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An essential role for connexin43 gap junctions in mouse coronary artery development

W. E. I. Li[1], K. Waldo[2], K. L. Linask[1,3], T. Chen[1], A. Wessels[4], M. S. Parmacek[5], M. L. Kirby[2] and C. W. Lo[1,3,*]

[1]Biology Department, Goddard Laboratories, University of Pennsylvania, Philadelphia, PA, USA
[2]Division of Neonatology, Department of Pediatrics, Duke University, Durham, NC, USA
[3]Laboratory of Developmental Biology, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA
[4]Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC, USA
[5]Department of Medicine, University of Pennsylvania Medical School, Philadelphia, PA, USA
*Author for correspondence (e-mail: clo@nhlbi.nih.gov)

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SUMMARY

Connexin43 knockout mice die neonatally from conotruncal heart malformation and outflow obstruction. Previous studies have indicated the involvement of neural crest perturbations in these cardiac anomalies. We provide evidence for the involvement of another extracardiac cell population, the proepicardial cells. These cells give rise to the vascular smooth muscle cells of the coronary arteries and cardiac fibroblasts in the heart. We have observed the abnormal presence of fibroblast and vascular smooth muscle cells in the infundibular pouches of the connexin43 knockout mouse heart. In addition, the connexin43 knockout mice exhibit a variety of coronary artery patterning defects previously described for neural crest-ablated chick embryos, such as anomalous origin of the coronary arteries, absent left or right coronary artery, and accessory coronary arteries. However, we show that proepicardial cells also express connexin43 gap junctions abundantly. The proepicardial cells are functionally well coupled, and this coupling is significantly reduced with the loss of connexin43 function. Further analysis revealed an elevation in the speed of cell locomotion and cell proliferation rate in the connexin43-deficient proepicardial cells. A parallel analysis of proepicardial cells in transgenic mice with dominant negative inhibition of connexin43 targeted only to neural crest cells showed none of these coupling, proliferation or migration changes. These mice exhibit outflow obstruction, but no infundibular pouches. Together these findings indicate an important role for connexin43 in coronary artery patterning, a role that probably involves the proepicardial and cardiac neural crest cells. We discuss the potential involvement of connexin43 in human cardiovascular anomalies involving the coronary arteries.

Key words: Connexin43, Gap junctions, Coronary artery, Cell proliferation, Cell migration, Proepicardial cell

INTRODUCTION

Gap junctions are intercellular junctions containing membrane channels that mediate the movement of ions, metabolites and secondary messengers below 1.2 kDa. They are formed by a protein family of at least 16 members termed connexins (Bruzzone et al., 1996; Condorelli et al., 1998; Itahana et al., 1998; Manthey et al., 1999). Given these properties, gap junctions are thought to have a role in maintaining tissue homeostasis, and regulating cell proliferation and differentiation, and embryonic development (Goodenough et al., 1996; Kumar and Gilula, 1996; Lo, 1999; Newman, 1985). Mutations in connexin genes have been associated with a variety of human diseases, including heart anomalies, cataract, deafness, skin disorders and X-linked Charcot-Marie-Tooth disease (Britz-Cunningham et al., 1995; Kelsell et al., 1997; Richard et al., 1998; Shiels et al., 1998; Xia et al., 1998). The essential role of gap junctions also has been revealed by the analysis of connexin knockout mice (reviewed by White and Paul, 1999; Lo, 1999). Furthermore, connexin knock-in mouse models demonstrated that the phenotypes resulting from eliminating one connexin gene cannot be fully corrected by the replacement of another connexin gene (Plum et al., 2000). This suggests that each connexin isotype may have unique function that is cell- or tissue type-specific.

The connexin gene known as connexin43 or α_1 connexin (referred to as Cx43α1; Gja1 – Mouse Genome Informatics) plays a crucial role in cardiac development, as Cx43α1 knockout mice die shortly after birth due to pulmonary outflow obstruction (Reaume et al., 1995). Typically, the Cx43α1 knockout mouse heart exhibits two prominent pouches at the base of the pulmonary outflow tract, a region known as the infundibulum of the heart. Studies using transgenic mouse models to manipulate Cx43α1 function indicate that the

outflow obstruction probably involves the perturbation of cardiac neural crest cell migration. Thus, when Cx43α1-mediated gap junction communication in cardiac neural crest cells was up- or downregulated in the CMV43 (overexpression) and FC (dominant negative) transgenic mice, respectively, right ventricular cardiac defects associated with outflow obstruction were observed (Ewart et al., 1997; Sullivan et al., 1998). In the CMV43 transgenic mice, an increase in gap junction communication was associated with an increase in the rate of neural crest cell migration and an elevation in the abundance of neural crest cells in the heart outflow tract (Huang et al., 1998a). By contrast, in the FC transgenic and Cx43α1 knockout mice, a reduction in coupling was associated with a reduction in cell migration rate and a lower abundance of neural crest cells in the heart (Huang et al., 1998a). Although the FC and CMV43 transgenic mice both exhibited defects involving the heart outflow tract, the same region affected in the Cx43α1 knockout mice, neither animal model exhibited the pouches typically seen in Cx43α1 knockout mouse heart (Ewart et al., 1997; Huang et al., 1998b; Sullivan et al., 1998). This was surprising, as the cardiac neural crest cells from the FC transgenic mice showed similar reductions in gap junction coupling and cell locomotion as that found in the Cx43α1 knockout mice (Huang et al., 1999a; Xu et al., 2002). Our present study suggests that this discrepancy may reflect the fact that formation of the pouches involves not only the cardiac neural crest cells, but also another extra-cardiac migratory cell population, the proepicardial cells.

The proepicardial cells originate from the proepicardial organ (PEO), a grape-like cluster of cells derived from the hepatic primordium and found at E9.0-E9.5 at the ventral region of the sinus venosus (Viragh and Challice, 1981; Viragh et al., 1993). These cells rapidly migrate over the primitive tubular heart to form the epicardium, and subsequently, cells from the epicardium undergo an epithelial-mesenchymal transformation and infiltrate the heart, giving rise to the vascular smooth muscle cells of the coronary arteries, and also fibroblasts in the heart (Dettman et al., 1998; Mikawa and Gourdie, 1996; Vrancken-Peeters et al., 1997; Vrancken-Peeters et al., 1999). Although proepicardially derived cells also have been suggested to contribute endothelial cells in the heart, this possibility remains unresolved (Poelman et al., 1993; Mikawa and Gourdie, 1996). When the epicardium is not maintained or does not form, such as in the α4 integrin and VCAM-1 knoockout mice, coronary arteries fail to develop (Kwee et al., 1995; Yang et al., 1996). The phenotype of these knockout mice strongly suggests that cell-cell adhesion and cell-matrix interactions play an important role in the invasion and subsequent deployment of the epicardial cells.

