium and AV canal were determined by comparing the number of autoradiographic grains per cell in the respective region of the same thin section (see Materials and Methods). Compared with its even distribution in the E9.5 heart tube, by day E10.5 VEGF expression in the AV canal was upregulated to a level 2.3-fold higher than in the ventricle and fourfold higher than in the atrium (Fig. 1F).

VEGF receptor 1 (Fig. 1G,H) and VEGF receptor 2 (not shown) were both expressed in the endocardium throughout its entire length, suggesting that VEGF secreted by the myocardium can indeed transduce signals in the adjacent endocardium. Importantly, however, VEGF receptors were rapidly downregulated upon mesenchymal transformation, implying that following delamination of endocardial cells, these cells are no longer VEGF responsive. Together, these findings are consistent with a role for locally induced VEGF in cardiac cushion formation, by acting on endocardial cells prior to their invasion of the cardiac jelly. The fact that VEGF is induced only after the onset of mesenchymal transformation

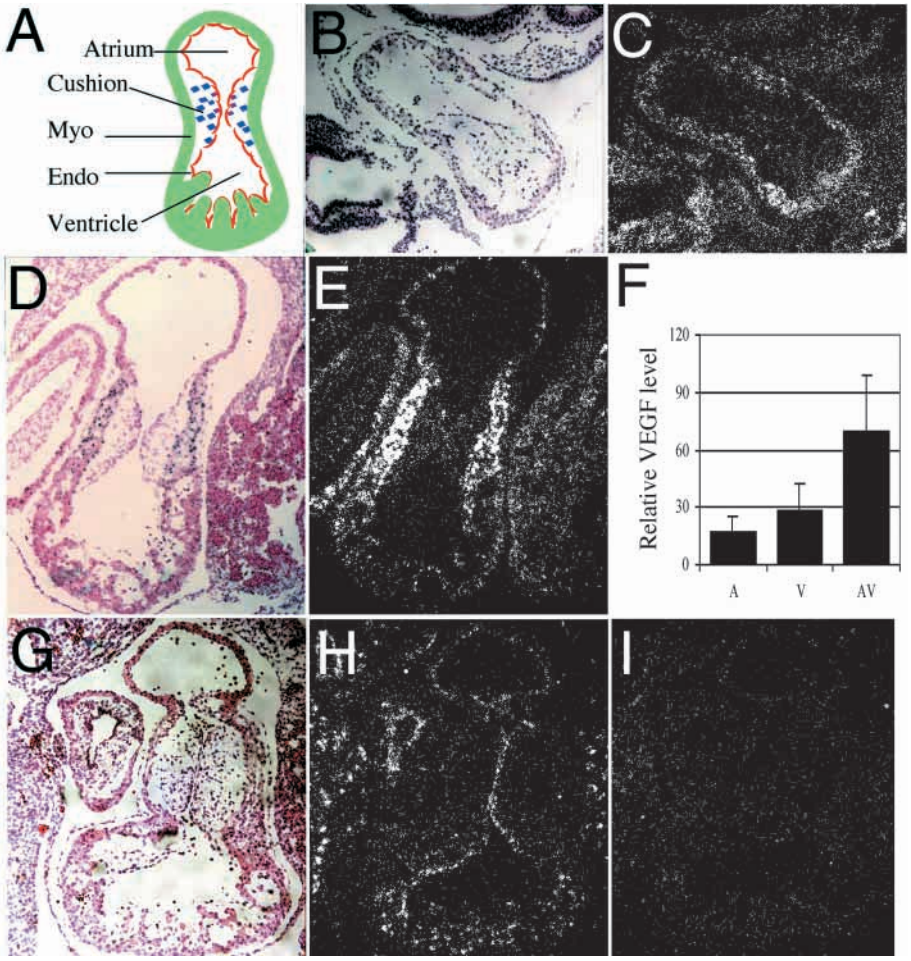

Fig. 1. Expression patterns of VEGF and its receptors during normal heart partitioning. (A) The partitioning process of the common chamber into a ventricle and atrium via mesenchymal transformation of endocardial cells and formation of cardiac cushions. (B,C) In situ hybridization with VEGF-specific probe of a E9.5 heart (brightfield and darkfield images). Note that myocardial VEGF expression is similar throughout the heart tube. (D,E) VEGF expression in a E10.5 heart. Note upregulated VEGF expression, specifically in the myocardium underlying sites of endocardial cushion formation. (F) Quantification of relative levels of VEGF mRNA in the atrium, ventricle and AV canal, determined as described in 'Materials and Methods'. Data shown are the average (±s.d.) of autoradiographic grain density in three sections of each of seven different embryos. (G-I) In situ hybridization of a E10.5 heart with a VEGFR1-specific probe: antisense (H) and sense (I). (G) Brightfield, (H,I) darkfield images.

