Role of Mutation and Pharmacologic Block of Human KCNH2 in Vasculogenesis and Fetal Mortality

Partial Rescue by Transforming Growth Factor-β

Guoqi Teng, PhD; Xiang Zhao, PhD; James P. Lees-Miller, PhD; Darrell Belke, PhD; Chunhua Shi, PhD; Yongxiang Chen, PhD; Edward R. O’Brien, MD, PhD; Paul W. Fedak, MD, PhD; Nathan Bracey, PhD; James C. Cross, PhD, DVM; Henry J. Duff, MD

Background—N629D KCNH2 is a human missense long-QT2 mutation. Previously, we reported that the N629D/N629D mutation embryos disrupted cardiac looping, right ventricle development, and ablated Ikr activity at E9.5. The present study evaluates the role of KCNH2 in vasculogenesis.

Methods and Results—N629D/N629D yolk sac vessels and aorta consist of sinusoids without normal arborization. Isolated E9.5 +/- first branchial arches showed normal outgrowth of mouse ERG–positive/α-smooth muscle actin coimmunolocalized cells; however, outgrowth was grossly reduced in N629D/N629D. N629D/N629D aortas showed fewer α-smooth muscle actin positive cells that were not coimmunolocalized with mouse ERG cells. Transforming growth factor-β treatment of isolated N629D/N629D embryoid bodies partially rescued this phenotype. Cultured N629D/N629D embryos recapitulate the same cardiovascular phenotypes as seen in vivo. Transforming growth factor-β treatment significantly rescued these embryonic phenotypes. Both in vivo and in vitro, dofetilide treatment, over a narrow window of time, entirely recapitated the N629D/N629D fetal phenotypes. Exogenous transforming growth factor-β treatment also rescued the dofetilide-induced phenotype toward normal.

Conclusions—Loss of function of KCNH2 mutations results in defects in cardiogenesis and vasculogenesis. Because many medications inadvertently block the KCNH2 potassium current, these novel findings seem to have clinical relevance.

Key Words: aorta ■ embryonic development ■ gene targeting ■ mice ■ organogenesis ■ potassium channels ■ transforming growth factor beta

Patients with mutations in KCNH2, also known as hERG, the human Ether-a-go-go Related potassium channel, produce long-QT2 syndrome.1–4 Mutations in hERG can be associated with embryonic lethality and sudden infant death syndrome.5–7 Although long-QT2 syndrome generally occurs in individuals heterozygous for the mutant allele, individuals homozygous for the exon 4 duplication manifest embryonic lethality.8 Although not widely recognized, mutations of KCNH2 are associated with structural congenital cardiovascular anomalies, including Tetralogy of Fallot, atrial-septal defects, ventricular-septal defects, and patent ductus arteriosus.9–11 In rodent models, antiepileptic drugs that block hERG cause fetal death and birth defects.12 In addition, mouse ERG (mERG) potassium current is the dominant repolarizing current during critical phases of cardiac development.13 We previously reported that the cardiac characteristics of a mouse bearing the human N629D long-QT2 syndrome mutation is inserted in situ (knocked-in).14 The homozygous N629D missense mutation produced developmental defects in the secondary heart field and a loss-of-function Ikr phenotype.15 The earlier study focused on cardiac looping defect and the electrophysiology of the heart. The present focuses on a defect in vasculogenesis in these N629D/N629D embryos. In this study, we discovered a defect in transforming growth factor (TGF)-β ligand expression in N629D/N629D mice, and that exogenous treatment with TGFβ ligand at least partially rescues these phenotypes.