Our present study suggests that cell-cell interactions mediated by Cx43α1 gap junctions also play an important role in the deployment of the proepicardial cells. The cardiac defects in the Cx43α1 knockout mice are more subtle than that seen in the α4 integrin or VCAM-1 knockout mice, as formation of the epicardium does not appear to be affected by the loss of Cx43α1 function. In the Cx43α1 knockout mouse heart, coronary arteries form but are abnormally patterned. Histological analysis suggested that the coronary artery anomalies and the infundibular pouches involve the abnormal deployment of the proepicardially derived vascular smooth muscle cells. Analysis of the proepicardially derived cells revealed that they are functionally well coupled, and they also have an abundance of Cx43α1 gap junction contacts. In the Cx43α1-deficient proepicardial cells, coupling is greatly reduced, but in contrast to Cx43α1-deficient neural crest cells, this was associated with an elevation in the rate of cell locomotion and cell proliferation. These and other findings suggest that Cx43α1 gap junctions play an essential role in normal coronary artery patterning. We propose that this may involve a dual role for Cx43α1 gap junctions in modulating the deployment of the proepicardial and neural crest cells. We further discuss the possible involvement of Cx43α1 in human congenital cardiovascular anomalies and cardiovascular disease.

MATERIALS AND METHODS

Breeding and genotyping of mice

The Cx43α1 knockout mice are in a mixed B6/129 background, and offspring obtained from one generation outcross to CD1 were used for experiments. The FC transgenic mice, maintained in a B6/SJL background, were intercrossed with CD1 to obtain hemizygous transgenic and nontransgenic embryos for experiments. Genotyping was carried out by PCR analysis of yolk sac or tail DNA. For genotyping the Cx43α1 knockout mice, primers used were to the wild-type Cx43α1 allele and/or the neo insert in the Cx43α1 knockout allele (Huang et al., 1998a; Reaume et al., 1995). For the FC transgenic mice, the primers used were for the *lacZ* region of the Cx43α/lacZ fusion protein construct (Sullivan et al., 1998).

Immunohistochemical analysis

Hearts from mouse fetuses and neonates were fixed in 4% paraformaldehyde (PF) overnight, and after dehydration in a graded series of alcohol, they were paraffin embedded, cut into serial sections, de-paraffinized and subjected to immunostaining as previously described (Waldo et al., 1999; Epstein et al., 2000). Smooth muscle cells were identified using antibodies to a smooth muscle cytoskeletal protein, SM22α (Zhang et al., 2001) or smooth muscle myosin heavy chain (Biomedical Technologies, Stoughton, MA). Anti-proliferating cell nuclear antigen (PCNA) antibody (Zymed Laboratories, San Francisco, CA) were used to examine cell proliferation, and to examine apoptosis, a TUNEL assay was carried out using the APOPTAG kit from Intergen Company (Purchase, NY).

For immunohistochemical analysis of proepicardial organ (PEO) explant cultures, fixation and subsequent immunostaining were carried out as previously described (Li and Nagy, 2000). For whole-mount Cx43α1 immunostaining, E9.5 wild-type mouse embryos were incubated with primary and secondary antibodies for 48 hours and 20 hours, respectively. After washing, the embryos were dehydrated, plastic embedded, cut into 5 μm sections and mounted on slides using a Vectorshield mounting medium (Vector Laboratory, Inc.).

Proepicardial organ and heart explants

PEO explants were obtained from E9.0-E9.5 mouse embryos, with E0.5 designated as the morning a vaginal plug was found. Only the top part of the protruding PEO cluster was removed, in order to minimize contamination from hepatic primordium. The explants were plated on glass coverslips coated with Type I rat tail collagen and maintained at 37°C in Dulbecco's modified essential medium (high glucose) supplemented with 10% fetal bovine serum, unless otherwise noted. To obtain epicardial explants, E11.5 mouse hearts were similarly plated onto collagen coated coverslips. A polyclonal anti-cytokeratin antibody (Dako) was used to examine the identity and purity of cultured PEO explants. For the study of proepicardial cell

