Development 136, 3335-3345 (2009) doi:10.1242/dev.037903

Spatial and temporal regulation of coronary vessel formation by calcineurin-NFAT signaling

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Formation of the coronary vasculature requires reciprocal signaling between endothelial, epicardially derived smooth muscle and underlying myocardial cells. Our studies show that calcineurin-NFAT signaling functions in endothelial cells within specific time windows to regulate coronary vessel development. Mouse embryos exposed to cyclosporin A (CsA), which inhibits calcineurin phosphatase activity, failed to develop normal coronary vasculature. To determine the cellular site at which calcineurin functions for coronary angiogenesis, we deleted calcineurin in endothelial, epicardial and myocardial cells. Disruption of calcineurin-NFAT signaling in endothelial cells resulted in the failure of coronary angiogenesis, recapitulating the coronary phenotype observed in CsA-treated embryos. By contrast, deletion of calcineurin in either epicardial or myocardial cells had no effect on coronary vasculature during early embryogenesis. To define the temporal requirement for NFAT signaling, we treated developing embryos with CsA at overlapping windows from E9.5 to E12.5 and examined coronary development at E12.5. These experiments demonstrated that calcineurin-NFAT signaling functions between E10.5 and E11.5 to regulate coronary angiogenesis. Consistent with these in vivo observations, endothelial cells exposed to CsA within specific time windows in tissue culture were unable to form tubular structures and their cellular responses to VEGF-A were blunted. Thus, our studies demonstrate specific temporal and spatial requirements of NFAT signaling for coronary vessel angiogenesis. These requirements are distinct from the roles of NFAT signaling in the angiogenesis of peripheral somatic vessels, providing an example of the environmental influence of different vascular beds on the in vivo endothelial responses to angiogenic stimuli.

KEY WORDS: Calcineurin (PPP3CA; PPP3R1), NFAT, VEGF, Endothelial cell, Coronary vessel, Heart development, Hedgehog, Mouse

INTRODUCTION

Development of the coronary vasculature is a unique process during embryogenesis (Reese et al., 2002). Early coronary vessels develop through vasculogenesis as angioblasts coalesce to form a primitive vascular plexus on the surface of developing hearts (Kattan et al., 2004; Perez-Pomares et al., 2002; Tomanek et al., 2002; Tomanek et al., 1999; Tomanek and Zheng, 2002; Tomanek et al., 2001; Yue and Tomanek, 2001). The final growth of the coronary tree occurs by angiogenesis as new capillaries extend from these preformed blood vessels (Tomanek et al., 2001; Yue and Tomanek, 2001). Later, these endothelial channels remodel and evolve into mature coronary vessels as smooth muscle cells and fibroblasts are recruited to the vessel wall. Smooth muscle cells and fibroblasts in the coronaries are derived from epicardial cells through an epicardial-to-mesenchymal transformation (Lu et al., 2001; Wada et al., 2003) and subsequent differentiation within the myocardium. Signals from epicardial and myocardial cells are thus crucial for the formation of a mature coronary vasculature. Mice with mutations in certain genes that are expressed in epicardial or myocardial cells, such as *Wt1* (Moore et al., 1998), *Zfpm2* (also known as *FOG-2*) (Tevosian et al., 2000), *Gata4* (Crispino et al., 2001) and *Bves* (Wada et al., 2001), among others, have impaired coronary vessel development, indicating that there is a close interaction between myocardial and epicardially derived cells and endothelial cells to regulate coronary formation.

Endothelial cells play a central role in coronary and peripheral vascular patterning. Multiple cell surface receptors and their ligands have been shown to regulate endothelial cell differentiation, vasculogenesis and angiogenesis (Carmeliet, 2000; Yancopoulos et al., 1998). Most of the receptors involved in vascular development, such as VEGFR2 (also known as FLK1 or KDR – Mouse Genome Informatics) (Shalaby et al., 1995), VEGFR1 (FLT1 – Mouse Genome Informatics) (Fong et al., 1995) and VEGFR3 (FLT4 – Mouse Genome Informatics) (Dumont et al., 1998), are tyrosine kinases that activate MAP kinase cascades and Ca^{2+} signaling. Ca^{2+} signals can lead to the activation of calcineurin, a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase composed of catalytic (CnA) and regulatory B (CnB) subunits (Klee et al., 1998). Calcineurin activation results in the rapid dephosphorylation of NFATc proteins (c1 to c4), causing them to translocate into the nucleus (Beals et al., 1997a; Clipstone and Crabtree, 1992; Crabtree, 1989; Crabtree and Olson, 2002; Flanagan et al., 1991). Once in the nucleus, NFATc proteins cooperate with nuclear partners (referred to here as NFATn) to form NFAT transcriptional complexes on target genes (Crabtree, 1989; Flanagan et al., 1991). The NFAT pathway is opposed by DYRK1A and GSK3 (GSK3β – Mouse Genome Informatics) kinases, which act sequentially to actively export NFATc proteins from the nucleus (Arron et al., 2006; Beals et al., 1997b). A second level of opposition to the pathway occurs through inhibitors of calcineurin, such as DSCR1 (also known as RCAN1, calcipressin1 or MCIP1 – Mouse Genome Informatics) (Gorlach et al., 2000; Kingsbury and Cunningham, 2000; Rothermel et al., 2001). Furthermore, the activity of calcineurin and NFATc can be specifically blocked by the immunosuppressive drugs cyclosporin A (CsA) and FK506 (Emmel et al., 1989; Liu et al., 1991). Although biochemical studies have suggested that calcineurin has many substrates (Aperia et al., 1992), genetic studies indicate that calcineurin is

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Accepted 7 July 2009

rather dedicated to NFATc proteins during early embryonic development (Chang et al., 2004; de la Pompa et al., 1998; Graef et al., 2001; Graef et al., 2003; Ranger et al., 1998; Wu et al., 2007).

Calcineurin-NFAT signaling has been shown to regulate important processes during cardiovascular development, including heart valve morphogenesis (Chang et al., 2004; de la Pompa et al., 1998; Ranger et al., 1998), myocardial development (Bushdid et al., 2003; Chang et al., 2004; Schubert et al., 2003) and peripheral vascular patterning (Graef et al., 2001). Mice with *Nfatc3/c4* or *Cnb1* (also known as *Ppp3r1* – Mouse Genome Informatics) loss-of-function mutations exhibit excessive vessel growth into normally avascular tissues and a failure to form mature vessels, leading to embryonic lethality at E11.5 (Graef et al., 2001). These peripheral vascular defects are caused by the excessive production of vascular endothelial growth factor A (VEGF-A; also known as VEGFA) by perivascular tissues, a result of the derepression of VEGF-A expression due to a lack of calcineurin or NFATc3/c4 (Chang et al., 2004; Graef et al., 2001). Despite the importance of NFAT signaling in peripheral vessel angiogenesis, its role in coronary vascular formation is unknown, as *Cnb1*- or *NFATc2/c4*-null embryos die at E11.5 before the formation of coronary vessels. To overcome the early lethality of *Cnb1*-null mice, we have used pharmacological inhibition and tissue-specific disruption of calcineurin function to define the temporal and spatial requirements of calcineurin-NFAT during coronary angiogenesis. Our studies demonstrate that NFAT signaling regulates coronary angiogenesis by a mechanism distinct from its roles in the peripheral somatic vasculature, and provide a model to understand the interactions between endothelial cells and perivascular tissues in the angiogenic production of different vascular beds.

MATERIALS AND METHODS

Mice

All mouse strains were maintained in outbred backgrounds. The *Cnb1*^{f} (Neilson et al., 2004), *Tie2-Cre* (Kisanuki et al., 2001), *Sm22a-Cre* (Holtwick et al., 2002; Stankunas et al., 2008) and *Gata5-Cre* (Merki et al., 2005) strains are previously described. The date of observing a vaginal plug was set as E0.5, and embryonic development was confirmed by ultrasonography before sacrificing pregnant mice (Chang et al., 2003).