Editorial see p 261

The TGFβ signaling system plays pivotal roles in vasculogenesis. Multiple TGFβ ligands exist, which interact with 3 primary receptors and multiple coreceptors including activin-A-type 1 and endoglin.16 In chick embryo, TGFβ 2,3 receptors are expressed in the outflow tract.17 Ablation of endoglin results in defective angiogenesis,18 whereas TGFβ is required for creation of the primary capillary network. There are a myriad of proteins that mediate signaling down-stream signaling of TGFβ, including VEGF (vascular endothelial growth factor) proteins, SMAD 2,3, mapKinase (mitogen-activated protein kinase),

Received May 5, 2014; accepted January 20, 2015.
From the Libin Cardiovascular Institute, Faculty of Medicine (G.T., J.P.L.-M., D.B., C.S., Y.C., E.R.O’B., P.W.F., N.B., H.J.D.) and Department of Comparative Biology and Experimental Medicine and Faculty of Veterinary Medicine (X.Z., J.C.C.), University of Calgary, Calgary, Alberta, Canada.

Correspondence to Henry J. Duff, MD, Libin Cardiovascular Institute, University of Calgary, HRIC GC73, 3330 Hospital Dr NW, Calgary, Alberta T2N 4N1, Canada. E-mail hduff@ucalgary.ca
© 2015 American Heart Association, Inc.

Circ Arrhythm Electrophysiol is available at http://circcep.ahajournals.org DOI: 10.1161/CIRCEP.114.001837

420
Teng et al
KCNH2 Channel Knockout and Embryonic Development

**WHAT IS KNOWN**
- Embryonic exposure to pharmacological blockers of the hERG K+ current over a very narrow window of time is lethal and teratogenic.

**WHAT THE STUDY ADDS**
- Some mutations of KCNH2 (hERG) result in embryonic death and congenital malformations of the outflow tract.
- Loss of ERG1 potassium channel expression results in primary defects in vasculogenesis apparently independent of abnormalities of flow or arrhythmias.
- Disruption of the coimmunolocalization of hERG and α-smooth muscle actin is associated with the defect in vasculogenesis.
- Transforming growth factor-β treatment partially rescues the defect of vasculogenesis in KCNH2 knockout mice.

**WHAT IS KNOWN**
- Embryonic exposure to pharmacological blockers of the hERG K+ current over a very narrow window of time is lethal and teratogenic.

**WHAT THE STUDY ADDS**
- Some mutations of KCNH2 (hERG) result in embryonic death and congenital malformations of the outflow tract.
- Loss of ERG1 potassium channel expression results in primary defects in vasculogenesis apparently independent of abnormalities of flow or arrhythmias.
- Disruption of the coimmunolocalization of hERG and α-smooth muscle actin is associated with the defect in vasculogenesis.
- Transforming growth factor-β treatment partially rescues the defect of vasculogenesis in KCNH2 knockout mice.

**Methods**

**Creation of mERG-N629D Mice**
The N629D/N629D mouse has been previously reported. All animals were treated in accordance with guidelines established by the Canadian Council on Animal Care. Institutional review board approval was obtained.

**N629D/N629D Homozygous Embryonic Stem Cells**
Male and female heterozygous +/N629D mERG1 mice were mated. At the 8-cell stage, morulas were flushed and grown in culture. Polymerase chain reaction (PCR) was used to recognize morulas containing a +/+ allele. The N629D/N629D alleles were also confirmed by PCR. Cells were then karyotyped.

**Immunohistochemistry and Immunofluorescence**

Immunochemistry of whole-mount embryos was performed as described earlier. Whole embryos and yolk sac were fixed in 4% paraformaldehyde, dehydrated through a series of methanol, and bleached in 5% H2O2/100% methanol. The samples were then rehydrated into PBS and incubated in blocking solution (2% normal goat serum, 1% BSA, 0.1% Tritonx-100, 0.05%Tween20 in PBS). Samples were then exposed to 1:100 dilution of rat antimouse CD31 (BD Biosciences Pharmingen, San Jose, CA) overnight at 4°C. After extensive washing (0.05% Tween10 in PBS), samples were treated as follows:

- +/+ (A and B): Bar, 600 μm.
- N629D/N629D (C and D): Bar, 200 μm.
- +/+ (E and F): Bar, 50 μm.