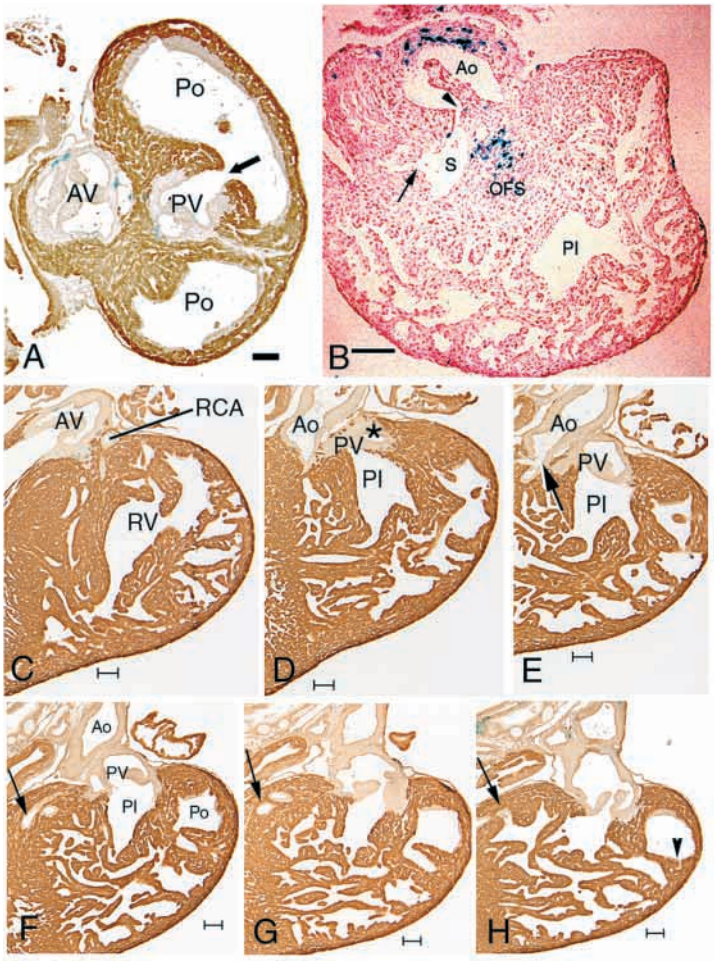

Fig. 1. Coronary artery defects in Cx43α1 knockout mouse hearts. (A) Transverse sections through the base of the heart of an E17.5 Cx43α1$^{-/-}$ embryo stained with an antibody to α-cardiac myosin. A large pouch (Po) breached the pulmonary valve ring. Note the myosin-free subendocardial layer in the wall of the pouch. The right coronary artery stem was absent in this animal (not shown). (B) In this E14.5 Cx43α1$^{-/-}$ mouse heart, the mouth of the coronary artery (arrowhead) is narrowed and the stem of the coronary artery is continuous with a large thin-walled sinus (s) that empties into numerous intertrabecular sinusoids. Note the base of the right ventricle is filled with trabecula that obscures the right ventricular outflow. The *lacZ*-positive cells (blue) represent neural crest cells labeled with a Cx43α1 promoter driven *lacZ* transgene (Lo et al., 1997). (C-E) A caudal-to-cranial sequence of an E17.5 Cx43α1$^{-/-}$ heart stained with an antibody to α-cardiac myosin. The left coronary artery exits the aorta normally (arrow in E) but the right coronary artery exits a sinus of the pulmonary trunk rather than the aorta. In C, the mouth of the right coronary artery (RCA) is just opening into the coronary sinus of the pulmonary trunk, seen more clearly in D (asterisk). (F-H) E17.5 Cx43α1$^{-/-}$ heart stained with an antibody to myosin. In a caudal-to-cranial sequence, a branch of the left coronary artery (arrow) empties into and becomes continuous with an enlarged intertrabecular sinusoid that is in the early stages of forming a pouch in the right ventricle. Ao, aorta; Av, aortic valve; PI, pulmonary infundibulum (right ventricular outflow tract); Po, pouch; Pv, pulmonary valve; OFS, remnant of the outflow tract septum; RV, right ventricle; RCA, right coronary artery. Scale bars: 100 μm.

differentiation, a cocktail of two monoclonal antibodies against smooth muscle calponin was used (clone CP93 and hCP, Sigma).

Analysis of dye coupling and cell motility in the PEO explants

To monitor dye coupling, coverslips containing 24 hours PEO explants were transferred into phosphate buffered L-15 medium (Sigma) supplemented with 10% fetal bovine serum, and maintained at 37°C on a heated stage of a Leitz Diavert microscope equipped for epifluorescent illumination. Single proepicardial cells were impaled with a glass microelectrode filled with 5% carboxyfluorescein and iontophoretic dye injection was carried out for 2 minutes using 1 nA hyperpolarizing current pulses of 0.5 second duration at 1 Hz. The number of dye-filled cells at the end of 2 minutes of iontophoresis was recorded. To analyze proepicardial cell motility, images of the PEO explant cultures were recorded at 5 minute intervals for 24 hours. Time lapse movies generated in this manner were used to trace the migration paths of individual proepicardial cells, and from which the speed and directionality of cell movement were determined using the Dynamic Imaging Analysis Software (Solltech, Oakdale, IA).

Cell proliferation analysis with bromodeoxyuridine (BrdU) labeling

To examine the proliferation rate of proepicardial cells, 24 hours explant cultures were incubated with 10 μM BrdU for 1 hour, fixed in 4% PF for 30 minutes and immunostained with an anti-BrdU antibody (Calbiochem, San Diego, CA) and further counterstained using Hematoxylin. Images of the stained PEO explant cultures were captured digitally, and the labeled nuclei were quantitated using the Openlab software (Improvision, Coventry, UK).

RESULTS

Coronary artery defects and pouches in the Cx43α1 knockout mouse heart

Examination of pouches in the infundibulum of the fetal and newborn Cx43α1 knockout mouse heart by histology revealed an unusual subendocardial layer of α-cardiac myosin free tissue lining the pouches (Fig. 1A). In some regions, distal coronary artery branches were directly continuous with the pouches (Fig. 1F-H). Interestingly, the tunica media of the coronary artery, which comprises vascular smooth muscle cells, continued seamlessly into the tissue lining the pouch. Further examination of the coronary arteries revealed only a single major coronary artery (either right or left) in some knockout mouse hearts. In a few animals, we observed an anomalous origin of the coronary artery, i.e. where one of the two major coronary arteries arose from the pulmonary rather then aortic trunk (Fig. 1C-E). In some cases, the stem of the coronary artery was continuous with a thin-walled sinus that fed into a complex of intertrabecular sinusoids (Fig. 1B). As the infundibulum is also the region of the heart where the major coronary arteries arise from the aorta, these observations

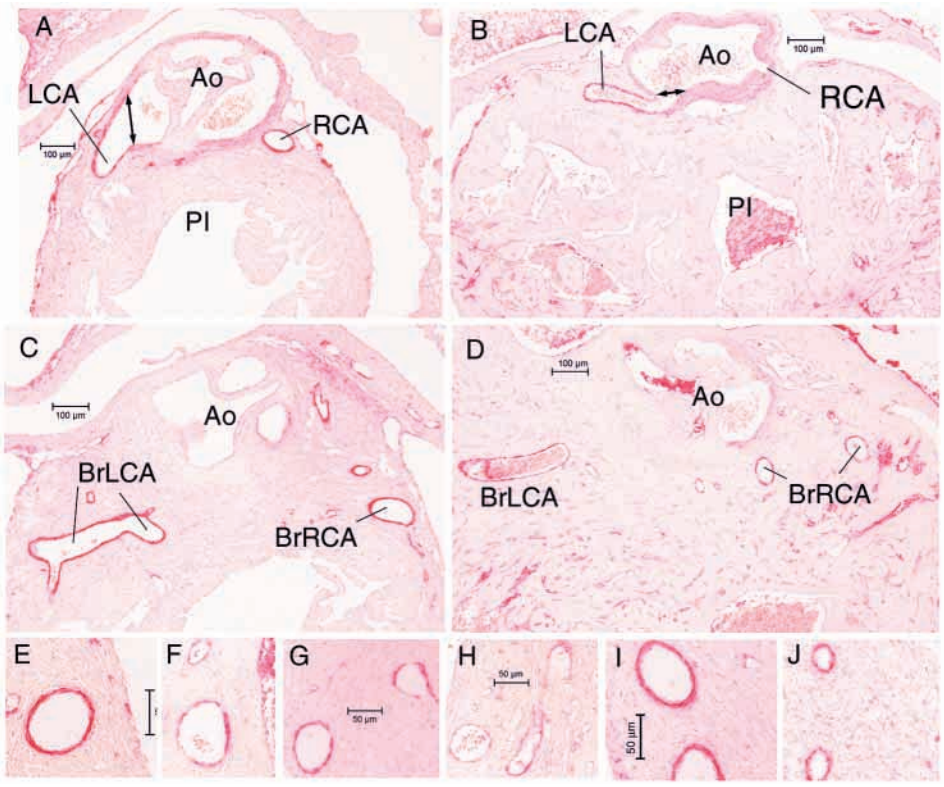

Fig. 2. Reduction of vascular smooth muscle myosin expression in the coronary arteries of Cx43α1 knockout hearts. Transverse sections of neonatal mouse hearts stained with anti-vascular smooth muscle myosin antibody. (A,B) As the coronary artery exits the aorta (LCA), the coronary artery stem and its mouth (double-headed arrow) are greatly reduced in the Cx43α1$^{-/-}$ (B) versus wild-type (A) heart. Ao, aorta; PI, pulmonary infundibulum (right ventricular outflow tract); RCA, right coronary artery; LCA, left coronary artery. (C,D) A reduction in vascular smooth muscle myosin expression in large branches of the right and left coronary arteries (BrRCA or BrLCA) of the Cx43α1$^{-/-}$ (D) versus wild-type (C) heart. (E-J) A similar reduction in smooth muscle labeling is seen in the mural arteries of the right (E,F) and left ventricular walls (I,J), and in arteries of the ventricular septum (G,H). (E,G,I) Wild-type vessels; (F,H, J) Cx43α1$^{-/-}$ vessels. Note the reduction in size of the Cx43α1 knockout vessels and the interruption of vascular smooth muscle myosin immunolabeling in the walls of the Cx43α1 knockout vessels.

suggest the possibility that formation of the pouches may be related to defects in the deployment of the coronary arteries.

