N-cadherin acts upstream of VE-cadherin in controlling vascular morphogenesis

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Endothelial cells express two classic cadherins, VE-cadherin and N-cadherin. The importance of VE-cadherin in vascular development is well known; however, the function of N-cadherin in endothelial cells remains poorly understood. Contrary to previous studies, we found that N-cadherin localizes to endothelial cell–cell junctions in addition to its well-known diffusive membrane expression. To investigate the role of N-cadherin in vascular development, N-cadherin was specifically deleted from endothelial cells in mice. Loss of N-cadherin in endothelial cells results in embryonic lethality at mid-gestation due to severe vascular defects. Intriguingly, loss of N-cadherin caused a significant decrease in VE-cadherin and its cytoplasmic binding partner, p120ctn. The down-regulation of both VE-cadherin and p120ctn was confirmed in cultured endothelial cells using small interfering RNA to knockdown N-cadherin. We also show that N-cadherin is important for endothelial cell proliferation and motility. These findings provide a novel paradigm by which N-cadherin regulates angiogenesis, in part, by controlling VE-cadherin expression at the cell membrane.

Introduction

Vascular remodeling is the end result of a complex series of dynamic cell–cell and cell–ECM interactions driven by fluid shear stress and angiogenic growth factors (Carmeliet, 2000; Yancopoulos et al., 2000; Jain, 2003). The adherens junction provides strong cell–cell adhesion mediated by the cadherin–catenin complex via its linkage to the actin cytoskeleton (Wheelock and Johnson, 2003). Most cells express multiple cadherin subtypes; however, the interrelationship and functional contribution of the individual cadherins in cell adhesion and signaling is poorly understood. N-cadherin is normally concentrated at cell–cell contact sites in most cell types where it is expressed. However, in endothelial cells (ECs) N-cadherin displays an unusual nonjunctional distribution (Navarro et al., 1998) thought to be important for EC–mural cell (i.e., pericyte and smooth muscle) interactions (Gerhardt and Betsholtz, 2003; Dejana, 2004). Injection of N-cadherin function-blocking antibodies into the developing chick brain disrupted EC–pericyte interactions, leading to vascular abnormalities and hemorrhaging (Gerhardt et al., 2000). Recent evidence indicates that the bioactive lipid, sphingosine1-phosphate, plays an important role in stabilizing N-cadherin–mediated EC–mural cell interactions both in vitro and in vivo (Paik et al., 2004). Hence, the prevailing view is that N-cadherin's primary function in ECs is to promote heterotypic cell interactions with mural cells.

In this study, we genetically manipulate N-cadherin expression in vivo and in vitro to examine its function in ECs. Endothelial-specific deletion of N-cadherin from the developing vascular system led to an unexpected phenotype reminiscent of the VE-cadherin knockout mouse phenotype (Carmeliet et al., 1999; Gory-Faure et al., 1999). Indeed, VE-cadherin was significantly down-regulated in the mutant embryos, demonstrating that N-cadherin is genetically upstream of VE-cadherin.

Results and discussion

Endothelial-restricted deletion of N-cadherin leads to vascular defects

We report here that N-cadherin is also found at intercellular junctions colocalized with VE-cadherin in human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC), suggesting an unappreciated role for N-cadherin in EC–EC interactions (Fig. 1). To determine N-cadherin's function in ECs during vascular development, N-cadherin floxed mice (Kostetskii et al., 2005) were bred to Tie2-Cre transgenic mice (Kisanuki et al., 2001) to delete N-cadherin specifically from the EC lineage. Unexpectedly, N-cadherin loss in ECs caused recessive embryonic lethality at

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Abbreviations used in this paper: EC, endothelial cell; HMVEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; PECAM, platelet endothelial cell adhesion molecule; siRNA, small interfering RNA.

