Abstract

Protein Kinase C delta and epsilon are mediators of important cellular events, such as cell proliferation, migration or apoptosis. The formation of blood vessels, i.e., vasculo- and angiogenesis, is a process where these isoforms have also been shown to participate. However, mice deficient in either Protein Kinase C delta or epsilon are viable and therefore their individual contribution to the formation of the vasculature appeared so far dispensable. In this study, we show that double null mutation of Protein Kinase C delta and epsilon causes embryonic lethality at approximately E9.5. At this stage, whole mount staining of the endothelial marker CD31 in double null embryos revealed defective blood vessel formation. Moreover, culture of double deficient mouse allantois showed impaired endothelial cell organization, and analyses of double deficient embryo sections showed dilated vessels, decreased endothelial-specific adherent junctions, and decreased contact of endothelial cells with mural cells. Protein kinase C delta and epsilon also appeared essential for vascular smooth muscle cell differentiation, since α-smooth muscle actin, a classical marker for vascular smooth muscle cells, was almost undetectable in double deficient embryonic aorta at E9.5. Subsequent qPCR analyses showed decreased VE-cadherin, Vegfr2, Cd31, Cdh2, Ets1, and Fli-1, among other angiogenesis related transcripts in double deficient embryos. Taken together, these data suggest for the first time an in vivo redundant role between members of the novel Protein Kinase C subfamily that allows for mutual compensation during mouse embryonic development, with vasculogenesis/angiogenesis as an obvious common function of these two Protein Kinase Cs. Protein Kinase C delta and epsilon might therefore be useful targets for inhibiting vascular- and/or angiogenesis.

Introduction

The protein kinase C (PRKC) family consists of ten serine/threonine kinases grouped into three subfamilies according to their dependence on their biochemical properties and sequence homologies: classical PRKCs (cPRKCs, α, β, βII, and γ), dependent on diacylglycerol (DAG) and Ca²⁺; novel PRKCs (nPRKCs, δ, ε, η and θ), dependent on DAG but not Ca²⁺; and atypical PRKCs (aPRKCs, ζ, t/λ), DAG and Ca²⁺ independent. PRKC delta and epsilon (PRKCD and PRKCE) have generally different or even opposite roles [1], but both are important in several pathological scenarios, such as diabetic retinopathy [2] and fibrosis [3], respectively. However, their in vivo role in the formation of blood vessels is not completely understood. Previous studies on endothelial PRKCD and PRKCE have suggested roles for nPRKCs in the formation of blood vessels, such as sprouting angiogenesis [4,5], endothelial lumen formation [6], basal barrier function [7], as well as cell migration [8,9] and proliferation [5,10] in different species. Regarding endothelial cell signalling, PRKCE is suggested to induce FGF-2 exocytosis (and in turn endothelial cell proliferation and sprouting [3]), as well as VEGFR2 expression and activation, thus affecting downstream targets via Akt [11]. Activation of PRKCD by VEGF via PI3K regulates vasculogenesis in embryonic stem cells [4]. PRKCD is also important in blood vessel formation under pathological conditions, such as retinopathy [2] or ischemic limbs [12] in diabetic mice. However, PRKCD activation prevents instead of promoting vessel formation in diabetes.

The formation of blood vessels can be a de novo synthesis, which is known as vasculogenesis, or instead derive from pre-existing vessels, which is known as angiogenesis. During embryonic vasculogenesis, endothelial cells develop from both angioblasts present within the blood islands formed in the extraembryonic mesoderm of the yolk sac and angioblasts differentiated from mesodermal progenitors within the embryo [13,14]. During angiogenesis, the formation of blood vessels can occur via different mechanisms, such as intussusception, where a pre-existing vessel splits into new vessels by the insertion of columns of tissue, or sprouting angiogenesis, where a new vessel will branch out from a pre-existing one. Different signaling pathways, such as FGF, VEGF and TGFβ are involved in the formation of blood vessels [14]. Consistently with roles for VEGF and FGF in angiogenesis, ERK signalling appears essential in this scenario during mouse embryogenesis, since its deficiency leads to lethality due to an angiogenic phenotype [15]. Among transcription factors, ETS1...
and FLI-1 are also important in angiogenesis [16,17]. In this study, we show for the first time that PRKCD and PRKCE have an in vivo redundant role in the formation of blood vessels during mouse embryogenesis involving endothelial proliferation and vascular smooth muscle cell (VSMC) differentiation. Our data define PRKCD and PRKCE as potential targets for inhibiting vasculo- and/or angiogenesis.