Coronary artery patterning defects

To examine coronary artery anomalies further in the Cx43α1 knockout mice, we carried out a more detailed analysis of coronary arteries and their pattern of deployment in the E16.5 and E17.5 mouse heart. For these studies, vascular smooth muscle myosin (VSMM) was used as a marker to label the coronary arteries. Unexpectedly, we found a reduction in VSMM expression in the coronary arteries of the Cx43α1 knockout mice (Fig. 2). This can be seen in the left and right branches of the coronary arteries (Fig. 2A-D), as well as in the septal and mural coronary arteries in the left and right ventricular wall (Fig. 2E-J). In addition, we noted discontinuities in the VSMM staining, suggesting that vascular smooth muscle cells may not completely line the wall of the coronary arteries in the Cx43α1 knockout mouse heart (for example, compare Fig. 2C with Fig. 2D and Fig. 2E with Fig. 2F). Also striking was the finding of a significant narrowing of the mouth and stem of the coronary arteries as they exited the aorta (compare double headed arrows in Fig. 2B with those in 2A), and a general reduction in the size of the coronary arteries (Fig. 2). It was not possible to analyze this quantitatively, given the combination of the very small size of the coronary arteries and the difficulty in achieving identical alignment of the sectioning planes of the wild-type and knockout mouse hearts. The small size of the coronary arteries also excluded the use of a casting method for three-dimensional reconstructions.

Nevertheless, a systematic analysis of serial sections of wild-type, and heterozygous and homozygous Cx43α1 knockout mouse hearts revealed a variety of coronary artery patterning defects. In wild-type mice, the right and left coronary arteries exited the aorta from the right and left aortic sinuses respectively (R, L in Fig. 3), while no coronary artery arises from the third aortic (non-coronary) sinus (N in Fig. 3). Each coronary artery stem divides into major branches that feed the ventricular septum (S in Fig. 3), the free walls of the heart (M in Fig. 3), the base of the heart (C in Fig. 3) and the atria. In the heterozygous and homozygous knockout hearts, there were numerous alterations in the pattern of coronary artery development. For example, in addition to a main coronary artery, there were often small accessory coronary arteries either exiting from the non-coronary aortic sinus (8065, 8464, 8462 and 8463 in Fig. 3), or the right or left coronary sinus (8065 in Fig. 3). The right coronary artery sometimes tunneled some distance caudally through the wall of the aorta before exiting (8048, 8462, 8463 and 8065 in Fig. 3). As described above, occasionally a stem would be missing and only one coronary artery arose from the aorta (7949 and 8489 in Fig. 3). The ostia or mouths of the coronary artery were often very small (8465 in Fig. 3), and in some cases, the main branches of a coronary artery exited the aorta separately rather than branch from a main stem (8065 in Fig. 3), or the stem was so short that it divided in the wall (8064 in Fig. 3). In several hearts, a branch of the coronary artery became sinusoidal with very thin walls (8489 and 8488 in Fig. 3). Surprisingly, we found the same set of coronary arterial anomalies in the heterozygous and homozygous knockout mouse hearts (Fig. 3). The data summarized in Fig. 3 represents 85% of the hearts analyzed and are representative of the defects typically observed (except for the heart with anomalous origin of the coronary artery, which is illustrated only in Fig. 1B). Given these observations, it is interesting to note that although the infundibular pouches