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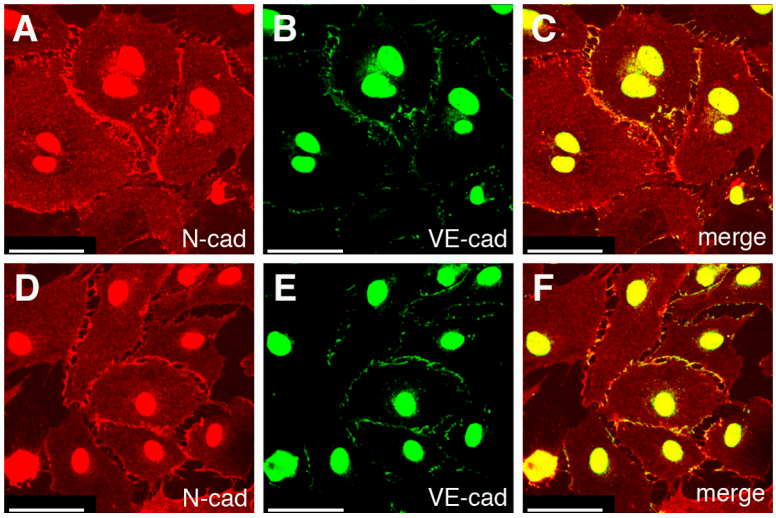

Figure 1. **Cellular localization of cadherins in endothelial cells.** Double immunostaining of N-cadherin and VE-cadherin in HUVEC (A–C) and HMVEC (D–F). N-cadherin exhibits both diffuse, nonjunctional as well as junctional staining (A and D). In contrast, VE-cadherin is restricted to regions of cell–cell contact (B and E) where it colocalizes with N-cadherin (C and F). The nuclei staining is due to nonspecific background. Bars, 50 μm.

mid-gestation, before mural cell investment, raising the possibility of an important role for N-cadherin in EC–EC interactions. Mutant embryos were developmentally delayed compared with their wild-type littermates at E9.5, with lack of blood circulation accompanied by pericardial effusion (Fig. 2, A and B). By E10.5, mutant embryos were deteriorated (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200411127/DC1). Mutant embryos exhibited defective embryonic and yolk sac vasculature, including the lack of large vessels in the yolk sac. In contrast, wild-type yolk sac displayed great vessels at this stage (Fig. 2, C and D). Embryos also were examined by whole-mount staining with platelet endothelial cell adhesion molecule (PECAM) antibody to show the emerging vasculature. Mutant embryos exhibited an abnormal vascular plexus with smaller, less developed vessels in the brain and intersomitic region (Fig. 2 C; Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200411127/DC1). Sectioning of the PECAM-stained mutant embryo showed smaller dorsal aorta and multiple endothelial vesicles instead of one large cardinal vein compared with the wild-type embryo (Fig. 2, D and E). Interestingly, endocardial cells in the heart tube appeared enlarged or swollen in the mutant embryo compared with the elongated morphology

