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 recapitulated 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 hERG are associated with structural congenital cardiovascular anomalies, including Tetralogy of Fallot, atrial-septal defects, ventricular-septal defects, and patent ductus arteriosus.9–13 In rodent models, antiepileptic drugs that block hERG cause fetal death and birth defects.14 In addition, mouse ERG (mERG) potassium current is the dominant repolarizing current during critical phases of cardiac development.15 We previously reported that the cardiac characteristics of a mouse bearing the human N629D long-QT2 syndrome mutation is inserted in situ (knocked-in).16 The homozygous N629D missense mutation produced developmental defects in the secondary heart field and a loss-of-function Ikr phenotype.16 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.17 In chick embryo, TGFβ 2,3 receptors are expressed in the outflow tract.18 Ablation of endoglin results in defective angiogenesis,19 whereas TGFβ is required for creation of the primary capillary network. There are a myriad of proteins that mediate signaling down-steam 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, HRC 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://circep.ahajournals.org DOI: 10.1161/CIRCEP.114.001837
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.

BMPs (bone morphogenic proteins).20,21 Moreover, many transcription factors, downstream of TGFβ, work cooperatively22 to mediate smooth muscle differentiation and branchial arch remodeling.23 TGFβ also regulates the epithelial–mesenchymal cell transition and differentiation of endothelial cells.24

Methods

Creation of mERG-N629D Mice

The N629D/N629D mouse has been previously reported.16 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

Immunohistochemistry of whole-mount embryos was performed as described earlier.25 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 stained for CD31-Pecam-1 staining.

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. Torrence, CA) 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**

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 18 s ribosomal mRNA (Quantitect Cat QT00199367) as the house-keeping reference gene. Integrin-β1 was measured using primers F: ggcaggctgtgatactagca, R: ttcacatggcacacaggttt, whereas TGFβ1 was measured using primers F: gccagggctggttatacaga, 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, exposed to primary antibodies: anti-integrin-β1 (RabMAB), anti-TGFβ (Santa Cruz Biotechnology), and anti-FAKpY397 (Invitrogen) and corresponding secondary antibodies and quantified using chemiluminescence.

**Culture of Whole Embryos**

After pentobarbital 0.2 mL IP (65 mg/mL), embryos were euthanized at E8.0. Embryos with visceral yolk sacs and ectoplacental cones were cultured for 48 hours in an incubator on a rotated platform. Embryos were randomized to control (saline) and TGFβ treatment (10 ng/mL). The incubator was modified to allow simultaneous O2 entry with regulated CO2.

**Apoptosis Annexin V staining**

E9.5 wild-type and N629D/N629D embryos were incubated in 0.25% trypsin at 4°C for 3 hours, and then the cells were isolated. After 1 week, cells were trypsinized and stained against fluorescein isothiocyanate-conjugated Annexin-V (in the dark) and propidium iodide (BD Pharmingen; Becton Dickinson, San Jose, CA). Flow cytometry analysis was performed by a fluorescence-activated cell sorter (Coulter; Becton Dickinson).

**Morula Aggregation to Create Chimeric Mice**

Transgenic Rosa26 mice ubiquitously expressing β-galactosidase in a 129 strain background were obtained from Jackson Laboratories. Eight-cell stage Rosa26 embryos were aggregated with N629D/N629D ES cells (or +/+ cells in the negative control studies) using the morula aggregation technique. The aggregates were cultured overnight, and resultant blastocysts were transferred into the uteri of pseudopregnant CD-1 females. Chimeric embryos were dissected at E9.5. The relative contribution to the embryo of mutant ES cells and β-galactosidase-positive +/+ cells was determined by whole-mount β-galactosidase staining. Sections were counterstained with 0.5% eosin. Chimeric mice were selected with substantial overall mutant cell representation (~40%–50%) but with no cardiovascular phenotype. We expected that no developmental phenotype would be observed only when the N629D/N629D cells were under-represented in the tissues critical for normal embryogenesis.