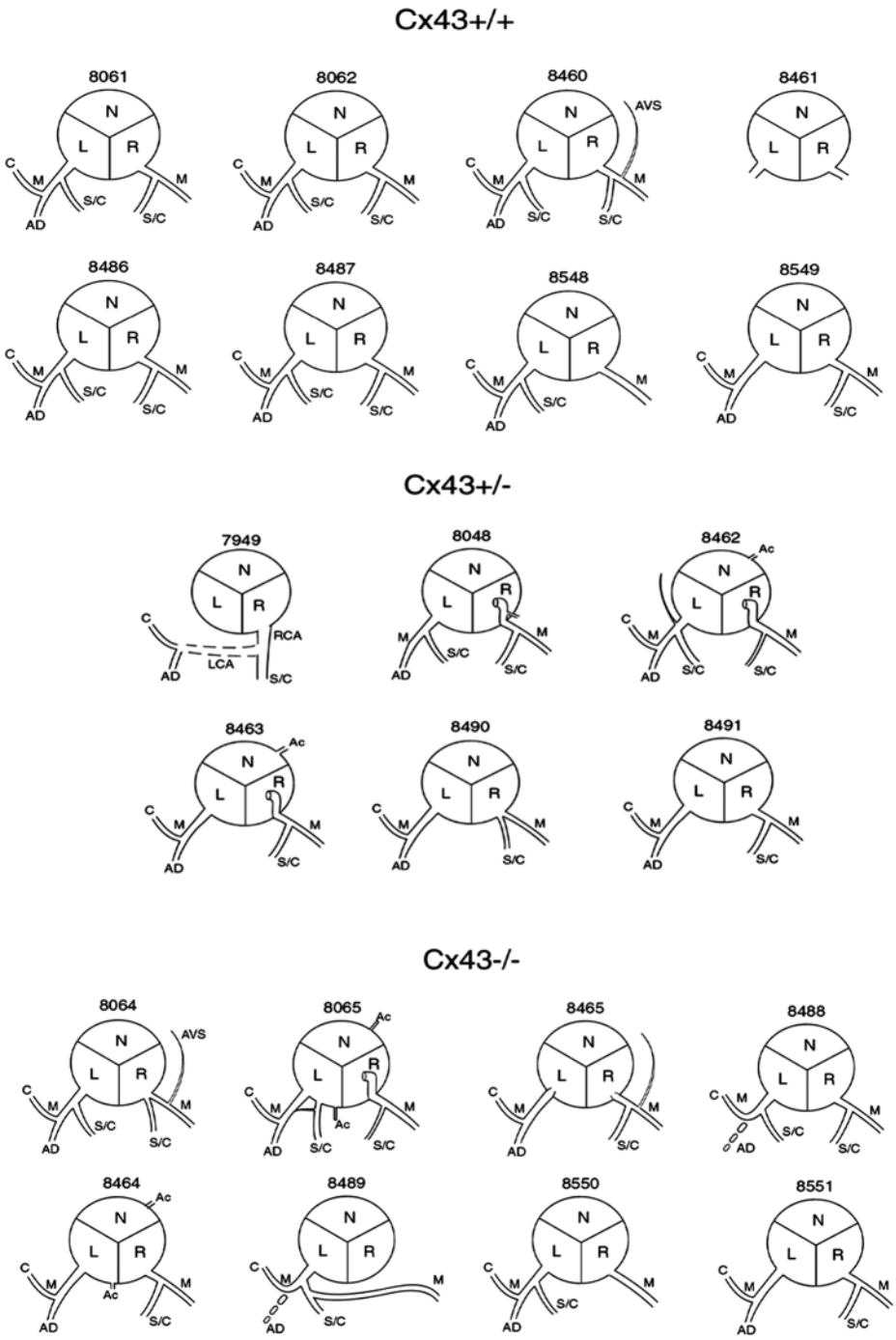

Fig. 3. Perturbation in major coronary artery patterning in the Cx43α1 knockout mouse. Major coronary arteries in wild type ($Cx43\alpha1^{+/+}$) littermates: the coronary arterial pattern of Cx43α1 wild-type hearts at E16.5-17.5 diagrammed from sectioned hearts. The aorta is viewed as if looking caudally. The three aortic sinuses are labeled 'R' and 'L' for right and left, and 'N' for the third non-coronary sinus. Coronary artery branches feed the ventricular septum (S), the free walls of the heart (M) and the base of the heart (C). The mural branch of the left coronary artery divides into a circumflex artery, which feeds the dorsal side of the heart, and the anterior descending branch (AD) that follows the interventricular groove on the ventral side of the heart. In hearts sectioned favorably, the mural branch of the right coronary artery gives off a slender artery that feeds the base of the atrial septum (AVS). Coronary artery patterns in the hearts of heterozygous (+/–) and homozygous (–/–) Cx43α1 knockout mice. Some of the changes in coronary artery pattern observed in the E16.5-17.5 heterozygous and homozygous Cx43α1 knockout mouse hearts are shown: small accessory coronary arteries (Ac) exiting the non-coronary aortic sinus (8462, 8463, 8065 and 8464) or the right or left coronary sinus (8065 and 8048), tunneling of the right coronary artery caudally through the wall of the aorta (8048, 8462, 8463 and 8065), main branches of a coronary artery exiting the aorta separately rather than branching from a main stem (8065), and in one case, the coronary artery stem divided in the wall (8064). In several hearts, a branch of the coronary artery became sinusoidal with thin walls (8489 and 8488).

are usually seen only in homozygous knockout mice, pouches were also observed in a few heterozygous knockout mouse hearts (Fig. 4H).

Expression of vascular smooth muscle markers in the pouches

To further determine the nature of tissue lining the pouches and its possible relationship to the coronary artery anomalies, we examined the expression of myocyte and vascular smooth muscle differentiation markers in fetal (E14.5 to E17.5) and newborn Cx43α1 knockout mouse hearts. These studies showed that the walls of the pouches are not only cardiac myosin free (Fig. 4A), but also deficient in MLC-2V, another cardiomyocyte marker (Fig. 4B). Instead, we detected expression of SM22α, an early marker of vascular smooth muscle cells (Fig. 4D) (Li et al., 1996). This was observed in pouches of E16.5 and E17.5 knockout mouse heart. In the newborn knockout mouse heart, we also detected a low level expression of VSMM (Fig. 4F). These results are consistent with the notion that the lining of the pouch may contain vascular smooth muscle-like cells, although we note that SM22α and VSMM expression was observed only in a thin layer of tissue immediately subjacent to the endocardium. Further analysis of the knockout mouse heart by Masson

Goldner trichrome stain for extracellular matrix deposition indicated the presence of heavy matrix deposition in the pouches, but unlike the SM22α and VSMM staining, this was distributed a few cell layers beneath the endocardium (Fig. 4G). This suggests the abnormal presence of fibroblasts in the pouches. Immunostaining also revealed strong PCNA expression in pouch tissue subjacent to the endocardium, suggesting an elevation of cell proliferation (Fig. 4C). In this same region, TUNEL labeling indicated a modest increase in apoptosis (Fig. 4E). Together with the coronary artery anomalies observed above, these observations suggest that formation of the pouches in the Cx43α1 knockout mouse probably involves the abnormal distribution of vascular smooth muscle and fibroblast cells, both of which are derived from the PEO.