**Figure 1.** Yolk sac and intraembryonic vascular defects. Microscopic and histological assessment of extra- and intraembryonic vascular formation in wild-type (WT; +/+) vs N629D/N629D. Whole-mount assessment of +/+ (A) and N629D/N629D (B) yolk sacs at E9.5 (N=10 for each, consistent results are seen in each/every experiment). Whole-mount yolk sacs CD31 immunostaining of +/+ (C) and N629D/N629D (D). Blood vessels have remodeled to form large and small vessels (white arrows in C in +/+ yolk sacs), whereas that in N629D/N629D is a primitive meshwork (D). Paraffin wax–embedded sections from whole-mount yolk sacs CD31 immunostaining +/+ (E) and N629D/N629D after CD31 immunostaining (F). Separation of mesodermal and endodermal layers was observed in the N629D/N629D yolk sac (F). Whole-mount CD31 immunostaining of +/+ (G–I) and N629D/N629D (J–L) for CD31 at E9.5 are shown. Green arrows in (G, H, and I) point out the normal developed dorsal aorta in an E9.5+/+ embryo. Green arrows in (J, K, and L) point out the lack of organization of cranial and intersegmental vessels in N629D/N629D embryos when compared with that in the +/+ embryos. Sections through first branchial arch of E9.5 +/+ (M) and N629D/N629D (N) embryos after whole-mount CD31 staining are shown, black arrows and white arrowheads show dorsal aorta and first branchial arch artery (N), both dorsal aorta and first branchial arch artery are dilated in N629D/N629D relative to +/+ (M). A and B, Bar, 600 μm. C, D, and G–L, Bar, 200 μm. E and F, Bar, 50 μm. For CD31-Pecam-1 staining N=6 for both WT and N629D/N629D, results were absolutely consistent between experiments.

BMPs (bone morphogenic proteins). Moreover, many transcription factors, downstream of TGFβ, work cooperatively to mediate smooth muscle differentiation and branchial arch remodeling. TGFβ also regulates the epithelial–mesenchymal cell transition and differentiation of endothelial cells.

**Figure 1.** Yolk sac and intraembryonic vascular defects. Microscopic and histological assessment of extra- and intraembryonic vascular formation in wild-type (WT; +/+) vs N629D/N629D. Whole-mount assessment of +/+ (A) and N629D/N629D (B) yolk sacs at E9.5 (N=10 for each, consistent results are seen in each/every experiment). Whole-mount yolk sacs CD31 immunostaining of +/+ (C) and N629D/N629D (D). Blood vessels have remodeled to form large and small vessels (white arrows in C in +/+ yolk sacs), whereas that in N629D/N629D is a primitive meshwork (D). Paraffin wax–embedded sections from whole-mount yolk sacs CD31 immunostaining +/+ (E) and N629D/N629D after CD31 immunostaining (F). Separation of mesodermal and endodermal layers was observed in the N629D/N629D yolk sac (F). Whole-mount CD31 immunostaining of +/+ (G–I) and N629D/N629D (J–L) for CD31 at E9.5 are shown. Green arrows in (G, H, and I) point out the normal developed dorsal aorta in an E9.5+/+ embryo. Green arrows in (J, K, and L) point out the lack of organization of cranial and intersegmental vessels in N629D/N629D embryos when compared with that in the +/+ embryos. Sections through first branchial arch of E9.5 +/+ (M) and N629D/N629D (N) embryos after whole-mount CD31 staining are shown, black arrows and white arrowheads show dorsal aorta and first branchial arch artery (N), both dorsal aorta and first branchial arch artery are dilated in N629D/N629D relative to +/+ (M). A and B, Bar, 600 μm. C, D, and G–L, Bar, 200 μm. E and F, Bar, 50 μm. For CD31-Pecam-1 staining N=6 for both WT and N629D/N629D, results were absolutely consistent between experiments.
exposed to 1:200 dilution of alkaline-phosphatase-conjugated goat antirat IgG (Sigma). Color reaction was performed using BCIP/NBT Development Substrate (Promega Corporation). Embryos were embedded in paraffin and sectioned at 10 μm.