**Materials and Methods**

**Animals and embryo collection**

*Prkcd* and *Prkce* single mutant mice have been previously described [18,19]. Generation of mice carrying the mutated allele for both *Prkcd* and *Prkce* was achieved by intercrossing double heterozygous mice for the corresponding isoforms. All animal work conducted for this study was approved by The Norwegian Institute of public health (Nasjonalt folkehelseinstitutt) and performed according to Norwegian legislation. The different pregnancy stages were established upon observation of vaginal plug at midday, which was considered as E0.5.

**Immunohistochemistry and immunocytochemistry**

4 μm sections were serially dewaxed 3×10 min in Histochoice clearing agent (Sigma), 2×5 min in 100% ethanola, and 3 min in 90%, 70% and 50% ethanol. Sections were then immersed in water for at least 5 min and boiled in citrate buffer (pH 6.0) for 2 min. Next, sections were rinsed in PBS and blocked overnight with 30% FCS in PBS 0.1% Triton X-100. Sections were then incubated with the corresponding primary antibodies in PBS 10% FCS: Rabbit anti mouse CD31 (1:100, Abcam), rabbit anti mouse VE-cadherin (1:800, Abcam) or monoclonal Cy3-conjugated antimouse α-SMA (1:1000, Sigma). CD31 was detected using the DAB method (Biogenex), whereas VE-cadherin was incubated in cy3-conjugated goat anti rabbit (1:500, Life Technologies). For immunocytochemistry, MEECs and MEFs were cultured for two days in gelatin coated coverslips, fixed for 15 min in 4% PFA, permeabilized for 10 min with PBS 0.25% Triton X-100, blocked in PBS 10% FCS and 0.05% Tween 20 (PBST) for one hour at room temperature, incubated with PBST containing rabbit antimouse CD31 (1:50, Abcam) overnight at 4°C, washed 3×10 min in PBST, and incubated with cy3-conjugated goat anti-rabbit (1:500, Life Technologies) for two hours at room temperature. Cells were photographed upon mounting with Dapi containing medium (Life Technologies).

**Whole mount immunostaining**

E9.5 embryos were fixed overnight at 4°C in a methanol-DMSO mix (4:1), bleached next day in a methanol-DMSO-30% H₂O₂ mix (4:1:1) for 2 h at 4°C, washed three times in methanol, rehydrated in PBS containing decreasing methanol concentrations (75–50–25%), washed twice in PBS, digested for 5 min in PBS 0.1% Triton X-100 containing 20 μg/ml protease K, washed two
times in PBS 0.1% Triton X-100, incubated in 0.1% GDA in PBS 0.1% Triton X-100 for 15 min, washed in PBS 0.1% Triton X-100, incubated in 2% milk glycine in PBS, incubated with blocking buffer (2% skimmed milk in PBS 0.1% Triton X-100) for 2 h at room temperature, incubated overnight at 4°C with rat antiserum CD31 (BD Pharmingen) or α-SMA (Sigma) in blocking buffer at a dilution of 1:200 and 1:500 respectively, washed next day 5 times for one hour each time in blocking buffer, incubated in peroxidase-conjugated goat antirat and antimouse IgGs, respectively (1:200, Jackson Immunoresearch), and developed using the DAB method according to the manufacturer’s instructions (Biogenex). Embryos were postfixed in 0.1% GDA in PBS, passed for 2 min, rinsed in running water for 5 min, dehydrated serially (75%) and stored in 100% methanol until photographed.

Cell culture
WT and Prkcd−/− MEECs were generated and cultured as previously described [20]. Their purity was assessed by detection of CD31 (described above in the immunocytochemistry method). MEFs used as negative control for immunodetection of CD31 were isolated as previously described [24].