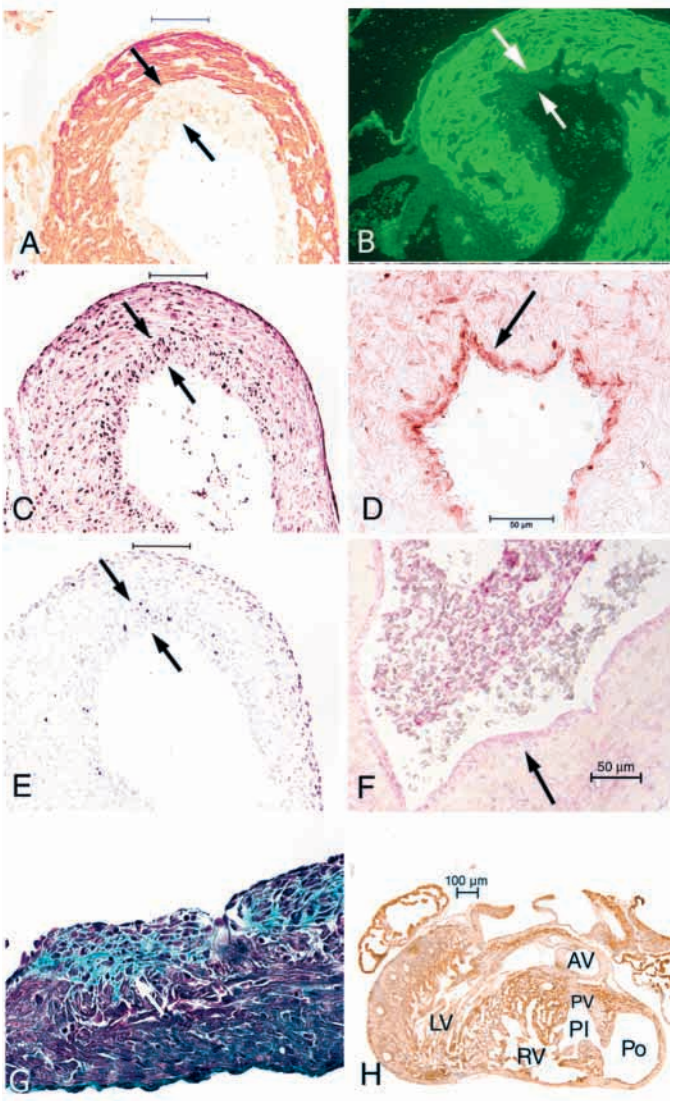

Fig. 4. Pouches show elevated proliferation and express markers of vascular smooth muscle cells. (A,C,E) A pouch from an E17.5 Cx43α1 knockout heart was consecutively sectioned and stained with cardiac myosin antibody (A) and PCNA antibody (C), and TUNEL labeled to detect apoptosis (E). The walls of the pouches consist of a myosin-free subendocardial layer of tissue (between arrows in A,C,E) that exhibits increased PCNA staining and TUNEL labeling (see dark nuclei between arrows in C,E, respectively). (B) Ventricular myosin light chain 2 (MLC2V) is also absent in the subendocardial layer of a pouch from an E17.5 Cx43α1$^{-/-}$ mouse heart. (D,F) SM22α (D) is expressed subendocardially in the pouches of E16.5 hearts. After birth, vascular smooth muscle myosin (F) is also observed in this subendocardial layer. (G) The Masson-Goldner trichrome stain shows abnormal extracellular matrix deposition in the pouches of the Cx43α1 knockout mouse heart (pale blue staining denoted by white arrow). (H**)** A heterozygous E16.5 knockout mouse heart stained with anti-smooth muscle α-actin exhibits a large pouch breaching the wall of the right ventricular outflow just beneath the pulmonary valve. AV, aortic valve; LV, left ventricle; Po, pouch; PV, pulmonary valve; RV, right ventricle. Scale bars in A,C,E,H, 100 μm; D,F, 50 μm.

Cx43α1 gap junctions in the mouse PEO and fetal epicardium

To determine if Cx43α1 is expressed in the PEO, we carried out whole-mount immunostaining of E9.5 mouse embryos using a rabbit polyclonal Cx43α1 antibody. Semi-thin plastic sections of the immunostained embryos were examined by darkfield immunofluorescence microscopy. This analysis revealed that the PEO consists of mesenchymal cells with a dendritic morphology (Fig. 5A). They exhibit punctate cell surface immunolabeling typical of gap junctions. In some regions, particularly along cell processes, very long profiles of Cx43α1 immunostaining were observed (Fig. 5B,C). As the PEO is a transient structure that quickly delaminates to form the epicardium, we further examined the epicardium of E12.5 mouse embryos, and found the continued expression of Cx43α1 gap junctions in the epicardial cells (purple in Fig. 6A,B). A similar analysis of the Cx43α1 knockout mouse heart showed that the epicardium is present and exhibits normal tissue morphology, including expression of cytokeratin (blue

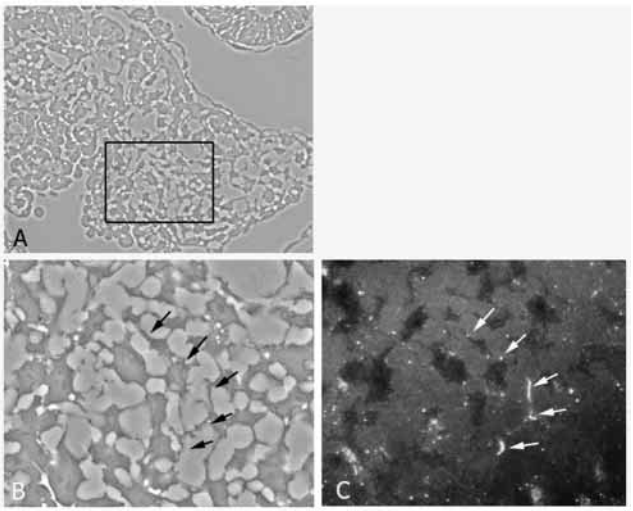

Fig. 5. Cx43α1 expression in the proepicardial organ. **(**A) E9.5 embryo whole mount immunostained with a Cx43α1 antibody was embedded and sectioned. (A) Phase contrast image of a section which includes the PEO. The boxed region is magnified in B,C. Note the dendritic mesenchymal cell morphology (B, phase contrast). Cx43α1 immunostaining (C, darkfield) show punctate and long profiles of gap junction contacts between cell processes (arrows in B,C).

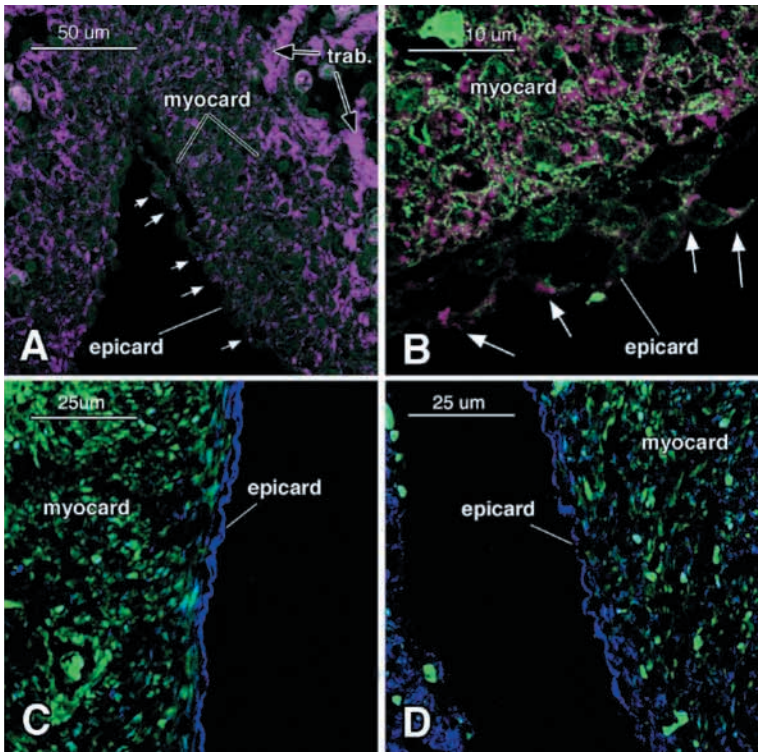

Fig. 6. Expression of Cx43α1 gap junctions in the epicardium of the fetal mouse heart. (A,B) Immunostaining shows Cx43α1 expression in the compact layer of the ventricular myocardium (myocard), in the ventricular trabeculae (trab) and in the epicardium (white arrows) in the interventricular sulcus of an E12.5 mouse heart. Cx43α1 expression is depicted in purple. The green background is autofluorescence recorded in the FITC (green) channel to delineate the myocardium and epicardium. Cx43α1 expression in the epicardium is comparable with that of the compact myocardium, while expression in the trabeculae is much higher. Cx43α1 expression in the ventricular myocardium and epicardium (white arrows) is shown at higher magnification in B. (C,D). Immunostaining with a cytokeratin antibody revealed abundant cytokeratin (blue) expression in the epicardium of Cx43α1$^{+/-}$ (C) and Cx43α1$^{-/-}$ (D) hearts.

in Fig. 6C,D), a marker specific for epicardial cells (Dettman et al., 1998).