Cyclosporin treatment

Pregnant females were injected with cyclosporin A (CsA) during the time windows indicated in Fig. 2D. CsA was administered at a dose of 50 mg/kg through intraperitoneal injection twice a day at 09.00-10.00 h and at 19.00-20.00 h. Embryos were harvested at E11.5 or E12.5 for PECAM1 whole-mount staining. Control females were injected with phosphate-buffered saline (PBS) at the same time points.

Whole-mount staining

PECAM1 whole-mount staining was performed on whole embryos or embryonic hearts as described previously (Graef et al., 2001; Stankunas et al., 2008). These tissues were stained with rat anti-PECAM1 antibody (1:100, Pharmingen). A donkey anti-rat HRP-conjugated secondary antibody was used to detect the signal with DAB substrate. Tissues were postfixed in 4% paraformaldehyde (PFA), imaged under a Leica dissecting microscope and then processed for sectioning. Paraffin sections were counterstained with nuclear Fast Red and imaged using a Nikon microscope.

Immunohistochemistry

For Ki67 and calcineurin immunostaining, paraffin sections were deparaffinized and treated with 3% hydrogen peroxide. Citrate (Ki67) or trypsin (calcineurin) antigen retrieval was followed by overnight incubation with anti-Ki67 (DAKO, Denmark) or anti-calcineurin (Sigma, St Louis, MO, USA) antibodies. HRP-conjugated secondary antibodies were used to detect the signal with DAB substrate. The TUNEL assay was performed according to manufacturer's guidelines (Roche). For frozen sections, embryos were fixed in PFA 4% for 1 hour at 4°C and then cryoprotected in 30% sucrose overnight at 4°C. The embryos were then embedded in OCT compound and kept at –20°C until sectioning in a Leica cryostat. Tissue was permeabilized with 0.3% Triton X-100 in PBS, blocked with 5% normal goat serum and incubated with the antibodies overnight at 4°C (anti-PECAM1, Chemicon; anti-PDGFRβ, eBioscience). Fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories) were used to detect the signal.

RNA in situ hybridization

This procedure was performed as described previously (Stankunas et al., 2008). Digoxigenin-labeled antisense transcripts were synthesized (Roche) from plasmid templates NFATc1 (de la Pompa et al., 1998), NFATc2 (Open Biosystems, Huntsville, AL), NFATc3 and NFATc4 (Graef et al., 2001; Graef et al., 1999), and VEGF-A (Chang et al., 2004).

Quantification of ventricular surface covered by endothelial tubes

The total ventricular area and area covered by the coronary endothelial bed were measured using the NIS-Elements program (Nikon). The ratio between the surface covered by endothelial cells and total ventricular surface in control embryonic hearts was set at 100% for comparison. This ratio in CsA-treated embryos or other mutant embryos was calculated as a percentage of the value in control littermates.

Cell culture and proliferation studies

Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) (Cambrex, NJ, USA) were grown in endothelial growth media (EGM-2; Lonza, MD, USA), containing hEGF, hydrocortisone, GA-1000 (gentamicin, Amphotericin B), fetal bovine serum (FBS), VEGF, hFGF-B, R3-IGF-1, ascorbic acid and heparin. H5V mouse endothelial cells (Garlanda et al., 1994) were grown in M199 medium with 10% FBS and penicillin-streptomycin. SVR40 mouse endothelial cells were grown in Dulbecco's Modified Eagle Medium with 10% FBS and penicillin-streptomycin. Cell proliferation studies were performed in growing HUVECs pulsed with 10 μM BrdU for 6 hours or 24 hours. Cells were stained for BrdU using a BrdU Kit, following the manufacturer's instructions (Zymed, CA, USA). Positively labeled cells were counted and calculated as a percentage of the total number of cells analyzed.

Matrigel tube formation assay

Dishes (with 24 wells) were coated with Matrigel (BD Pharmingen) and incubated at 37°C for 20 minutes followed by seeding with HUVECs, HCAECs or H5V cells in 1 ml of complete medium at 50,000 cells/ml. Recombinant VEGF (100 ng/ml) was added when cells were plated on the Matrigel. CsA (100 ng/ml) was added at the same time as seeding the cells on the Matrigel, or at different times after plating on the gels. Tube formation was assessed 24 hours after plating the cells on the Matrigel. Quantitation of tubular structures was performed by manual counting of tubes using light microscopy. The formation of tube-like structures in untreated control cells was considered as 100%, and the percentage of tube formation under other treatments was calculated relative to the control. A standard two-tailed Student *t*-test was used for statistical analysis.

Quantitative RT-PCR

HUVECs and HCAECs were cultured on multiwells or Matrigel-coated multiwells. RNA extraction was performed using TRIzol (Invitrogen) and 150 ng of purified RNA were used as a template to synthesize cDNA using the Superscript III Reverse Transcription System (Invitrogen). PCR was performed using the following primer sequences (all 5′ to 3′) for HUVECs and HCAECs: ESM1-F, GCTGAGGTGTCAGCCTTCTAAT; ESM1-R, CAGGTCTCTCTGCAATCCATC; CDH5-F, GGCTAGGCATAG-CATTGGATAC; CDH5-R, GGCCTCCACAGTCAGGTTATAC; ENG-F, AAACAGTCCATTGTGACCTTCA, ENG-R, TTTACACTGAG-GACCAGAAGCA; BMPR2-F, GGAAAGGATGGCTGAACTTATG; BMPR2-R, CGATGCTGTCAGTATGATGGAT; TIE2-F, ATG-GACTCTTTAGCCGGCTTA; TIE2-R, CCTTATAGCCTGTCCTCGAA; FLT1-F, AGCACTACACATGGAGCCTAAGA; FLT1-R, GTAGAAAC-CGTCAGAATCCTCCT; KDR-F, GTTAGTGACCAACATGGAGTCGT;

KDR-R, GCTGATCATGTAGCTGGGAATAG; hHPRT-F, CTGAGGATTTGGAAAGGGTGT; hHPRT-R, CTTGAGCACACAGAGGGCTAC. A Bio-Rad iCycler was used for quantitative analysis. The primer sets were first validated for use in quantitative PCR according to parameters recommended in a technical note ('Standard Curves in Real-Time Quantitative PCR') from Stratagene, using HUVEC or HCAEC cDNA as a template. Mean threshold cycles were determined for three repeats of each reaction using three control and three treated samples. The mean fold change in expression between the control and treated samples was calculated including correction for the efficiency of amplification of each primer set and normalization to *HPRT* levels.

RESULTS

Coronary vascular patterning during embryonic development

To observe the sequence of coronary vascular patterning, endothelial cell formation on the surface of the heart was analyzed in wild-type embryos at different developmental stages, from E10.5 to E13.5. PECAM1 (platelet-endothelial cell adhesion molecule 1) (Baldwin et al., 1994) whole-mount staining was performed on isolated embryonic hearts to analyze the nascent endothelial tube formation in vivo. At E10.5, a few PECAM1-positive cells were observed on the posterior surface of the heart (Fig. 1A). At E11.5, primitive, yet unrefined, endothelial networks or patches were observed in the atrioventricular junction (Fig. 1C). By E12.5, these endothelial tubes extended from the atrioventricular junction inferiorly and laterally to the apical and lateral regions of the ventricles, forming a more elaborate vascular network (Fig. 1E) that began to encroach upon the anterior surface of the heart (Fig. 1F). By E13.5, many endothelial tubes had coalesced to form a more distinct vascular pattern in the posterior surface (Fig. 1G). Endothelial tubes became apparent at the lateral border of the heart, and a number of endothelial nodules appeared on the anterior surface of the heart (Fig. 1H). This sequence of events suggests that cells at the distal end of endothelial tubes proliferate to expand the vascular formation or that new endothelial cells are recruited to maintain the progression of the vascular network. Retroviral cell tagging studies in chick hearts suggest that coronary vessels grow discontinuously by recruiting and coalescing new endothelial cells (Mikawa and Fischman, 1992).