**Figure 2.** Outgrowth of cells from isolated first branchial arches. Left: 2 columns show mERG staining (in red) and the right 2 columns show 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 9+ 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 (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 shows normal 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 1J). 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 1J). Figure 1M and 1N shows transverse sections of PECAM-1 stained +/+ and N629D/N629D through the first branchial arch. Figure 1N shows that the N629D/N629D fetuses manifest distorted first branchial arch architecture with the center being hypocellular.

To assess the character of cellular outgrowth, the first branchial arches were dissected free at E9 and grown in vitro for 24 hours. Figure 2 shows the immunocytochemical evaluation of the outgrowth expressing mERG (left panels) and α-smooth muscle actin (αSMA) in right panels. In +/+ first branchial arch, robust outgrowth of mERG-positive (Figure 2A, 2E, and 2I) and αSMA-positive staining cells (Figure 2C, 2G, and 2K) was observed. In contrast, outgrowth from the N629D/N629D first branchial arch showed substantially less outgrowth with much fewer mERG-positive cells (Figure 2B, 2F, and 2J). 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 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.

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

Thannickal et al. 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. 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

Figure 5. Relevant molecular mechanisms. Cell apoptosis was analysis by fluorescence-activated cell sorting using an Annexin V-Fluorescein Isothiocyanate Apoptosis Kit. A, Quadrant 4 cells indicate apoptotic cells. The results were expressed as a percentage of apoptosis cells. The individual results and the mean (shown as the bar) from 3 independent experiments are shown. The mRNA expression was examined by quantitative reverse transcriptase-polymerase chain reaction (B). The transforming growth factor (TGF)-β mRNA in N629D/N629D embryos was significantly decreased when compared with +/- embryos (+/+, N=3; N629D/N629D, N=3), whereas the integrin-β1 mRNA levels were not significantly changed. Western blot analysis of expression of TGFβ (C and C'); focal adhesion kinase (FAK) pY397 (D and D') and integrin-β1 (E and E) was compared in +/- vs N629D/N629D E9.5 embryos. The individual results and the mean (shown as the bar) from independent embryos are shown. *A significant difference compared with +/- using the nonparametric statistic. The n values for the individual embryos are N=4 for TGFβ for +/- and 5 for N629D/N629D; for FAK pY397 and integrin-β1 expression, N=3 for +/- and 4 for N629D/N629D. GAPDH was used as the sample loading controls.
Accordingly, we examined levels of these signaling molecules in N629D/N629D cells. Other studies indicate that hERG is physically linked to TGFβ expression and its bioactivity in glioblastoma. 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 when there is no heart, branchial arch, or circulation) and grown in culture for 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 reminiscent of the in vivo phenotypes including massive pericardial effusion (B) and a single chamber heart (H). TGFβ treatment during embryo culture partially restored embryonic development toward normal. The extent of the pericardial effusion is partially resolved (G), the branchial arches are more cellular (F compared with N629D/N629D E), and the heart has 2 chambers with a separate right (RV) and left ventricles (LV). Bar: A-C, 200 μm; others, 100 μm. N=6 for +/-, N=6 N629D/N629D and N=7 for TGFβ-treated N629D/N629D. The results were absolutely consistent between experiments.

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 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, left embryo) and the phenotype of a common ventricular chamber (Figure IIB 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 development.
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 al 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 al show that exogenous treatment with TGFβ induces differentiation of human mesenchymal cells into smooth muscle cells. Thankickal et al 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. 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-β1. Moreover, defects in integrin function produce a loss of TGFβ release and bioactivity. 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. 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

*Circulation: Arrhythmia and Electrophysiology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2015 American Heart Association, Inc. All rights reserved.

Print ISSN: 1941-3149. Online ISSN: 1941-3084

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circcep.ahajournals.org/content/8/2/420

Data Supplement (unedited) at:

http://circcep.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://circcep.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.