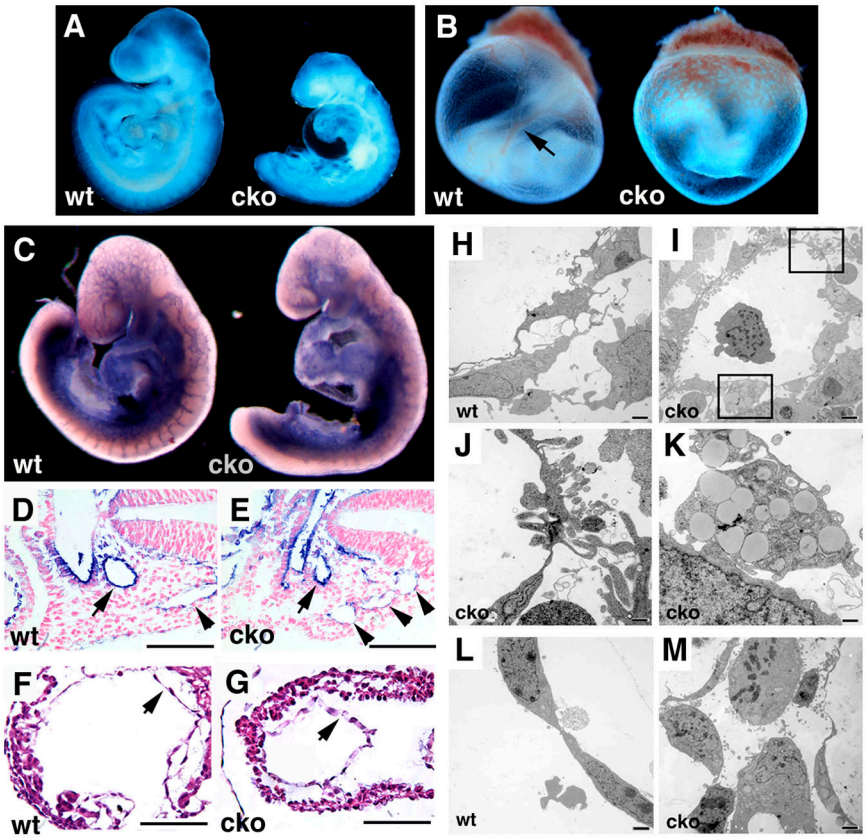

Figure 2. **Loss of N-cadherin in endothelial cells leads to embryonic lethality.** Whole-mount images of E9.5 embryos without (A) and with (B) yolk sac. Mutants (cko) lack well-defined blood vessels in yolk sac compared with large vessels (arrow) observed in wild-type (wt) embryos (B). Whole-mount PECAM stain of E9.0 embryos (C). Sections of PECAM-stained embryos (D and E) show dorsal aorta (arrow) and cardinal vein (arrowheads) in wild-type (D) and cko (E) embryos. Note the smaller dorsal aorta (arrow) in the cko (E) compare with wild-type (D) embryo. Instead of one large cardinal vein (arrowhead) in wild-type (D), three smaller vessels (arrowheads) are seen in the mutant (E). The endocardium (arrows) appears swollen in the mutant (G) compared with wild-type (F). Transmission EM of dorsal aorta (H–K) and endocardium (L and M) show the morphology of the wild-type (wt) and mutant (cko) cells. Higher magnification of boxed areas (I) illustrates the cell death process in the mutant endothelial cells (J and K). Endocardial cells have lost cell–cell contact in the mutant (M) compared with wild-type (L). Bars: D–G, 100 μm; H, I, L, and M, 2 μm; J and K, 500 nm.

of the wild-type cells (Fig. 2, F and G). Although the Tie2-Cre transgene is not active in the myocardium, the myocardial layer appeared less compact in the mutant embryo compared with the wild-type embryo, suggesting cross-talk between the endocardium and myocardium involving cadherin (Fig. 2, F and G).

To examine ECs at the ultrastructural level, transmission EM was performed on the E9.5 embryos. Somewhat unexpectedly, EC–EC contacts in the dorsal aorta appeared relatively normal in the mutant embryo (Fig. 2, H and I), although the lumen was smaller than that in the wild-type embryo where it was too large for the field of view (Fig. 2 H). Many endothelial cells in the N-cadherin CKO embryo, compared with the wild-type embryo, showed morphological signs of cell death including blebbing representative of programmed cell death and vacuoles representative of autophagic cell death (Fig. 2, J and K). In contrast to the dorsal aorta, endocardial cells in the mutant embryo appeared rounded and less cohesive compared with those in the wild-type embryo (Fig. 2, L and M).

VE-cadherin is dependent on N-cadherin

To confirm the efficiency of the Tie2-Cre–mediated deletion, we examined N-cadherin expression in the endocardium by immunofluorescence. Compared with myocardium, N-cadherin is weakly expressed in endocardium of the wild-type embryo (Fig. 3 A, inset); however, it is both present at cell–cell contacts and found diffusely distributed on the cell surface, similar to HUVEC (Fig. 1). In contrast, N-cadherin was absent from the endocardium in the mutant embryo (Fig. 3 B), but present in the myocardium. Although direct comparison of tissue-specific and constitutive knockout phenotypes can be difficult due to the timing of the deletion, the similarity between the N-cadherin CKO and VE-cadherin KO phenotypes (Carmeliet et al., 1999; Gory-Faure et al., 1999) led us to examine VE-cadherin expression in our CKO embryos using immunofluorescence microscopy. Surprisingly, the VE-cadherin signal was significantly decreased in the endocardium and dorsal aorta (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200411127/DC1) of the N-cadherin CKO embryo compared with the strong immunostaining seen in the wild-type (Fig. 3, C and D). The endothelial identity of the CKO tissue was confirmed by positive staining of an adjacent section with PECAM antibody (Fig. 3 E). Furthermore, Western blot analysis of yolk sac lysates derived from CKO embryos showed a dramatic reduction of VE-cadherin compared with wild-type (Fig. 3 H). Thus, our results provide the first evidence that N-cadherin can regulate the cellular level of VE-cadherin.