**Culture and Cryosection of Embryoid Bodies and Isolated First Branchial Arch**

Either +/+ or N629D/N629D ES cells were cultured on a mouse feeder layer. The hanging drop method (1000 cells/20 μL per drop) was used to induce embryoid body (EB) differentiation. The hanging drops were cultured for 2 days in medium without leukemia inhibitory factor, yielding EBs. EBs were transferred into suspension in ultra low-adherence dishes (Corning Inc, Corning, NY), and TGFβ treatment was started at day 6 (10 ng/mL). At day 13, EBs were fixed with 4% paraformaldehyde solution for 30 minutes. EBs were placed in 10% PBS-buffered sucrose solutions for 1 hour and 20% sucrose overnight and then had OCT (Proprietary Compound for Cryostat embedding for cryosection. Over night and then had OCT (Proprietary Compound for Cryostat embedding for cryosection. The first branchial arches were dissected and isolated from E9 embryos and grown in vitro for 24 hours on 0.1% gelatin-coated coverslips.

**Quantitative Reverse Transcriptase-PCR and Western Blot Analysis**

Quantitative reverse transcriptase-PCR of gene expression in mouse embryos was conducted using several kits from Qiagen (RNeasy kit; Quantitect reverse transcription and SYBR Green PCR kit; Qiagen, Montreal, Canada) and measured against 18s ribosomal mRNA (Quantitect Cat QT00199367) as the house-keeping reference gene. Integrin-β1 was measured using primers F: gccagggctggttatacaga, R: tgccttcagctccacagaga and R: tggttgtagagggcaaggac.

Western Blot Analysis

The expression of integrin-β1, TGFβ, and the phosphorylation status of focal adhesion kinase (FAK) was determined in embryo homogenates using protein separation via SDS-PAGE followed by transfer to polyvinylidene fluoride membrane. Membranes were blocked using 5% skim milk, incubated with primary antibodies (α-smooth muscle actin (SMA) staining (in green), 4′,6-Diamidino-2-phenylindole (DAPI) nuclear staining is used to show cell number (blue in top row of slides) and merged immunofluorescence is displayed in the bottom row. Cellular outgrowth from +/+ branchial arches shows robust mERG-positive staining (E), whereas for N629D/N629D branchial arches cell outgrowth of cells manifest much less mERG-positive staining cells (F) and the mERG staining cells appear limited to the rim of the branchial arch. The right 2 columns of data show αSMA expression patterns in outgrowth of cells from +/+ branchial arch (G and K) vs N629D/N629D arches (H and L). Outgrowth from +/+ branchial arch shows robust outgrowth of αSMA cells. In N629D/N629D, there are fewer cells (αSMA) outgrowing from explants (see DAPI stains C vs D). The αSMA growth pattern in N629D/N629D shows a honeycomb (cystic) pattern with few DAPI-positive cells in the center of the cysts. Bar, 100 μm. N=6 for both wild-type and N629D/N629D; results were absolutely consistent between experiments.
nonadjacent serial sections. For control experiments, +/+ ES cells were aggregated with β-galactosidase positive +/+ cells.

Statistical Analysis
Direct comparison between data obtained from wild-type and N629D/N629D from both cells and embryos was evaluated using SIGMA STAT 3.5 (Systat, San Jose, CA) and the non-parametric Mann–Whitney rank-sum test.