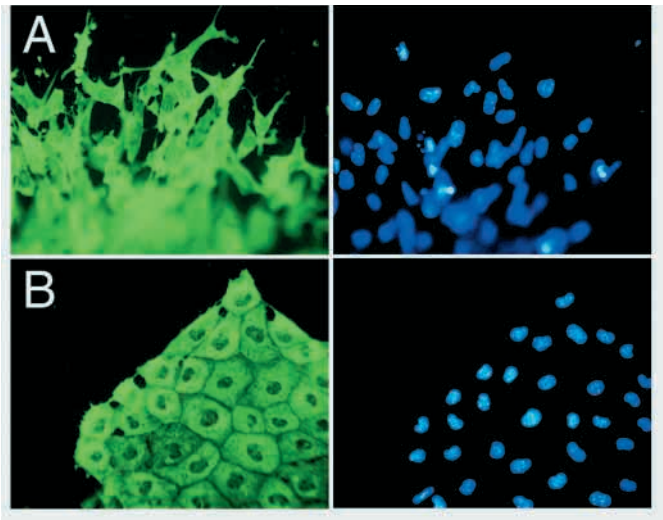

Fig. 2. VEGF inhibits endocardium-to-mesenchyme transformation in vitro. (A,B) AV explants from E9.5 wild-type mouse embryos were cultured on collagen gels in the absence (A) or presence (B) of 100 ng/ml recombinant hVEGF165 (Pepro Tech). After 24 hours, explants were fixed and stained with phalloidin-FITC (left) to view cell morphology and with DAPI (right) to view cell nuclei.

is consistent with a role in a negative feedback regulation rather than in a role in transformation per se (see below).

VEGF inhibits endocardial-to-mesenchymal transformation in vitro

To examine the hypothesis that VEGF plays a negative regulatory role in mesenchymal transformation, we used the in vitro model system for cushion tissue formation developed by Markwald and co-workers (Bernanke and Markwald, 1982). In this model the transformation of endothelium to mesenchyme can be replicated by culturing AV explants of the appropriate embryonic age atop a collagen gel. Endothelial cells that transform into mesenchymal cells migrate away from the explant and subsequently invade the collagen gel, thus allowing quantification of transformation and invasion events.

As can be seen in the representative example shown in Fig. 2, VEGF added to E9.5 AV explant cultures inhibited almost completely mesenchymal transformation and gel invasion. This result suggests that surplus VEGF may indeed prevent formation of cushion tissue.

Premature induction of myocardial VEGF inhibits endocardial cushion formation *in vivo:* a transgene switching approach

To extend the in vitro observations, we next determined the effects of VEGF misexpression on endocardial cushion formation in vivo, using a transgenic mouse system. Anticipating that perturbation of normal VEGF expression might be detrimental to a number of crucial developmental processes and, hence, lead to embryonic lethality, we resorted to tetracycline-regulated conditional transgenic system. (Gossen and Bujard, 1992; Passman and Fishman, 1994; Yu et al., 1996).

Transgenic mice expressing the tetracycline-controlled transactivator protein (tTA) exclusively in the myocardium were used (in which tTA expression is driven by a myosin heavy chain α (MHCα) heart-specific promoter (Yu et al., 1996; driver line)). These mice were crossed with a transgenic line that harbored a VEGF165 transgene driven by a tetracycline-responsive promoter (responder line). The level of VEGF expression in embryos carrying both transgenes can be tightly controlled in this system by either adding or omitting tetracycline from the drinking water of the mother ('off' and 'on' states, respectively).