The catenin family consists of several members, including α-, β-, and γ-catenin, and p120ctn, which bind the highly conserved cytoplasmic domain and regulate cadherin adhesive activity (Lilien et al., 2002). Recent data indicate that the juxtamembrane-binding catenin, p120ctn, plays a critical role in controlling cadherin turnover by regulating the entry of cadherins into a degradative endocytic pathway (Davis et al., 2003; Xiao et al., 2003; Kowalczyk and Reynolds, 2004). Previous work from our laboratory revealed that the level or cellular distribution of p120ctn is regulated by N-cadherin in cultured

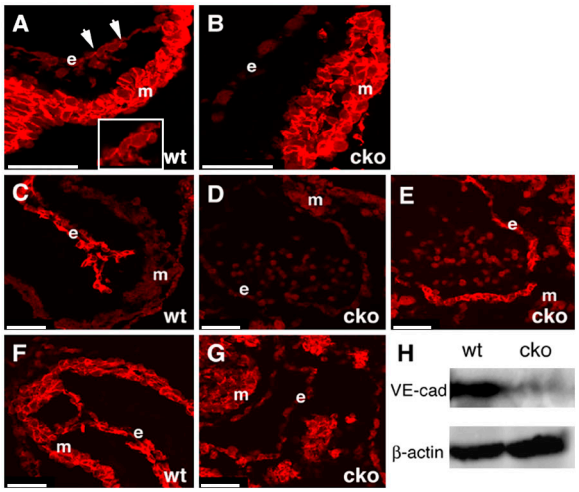

Figure 3. **Cadherin/catenin expression in endothelial-restricted N-cadherin CKO embryos.** Immunofluorescence was performed on E9.5 embryos for N-cadherin (A and B), VE-cadherin (C and D), PECAM (E), and p120ctn (F and G). In addition to its diffuse cell surface localization, N-cadherin is also observed at cell–cell contacts (arrows) between endocardial cells (A, inset). Western analysis of VE-cadherin in yolk sacs from wild-type (wt) and mutant (cko) embryos (H). m, myocardium; e, endocardium. Bars, 50 μm.

embryonic myocytes (Luo and Radice, 2003) and neural crest cells (Xu et al., 2001). Interestingly, p120ctn also showed a significant decrease in endocardium in N-cadherin CKO embryo compared with wild-type embryo (Fig. 3, F and G). These in vivo data indicate N-cadherin is critical for the normal regulation of VE-cadherin expression, possibly through controlling p120ctn levels. This may explain the similar vascular phenotypes seen in EC-specific N-cadherin CKO and VE-cadherin KO mice.

N-cadherin regulates VE-cadherin expression in human endothelial cells

To further examine the function of N-cadherin in endothelial cells, a small interfering RNA (siRNA) approach was used to knockdown N-cadherin in HUVEC. Consistent with our in vivo findings, knockdown of N-cadherin resulted in loss of VE-cadherin from endothelial cell–cell junctions (Fig. 4 E). Furthermore, both junctional and nonjunctional p120ctn immunostaining was significantly reduced after knockdown of N-cadherin in HUVEC (Fig. 4 F). Western blot analysis confirmed the reduction of both VE-cadherin (60%) and p120ctn (65%) in cultured cells (Fig. 4 M). Beta-catenin levels also were reduced (unpublished data). In contrast, siRNA knockdown of VE-cadherin did not affect N-cadherin distribution or expression level (Fig. 4 K). In addition, p120ctn remained localized at cell–cell contacts after VE-cadherin depletion (Fig. 4 L). Western blot analysis confirmed that VE-cadherin did not affect N-cadherin expression level; however, a modest reduction (35%) of p120ctn was observed consistent with decreased VE-cadherin levels (Fig. 4 N). Thus, the ability of N-cadherin to regulate VE-cadherin and p120ctn expression was observed in both mouse and human endothelial cells, and this is a nonreciprocal relationship because VE-cadherin knockdown had no effect on N-cadherin expression.