Western Blotting
Cells were lysed on a solution containing Tris/HCl 50 mM, EDTA 2 mM, EGTA 10 mM, protease inhibitor cocktail (Sigma) and 0.3% beta-mercaptoethanol (Biorad), and centrifuged at 300 rcf to collect the cytosolic fraction, upon which SDS-PAGE was performed. After protein transfer, nitrocellulose membranes (GE Healthcare) were blocked with 3% non-fat dry milk (Marvel) in PBS 0.1% Tween 20 (TBS-T) for one hour. Next, membranes were incubated with rabbit anti mouse PKCD (Santa Cruz Biotechnologies) and rabbit anti GAPDH (Cell Signalling Technology) were used overnight at 4°C at a concentration of 1:1000 and 1:5000, respectively, and incubation of membranes in goat anti-rabbit horseradish peroxidase-conjugated secondary IgGs (Jackson ImmunoResearch, 1:5000) was done for two hours at room temperature. Upon washing with PBS 0.1% Tween 20, membranes were developed using SuperSignal West Pico kit (Thermo-Scientific Pierce) and X-ray films (GE-Healthcare), which were further scanned to create the corresponding figure.

Quantitative Polymerase Chain Reaction (qPCR)
Total mRNA from embryos was isolated by using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Sixty ng of total RNA was used to generate total cDNA using the iScript cDNA synthesis kit (Biorad). Subsequently, 1 μl cDNA was used to amplify the pertinent genes via qPCR using iCycler IQ Real-Time PCR Detection System and IQ SYBR green master mix (Biorad) according to the manufacturer’s instructions. Absolute mean quantifications of triplicates for each gene and genotype were normalized to those corresponding to GAPDH, and fold changes in mutant embryos were calculated by normalizing their GAPDH normalized values to those obtained in wild type embryos. Bars for each gene plotted in the graph represent mRNA fold change of normalized double deficient versus wild type values obtained in three different experiments. cDNA from independent embryos was used in each experiment. Error bars represent standard errors for normalized values from three different experiments. Two-tailed t-test was used to determine significant difference between wild type and mutant embryo values. Primer sequences for each gene were obtained from http://mouseprimerdepot.nci.nih.gov/ and are provided as Table S1.

Results
Embryonic lethality in Prkcd and Prkce double deficient embryos at approximately E9.5
We have recently shown that Prkcd and Prkce have partly overlapping expression patterns during embryogenesis [20,21]. Since the individual knockout mutants of these two genes do not display obvious embryonic phenotypes [18,19] we established a
double deficient line in order to address potential in vivo redundancy. Interestingly, whereas wild type, Prkcd⁻/⁻, Prkce⁻/⁻, Prkcd+/⁻/Prkce+/-, Prkcd⁻/-/Prkce+/- and Prkcd+/⁻/Prkce−/− embryos developed normally, no double homozygous Prkcd⁻/-/Prkce−/− offspring was observed, thus indicating an embryonic lethal phenotype (out of 150 newborns, 6% wild type, 6% Prkcd⁻/-, 5% Prkce⁻/-, 37% Prkcd+/⁻/Prkce+/-, 16% Prkcd+/-, Prkce+/-, 7% Prkcd⁻/-/Prkce+/- and 5% Prkcd+/-/Prkce−/− were identified). A subsequent analysis of various embryonic stages from double heterozygous crosses revealed that Prkcd and Prkce double deficiency lead to developmental defects at approximately E9.5. Unlike wild type (Fig. 1A), they displayed obvious growth retardation, mainly indicated by a reduced head size and a shortened tail, as well as swollen pericardium (Fig. 1B). Such developmental defects became more pronounced at E10.5 (Fig. 1D), whereas at later stages (e.g. E12.5) no double deficient embryos were detected due to absorption of affected embryos by the mother. To address whether the observed morphological abnormalities involved a vascular defect, we next performed whole mount and section immunostaining of wild type and Prkcd and Prkce double deficient embryos in order to compare the overall structure and distribution of the vasculature. Thus, through immunodetection of the endothelial marker CD31 in whole embryos, clearly detectable capillaries branching out from larger vessels could not be observed in the absence of Prkcd and Prkce (Figure 2B). Consistently, fewer vessels were detected in the corresponding sections (Figures 2D and 2F), as well as weaker positive immunosignal for CD31 in double mutants (Fig. 2F). Wild type embryos however showed a clearly defined vessel structure overall, as expected (Figures 2A, 2C and 2E). Thus, considering the viability of Prkcd and Prkce single deficient mice, these data show that PRKCD and PRKCE have at least one redundant role in vivo, and that double deficiency of these isoforms leads to impaired formation of the vasculature.