As shown in Fig. 3A,B, and confirming earlier studies (Yu et al., 1996), the transactivator protein was indeed expressed exclusively in the myocardium. Since the MHCα promoter is active from embryonic day 7.5 onwards (Lyons et al., 1990), the responder VEGF transgene could potentially be activated during early stages of heart development and, in our hands, could clearly be seen in E9.5 embryos. In embryos that harbored both transgenes, in the absence of suppressive dose of tetracycline, there was massive upregulation of VEGF in the

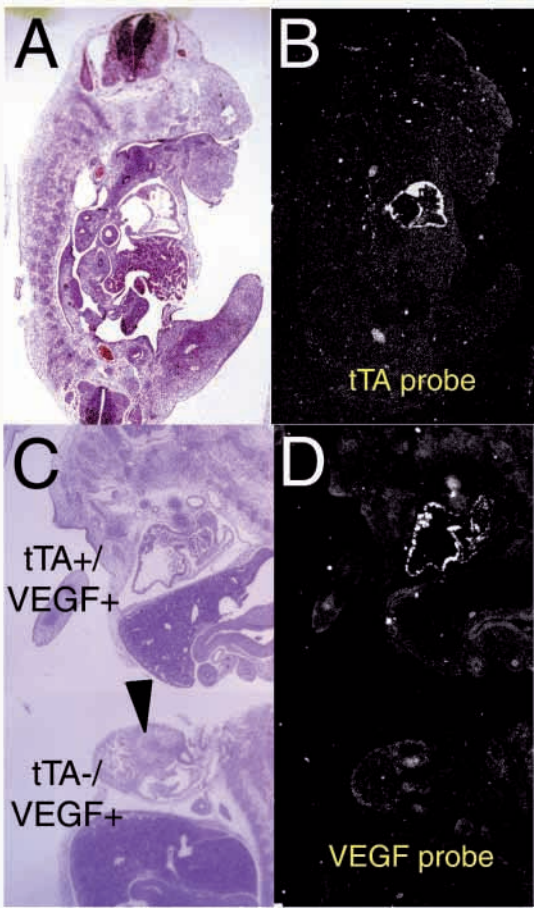

Fig. 3. Conditional switching of VEGF expression in the heart myocardium .(A,B) In situ hybridization with a tTA-specific probe of a E12.5 embryo from the driver line demonstrating myocardial-specific expression of the transactivator. (C,D) In situ hybridization with a VEGF-specific probe detecting both endogenous and transgenic mRNAs. Shown are $tTA^{-}/VEGF^{+}$ and $tTA^{+}/VEGF^{+}$ E12.5 littermates hybridized on the same slide. Note that, whereas $tTA^{-}/VEGF^{+}$ embryos maintained a restricted distribution of myocardial VEGF (arrowhead), in $tTA^{+}/VEGF^{+}$ embryos VEGF was overexpressed throughout the myocardium. In this particular experiment, VEGF was switched on by tetracycline withdrawal at day E9.5.