Calcineurin inhibition leads to abnormal coronary angiogenesis

To study the role of calcineurin-NFAT signaling in coronary vessel development, we used cyclosporin A (CsA) to inhibit calcineurin activity in embryos at different gestational ages. As we have previously described, CsA effectively inhibits calcineurin activity in embryos within 3 hours of administering to pregnant mice (Chang et al., 2004). Its level in embryos drops to the control background levels within 24 hours of CsA treatment, providing the precision of 1 developmental day to define the window of calcineurin-NFAT action.

Pregnant mice were treated with CsA (50 mg/kg by intraperitoneal injection, twice a day) starting from E9.5, E10.5 or E11.5, and embryos were harvested at E12.5 for analysis of coronary vasculature patterning. Embryos exposed to CsA in utero were grossly indistinguishable from untreated embryos at E12.5, regardless of the starting time of CsA treatment (Fig. 2A). Hearts from CsA-treated embryos were also similar in size to non-treated littermate controls (Fig. 2B,C), suggesting that there was no gross developmental delay at E12.5 as a result of CsA treatment. Coronary vessel development was analyzed by PECAM1 whole-mount staining of E12.5 hearts. E12.5 was chosen as the time point for analysis because embryos treated with CsA died at early E13 owing to heart valve defects (Chang et al., 2004), precluding further examination of coronary development at or beyond E13. PECAM1 whole-mount analysis showed an extensive network of coronary endothelial cells in untreated embryos at E12.5, whereas embryos exposed to CsA at E10.5 showed limited endothelial patterning (Fig.

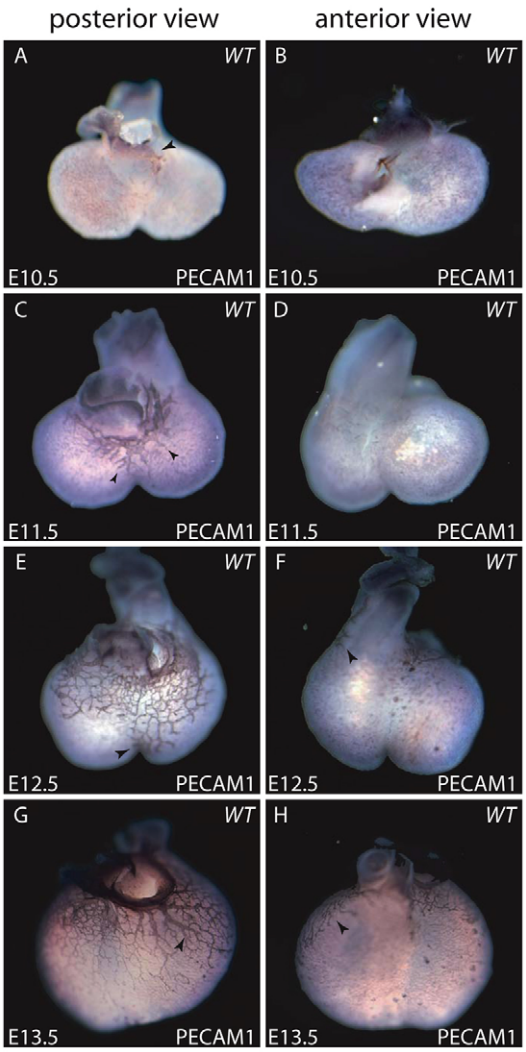

Fig. 1. Vascular patterning of the coronaries during mouse embryonic development. (**A**,**B**) PECAM1-positive cells (purple) observed on the posterior (A) and anterior (B) surface of the heart by E10.5. Some endothelial cells are present at the atrioventricular junction (arrowhead in A). (**C**,**D**) PECAM1 staining showing nascent coronary vessels on the posterior (C) and anterior (D) surface of the heart at E11.5. Arrowheads in C indicate the distal end of the endothelial tubes extending from the atrioventricular junction to the apical region of the heart. (**E**,**F**) PECAM1 staining of the endothelial network on the posterior (E) and anterior (F) surface of E12.5 embryonic hearts. By E12.5, the vascular endothelial network has reached more apically to the interventricular region of the heart (arrowhead in E). Also, some vessels appear laterally and begin to extend to the anterior surface of the heart (arrowhead in F). (**G**,**H**) PECAM1 staining showing the pattern of coronary endothelial cells on the posterior (G) and anterior (H) surface of the heart at E13.5. More mature coronary vessels are formed on the posterior surface by E13.5 (arrowhead in G), and some vessels have extended to the anterolateral surface of the heart (arrowhead in H).

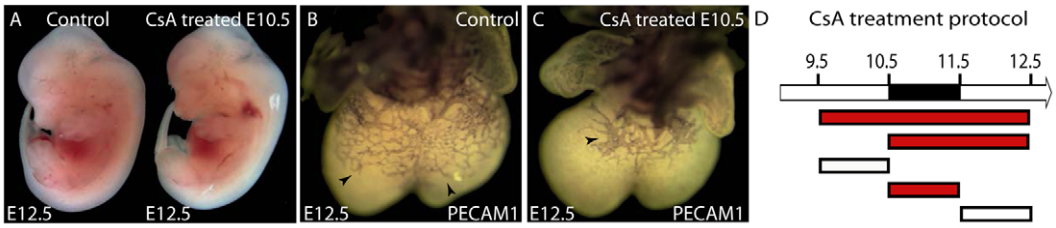

Fig. 2. Calcineurin inhibition leads to abnormal coronary angiogenesis. (**A**) Gross morphology of a wild-type control embryo (left) and an embryo exposed to CsA (50 mg/kg by intraperitoneal injection, twice daily) from E10.5 to E12.5 (right). (**B**,**C**) PECAM1 staining showing coronary endothelial networks in control hearts at E12.5 (B) and in hearts exposed to CsA from E10.5 to E12.5 (C). Arrowheads indicate developing endothelial tubes. (**D**) Summary of the CsA treatment protocol and time window of calcineurin requirement for coronary vessel patterning. Red bars show time windows of CsA administration that resulted in impaired coronary endothelial cell assembly. White bars indicate treatments that did not affect coronary endothelial assembly. The critical window for calcineurin requirement (E10.5-11.5) is highlighted in the time-line in black.

2B,C). The endothelial tubes were fused and restricted to the atrioventricular junction, failing to form an elaborate endothelial network. By contrast, embryos exposed to CsA at E9.5-10.5 or E11.5-12.5 displayed normal coronary endothelial patterning at E12.5 (Fig. 2D). Also, embryos exposed to CsA during these windows from E9.5 to E12.5 showed no endothelial patterning abnormalities in the peripheral vascular beds of cranial, intersomitic and dorsal regions of the embryo (data not shown), consistent with previous reports that calcineurin functions at E7.5-8.5 to regulate peripheral angiogenesis (Graef et al., 2001). Taken together, these observations indicate a specific temporal requirement of calcineurin at E10.5 for normal coronary vessel development.

Calcineurin-NFAT signaling is not required in epicardial or myocardial cells for embryonic coronary angiogenesis

The next question concerns where calcineurin-NFAT signaling functions to regulate coronary development. Coronary development requires reciprocal signaling between neighboring cells or tissues that include epicardial, myocardial, vascular smooth muscle cells and endothelial cells. Since calcineurin is widely expressed in all of these cells (Chang et al., 2004), calcineurin could function in any of these cell types to regulate coronary vessel formation. To determine the cellular site of calcineurin action during coronary angiogenesis, we performed tissue-specific gene deletion of the calcineurin regulatory subunit (CNB1) using the murine Cre-lox genetic method.