**Figure 3. PRKCD and PRKCE are both expressed in mouse embryonic endothelium at E9.5.** A, whole mount LacZ staining suggests weak expression of PRKCD in few endothelial cells. B, PRKCE appears broadly expressed in endothelium in whole mount LacZ stained embryos. C and D, LacZ and eosin stained sections confirmed the endothelial expression pattern observed for PRKCD and PRKCE in whole embryos, respectively. E, western blotting shows that wild type mouse embryonic endothelial cells (MEECs) express PRKCD. Prkcd⁻/- MEECs were used as a negative control. F, CD31 positive signal in virtually all MEECs confirms the purity of WT and Prkcd−/− cell populations. Mouse embryonic fibroblasts (MEFs) were used as a negative control. Expression of PRKCE in MEECs was previously demonstrated in a similar manner [20]. Scale bars = 500 μm (A and B), 25 μm (C and D) and 200 μm (F).

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**PKD and PRKCE are co-expressed in mouse endothelium**

Given the impaired formation of the vasculature found in our double deficient embryos (Fig. 2), we hypothesized that impaired vascular development in the absence of Prkcd and Prkce double deficiency could involve an endothelial-related defect. This would imply that both PRKCD and PRKCE would need to be expressed in endothelium. Thus, since both Prkcd and Prkce single deficient embryos contain the LacZ reporter gene under the control of the Prkcd and Prkce promoters, respectively (see material and methods), we aimed to identify the endothelium as a novel potential redundant mediator of vascular development during mouse embryogenesis.

**Impaired cell organization in cultured Prkcd and Prkce double deficient allantois**

In the mouse embryo, the allantois consists of mesodermal tissue that differentiates into the umbilical vein and artery [25], and has been used as an *in vitro* model for vascular network formation and angiogenesis [26]. Given the lack of vessel branching in our double deficient embryos, we cultured E8.5 embryonic allantois to further confirm the vascular phenotype suggested by CD31 immunostaining in whole embryos (Fig. 2). Wild type murine allantois led to the formation of a vascular plexus *in vitro* (Fig. 4A), as previously reported [23]. However, Prkcd and Prkce double deficient allantois failed to form a defined vascular network under the same conditions, as indicated by impaired formation of larger and smaller vessels (Fig. 4B). Prkcd and Prkce double heterozygosity did not significantly affect blood vessel formation in cultured allantois (Figs. 4C and D), which is consistent with the viability of the respective mice. Taken together, these data suggest that there is a key redundant role for PRKCD and PRKCE in embryonic endothelium, and therefore in the formation of embryonic vasculature.

**Impaired vessel integrity in Prkcd and Prkce double deficient embryos**

Since we observed impaired vessel formation in Prkcd and Prkce deficient embryos and allantois (Figs. 2 and 4, respectively), we next performed a more detailed histological analysis to obtain further information regarding blood vessel structure (Fig. 5). Via electron microscopy, wild type embryo sections showed that endothelial cells were properly interconnected and physically interacting with surrounding mural mesenchymal cells (Figs. 5A and 5B). In contrast, Prkcd and Prkce double deficient sections showed disassembled endothelial tubes with barely detectable endothelial-specific cell-cell adhesion molecules, i.e adherens junctions, as well as decreased contact with surrounding mural mesenchymal cells (Figs. 5C and 5D). Extracellular matrix-cell adhesion molecules were however detected at hemidesmosomes in both wild type and PRKCD and PRKCE double deficient embryo sections (Fig. 5B and 5D, respectively). In addition, while apparently normal in wild type sections, histochemical analyses showed a small dorsal aorta in double deficient embryo sections (Figs. 5E and 5F, respectively), which was also suggested by our
Figure 4. Impaired cell organization in cultured Prkcd and Prkce double deficient murine allantois. A, in vitro culture of wild type allantois led to the formation of a vascular plexus. B, Prkcd and Prkce double deficient allantois in culture displayed impaired formation of a vascular network. C, blood vessel formation in cultured Prkcd and Prkce double heterozygous allantois does not significantly differ from wild type counterparts. D, quantifications with the software angiotool and student t-test analyses of 3 allantoises per genotype show significantly decreased average value for total number of junctions in Prkcd and Prkce double deficient allantoises versus their wild type counterparts. Scale bars = 500 μm. * and n.s. = Difference between average values is (p<0.01) and is not statistically significant, respectively.