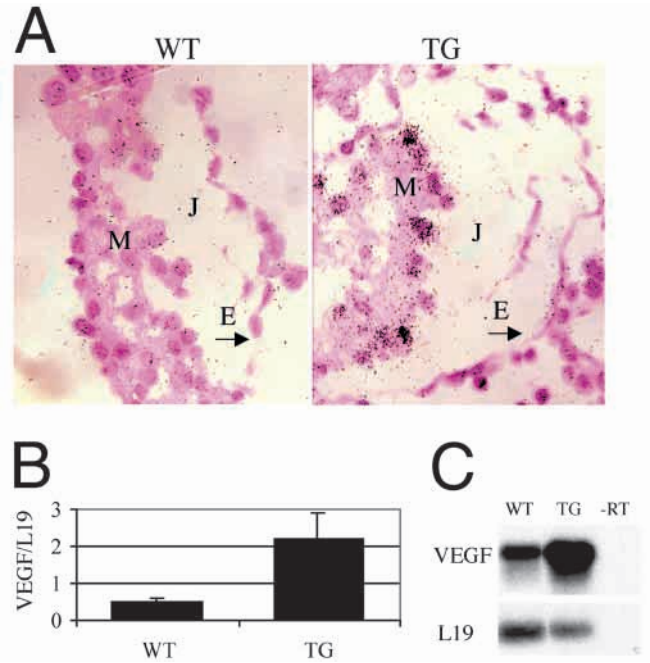

Fig. 4. Quantification of VEGF levels induced in the E9.5 myocardium. In situ hybridization with VEGF, focusing on the presumptive AV-canal region. Images shown are of a representative control (wild type, WT) and double transgenic (TG) littermate embryos that were sectioned and hybridized on the same slide. Note increased signal intensity in myocardial cells (M) adjacent to the swollen cardiac jelly (J). Arrow indicates the overlying endocardium (E). Autoradiographic signal intensities were determined as described in Materials and Methods using three different co-analyzed embryo pairs. An average 4.4-fold increase in signal intensity was measured in double transgenic animals with the switch 'on'. (B,C) E9.5 hearts were microdissected and the AV-canal region was isolated from control (n=4) and double transgenic (n=6) embryos. RNA extraction and a quantitative RT-PCR analysis of each specimen were carried out as described in Materials and Methods. A representative embryo pair is shown in C. On average, a 4.6-fold increase in VEGF mRNA level in transgenic animals was observed (values standardized for the amount of ribosomal protein L19).

myocardium (Fig. 3C,D). Control tTA$^-$/VEGF$^+$ littermates maintained the normal pattern and level of VEGF expression, confirming that the TET promoter is not leaky in the absence of tTA. In addition, tTA$^+$/VEGF$^-$ mice appeared normal, indicating that expression of the transactivator itself had no adverse effects (Fig. 3A). In VEGF$^+$/tTA$^+$ embryos VEGF165 was uniformly induced throughout the myocardium, thereby disrupting the spatial and temporal pattern of myocardial expression typifying E10.5 embryos (Fig. 3D, compare with Fig. 1).

To examine whether premature induction of VEGF in the myocardium would indeed inhibit the process of endocardial cushion formation, transgenic expression of VEGF was allowed to take place from an early stage and the heart was examined at embryonic day E.9.5, i.e. 1 day before it is upregulated naturally at sites of endocardial cushion formation. Fig. 4 highlights the relevant region of the myocardium, demonstrating that VEGF was indeed induced in the myocardium underlying the site of cushion formation. Two independent methods of quantification were used to determine the extent of VEGF induction following the switch: measurement of the in situ hybridization signal intensity (Fig. 4A) and quantitative RT-PCR on RNA extracted from E9.5 hearts (Fig. 4B). Both methods revealed about 4.5-fold more VEGF mRNA in transgenic hearts compared with control hearts. Switching myocardial VEGF at later developmental timings resulted in a 10-20-fold level of induction, presumably reflecting changes in the activity of the driving MHCα promoter (data not shown). Notably, the magnitude of induction of myocardial VEGF mRNA that results from the genetic switch is comparable with the VEGF increase that results from cardiac hypoxia (Banai et al., 1994; Levy et al., 1995).

To determine the cellular consequences of premature induction of VEGF in the myocardium, E9.5 and E10.5 littermate embryos of both genotypes (i.e. with the 'on' switch operative or not) were compared by histological and marker analyses (Fig. 5). At E9.5, the cardiac jelly was still mostly acellular in both control and double transgenic animals (Fig. 5A,B). Although development of cardiac cushions proceeded normally in control embryos, mesenchymal transformation and cellularization of the cardiac jelly were completely blocked in about half of E10.5 embryos overexpressing VEGF (Fig. 5C,D). Proper development of the outflow tract (OFT) was also impaired. OFT development is also distinguished by endocardial-to-mesenchymal transformation and cushion tissue formation. Here too, the cardiac jelly remained empty by the time that it was already colonized with mesenchymal cells in the controls (arrowheads in Fig. 5C,D). Thus, premature induction of VEGF resulted in a failure to form endocardial cushions in two distinct anatomical locations.

Another striking phenotype observed in a large fraction of double transgenic embryos was the formation of a multi-layered endocardium (Fig. 5E,F). Thus, it appears that the exceedingly high levels of VEGF emanating from the adjacent myocardium have induced abnormal expansion of the endocardium that is, however, not accompanied by mesenchymal transformation and invasion into the cardiac jelly. Rather, these endocardial cells maintain tight cell-cell associations, maintain expression of endothelial markers such as VEGFR1 (FLT – Mouse Genome Informatics; Fig. 5F), and do not switch to expressing cushion-specific markers like fibulin (Zhang et al., 1995; data not shown). These findings thus substantiate the ex vivo results shown above, supporting the notion that VEGF plays a negative regulatory role in endocardial-to-mesenchymal transformation.