To study the roles of calcineurin in epicardial or myocardial cells for coronary vascular patterning, we deleted *Cnb1* in epicardial and myocardial cells, using *Gata5-Cre* and *Sm22a-Cre* (also known as *Tagln-Cre*) mouse lines, respectively. The *Gata5* promoter in the *Gata5-Cre* mouse line directs the expression of Cre recombinase to epicardial cells (Merki et al., 2005), which give rise to the vascular smooth muscle cells of coronary vessels (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Perez-Pomares et al., 2002). *Gata5-Cre* activity is evident by E9.25 in the pro-epicardial cells and, by E9.5-10.0, in the epicardial cells (Merki et al., 2005). Epicardial-restricted *Cnb1* mutant mice (*Gata5-Cre;Cnb1*$^{f/f}$) were grossly normal (Fig. 3A) and displayed no defects in endothelial patterning at E12.5 by whole-mount PECAM1 staining (Fig. 3B,C). Furthermore, *Gata5-Cre;Cnb1*$^{f/f}$ mice lived to adulthood, and they were indistinguishable from their wild-type littermates at birth (data

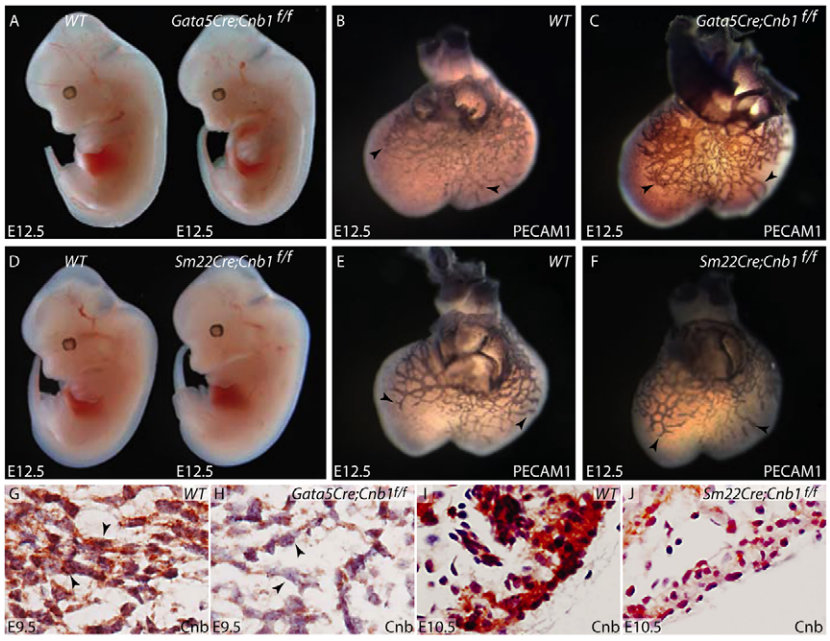

Fig. 3. Calcineurin-NFAT signaling is not required in epicardial cells or myocardial cells for coronary vessel patterning. (**A**) Gross morphology of wild-type (left) and *Gata5-Cre;Cnb1*$^{f/f}$ littermate (right) embryos at E12.5. (**B**,**C**) PECAM1 staining of coronary endothelial cells in wild-type (B) and *Gata5-Cre;Cnb1*$^{f/f}$ littermate (C) hearts at E12.5. Arrowheads indicate endothelial cells. (**D**) Gross morphology of wild-type (left) and *Sm22a-Cre;Cnb1*$^{f/f}$ littermate (right) embryos at E12.5. (**E**,**F**) PECAM1 staining of coronary endothelial cells in wild-type (E) and *Sm22a-Cre;Cnb1*$^{f/f}$ littermate (F) hearts at E12.5. Arrowheads indicate endothelial cells. (**G**,**H**) Calcineurin staining in wild-type (G) and *Gata5-Cre;Cnb1*$^{f/f}$ (H) pro-epicardial cells at E9.5. Arrowheads indicate pro-epicardial cells. Brown, calcineurin; blue, counterstaining with Hematoxylin. (**I**,**J**) Calcineurin staining in wild-type (I) and *Sm22a-Cre;Cnb1*$^{f/f}$ mutant (J) myocardium at E10.5. Arrowheads indicate myocardial cells. Brown, calcineurin; blue, counterstaining with Hematoxylin.

not shown). This observation indicates that calcineurin in epicardial or in epicardially derived cells is not required for coronary vessel formation.

To study the role of calcineurin in myocardial cells during coronary development, we used a *Sm22a-Cre* mouse line to delete *Cnb1* in myocardial cells (Fig. 3D). Cre recombinase-driven by the *Sm22a* promoter is active in both vascular smooth muscle cells and myocardial cells. *Sm22a-Cre* expression in myocardial cells occurs as early as E9.0, as described previously (Stankunas et al., 2008; Umans et al., 2007) *Sm22a-Cre;Cnb1*$^{f/f}$ embryos were grossly normal (Fig. 3D) and showed normal coronary endothelial patterning at E12.5 (Fig. 3E,F). *Sm22a-Cre;Cnb1*$^{f/f}$ mice also lived to adulthood, with no evidence of coronary patterning defects at birth (data not shown). These findings indicate that calcineurin is not essential in myocardial or vascular smooth muscle cells for coronary angiogenesis.

To verify the deletion of *Cnb1* from epicardial and myocardial cells, we performed calcineurin immunostaining in *Gata5-Cre;Cnb1*$^{f/f}$ and *Sm22a-Cre;Cnb*$^{f/f}$ embryos. We found that calcineurin was indeed removed from the pro-epicardial cells of *Gata5-Cre;Cnb1*$^{f/f}$ embryos by E9.5 (Fig. 3G,H) and from the myocardial cells of *Sm22a-Cre;Cnb1*$^{f/f}$ embryos by E10.5 (Fig. 3I,J), before the critical window when calcineurin is required for coronary angiogenesis (Fig. 2D). These observations thus validate the conclusion that calcineurin does not function in epicardial, myocardial or vascular smooth muscle cells to regulate early coronary development.

Endothelial calcineurin-NFAT signaling is essential for coronary vascular patterning

To examine endothelial calcineurin function, we used a *Tie2-Cre* (also known as *Tek-Cre*) mouse line to direct the deletion of *Cnb1* in endothelial cells (Chang et al., 2004; Kisanuki et al., 2001). As we reported previously, this *Tie2-Cre* activity is detectable in endothelial cells by E8.0, and calcineurin is deleted in endocardial cells by E9.5 (Chang et al., 2004). *Tie2-Cre;Cnb1*$^{f/f}$ embryos showed no gross developmental defects compared with their wild-type littermates at E12.5 (Fig. 4A). PECAM1 whole-mount staining of *Tie2-Cre;Cnb1*$^{f/f}$ hearts at E12.5 showed limited endothelial branching, in contrast to an extensive network of endothelial tubes in the wild-type littermate control (Fig. 4B,C). Coronary endothelial cells in *Tie2-Cre;Cnb1*$^{f/f}$ hearts were fused and limited to the atrioventricular junction. These cells were unable to extend apically or laterally in the heart (Fig. 4C). Histological sections of wild-type hearts showed that PECAM1-positive endothelial cells assembled to form tubes that extended to the lateral and apical parts of the heart, whereas in the *Tie2-Cre;Cnb1*$^{f/f}$ hearts PECAM1-positive cells were limited to the basal portion of the ventricles, with no endothelial cells reaching the apical region of the heart (Fig. 4D). These endothelial patterning defects in *Tie2-Cre;Cnb1*$^{f/f}$ embryos phenocopy the defects observed in CsA-treated embryos (Fig. 2C). Combined with the data obtained from mice lacking *Cnb1* in epicardial and myocardial cells, these findings demonstrate that calcineurin is specifically required in endothelial cells to regulate endothelial assembly in early coronary vascular development.