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PRKCD and PRKCE in Vasculo- and Angiogenesis

Electron microscopy

H&E

VE-cadherin

Dapi
findings via CD31 (Fig. 2E and 2F). Moreover, immunofluorescent detection of the VE-cadherin (CDH5), an endothelial marker expressed at endothelial adherens junctions, appeared expressed at lower levels in the absence of PRKCD and PRKCE (Fig. 5H). Taken together, these data suggest that PRKCD and PRKCE are necessary for proper assembly and development of the vasculature, and for expression of CDH5 at the endothelial cell membrane.

Lack of differentiated vascular smooth muscle cells in Prkd and Prkce double deficient aorta

During embryonic blood vessel formation, endothelial cells interact with surrounding mesenchymal cells and induce VSMC/pericyte differentiation [27]. Given that endothelial-mesenchymal cell interactions appeared impaired in the absence of PRKCD and PRKCE (Fig. 5), we next analyzed in vivo mesenchymal differentiation and therefore vessel maturation in the absence of PRKCD and PRKCE. Thus, immunostaining of whole mount embryos with antibodies to alpha-smooth muscle actin (α-SMA), a marker for pericytes and VSMCs [27], allowed for detection of positive staining at dorsal aorta in wild type but not in Prkd and Prkce double deficient embryos at E9.5 (Fig. 6A and 6B, respectively). This was further confirmed by immunofluorescent detection of α-SMA in E9.5 wild type and double deficient embryo sections (Figs. 6 C–F). Thus, these data suggest that PRKCD and PRKCE are needed for proper VSMC differentiation and therefore blood vessel stability and maturation.

Decreased levels of endothelial markers and vasculo/angiogenic related genes in Prkd and Prkce double deficient embryos

Since our data at the protein level suggested impaired vasculature formation in the absence of PRKCD and PRKCE, we next performed a pre-screening of vascular/angiogenic related transcripts in double deficient embryos, and found downregulation of cdh5, N-cadherin (Cdhl2), and the transcription factors Ets1 and Fli1, both involved in vascular development [16] (data not shown). The transcriptional regulation of these genes was confirmed via qPCR (Fig. 7). In addition, since ERK is a known downstream target for PRKCD [28] and PRKCE [11], and mouse embryos with conditional deletion of Erk1/2 in the endothelium are not viable due to an angiogenic phenotype at approximately the same developmental stage as Prkd and Prkce double deficient embryos, we also analyzed mRNA levels of angiogenic genes that were found most significantly regulated in embryos lacking endothelial ERK1/2 [15]. Indeed, Fli1, Ccne, Aurka, and Mecn all appeared downregulated in the absence of PRKCD and PRKCE. However, Ihatl, also significantly downregulated in the absence of endothelial ERK1/2, did not seem to be dependent on the expression of PRKCD and PRKCE. This is consistent with the detection of hemidesmosomes (which contain ITGB1) in Prkd and Prkce double deficient embryo sections (Fig. 5). Thus, these data suggest that PRKCD and PRKCE regulate genes involved in blood vessel formation at the transcriptional level.