VEGF mediates hypoxia-induced defects in endocardial cushion formation

The fact that hypoxia induces high levels of VEGF, on the one hand, and that surplus VEGF impairs proper development of endocardial cushions, on the other hand, suggests that VEGF

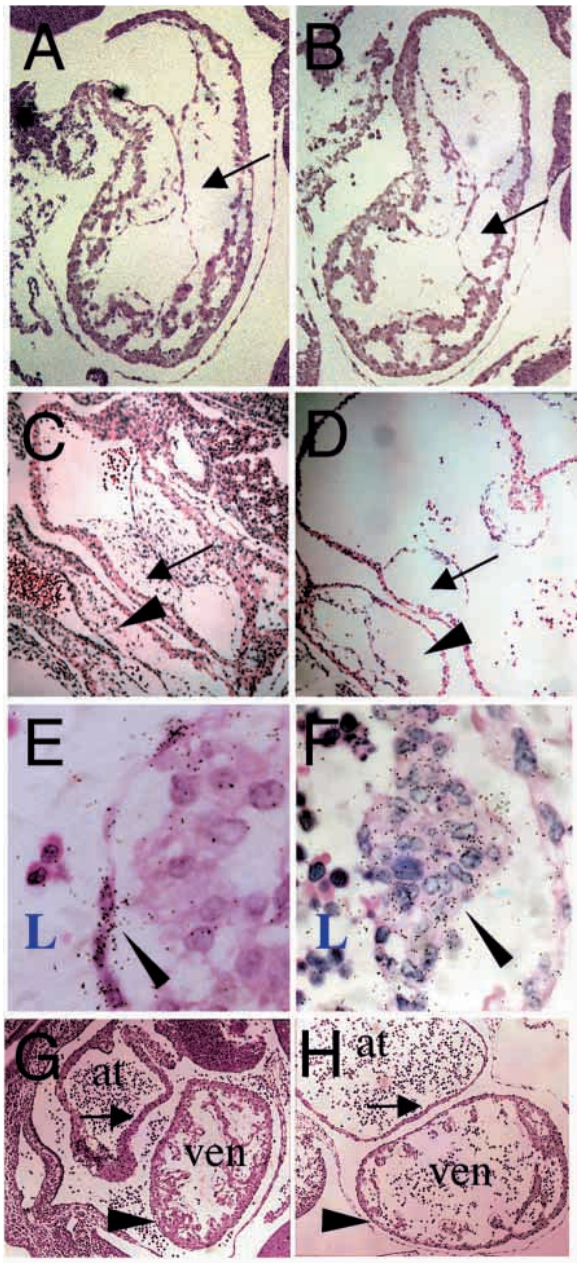

Fig. 5. Failure to form endocardial cushions in transgenic mice manipulated to prematurely induce myocardial VEGF. Wild-type (images on left) and a $tTA^+/VEGF^+$ (images on right) hearts sectioned at the level of the AV-canal (Hematoxylin and Eosin staining). **(**A,B) E9.5 hearts. Note that invasion of the cardiac jelly has not yet occurred (arrows) and that both hearts appear similar. **(**C,D) E10.5 hearts. Note the difference in cellularization of the cardiac jelly (arrows) that in the double transgenic heart has remained almost empty. As a result, the ventricle and atrium are not properly separated. Also note failure to colonize the cardiac jelly of the outflow tract (arrowheads). **(**E,F) E10.5 hearts hybridized in situ with a VEGFR1 probe. A small portion of the heart wall is shown at a high magnification where (L) denotes the lumen. Arrowheads indicates the endocardium, the assignment of which is aided by the autoradiographic grains of the VEGFR1 signal. Note the multi-layered endocardium in the heart overexpressing VEGF. (G,H) E10.5 heart sections demonstrating thinning of the atrial wall (arrows) and underdeveloped trabeculae (arrowheads) in the double transgenic animal. at, atrium; ven, ventricle.