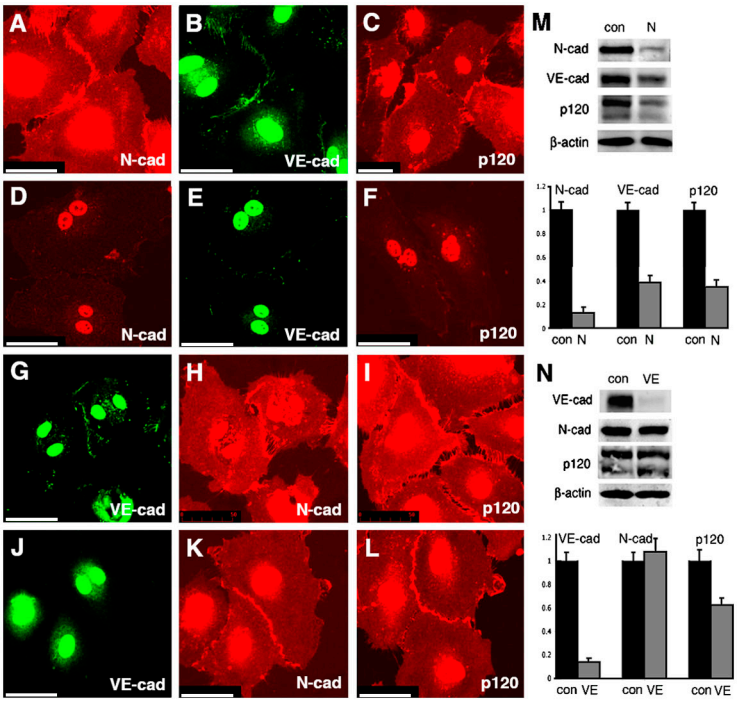

Figure 4. **N-cadherin regulates VE-cadherin levels in human endothelial cells.** Immunofluorescence of endothelial cells after cadherin knockdown. HUVEC were transfected with control siRNA (A–C and G–I), N-cadherin siRNA (D–F), or VE-cadherin siRNA (J–L). After 48 h, the cells were double stained for N-cadherin (A, D, H, and K) and VE-cadherin (B, E, G, and J), or p120ctn (C, F, I, and L). Note loss of VE-cadherin (E) and p120ctn (F) after knockdown of N-cadherin. In contrast, knockdown of VE-cadherin did not significantly affect N-cadherin (K) or p120ctn (L). Western analysis of HUVEC after transfection with control (con), N-cadherin (N), or VE-cadherin (VE) siRNA (M and N). Quantification of the knockdown from three independent experiments. Bars, 50 μm.

To determine whether VE-cadherin expression was affected at the mRNA level, real-time PCR analysis was performed on HUVEC. VE-cadherin mRNA levels were not changed after knockdown of N-cadherin (Fig. S3), indicating posttranscriptional regulation is responsible for the down-regulation of VE-cadherin.

N-cadherin is important for endothelial cell proliferation and motility

We observed fewer cell numbers 24 h after knockdown of N-cadherin compared with control, or VE-cadherin knockdown. No significant increase in apoptosis was observed after transfection of the N-cadherin siRNA (unpublished data). Therefore, we examined cellular proliferation in HUVEC cultures. We found that knockdown of N-cadherin caused a significant growth arrest by BrdU incorporation and Ki67 labeling (50% reduction in proliferation rate compared with control; Fig. 5 A). The growth inhibitory effect was attributed to loss of N-cadherin, rather than a decrease in VE-cadherin, because VE-cadherin knockdown alone had no effect on cellular proliferation. Hence, a decrease in EC proliferation may be responsible, at least in part, for the lack of vascular development in the N-cadherin CKO embryos.

In addition to EC proliferation, vascular remodeling requires migration and assembly of ECs into tubular structures. We previously showed that loss of N-cadherin in neural crest cells led to faster migration accompanied by a loss of directionality (Xu et al., 2001). Therefore, we asked whether loss of N-cadherin would influence EC motility using a transwell migration assay. We found that knockdown of N-cadherin resulted in enhanced EC migration (50%) compared with control (Fig. 5 B), consistent with our study on neural crest cells (Xu et al., 2001). The increased motility was attributed to loss of N-cadherin rather than a decrease in VE-cadherin, because VE-cadherin knockdown alone had no effect on cell motility.