Discussion

We recently showed that PRKCD and PRKCE display similar expression patterns in the mouse embryo during midgestation [20,21]. Such similarity lead us to hypothesize that PRKCD and PRKCE could have redundant function(s) in vivo that would explain the viability of both Prkd and Prkce single deficient mice [18,19]. For this reason, a mouse line containing null mutations for both Prkd and Prkce was generated. Given the strong expression of these isoforms within the nervous system [20,21], finding an obvious phenotype within this domain would not have come as a surprise. However, we found that Prkd and Prkce double deficiency caused lethality in mouse at E9.5, a stage at which the nervous system has not started to develop yet. The main observed phenotypes were growth retardation and swollen pericardium, which suggested impaired vascular development at this stage and a potential contributor to embryonic lethality (Fig. 1). This was further supported by immunodetecting impaired vascular network in double deficient embryos (Fig. 2). Thus, it seems obvious that either PRKCD or PRKCE must be present in the mouse embryo for adequate development of the vasculature. An endothelial related defect implies that both PRKCD and PRKCE need to be expressed in endothelium. However, whereas we previously reported obvious expression of PRKCE in endothelium from E.12.5 and onwards via a LacZ reporter gene under the control of the Prkce promoter and western blotting [20], a similar in vivo expression analysis for PRKCD did not suggest clear expression of this isoform in endothelial cells during mouse midgestation [29]. Some modifications in the LacZ staining protocol (see materials and methods) allowed us to obtain more prominent LacZ signal for both PRKCD and PRKCE (Fig. 3), and thus better detect indirect expression of these isoforms at E9.5. The detection of PRKCD at discrete spots throughout the endothelium may indicate that it has a significant role in a small endothelial cell subpopulation, perhaps tip cells, and therefore in endothelial migration during sprouting angiogenesis, although further studies are needed in this regard. Indeed, absence of endothelial PRKCD has been shown to delay reendothelialization in vivo via decreased vasohibin-1 in a mouse model of injured artery [8]. PRKCE has also been shown to take part in endothelial function, for example in a novel mechanism where FGFP2, a known pro-angiogenic factor, has been linked to endothelial cell sprouting upon PRKCE activation via VEGF signaling [5]. This provides a novel mechanism that might at least partly contribute to the development of murine vasculature in vivo. Downstream effectors of signaling cascades involved in PRKCE-dependent formation of blood vessels may also include AKT and eNOS [11].

Our observations that Prkd and Prkce double deficient vessels a) did not appear adequately assembled into a vascular network (Figs. 2 and 4), and b) showed decreased detectable cell-cell adhesion molecules at the cell membrane that could allow for endothelial/mesenchymal cell interaction and endothelial interconnection respectively (Fig. 5), strongly suggested impaired vessel maturation in the absence of PRKCD and PRKCE. This was
Figure 6. Undetectable vascular smooth muscle cell differentiation in Prkcd and Prkce double deficient dorsal aorta. A and B, immunodetection of α-SMA in whole embryos resulted in positive staining of dorsal aorta in wild type (A) but not Prkcd and Prkce double deficient (B) embryos at E9.5. C and D, wild type sections showed positive staining for α-SMA at the dorsal aorta (C and D) and heart (C) at E9.5. E and F, Prkcd and Prkce double deficient sections showed no immunosignal over the background at dorsal aorta at E9.5. Arrows indicate dorsal aorta. DA, dorsal aorta; U, umbilical cord. Scale bars = 250 μm (A and B), 100 μm (C and E), and 50 μm (D and F).

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Therefore, the downregulated transcriptional levels for cadherin (CDH2) given the vascular phenotype and lethality [36], which are downregulated in Prkcd and Prkce double deficient embryos. This further confirms the absence of immunodetectable z-SMA, a marker for VSMCs and pericytes [30], in double deficient aorta (Fig. 6). Thus, these data suggest a potential role for PRKCD and PRKCE in the crosstalk between endothelial and mesenchymal cells in order to allow for recruitment of the latter, their further differentiation into VSMCs/pericytes, and hence the formation of a mature and functional vascular network.

We also wanted to address whether the apparent decrease of detectable CDH5 in embryo sections was the result of lower transcription levels of the corresponding gene. Through qPCR analysis (Fig. 7) we could confirm a previous screening of transcripts (data not shown) and detect downregulated cdh5 (Fig. 7). However, it is likely that the observed lower levels of Cdhd5 in double deficient embryos not only reflect lower expression of these molecules in endothelial cells, but also a lower endothelial cell number in double deficient embryos, given the observed underdeveloped vasculature. Nevertheless, our data via electron and fluorescent microscopy (Fig. 5) together with the fact that Cdhd5 deficient mice also display a vascular lethal phenotype [31] suggest that PRKCD and PRKCE redundantly act upstream CDH5 expression. Such redundancy may also exist for N-cadherin (CDH2) given the vascular phenotype and lethality observed in the corresponding deficient mice [32], and that it mediates VSMC survival and migration, as well as pericyte recruitment by endothelial cells [33,34]. However, although Cdhd2 also appeared downregulated in Prkd and Prkce deficient embryos (Fig. 7), immunofluorescent detection of CDH2 in mouse embryo sections showed nervous system and heart as the main sites of expression, whereas endothelium/mural cell interconnections did not show an obvious positive signal over the background in vivo (see Figure S1). This is in agreement with previous data regarding the expression pattern of CDH2 in mouse embryos [35]. Therefore, the downregulated transcriptional levels for cdh2 found in double deficient embryos via qPCR is likely addressing a potential downregulation in other embryonic domains than endothelium in Prkd and Prkce deficient embryos.