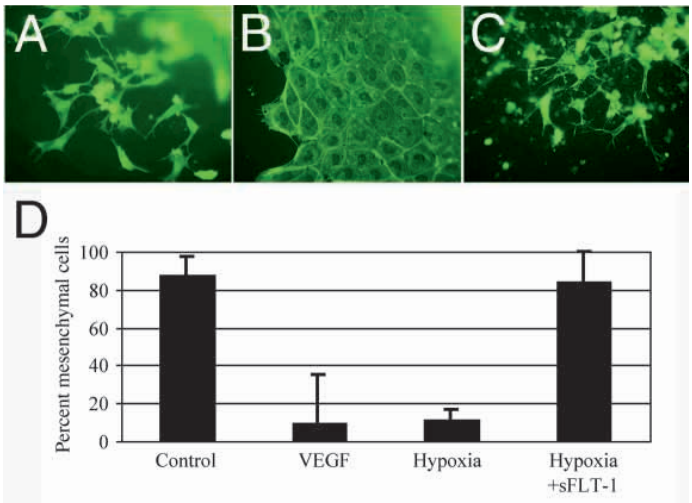

Fig. 6. Hypoxic inhibition of endocardium-to-mesenchyme is mediated by VEGF. AV explants from E9.5 wild-type mouse embryos were cultured on collagen gels under conditions of normoxia (A), hypoxia (B) or hypoxia in the presence of 25 μg/ml sFlt1 (C). After 24 hours, explants were fixed and stained with phalloidin-FITC. (D) Quantification of the effect of VEGF (a representative data shown in Fig.2), hypoxia and sFlt1 on mesenchymal transformation. Mesenchymal cells (identified by morphology and identification verified by staining with αSMA (not shown)) and endocardial cells in each AV explant were enumerated by multiple independent observers. Data are presented as percent mesenchymal cell over the total cell number in the field and are the average of values obtained from eight control explants and five VEGF-treated explants, 15 hypoxia-treated explants and six hypoxia+sFlt1-treated explants. Calculated *P* values: VEGF versus control, $P=1.8\times10^{-7}$; hypoxia versus control, $P=4.5\times10^{-8}$; hypoxia versus sFlt1, $P=3.1\times10^{-6}$.

might be a crucial factor linking gestational ischemia and congenital heart defects. The in vivo analysis outlined above indeed showed that surplus VEGF at levels comparable with those induced by hypoxia during a crucial developmental stage is associated with the generation of a major defect in formation of the AV-canal and outflow tract. To directly establish a cause-effect relationship between hypoxia, VEGF and developmental heart defects, we again resorted to an ex vivo model of endocardium-to-mesenchyme transformation. E9.5 AV explants were cultured as described above and transformation allowed to proceed under normoxic or hypoxic conditions. As shown in Fig. 6, hypoxia completely inhibited mesenchymal transformation, thus reproducing the effect of recombinant VEGF. To determine whether the inhibitory effect of hypoxia on mesenchymal transformation could be attributed to VEGF, a soluble Ig-VEGFR1 chimeric protein (sFlt) was added to the hypoxic cultures. sFlt acts as a dominant negative inhibitor of VEGF signaling through sequestration of the growth factor and has been shown to be an effective and highly specific inhibitor of VEGF signaling (Gerber et al., 1999). As shown in Fig. 6, sFlt negated the inhibitory effect of hypoxia and fully restored mesenchymal transformation and gel invasion. This result indicates that VEGF is the key mediator of hypoxia-induced defects in the formation of endocardial cushions and, by extrapolation, suggests that VEGF might mediate additional teratogenic effects of hypoxia on heart development.

DISCUSSION

Several lines of evidence provided by this study establish a novel role for VEGF in heart morphogenesis, namely, in negatively regulating the formation of endocardial cushions. First, endogenous VEGF is specifically upregulated in the myocardium that underlies the forming endocardial cushions shortly after the onset of this process. Second, VEGF secreted by this subset of myocardial cells activates the adjacent endocardium, including increased expression of its own receptors (this work; Barleon et al., 1997; Flamme et al., 1995; Kremer et al., 1997; Shen et al., 1998), but receptor expression is downregulated immediately upon detachment from the endocardial layer and transformation. Third, overexpression of myocardial VEGF in vivo at the time that mesenchymal transformation normally takes place inhibits the process and abrogates subsequent cellularization of the cardiac jelly with cushion tissue. Fourth, VEGF prevents endocardium-to mesenchyme transformation in vitro. Taken together, the following scenario regarding the role of VEGF is envisaged: a myocardial signal, possibly TGFβ (Boyer et al., 1999; Brown et al., 1999; Eisenberg and Markwald, 1995) induces endocardial-to-mesenchymal transformation. Following cellularization of the cardiac jelly, VEGF is locally induced in the underlying myocardium, perhaps by a signal emanating from the transformed cells. VEGF then acts upon the adjacent endocardium, enforcing an epithelial phenotype and, thereby, terminating the endocardial competence for mesenchymal transformation. In this way, VEGF may restrict the extent of endocardial cell delamination and, in concert with independent controls that affect the magnitude of cushion cell proliferation (Lakkis and Epstein, 1998), participate in determining the overall cellularity of cushion tissue. VEGF might also induce local proliferation of endocardial cells, thereby acting to fill the gaps created by the abandonment of transformed cells. It should be pointed out that while extensive studies have provided important insights regarding factors that promote epithelial-to-mesenchymal transformation in diverse biological systems, little is known about mechanisms that keep transformation in check to prevent excessive transformation. In this respect, our findings regarding negative control by VEGF on endocardial-to-mesenchymal transformation might set an example.