We also examined Cx43α1 expression in proepicardial cells derived from PEO explants plated on a collagen substratum (Fig. 7). Within 24 hours, a monolayer of epithelial cells emerged from the explant expressing cytokeratin, confirming that they are indeed proepicardial cells (Fig. 7B). As in the PEO, Cx43α1 gap junctions were found in abundance in the explanted proepicardial cells, being localized along the cell borders (Fig. 7C). Cx43α1 expression was reduced in proepicardial cells derived from the heterozygous knockout embryos (not shown), and completely absent in homozygous knockout proepicardial cells (Fig. 7D). Note, in contrast to the mesenchymal morphology of cells in the PEO, the proepicardial cells in the explant cultures were epithelial, reflecting the mesenchymal to epithelial transition associated with formation of the epicardium. An examination of PEO explants derived from the Cx43α1 knockout mouse showed no change in this epithelial cell transition nor in the apparent packing of cells in the epithelial sheet (Fig. 9).

Cx43α1 gap junctions required for efficient functional coupling of proepicardial cells

Gap junction communication in the proepicardial cells was examined by monitoring dye coupling. This entailed using microelectrode impalement and the iontophoretic injection of 6-carboxylfluorescein to quantitate the extent of dye spread. This analysis showed that wild-type proepicardial cells are

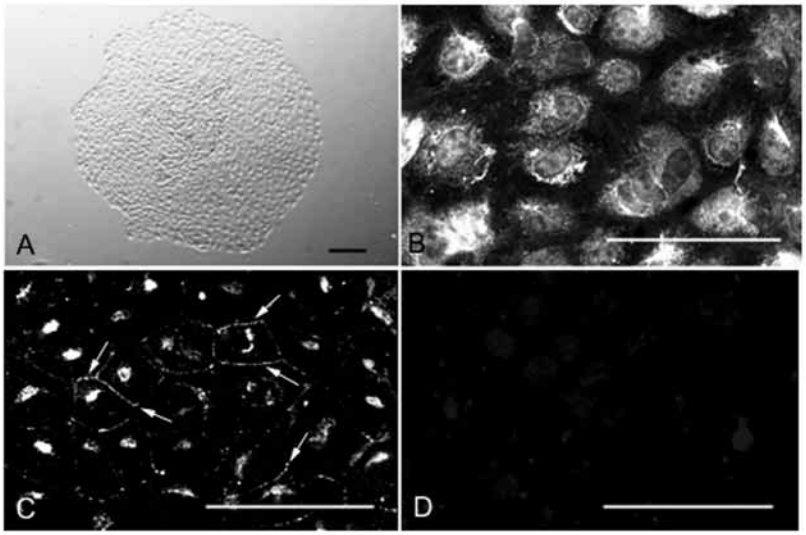

Fig. 7. Cx43α1 expression in a PEO explant. A 24 hour PEO explant culture (A) is comprised of a monolayer of cells with epithelial morphology. These cells express cytokeratin (B) and Cx43α1 (C). Punctate Cx43α1 immunolabeling is found in abundance along regions of cell-cell contact (arrows in C). In many cells, the Cx43α1 immunostaining occupied much of the peripheral cell membrane, outlining the polygonal shape of the proepicardial cells (C). By contrast, Cx43α1 immunolabeling is absent in proepicardial cells from a Cx43α1 knockout mouse embryo (D). Scale bars: 200 μm in A; 80 μm in B-D.

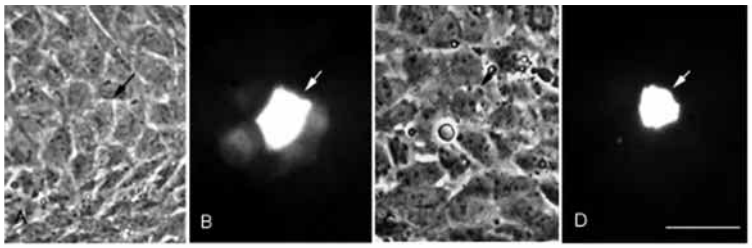

Fig. 8. Proepicardial cells are functionally coupled by gap junctions. Single cells in a wild-type (A) and a Cx43α1 knockout (C) proepicardial explant culture were impaled with a microelectrode (arrow) and iontophoretically injected with carboxyfluorescein. The gap junction mediated spread of carboxyfluoescein to adjacent cells can be seen in the darkfield images of a wild-type PEO explant (B), but not in a Cx43α1 knockout explant (D). Scale bar: 60 μm.

functionally well coupled by gap junctions (Fig. 8). Significantly, the level of dye coupling is reduced in a stepwise manner in the heterozygous and homozygous knockout proepicardial cells (Table 1). These observations indicate that Cx43α1 plays a significant role in mediating gap junctional communication in the proepicardial cells. However, as a low level of dye coupling remained, it is likely that at least one other connexin isotype is expressed in the proepicardial cells.

Motility and proliferation altered in Cx43α1 knockout proepicardial cells

Given our previous studies showing abnormal migration of cardiac neural crest cells in the Cx43α1-deficient embryos, we analyzed the migration paths of individual proepicardial cells (Fig. 9). This was carried out by capturing images of PEO explant cultures over a 20 hour interval and using the resulting time lapse movies to determine the speed and directionality of cell movement. Directionality reflects the frequency of direction change during migration and is defined as the net distance divided by the total distance traveled.

We observed that the migration of proepicardial cells usually began with the extension of lamellipodia, and occasionally filopodia were seen. After emergence from the explants, the proepicardial cells migrated as a coherent sheet, with cells at the periphery maintaining their leading position (Fig. 9). A significant elevation in the speed of cell locomotion was observed in both the heterozygous and homozygous Cx43α1 knockout proepicardial cells. It is important to note that the same increases in speed were observed in the heterozygous and homozygous Cx43α1 knockout proepicardial cells, whether cell motility was examined over the first 4 hours or the entire 20 hour interval of the experiment (Table 2).

Given the observed increased PCNA immunostaining in cells lining the pouches of the Cx43α1 knockout mouse heart, we also used BrdU incorporation to assess the rate of cell proliferation in the PEO explants. A significant increase in BrdU incorporation was observed in the heterozygous and homozygous Cx43α1 knockout proepicardial cells (Table 3). Again, the heterozygous and homozygous knockout proepicardial cells exhibited the same elevation in cell proliferation rate (Table 3). We also monitored the cell density in these explant cultures, but observed no consistent change (Table 3). These results indicate that two copies of the Cx43α1 gene are required for the normal regulation of proepicardial cell motility and cell proliferation.