Modulation of integrin adhesive activity is important for vascular morphogenesis (Byzova et al., 2000). Recent evidence has shown that class 3 semaphorins (Sema3a) negatively regulate β1-integrin function and that loss of Sema3a leads to activation of β1-integrin and is associated with stimulation of endothelial cell migration and severe angiogenic defects (Serini et al., 2003). Interestingly, β1-integrin was up-regulated (threefold) after knockdown of N-cadherin (Fig. 5 C), providing a possible explanation for the increased migration rate observed in the transwell assay. In contrast, siRNA knockdown of VE-cadherin did not affect migration or β1-integrin expression. In support of cross-talk between N-cadherin and β1-integrin in vivo, we observed a similar increase in β1-integrin after deleting N-cadherin in adult myocardium using an inducible cardiac-specific Cre gene (Kostetskii et al., 2005). Thus, the up-regulation of β1-integrin after loss of N-cadherin may interrupt finely controlled EC migration, as well as directionality, contributing to the vascular defects observed in N-cadherin CKO embryos.

Conclusions

It was previously thought that N-cadherin's primary function in ECs was to facilitate EC–mural cell interactions (Dejana, 2004); however, our in vivo and in vitro studies indicate that N-cadherin plays a fundamental role in angiogenesis by modulating adherens junction components and EC behavior. The vascular phenotype observed in N-cadherin CKO embryos is similar to that of VE-cadherin–null mice (Carmeliet et al., 1999; Gory-Faure et al., 1999), consistent with N-cadherin acting upstream of VE-cadherin. However, the vascular phenotype is unlikely explained by reduction of VE-cadherin alone because

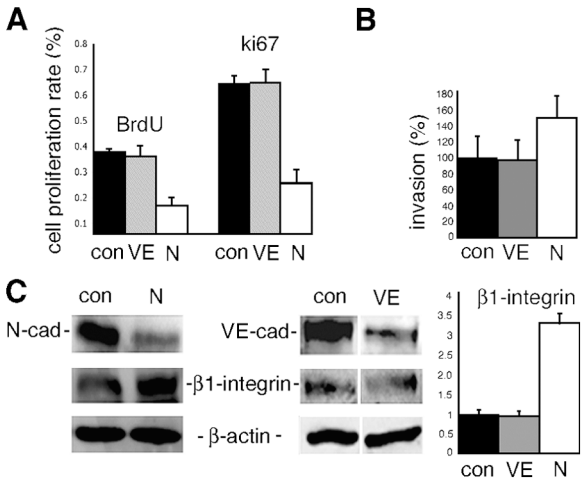

Figure 5. **Endothelial cell behavior is regulated by N-cadherin.** Quantification of BrdU incorporation and Ki-67 immunostaining in HUVEC after transfection with control (con), VE-cadherin (VE), or N-cadherin (N) siRNA (A). Quantification of EC migration using a transwell cell invasiveness assay after transfection with control, VE-cadherin, or N-cadherin siRNA (B). Western analysis of β1-integrin expression after transfection with control, VE-cadherin, or N-cadherin siRNA (C). Bar graphs (B and C) were normalized to control. White lines indicate that intervening lanes have been spliced out. Data represent the average for three independent knockdown experiments.

comparison of N-cadherin and VE-cadherin function in HUVEC suggests unique functions for N-cadherin in cellular proliferation and motility.

The myocardial defect observed in the EC-specific N-cadherin CKO embryo was unexpected. Interestingly, a similar myocardial defect was also observed in VE-cadherin–null embryos (Carmeliet et al., 1999), which could only result from VE-cadherin loss in the endocardium because it is not expressed in the myocardium. We previously demonstrated that myocardial-specific expression of cadherin was sufficient to postpone embryonic lethality of the N-cadherin–null embryo in the absence of EC expression (Luo et al., 2001). However, the vascular phenotype could not be examined definitively in these partially rescued embryos because of N-cadherin's requirement in other noncardiac cell lineages. We conclude that both the EC-specific N-cadherin CKO and VE-cadherin KO phenotypes are more complicated than simply perturbing EC function, and likely involve cross-talk between the endocardium and myocardium via cadherin-mediated signaling.