Results
Vascular Developmental Defects in N629D/N629D Embryos
Yolk sac vessels (Figure 1A for +/+ versus Figure 1B for N629D/N629D) and whole-mount CD31 stained yolk sac vessels are shown (Figure 1C for +/+ and Figure 1D for N629D/N629D). The +/+ yolk sac vessels show a highly organized arborization. In contrast, N629D/N629D yolk sac vessels consist of dilated and disorganized primitive sinusoids (Figure 1D). Transverse sections of yolk sacs in Figure 1E and 1F show that the +/+ vitelline vessels has differentiated into both small and large vitelline vessels, whereas N629D/N629D yolk sac consisted of a disorganized confluent plexus. Examples of whole embryo CD31 staining are shown in Figure 1G–1I for +/+ and Figure 1J–1L for the N629D/N629D embryos. The +/+ embryos show a normal aorta with a normal arborization pattern (Figure 1G–1I). For example, in +/+ embryos, arrowheads (Figure 1H and 1I) show remodeled branched vessels in head (Figure 1H) and intersegmental vessels (Figure 1I). In contrast, N629D/N629D embryos (Figure 1J–1L) manifest a disorganized intraembryonic vascular plexus (Figure 1G versus 1I). The center of the arch also was depleted of 4',6-diamidino-2-phenylindole (DAPI)–positive staining cells. The N629D/N629D branchial arch outgrowth shows a plexiform, cystic outgrowth pattern (a honeycomb appearance) with a hypocellular centers (Figure 2D and 2H). The +/+ outgrowth showed substantially more cellularity with fewer cysts (Figure 2C and 2G).
Disruption of αSMA and mERG Coimmunolocalization Is Associated With Defective Vasculogenesis

Expression and colocalization of mERG and αSMA were evaluated in embryonic aorta and vitelline vessels. The coimmunolocalization of mERG and αSMA staining cells in dorsal aorta and in vitelline vessels at E9.5 to 10.5 from +/+ embryos is shown in Figure 3. Colocalized or tight apposition of mERG and αSMA staining cells is shown in the dorsal aorta (merged staining -right caption of Figure 3A) at E10.5. In contrast, there is little or no αSMA staining in dorsal aorta of N629D/N629D embryos (E9.5; Figure 3B). Similar findings were observed in the vitelline vessels (Figure 3C). In +/+ vitelline vessels, there is tight physical colocalization of mERG and αSMA staining cells. In contrast, in N629D/N629D vitelline vessels, the mERG staining cells are physically separated (spatial dissociated) from the few αSMA staining cells. This was a consistent finding.

Defect in Colocalization Occurs in Isolated EBs

To provide evidence that defective colocalization of mERG and αSMA-positive staining cells in N629D/N629D was a primary defect and not secondary to abnormalities of flow, we assessed the character of cell staining in +/+ versus N629D/N629D EBs at E13. In +/+ EBs (Figure 4G), the mERG and αSMA-positive staining was tightly apposed or colocalized. However, N629D/N629D EB manifest a paucity of αSMA and mERG staining; more importantly, αSMA and mERG staining cells were spatially dissociated (Figure 4H).

Partial Recovery of the Vasculogenesis Defect in N629D/N629D by TFGβ Treatment

Thannickal et al30 reported that exogenous treatment with TGFβ induces FAK phosphorylation and αSMA. Other studies indicate that cell-to-cell contact activates FAK phosphorylation in a hERG potassium channel–dependent manner.31 This suggests the possibility of a common signal transduction link between hERG and TGFβ. Accordingly, we tested the hypothesis that exogenous treatment with TGFβ would restore αSMA expression in the N629D/N629D EBs. Exogenous treatment with TGFβ partially restored mERG and αSMA expression (Figure 4I with TGFβ compared with Figure 4H) in N629D/N629D EBs.

To further understand the hypocellular phenotype, we compared apoptosis rates in N629D/N629D embryonic cells versus +/+ when grown in culture for 2 weeks. Figure 5A shows...
the distribution of annexin-positive apoptotic cells in these 2
groups. Quadrant 4 shows the distribution of cells undergoing
apoptosis. Mean data are shown in the dot plot. Apoptotic rates
were significantly greater in N629D/N629D cells versus +/+ cells.