Further studies are required to address the signaling pathway downstream of VEGF during cardiac cushion formation. One possible mediator of VEGF signaling in the endocardium is the transcription factor NF-AT whose nuclear translocation in endothelial cells is induced by VEGF (Armesilla et al., 1999). Interestingly, it has recently been shown that the NF-ATc variant is expressed exclusively in the embryonic endocardium, that its nuclear translocation is restricted to cells overlying cardiac cushions and that mice deficient in NF-ATc die at E13.5-E17.5 from valvuloseptal defects (de la Pompa et al., 1998; Nolan, 1998; Ranger et al., 1998).

VEGF is a highly pleiotropic factor that plays multiple roles in both cardiovascular morphogenesis and homeostatic vascular responses to hypoxia. Whereas the first phenomenon is crucially dependent on precise levels and appropriately timed expression of VEGF, the latter is associated with a regional induction of VEGF to levels determined by the nature and magnitude of environmental stress. So far, these two aspects of VEGF activity have been dealt with in isolation from one another. We reasoned that an interplay between developmentally programmed and stress-induced VEGF expression under circumstances of gestational hypoxia or hypoglycemia might adversely effect the morphogenic process and, hence, be teratogenic. In this study we focused on heart development because the heart appears to be particularly prone to congenital defects in humans and as hypoxia greatly increases the frequency of cardiac malformations in experimental animals. The focus on cardiac cushion development is justified by the fact that the majority of congenital heart defects arise from abnormal development of valvuloseptal tissue (Eisenberg and Markwald, 1995; Olson and Srivastava, 1996), the primordia of which are the cardiac cushions. We have shown that endocardium-to-mesenchyme transformation underlying cardiac cushion formation is indeed abrogated by hypoxia and that this effect is entirely accounted for by surplus VEGF.

By way of extrapolation, we propose that additional heart morphogenic processes might be vulnerable to hypoxic insults associated with excessive VEGF production. Additional classes of congenital heart defects are recognized, including abnormal thinning of the heart wall and ventricular trabeculation defects (reviewed by Chien, 1999). Ongoing studies in our laboratory address the possibility that deregulated expression of VEGF can also produce cardiac malformations other than those reported here. In fact, switching on VEGF production during different stages of heart development resulted in a host of heart abnormalities that resemble human congenital defects, including extreme thinning of the atrial wall and impaired formation of ventricular trabeculae (Fig. 5G,H).

The use of a switchable transgenic system is instrumental for this type of analysis, as also illustrated in the following observation: when the VEGF switch was continuously ‘on’, embryonic death ensued around day E11.5, thus precluding analysis of cardiac phenotypes resulting from misexpression of VEGF at later stages. Induction of myocardial VEGF was, therefore, experimentally delayed by adding tetracycline to the drinking water of pregnant mice between E3.5 and E9.5, followed by further development in the absence of tetracycline. This procedure delayed accumulation of transgenic VEGF to around E10.5 and postponed embryonic death by at least 2 days. Inspection of these E12.5 embryos revealed that cardiac septa and valves, as well as ventricular trabeculae appeared normal. However, another abnormal phenotype was unmasked, namely formation of endocardial cell clusters penetrating the entire width of the myocardium (data not shown). Together, these findings show that the cardiac consequences of VEGF misexpression are crucially dependent on the developmental stage in which it occurs and suggest that embryonic hypoxia might produce a host of heart defects the nature of which depends on the developmental timing of the environmental insult.

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