To our knowledge, this is the first report of one classical cadherin controlling the fate of another classical cadherin. This regulatory scenario may be specific to ECs that undergo dynamic cell–cell rearrangement during vascular morphogenesis. In addition, regulation of EC–EC interactions by N-cadherin may be important for controlling vascular permeability. Both the unique nonjunctional distribution of N-cadherin and its dominant role in EC behavior suggest a novel mechanism for cadherin cross-talk in these cells. The reduction in p120ctn after N-cadherin depletion suggests this juxtamembrane-binding catenin may play a role in this process. In summary, our studies demonstrate that N-cadherin function is required for angiogenesis, and redefine the hierarchical relationship of the structural components of the interendothelial cell junction.

Materials and methods

Analysis of mutant embryos

To generate EC-specific deletion of N-cadherin, N-cad$^{null/+}$, Tie2-Cre mice were mated with N-cad$^{flox/flox}$ mice and embryos were recovered from timed pregnancies. Animal studies were approved by the University of Pennsylvania IACUC committee. All experiments were performed in accordance with National Institutes of Health guidelines and regulations.

Whole-mount PECAM staining on E9.0 embryos was performed as previously described (Cattelino et al., 2003). Transmission EM was performed on a transverse section through the thoracic region of E9.5 embryos according to standard protocol. Immunofluorescence of cryosections stained with antibodies for N-cadherin (Zymed Laboratories), VE-cadherin (BD Biosciences), p120ctn (BD Biosciences), or PECAM (BD Biosciences) were visualized with a confocal microscope.

RNA interference experiments

HUVEC (Cambrex), 6th generation or less, were cultured in EGM BulletKit medium (CC-3124; Cambrex) and HMVEC (Cambrex), 2nd generation, were cultured in EGM MV BulletKit medium (CC-3125; Cambrex) according to the manufacturer's recommendation. For RNA interference analysis, CDH2 (human N-cadherin) and CDH5 (human VE-cadherin) SMARTpools (Dharmacon) were delivered into 70% confluent cells with Targefect-siRNA kit (Targeting Systems) at the final concentration of 100 nM according to the manufacturer's protocol. In addition, VE-cadherin expression was reduced after transfection with individual N-cadherin siRNA derived from the SMARTpool. Nontargeting siRNA pool (Dharmacon) at the same concentration was used as a control for above RNA interference assays. After 48 h transfection, cell lysates were collected with RIPA buffer for Western blot analysis or cells were fixed with 2% PFA followed by 0.3% Triton X-100 for immunofluoresence. In addition to the above antibodies, β1-integrin (BD Biosciences) and VE-cadherin (Sigma-Aldrich) were used for the Western blots. For normalization of signals, blotting was also performed with β-actin antibody (Sigma-Aldrich), followed by alkaline phosphatase–conjugated secondary antibody and chemifluorescent processing (ECF; Amersham Biosciences). Densitometry of samples was performed via use of ImageQuant (Molecular Dynamics) software.

Cell proliferation and migration assays

BrdU (Sigma-Aldrich) incorporation and Ki-67 (DakoCytomation) labeling were used to monitor cell proliferation after 24 h transfection. For invasion assays, cells were transfected with N-cadherin SMARTpool for 24 h, and 2.5×10^5 cells were loaded into the top of a 24-well Matrigel invasion chamber assay plate (BioCoat; BD Biosciences). After 36 h in Matrigel, fluorometric detection was performed using a CytoFluor multi-well plate reader according to the manufacturer's protocol (BD ViaSante).

Statistics

Data are expressed as mean ± SEM. Comparisons between groups were performed with a two-tailed *t* test using Microsoft Excel software. $P < 0.05$ was considered statistically significant.

Online supplemental material

Fig. S1: whole-mount images of E10.5 embryos. Fig. S2: higher magnification of whole-mount PECAM-stained E9.0 embryos. Fig. S3: VE-cadherin and PECAM expression in dorsal aorta of E9.5 embryos; real-time PCR analysis of VE-cadherin mRNA expression after knockdown of N-cadherin in HUVEC. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200411127/DC1.

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