To further understand the molecular mechanisms, the impact of the N629D/N629D mutation on expression of relevant signaling proteins was evaluated at the mRNA and protein levels. The expression of TGFβ both at the mRNA and protein level was substantially and significantly reduced in the N629D/N629D embryos (Figure 5B, 5C, and 5C'). Studies by Cherubini et al. indicate that integrin-β1 regulates TGFβ expression and its bioactivity in glioblastoma cells. Other studies indicate that hERG is physically linked to integrin-β1. Accordingly, we examined levels of these signaling molecules. In N629D/N629D embryonic cells, a significant decrease in protein expression of integrin-β1 was noted (Figure 5E and 5E') but not at the mRNA level (Figure 5B). To provide further evidence to support this signaling pathway, the effect of N629D/N629D on protein levels of phospho-FAK was examined. The N629D/N629D mutation significantly decreased phospho-FAK levels (Figure 5D and 5D').

To address whether TGFβ restores a more normal architecture of the heart and branchial arch in the developing N629D/N629D fetuses, whole embryos excised at E8.0 (a time where there is no heart, branchial arch, or circulation) and grown in culture in vitro until E10. To allow drug-access to the embryo, a linear cut in the yolk sac was created. The gross morphology of the embryos grown in culture is shown in Figure 6. The in vitro growth and development pattern in +/+ embryos mimics normal in utero development. When placed in culture at E8.0, there was no evident heart or branchial arch, but after 24 to 30 hours of in vitro culture, all +/+ fetus manifest a looped heart and branchial arch and head development (Figure 6A, 6D, and 6G). In contrast, untreated N629D/N629D cultured embryos grow slowly; there is a massive pericardial effusion (Figure 6B), the hearts fail to loop (Figure 6E and 6H); there is a single common ventricular chamber and the center of the branchial arch is acellular (Figure 6E). Thus, the developmental defects of N629D/N629D fetuses when grown in vitro and
in vivo are nearly identical. Exogenous TGFβ treatment of cultured embryos partially restored the growth and development of N629D/N629D embryos toward normal (Figure 6C, 6F, and 6I).

To assess whether TGFβ treatment altered protein expression or coimmunolocalization of mERG and αSMA, immunofluorescent studies were performed. In +/+ embryos, there is robust mERG and αSMA expression with colocalization. In contrast, N629D/N629D embryos manifest less mERG expression and it is not colocalized with αSMA staining. During TGFβ treatment, there is partial colocalization of mERG and αSMA staining cells (Figure II compared with IH in the Data Supplement).

Dofetilide Treatment Phenocopies N629D/N629D and Exogenous TGFβ Partially Restores the Dofetilide Phenotype Toward +/+  
To provide evidence that functional mERG ion current expression was necessary for normal embryonic development, we assessed whether blockade of mERG by the specific and clinically used hERG blocker, dofetilide at 1 μmol/L, recapitulated the N629D/N629D defects in vitro. Isolated E8.0 embryos and branchial arches were cultured with dofetilide in vitro. Dofetilide treatment of cultured +/+ embryonic hearts (Figure II in the Data Supplement) recreated the pericardial effusion (Figure IIA in the Data Supplement) and that treatment with TGFβ partially rescued these dofetilide-induced phenotypes (Figure IIA in the Data Supplement, right embryo and Figure IIC). Figure IID versus IIE shows that in vitro dofetilide treatment substantially reduces cellularity of the branchial arch (DAPI staining) and decreased migration of cells out of the branchial arch. Interestingly, dofetilide also recreated the pattern of hypocellular, plexiform, and cystic outgrowth. In review, in vitro dofetilide treatment of cultured embryos, at a time when the heart or circulation still does not exist, recapitulated the N629D/N629D phenotypes.

To assess whether in vivo dofetilide also recreated the same vascular embryonic phenotype, pregnant +/+ females were treated in vivo with dofetilide (4 mg/kg per day IP) beginning at E5.5 and ending at E8.5; the embryos were harvested at E9.5. No cardiovascular defect was noted in these E9.5 mice (Figure 7A). However, when dofetilide treatment was initiated at E7.5 and treatment and continued until E9.5 or E10.5, the N629D/N629D cardiovascular phenotype was entirely replicated (Figure 7B). In review, there is a tight window of time over which dofetilide treatment recapitulates the entire N629D/N629D phenotype.