The next question that arose was whether endothelial calcineurin is also required for peripheral vascular patterning in developing embryos. We performed PECAM1 whole-mount immunostaining of embryos at E10.5 and E11.5, and observed no significant differences in the peripheral vasculature between wild-type and *Tie2-Cre;Cnb1*$^{f/f}$ embryos. Cranial, intersomitic and dorsal vessels of *Tie2-Cre;Cnb1*$^{f/f}$ mutant embryos appeared normal (Fig. 4E,F). Of note, these *Tie2-Cre;Cnb1*$^{f/f}$ embryos died at E13 owing to heart valve defects, consistent with previous reports (Fig. 4G) (Chang et al., 2004). These studies suggest that calcineurin in endothelial cells is not essential for peripheral vascular patterning. The differential effects of endothelial calcineurin in coronary versus peripheral vascular development suggest that distinct mechanisms are involved in the development of these two vascular beds.

We next examined whether *NFATc* genes are expressed in coronary endothelial cells at E12.5 when the endothelial tubes first became easily detectable (Fig. 1). By RNA in situ hybridization, we found that *NFATc3* and *NFATc4*, but not *NFATc1* or *NFATc2*, were expressed in coronary endothelial cells (Fig. 4H,I,J,K), suggesting that endothelial NFATc3 and NFATc4 are activated by calcineurin to transduce the signals required for coronary angiogenesis.

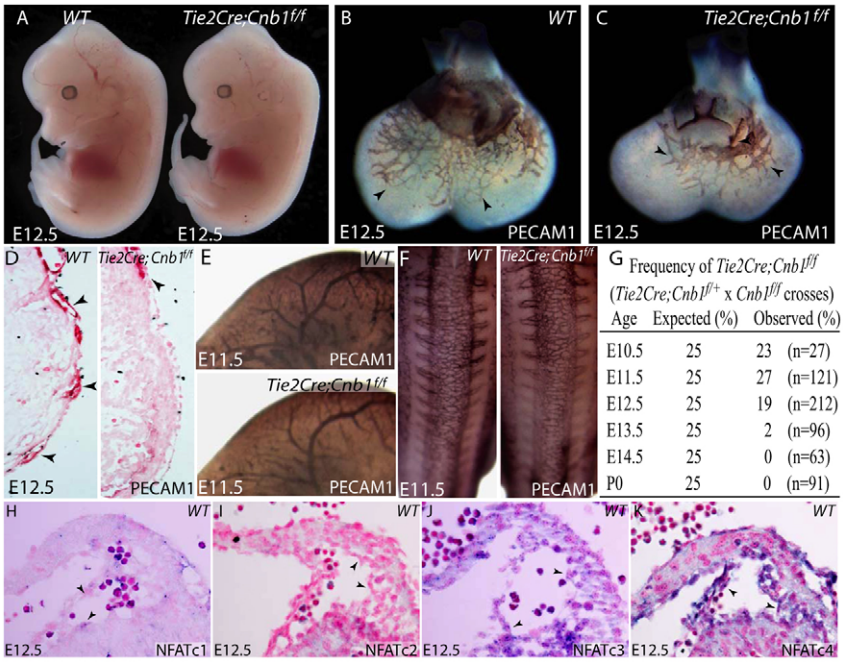

Fig. 4. Endothelial calcineurin-NFAT signaling is required for normal coronary vessel patterning. (**A**) Gross morphology of wild-type (left) and *Tie2-Cre;Cnb1*$^{f/f}$ (right) embryos at E12.5. (**B**,**C**) PECAM1 whole-mount staining of endothelial cells in wild-type (B) and *Tie2-Cre;Cnb1*$^{f/f}$ (C) hearts at E12.5. Arrowheads denote endothelial cells. (**D**) Sections of wild-type (left) and *Tie2-Cre;Cnb1*$^{f/f}$ (right) hearts showing distribution of nascent coronary vessels (arrowheads). Red, PECAM1 staining; pink, counterstaining with nuclear Fast Red. (**E**) PECAM1 staining of E11.5 wild-type (upper panel) and *Tie2-Cre;Cnb1*$^{f/f}$ (lower panel) embryos showing normal vasculature in the cranial region. (**F**) PECAM1 staining of E11.5 wild-type (left) and *Tie2-Cre;Cnb1*$^{f/f}$ (right) embryos showing normal vasculature in the dorsal region. (**G**) Frequency of *Tie2-Cre;Cnb1*$^{f/f}$ embryos harvested at different embryonic dates compared with the expected mendelian ratio. (**H-K**) RNA in situ hybridization for *NFATc1* (H), *c2* (I), *c3* (J) and *c4* (K) transcripts (blue) on coronary vessels of E12.5 wild-type embryos. Arrowheads point to coronary endothelial cells. Pink, counterstaining with nuclear Fast Red.

To determine the extent of the coronary vasculature defects observed at E12.5 in CsA-treated embryos or in the different genetic models, we measured the percentage of surface area of the ventricles covered by the endothelial network defined by PECAM1 staining (Fig. 5A). We found that CsA treatment resulted in a diminished endothelial network compared with that in the control embryos (42.2±5.7% versus 100±13.4%; $P<0.01$) (Fig. 5B). By contrast, the coronary endothelial network was not impaired in embryos lacking either epicardial (*Gata5-Cre;Cnb1$^{f/f}$*) or myocardial (*Sm22a-Cre;Cnb1$^{f/f}$*) *Cnb1* (Fig. 5B). However, in embryos lacking endothelial *Cnb1* (*Tie2-Cre;Cnb1$^{f/f}$*), the endothelial defects occurred to a similar extent as those caused by CsA treatment (40.6±3.8%), indicating that the primary effects of CsA treatment on coronary angiogenesis were mediated through endothelial calcineurin.

Next we asked whether the *Cnb1*-null endothelial cells were capable of recruiting pericytes to form a vessel wall. We analyzed the expression of a marker of pericytes, PDGFRβ, to assess the assembly of endothelial cells and pericytes in *Tie2-Cre;Cnb1$^{f/f}$* embryos. By co-immunostaining PECAM1 (to mark endothelial cells) and PDGFRβ (to mark pericytes), we observed that pericytes were recruited normally to the remaining coronary endothelial cells of *Tie2-Cre;Cnb1$^{f/f}$* embryos by E12.5 (Fig. 5C,D). Similarly, the recruitment of pericytes to endothelial cells occurred normally in the peripheral vessels of *Tie2-Cre;Cnb1$^{f/f}$* embryos (Fig. 5E,F). Together with previous reports showing that calcineurin-NFATc3/c4 is essential for endothelial-pericyte assembly in the cranial and somatic vasculature (Graef et al., 2001), our observations indicate that vessel wall formation in the peripheral vasculature is regulated by the non-endothelial or perivascular functions of calcineurin-NFAT. This is in contrast to the pericyte recruitment in the coronary vascular beds, as the peri-endothelial tissues lacking *Cnb1* in smooth muscle cells and the myocardium of *Sm22a-Cre;Cnb1$^{f/f}$* embryos had no effects on pericyte/smooth muscle cell recruitment to coronary vessels (data not shown). Unfortunately, vascular smooth muscle cell recruitment to coronary vessels in *Tie2-Cre;Cnb1$^{f/f}$* embryos could not be examined as these cells had not yet been recruited to the endothelial tubes before *Tie2-Cre;Cnb1$^{f/f}$* embryos died at early E13 (Fig. 4G; data not shown) (Mikawa and Gourdie, 1996).