Ets-1 belongs to the ets family, which consists of transcription factors involved in vasculo- and angiogenesis [16]. Although we found downregulated Ets-1, Ets-1 null mice are viable, probably due to redundancy among ETS members [16]. Therefore, the mechanism(s) that explain embryonic lethality in the absence of PRKCD and PRKCE cannot be explained through a pathway that only involves ETS-1. We thus looked further within the same family in an attempt to find a candidate(s) whose deficiency could be consistent with the phenotype displayed by Prkd and Prkce double deficient embryos, and were able to identify downregulated Fli-1 (Fig. 7), an ets family member whose deficiency in mice leads to embryonic lethality due to hemorrhage and disrupted vessel integrity at approximately E12.5 [16]. However, conditional deletion of Fli-1 in the endothelium does not prevent mouse viability, although it regulates genes involved in vascular homeostasis, such as Cdhd5 and Cd31 [36], which are downregulated in Prkd and Prkce double deficient embryos. This therefore opens the possibility to the existence of a mechanism involved, at least, in vascular homeostasis where Prkd and Prkce might regulate Cdhd5 and Cd31 via Fli-1 and Ets-1, since these genes are all regulated in Prkd and Prkce double deficient embryos (fig.7).

We also looked into mRNA levels via semiquantitative PCR for MeF2c, a transcription factor that contains essential ETS binding sites for vascular development and viability in mouse embryos at E9.5 [37], but did not observe any clear regulation in double deficient embryos (data not shown), which could be due to redundant function that exists within the Ets family [16]. The observed significant downregulation of the endothelial markers Cd31 and Vegfr2 (Flk-1) in double deficient embryos via qPCR (Fig. 7) could also be due to fewer endothelial cells present in double deficient embryos. Indeed, Flk-1 deficient mice die at E8.5 and display impaired vasculogenesis [38], so Flk1 seems to lie upstream Prkd and Prkce. Cd31 deficient mice, however, are viable and do not display a vascular phenotype [39], so PRKCD and PRKCE dependent Cd31 expression should not be crucial in mouse viability.

Regarding ERK, a downstream target in VEGF signaling, it can be activated by PRKCD [40] and PRKCE [11]. Moreover, endothelial specific Erk1/2 deficient mice are also embryonic lethal due to an angiogenic phenotype at approximately E9.5 [15]. For this reason we hypothesized that Prkd and Prkce could lie...
upstream ERK signaling in endothelium, and screened the most significantly regulated genes in the absence of endothelial ERK [15]. We thus found downregulation of the cell division/ proliferation related genes Aurka, Cenex and Mem2 (Fig. 7). However, the adhesion and migration mediating integrin subunit beta1 (Itgb1), which also appeared downregulated in Erkδ/ε null embryos, did not appear significantly regulated in our double deficient embryos (Fig. 7). Consistently, hemidesmosomes were detectable in the absence of PRKCD and PRKCE (Fig. 5F). Thus, a more complex mechanism that explains embryonic vessel formation may exist.

In summary, our data show for the first time that PRKCD and PRKCE have a redundant role in vivo that is necessary for blood vessel formation during mouse embryogenesis via a mechanism that may involve several angiogenic genes, such as Cdh2, Cdh5, Ets-1 and Fli-1. This vascular phenotype might be a major contributor to the observed embryonic lethality, although additional phenotypes that could also prevent further development and contribute to lethality might also exist at the same embryonic stage. Our data, however, still point at PRKCD and PRKCE as potentially good targets when considering inhibition of vascular- and/or angiogenesis.

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