Vascular Defect in N629D/N629D Embryos is Primary  
To provide further evidence that the defect in colocalization of mERG and αSMA-positive staining cells in N629D/N629D was primary and not secondary to a defect in the heart or flow, we created chimeric mice by morula aggregating Rosa26 embryos with N629D/N629D ES cells at the 8-cell stage. In the control experiments, +/+ ES cells were aggregated with the Rosa26 cells. Mice were selected with ≈50% overall β-galactosidase positive cell staining. The relative distribution of the β-galactosidase positive +/+ cells was determined by whole-mount β-galactosidase staining. We selected chimeric mice with substantial overall N629D/N629D cell representation (≈40% for the whole embryo) but with no cardiovascular phenotypes. We expected that no developmental phenotype would be observed only when the N629D/N629D cells were under-represented in the tissues where functional mERG (KCNH2) expression plays the critical role in normal

![Figure 8](image-url)  
Figure 8. Under-representation of N629D/N629D cells in first branchial and dorsal aorta in chimeric embryos without cardiovascular defects. Chimeric embryos that do not develop cardiovascular phenotypes had an uneven distribution of the β-galactosidase positive Rosa26 +/+ and β-galactosides negative N629D/N629D cells (N=3). In embryos without cardiovascular phenotypes, the β-galactosidase negative N629D/N629D cells were under-represented in the first branchial arch (A) and dorsal aorta (B). The results were entirely consistent between experiments. In contrast, the distribution of β-galactosidase negative N629D/N629D cells was equal to +/+ Rosa26 cells in the ventricle and early outflow tract in embryos with no cardiovascular phenotypes. In the negative-control experiments, β-galactosidase positive Rosa26 cells were aggregated with +/+ morula cells (N=3). The results were entirely consistent. D-F. The even distribution of the β-galactosidase-positive +/+ Rosa26 and β-galactosides-negative +/+ cells in these control experiments. Bar, 100 μmol/L.
embryogenesis. Importantly, we found that chimeric mice had no cardiovascular developmental phenotype only if their branchial arches (Figure 8A) and aortas (Figure 8B) were populated dominantly by β-galactosidase–positive, +/+ cells; that is N629D/N629D cells were under-represented in those tissues. Importantly, chimeric mice that phenocopy the N629D/N629D lethal phenotype always had >30% of N629D/N629D cells in their branchial arches and aorta. In the outflow tract and the right ventricle, the presence or absence of abundant of N629D/N629D cells did not determine the development phenotype (Figure 8C and 8F). Figure 8D and 8E shows an event distribution of the β-galactosidase–positive +/+ Rosa26 and β-galactosidase–negative +/+ cells in the control experiments.

Discussion

Clinical Relevance
Pharmacological blockade of hERG by dofetilide, over a narrow window of time, completely recapitulates the lethal defect in vasculogenesis seen in N629D/N629D embryonic mice. Pharmacological blockade of hERG 1 day before this critical window of time produced no deleterious effects. In human terms, avoidance of drug treatment that could inadvertently block the hERG current is important to normal fetal development.

Primary Vascular Defect Contributes to Embryonic Lethality
There are many reasons to believe that the vascular defect is primary and contributory to embryonic lethality: (1) N629D/N629D EBs and isolated branchial arches manifest abnormalities of outgrowth and colocalization of mERG and αSMA when grown in vitro, (2) exposure of isolated embryos to dofetilide at a time where there is no heart or circulation results in subsequent cardiovascular developmental defects, (3) chimeric studies provide evidence that the lethality of N629D/N629D on embryos development can be overcome when N629D/N629D cells are under-represented in the branchial arch and aorta.