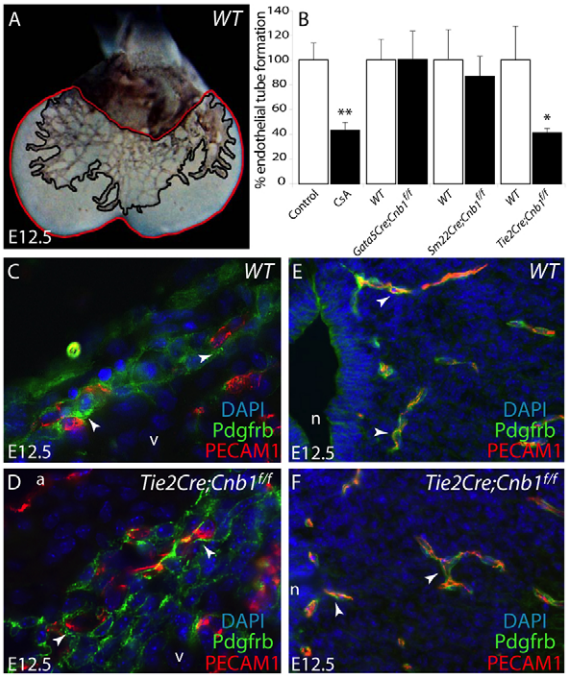

Fig. 5. Quantitation of coronary endothelial network formation and recruitment of perivascular supporting cells. (**A**) Quantitation of the extent of the coronary endothelial network: the ratio between the ventricular area covered by endothelial tubes (PECAM1 staining, outlined in black) and the total ventricular area (outlined in red) was calculated for three individual hearts in each of the described groups. (**B**) Percentage of the ventricular area covered by endothelial cells is represented for each of the groups of embryos analyzed. Statistical significance was calculated by *t*-test analysis (*, $P<0.05$; **, $P<0.01$). Error bars represent the standard deviation ($n=3$ wild-type and CsA-treated or mutant hearts). (**C**,**D**) PECAM1 (red) and PDGFRβ (green) staining in wild-type (C) and *Tie2-Cre;Cnb1$^{f/f}$* (D) embryos, showing recruitment of pericytes to nascent coronary vessels. Arrowheads denote coronary vessels. DAPI-labeled nuclei are blue. (**E**,**F**) PECAM1 and PDGFRβ staining indicating normal recruitment of pericytes to somatic vasculature in wild-type (E) and *Tie2-Cre;Cnb1$^{f/f}$* (F) embryos. Arrowheads point to vessels. a, atrium; n, neural tube; v, ventricle.

Calcineurin contributes to the initiation of endothelial tube formation

To understand the cellular defects underlying the coronary phenotype observed in the *Tie2-Cre;Cnb1$^{f/f}$* mutants, we first examined the effects of calcineurin inactivation on the proliferation and cell death of endothelial cells. By using Ki67 staining, we found no difference in the percentage of proliferating coronary endothelial cells between wild-type and mutant embryos at E12.5 (see Fig. S1A,B in the supplementary material). Nor was there significant apoptotic cell death in these embryos, as shown by TUNEL staining (see Fig. S1C,D in the supplementary material). Furthermore, human umbilical venous endothelial cells (HUVECs) treated with CsA did not show any significant difference in BrdU incorporation rate compared with untreated cells. The TUNEL assay showed no significant increase in cell death in HUVECs in the presence of CsA. Also, there was no difference in the number of HUVECs in the presence of CsA (data not shown). These findings suggest that calcineurin does not inhibit endothelial vascular network formation by reducing proliferation or inducing apoptosis of endothelial cells.

Previous studies suggest that calcineurin is required for endothelial cells to form tubular structures on Matrigel cultures (Hernandez et al., 2001; Rafiee et al., 2004). Endothelial cells, when cultured on Matrigel, spontaneously aggregate and assemble into multicellular capillary-like tubular structures (Folkman and Haudenschild, 1980; Grant et al., 1991), recapitulating many aspects of angiogenesis, including cellular migration, differentiation and metalloproteinase activation. However, it is not known whether there is a specific temporal requirement of calcineurin function for this process, similar to what we have observed in coronary angiogenesis in developing embryos.

To determine the temporal requirement for calcineurin function in endothelial tube formation, we used CsA to treat HUVECs on Matrigel-coated plates, starting at different time points in the presence or absence of additional VEGF-A treatment within a 24-hour observation period. HUVECs assembled into tube-like structures on the Matrigel (Fig. 6A), a process that was increased by 30% in the presence of VEGF-A (Fig. 6B,I). When CsA was administered at the time of plating the cells or within the first 6 hours of HUVEC culture, it significantly inhibited tube formation regardless of the presence of VEGF-A (Fig. 6C,D,I). CsA treatment inhibited tubular formation by 40% in VEGF-A-treated cultures, thus offsetting the additional tubular formation due to VEGF-A

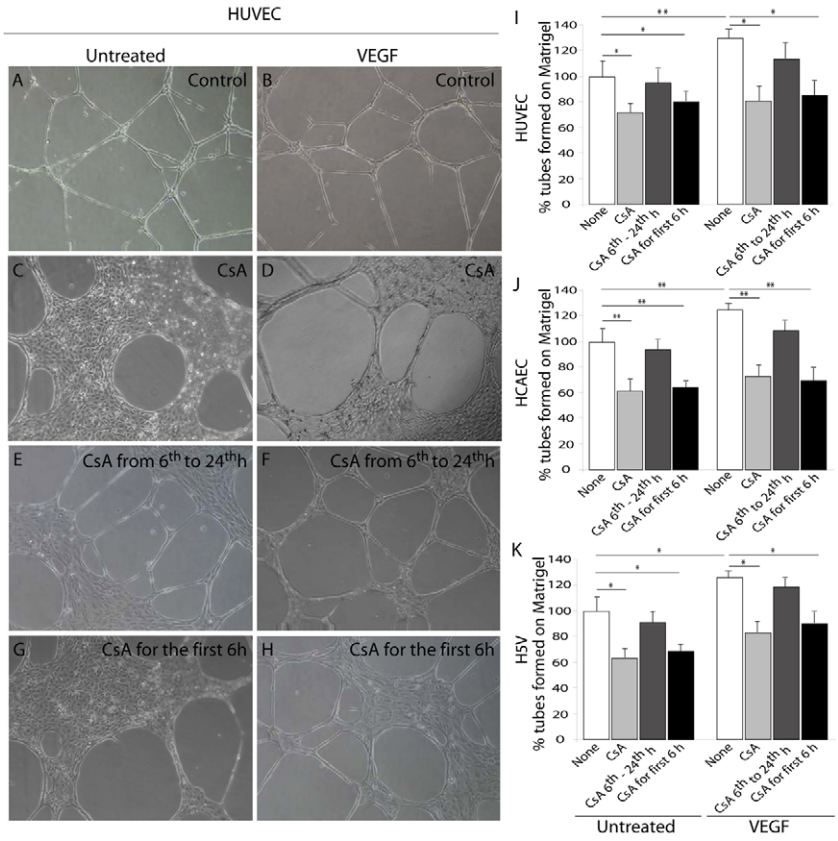

Fig. 6. Calcineurin-NFAT signaling contributes to the initiation of endothelial tube formation. (**A**,**B**) HUVECs cultured on Matrigel-coated plates assemble into tubular structures (A), a process that is enhanced by VEGF-A treatment (B). (**C**,**D**) CsA treatment inhibits HUVEC tube formation both in the absence (C) and presence (D) of additional VEGF-A treatment at the time of plating the cells on Matrigels. (**E**,**F**) Treatment of HUVECs with CsA from 6 hours to 24 hours after plating HUVECs on Matrigels does not affect tube formation whether additional VEGF-A is present (F) or not (E). (**G**,**H**) Treatment of HUVECs with CsA during the first 6 hours in culture is sufficient to inhibit tube formation (G), even in the presence of additional VEGF-A (H). (**I**) Quantitation of endothelial tube formation by HUVECs shown in A-H. The statistical significance is analyzed by Student's *t*-test (*, $P<0.05$, **, $P<0.001$). Error bars represent standard deviation ($n=3$ control and CsA-treated cells; three different fields of view of three independent experiments were counted and analyzed to determine statistical significance). (**J**,**K**) Quantitation of endothelial tube formation by HCAECs (J) and H5V cells (K). The statistical significance is analyzed by Student's *t*-test (*, $P<0.05$; **, $P<0.001$). Error bars represent standard deviation ($n=3$ control and CsA-treated cells). Image panels are detailed in Fig. S2 in the supplementary material.

treatment. Interestingly, many of the HUVECs exposed to CsA during this period aggregated to form large epithelial sheets on the Matrigel (Fig. 6C,D), which were never observed in the control HUVEC cultures (Fig. 6A,B). These epithelial sheets resembled the broad endothelial patches present in embryos treated with CsA (Fig. 2C) or lacking endothelial calcineurin (Fig. 4C). These observations suggest that HUVECs and embryonic coronary endothelial cells share similar cellular responses to CsA or calcineurin inhibition.