TGFβ protein expression is deficient in N629D/N629D embryos, and exogenous TGFβ treatment partially restores cardiovascular phenotypes. Previous studies have reported that vasculogenesis is defective in TGFβ knockout mice. Moreover, Molin et al44 have reported that apoptosis within the branchial arch of TGFβ knockout embryos was associated with malformations of the aortic arch. This is reminiscent of the N629D/N629D phenotype. Kim et al35 show that exogenous treatment with TGFβ induces differentiation of human mesenchymal cells into smooth muscle cells. Thaninckal et al39 have reported that exogenous treatment with TGFβ induces FAK phosphorylation and αSMA expression. In addition, cell-to-cell contact activates FAK phosphorylation in an ERG potassium channel–dependent manner.31 These studies suggest a common signal transduction link between the ERG potassium channel and TGFβ. The primary defect may be the absence of TGFβ protein expression in the N629D/N629D mice. Previous studies report that the hERG potassium channel creates a multiprotein complex with integrin-β127. Moreover, defects in integrin function produce a loss of TGFβ release and bioactivity.31 Thus, defects in this multiprotein complex may result in decreases in TGFβ expression with resultant dysfunctional vasculogenesis.

In conclusion, defects in ERG1 potassium channel expression results in defects of vasculogenesis in the embryo. The abnormalities of vasculogenesis are likely primary rather than secondary to abnormalities of flow.15 This vasculogenesis defect is recapitulated by pharmacological blockade by dofetilide. The clinical relevance of this work is that maternal exposure at a critical window of time creates lethal defects in cardiogenesis and vasculogenesis.

Acknowledgments
We thank Valerie Martin for editing and proofing assistance.

Sources of Funding
This work was funded by grants from the Heart and Stroke Foundation of Canada and by the Canadian Institutes of Health Research. Drs Duff and Cross are recipients of Alberta Innovates Health Solutions Medical Scientists Award.

Disclosures
None.

References


Role of Mutation and Pharmacologic Block of Human KCNH2 in Vasculogenesis and Fetal Mortality: Partial Rescue by Transforming Growth Factor-β

Guoqi Teng, Xiang Zhao, James P. Lees-Miller, Darrell Belke, Chunhua Shi, Yongxiang Chen, Edward R. O'Brien, Paul W. Fedak, Nathan Bracey, James C. Cross and Henry J. Duff

Circ Arrhythm Electrophysiol. 2015;8:420-428; originally published online February 3, 2015; doi: 10.1161/CIRCEP.114.001837

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/8/2/420

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2015/02/03/CIRCEP.114.001837.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Arrhythmia and Electrophysiology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Arrhythmia and Electrophysiology is online at:
http://circres.ahajournals.org//subscriptions/
**Supplemental Figure 1** Co-localization of mERG with αSMA in the heart of cultured embryos.

αSMA staining is shown in the top row, mERG staining in middle row and merged images in lower row. +/+ examples are shown in Panels A, D and G. In +/+ embryos, αSMA staining in the right ventricle (RV) and left ventricle (LV) co-localized with that of mERG. The N629D/N629D embryos are shown in Panels B, E and H showing diminished expression of mERG and αSMA, moreover they are not co-localized. In TGFβ treated N629D/N629D embryos there is a re-emergence of a separate RV, the expression of mERG and αSMA is increased and at least in some areas there appears to be co-localization. Bar=100 µM.
Supplemental Figure 2 Dofetilide Treatment Phenocopies N629D/N629D and Exogenous TGFβ

Supplemental Figure 2 Panel A shows gross morphology and H&E histology of dofetilide treatment of whole embryos culture in vitro (Panels B and C). Dofetilide treatment (1 µMol/L) created the same phenotype of pericardial effusion (Panel A left embryo) and a single ventricle (Panel B). Treatment with TGFβ partially rescued the dofetilide-induced phenotype (Panel A right embryo and Panel C). Panel D (+/+ untreated control) and E (+/+ dofetilide treatment) show that in vitro dofetilide treatment substantially reduces cellularity of the branchial arch and decreases migration of cells out of the branchial arch. Cellular outgrowth from +/+ untreated
control branchial arches show robust mERG positive staining, whereas +/+ BA-dofetilide treated branchial arches cell outgrowth manifest much less mERG positive staining cells. Outgrowth from +/+ untreated control branchial arch shows robust outgrowth of αSMA cells. In +/+ BA-dofetilide treated, αSMA there are fewer cells outgrowing from explants.