In comparison with early treatment, CsA added after the first 6 hours of HUVEC culture had no significant effect on HUVEC endothelial tube formation (Fig. 6E,F,I) within the 24-hour observation period, although there were some residual HUVEC epithelial patches. These findings indicate that calcineurin is required predominantly in the early phase of tubular transformation of HUVECs, consistent with a narrow temporal requirement of calcineurin in coronary angiogenesis (Fig. 2D). To further determine if an early, but narrow, time window of CsA exposure is sufficient to inhibit endothelial tubular formation, we treated HUVECs with CsA for the first 6 hours in culture, washed off CsA, and then continued the culture for another 18 hours to assess HUVEC tube formation. We found that HUVECs pulsed with CsA for the first 6 hours exhibited a 30-40% reduction in endothelial tubular formation, and these cells aggregated to form large epithelial sheets (either in the presence or absence of additional VEGF stimulation; Fig. 6G,H), compared with HUVECs treated with CsA between 6 and 24 hours of culture or HUVECs not exposed to CsA (Fig. 6A,B,E,F). The latter two experimental groups showed no difference in endothelial tubular formation. A quantification of endothelial tubular formation in these experiments is represented on Fig. 6I. Furthermore, FK506, another inhibitor of calcineurin activity, yielded similar results (data not shown). The reduction of endothelial tubular formation in HUVECs exposed to CsA in the first 6 hours was not caused by cell detachment from the Matrigel, as the culture supernatant collected after 7 hours and 24 hours of culture among all three experimental groups showed no difference in the number of detached cells (data not shown). Taken together, these findings indicate that calcineurin functions predominantly within the first 6 hours to transduce VEGF-A signaling and trigger endothelial tubular formation.

Similar temporal and cellular responses of endothelial tubular formation to CsA treatment were observed in two additional endothelial cell types: human coronary artery endothelial cells (HCAECs) and mouse embryonic heart endothelial (H5V) cells (Garlanda et al., 1994) (Fig. 6J,K; see Fig. S2 in the supplementary material). Thus, these studies demonstrate a narrow time window in which calcineurin-NFAT signaling is required for VEGF-A to trigger endothelial tube formation, similar to the presence of a defined developmental window when endothelial calcineurin is required for coronary angiogenesis.

Calcineurin inhibition does not impair endothelial cell differentiation

To further test the differentiation of CsA-treated endothelial cells and to determine whether endothelial expression of certain angiogenic factors could be compromised in the presence of CsA, we conducted a survey and examined the expression of several endothelial differentiation and angiogenic factors in CsA-treated HUVECs and HCAECs by quantitative RT-PCR. Included in our survey were the genes encoding endothelial cell-specific molecule 1 (ESM1), a proteoglycan involved in endothelial cell adhesion and integrity (Aitkenhead et al., 2002); cadherin 5 (CDH5), involved in the remodeling and maturation of endothelial cells (Carmeliet et al., 1999; Gory-Faure et al., 1999; Radice et al., 1997); endoglin

(ENG), an accessory protein for the TGF-β receptor complex that promotes endothelial cell proliferation (Lebrin et al., 2004; Li et al., 1999); bone morphogenic protein receptor, type 2 (BMPR2), a receptor for BMPs, which are members of the TGF-β superfamily of ligands involved in endothelial cell proliferation and angiogenesis (Nakaoka et al., 1997; Zhao, 2003); endothelial-specific receptor tyrosine kinase (TIE2, also known as TEK), an endothelial receptor tyrosine kinase involved in angiogenesis (Mustonen and Alitalo, 1995); and FLT1 and KDR, VEGF-A receptors required for angiogenesis (Peters et al., 1993; Quinn et al., 1993) (Fig. 7A,B). By quantitative RT-PCR, we observed no significant changes in the expression of these factors in CsA-treated endothelial cells compared with controls, suggesting that calcineurin inhibition does not alter the differentiation of endothelial cells. Furthermore, embryos lacking endothelial *Cnb1* (*Tie2-Cre;Cnb1$^{f/f}$*) had no significant changes in *VEGF-A* expression (see Fig. S3 in the supplementary material). Therefore, the blunted response of CsA-treated endothelial cells to VEGF-A was not due to the misregulation of VEGF or VEGF receptor expression, but due to the absence of calcineurin activity needed to transduce VEGF-VEGF receptor signaling.

DISCUSSION

NFAT signaling has a distinct role in coronary vascular formation

Previous studies have shown that calcineurin-NFATc3/c4 signaling prevents the aberrant growth of peripheral vessels by repressing VEGF-A expression in perivascular tissues (Graef et al., 2001), an important mechanism needed to confine peripheral vessels within anatomic boundaries. In contrast to NFAT signaling in the peripheral vasculature, our current studies demonstrate distinct spatial, temporal and molecular mechanisms of calcineurin-NFAT signaling during coronary vascular development (Table 1). Calcineurin acts in different tissues (perivascular versus endothelial cells), at different time windows (E7.5-8.5 versus E10.5-11.5) and through different mechanisms (repressing VEGF-A expression versus facilitating the endothelial response to VEGF-A) to regulate peripheral and coronary vascular patterning.

The specificity of VEGF isoforms and the spatiotemporal distribution of VEGF are crucial for proper coronary vascular development. Mice expressing solely VEGF120 (VEGF-A) show an impairment in coronary arterial and venous differentiation (van den Akker et al., 2008). Ablation of smoothened (a transducer of hedgehog signaling) in myocardial or perivascular cells in mice results in an arrest of coronary vessel formation due to the reduced expression of VEGF ligands (Lavine et al., 2008; Lavine and Ornitz, 2009). In view of the finding that VEGF signaling is transduced by endothelial calcineurin-NFAT during coronary angiogenesis (current study), we propose a model in which epicardial sonic hedgehog (Shh) signals myocardial or perivascular cells to produce different VEGF ligands that activate calcineurin and NFAT signaling in endothelial cells to control coronary vessel development (Fig. 7C). This model provides a framework for future studies of the signaling interactions between endothelial cells and their environment during coronary development. Further investigations into how hedgehog signals are initiated, how different VEGF ligands activate calcineurin-NFAT and what molecules results from NFAT signal transduction will be crucial for understanding the development of coronary vasculature.

Calcineurin-NFAT contributes to the programming of endothelial cells to form endothelial tubes

We show that calcineurin provides an early signal directing endothelial angiogenesis in an in vitro Matrigel culture model. Inhibition of calcineurin at an early, but not later, stage of HUVEC, HCAEC and H5V cell Matrigel cultures results in a failure in endothelial network formation. Once endothelial cells begin to form tubular networks, calcineurin is no longer required in this patterning process. Since calcineurin is dispensable for endothelial cell proliferation, survival and differentiation, these studies suggest that calcineurin-NFAT signaling is crucial for the initial programming of endothelial cells to undergo morphogenetic changes and form vascular networks, consistent with previous observations that NFAT is required for the initiation of pathological angiogenesis by VEGF-A (Hernandez et al., 2001).

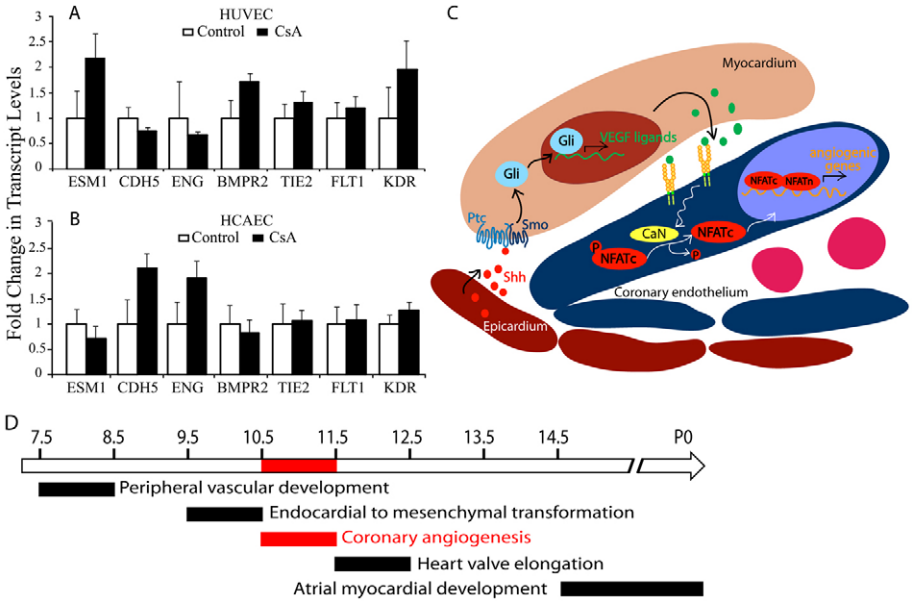

Fig. 7. Models of the spatial and temporal actions of calcineurin-NFAT during cardiovascular development. (**A**,**B**) Gene expression analyzed by quantitative RT-PCR in HUVECs A) and HCAECs (B) treated with CsA: genes encoding ESM1, CDH5, ENG, BMPR2, TIE2, FLT1 and KDR. Transcript levels were normalized to *HPRT* expression. Error bars represent the standard deviation (n=3 control and CsA-treated cells). Differences are not significant using a two-tailed Student's test. (**C**) Working model of molecular interactions among epicardial, myocardial and endothelial cells during coronary vessel development. Shh, sonic hedgehog; Smo, smoothened; Ptc, patched; Gli, Gli transcription factors; CaN, calcineurin; P, phosphate. (**D**) Calcineurin-NFAT signaling functions during distinct time windows to regulate cardiovascular development.

Table 1. Models of distinct in vivo actions of calcineurin-NFAT signaling during the development of peripheral versus coronary vasculature

	Peripheral somatic vasculature	Coronary vasculature
Temporal window of calcineurin-NFAT function	E7.5-8.5	E10.5-11.5
Cells where calcineurin-NFAT signals	Perivascular cells	Endothelial cells
Mechanism of regulating VEGF activity	Repressing VEGF-A expression to inhibit angiogenesis	Transducing VEGF-A signaling to promote angiogenesis

These findings also provide a cellular basis for the presence of a critical developmental window, within which calcineurin acts to initiate the formation of the coronary endothelial network.

Specific temporal and spatial requirements of calcineurin-NFAT signaling during cardiovascular development

The temporal and spatial expression of calcineurin-NFAT signaling components is tightly regulated during embryogenesis (Chang et al., 2004; de la Pompa et al., 1998; Graef et al., 2001; Schubert et al., 2003). NFAT signaling functions within narrow developmental windows and in specific tissues to regulate distinct processes in cardiovascular development (Fig. 7D). NFATc3/c4 signaling is required in perivascular tissues at E7.5-8.5 to control the patterning of peripheral vasculature in developing embryos (Graef et al., 2001). At E9.5, NFATc2/3/4 represses VEGF-A expression in cushion myocardium to initiate an endocardial-to-mesenchymal transformation (EMT) and heart valve morphogenesis (Chang et al., 2004). Later at E11.5, NFATc1 signaling functions in cushion endocardial cells to direct heart valve elongation and maturation (Chang et al., 2004; de la Pompa et al., 1998; Ranger et al., 1998). Furthermore, NFAT signaling is required for the development of atrial myocardium from E14.5 until birth (Schubert et al., 2003). Our current study defines a new role for calcineurin-NFAT signaling in cardiovascular development. NFAT signals in endothelial cells at E10.5 to regulate the development of coronary vasculature, further demonstrating the tight temporal and spatial control of calcineurin-NFAT signaling during development.

Implications of the temporal and spatial NFAT signaling in cardiovascular development

The temporal actions of calcineurin-NFAT signaling suggest that NFAT is crucial for the initiation of cardiovascular morphogenesis. NFAT signals occur 1-2 days before the morphological changes in specific cardiovascular tissues take place in development (Fig. 7D). Interestingly, NFAT is not required for sustaining these developmental processes once they are initiated. During heart valve morphogenesis, for example, NFAT signaling initiates an EMT in the endocardial cushion, but it is not necessary for the completion of the EMT process, a phenomenon observed in both zebrafish and mice (Beis et al., 2005; Chang et al., 2004). Also, after NFAT triggers the elongation of heart valves, the continuation of valve elongation and maturation does not depend on NFAT signaling (Chang et al., 2004). Similarly, calcineurin-NFAT signaling functions at the initial, but not later, phase of coronary angiogenesis (current study). These restricted temporal functions of NFAT signaling support the notion that a major function of the NFAT pathway resides in initiating, but not in sustaining, specific developmental processes. This model implies that NFAT signaling triggers precursor cells to undertake the morphogenetic path that leads to tissue formation.

The spatial patterns of NFAT signaling in development demonstrate that the initiation and control of tissue morphogenesis are accomplished by NFAT either cell-autonomously or non-cell-autonomously. For instance, NFAT functions within endothelial cells to activate their tubular transformation (current study); however, the mesenchymal transformation of endocardial cells requires NFAT activity in the neighboring myocardium (Chang et al., 2004). NFAT signaling thus operates in the target cells or their surrounding environment to control the target cell differentiation and tissue morphogenesis.

The temporal and spatial patterns of NFAT signaling could be defined by multiple mechanisms of control of the calcineurin-NFAT pathway during development. First, the activation of calcineurin could be determined by specific cell surface receptors that only respond to morphogenetic signals in particular tissues and during distinct time windows. Second, the distribution of NFAT activity could be established by developmental cues that trigger the expression or activation of the NFATn component of NFAT transcriptional complexes. Third, developmental signals might regulate the activity of other modulators of NFAT signaling, such as GSK3, DSCR1 and DYRK1a (Arron et al., 2006; Beals et al., 1997b; Gorlach et al., 2000; Kingsbury and Cunningham, 2000; Rothermel et al., 2001; Wu et al., 2007), to control the pattern of NFAT activity. These regulatory mechanisms are not mutually exclusive. Indeed, a combinatorial regulation of NFAT signaling at multiple levels might provide a sophisticated means to orchestrate the diverse and complex morphogenetic processes that occur in a wide range of tissues and developmental windows.

Acknowledgements

We thank K. Zhang for technical assistance. We are indebted to Dr P. Ruiz-Lozano for providing the *Gata5-Cre* mice and Dr Isabella Graef for advice. C.-P.C. was supported by funds from the National Heart Lung and Blood Institute (NHLBI; HL085345), American Heart Association (AHA; 0535239N), Children's Heart Foundation, March of Dimes Birth Defects Foundation and Office of the University of California (TRDRP). M.Z. was supported by a Stanford Dean's fellowship and a Marie Curie Outgoing International Fellowship; C.T.H. by an AHA predoctoral fellowship and an NIH training fellowship (5T32 CA09302); J.L.-G. by an NHLBI vascular medicine training fellowship (T32 HL007708); T.D. by a Stanford Bio-X fellowship and by a Stanford VPUE Faculty Grant for Undergraduate Research; and B.Z. by a NHLBI grant (HL078881). Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/19/3335/DC1

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