No apparent change in smooth muscle cell differentiation

To determine whether vascular smooth muscle cell differentiation might be altered by the loss of Cx43α1 function, we examined the PEO explant cultures for evidence of vascular smooth muscle cell differentiation. In quail and chick PEO explants, cells stained with antibodies against calponin, a smooth muscle marker, appeared after only 2 days in culture (Landerholm et al., 1999). Such cells exhibited a disruption of cell-cell contact resulting from their epithelial to mesenchymal cell transformation. However, in the mouse PEO explants, no calponin positive cells were found even after 8 days in culture (not shown). However, vascular smooth muscle cells were found in epicardial explants obtained from E11.5 fetal mouse hearts. Epicardial cells emerged soon after the establishment of stable heart-substratum adhesion. After 5 days in vitro, small, round or spindle shaped cells were observed (Fig. 10). These cells were immunostained by calponin (Fig. 10A,B) and SM22α antibodies (not shown), suggesting that they are smooth muscle cells derived from progenitors in the epicardium. A parallel analysis of epicardial outgrowths from Cx43α1-deficient fetal heart explants showed no discernible difference in the appearance or abundance of these

Table 1. Analysis of dye coupling between proepicardial cells from wildtype and Cx43α1 knockout mouse embryos

Genotypes	Number of injections/ number of explants	Number of coupled cells (mean±s.d.)
$Cx43\alpha1^{+/+}$	30/6	4.40±1.77
$Cx43\alpha1^{+/-}$	30/6	3.23±1.82*
$Cx43\alpha1^{-/-}$	41/6	$2.17\pm0.93^{\dagger,\ddagger}$

Analysis by ANOVA showed *P<0.01 when compared with wild type; $^{\dagger}P$<0.001 when compared with wild type; $^{\ddagger}P$<0.01 when compared with heterozygous.

Table 2. Time-lapse study of cell migration in PEO explants from wild-type and Cx43α1 knockout mouse embryos

Genotypes	Number of tracings/ number of explants	Speed (μm/minute)		Directionality	
		Total	After 4 hours	Total	After 4 hours
$Cx43\alpha1^{+/+}$	34/6	0.457±0.097	0.498±0.109	0.520±0.135	0.563±0.145
$Cx43\alpha1^{+/-}$	30/5	0.593±0.124*	$0.562\pm0.105^{\dagger}$	0.548±0.147	0.536±0.118
$Cx43\alpha1^{-/-}$	45/7	0.570±0.090*	$0.550\pm0.094^{\dagger}$	0.552±0.137	0.585±0.115

Values are mean±s.d. Analysis by ANOVA showed *P<0.001 and $^{\dagger}P$<0.05 when compared with wild type.

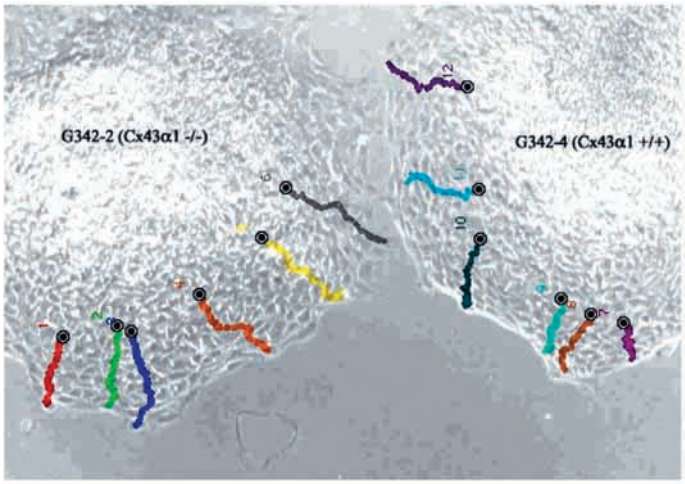

Fig. 9. The migratory paths of individual cells in the proepicardial explants. The colored lines represent the migratory paths of individual cells in the PEO explant as observed by time lapse videomicroscopy (images captured every 5 minutes for 20 hours). The Cx43α1-deficient proepicardial cells (left explant) exhibit longer migration paths, indicating a higher speed of cell locomotion.

calponin/SM22α-positive cells, suggesting that vascular smooth muscle cell differentiation is not altered by the loss of Cx43α1 function (Fig. 10C).

Normal motility and cell proliferation in proepicardial cells from FC transgenic mice

To distinguish between the contribution of neural crest versus proepicardial cells to the cardiac phenotype of the Cx43α1 knockout mouse, we examined proepicardial cells from the FC transgenic mouse model. The FC transgenic mice express a dominant negative Cx43α1/β-galactosidase fusion protein in subpopulations of neural crest cells, and although they exhibit outflow obstruction and right ventricular heart defects, infundibular pouches have never been observed (Sullivan et al., 1998). We further examined the proepicardial cells from the FC transgenic mice and found no significant alteration in dye coupling, proliferation rate or motility (Table 4). These results are consistent with the notion that proepicardial cell defects play an essential role in pouch formation in the Cx43α1 knockout mouse heart.

DISCUSSION

Cx43α1 gap junctions and coronary artery development

These studies showed for the first time that heart malformations in the Cx43α1 knockout mouse is associated with a variety of coronary artery defects. This was accompanied by an elevation in the rate of proepicardial cell migration and proliferation. Interestingly, heterozygous and homozygous knockout embryos exhibited the same coronary artery anomalies, and also similar increases in the rate of cell migration and proliferation. These findings are consistent with a previous study showing that proliferating vascular smooth muscle cells have diminished gap junction permeability (Kurjiaka et al., 1998). In the Cx43α1 knockout fetal hearts, VSMM expression in the coronary arteries was attenuated and patchy. However, the epicardium appeared normal, and analysis of epicardial explant cultures showed no change in vascular smooth muscle cell differentiation. Together these results suggest that the coronary artery defects in the Cx43α1 knockout mouse are not likely to be due to a deficiency in proepicardial or epicardial cells, nor to a defect in smooth muscle cell differentiation, but rather the abnormal proliferation and deployment of the proepicardially derived cells.

It is important to consider these findings in light of previous work showing that formation of the coronary arteries is also dependent on neural crest cells (Bartelings et al., 1993; Hood and Rosenquist, 1992; Hyer et al., 1999; Waldo et al., 1994). Unlike the proepicardial cells, neural crest cells do not contribute structurally to the coronary arteries and thus their role is presumably a regulatory one. In fact, our earlier studies had indicated the involvement of neural crest perturbations in the cardiac anomalies of the Cx43α1 knockout mouse (Ewart et al., 1997; Huang et al., 1998a; Huang et al., 1998b). We observed neural crest cells migrate into the outflow tract of the Cx43α1 knockout mouse heart, but in reduced abundance. In contrast to our findings with the proepicardial cells, significant alteration in cell locomotory behavior was observed only in the homozygous knockout neural crest cells (Xu et al., 2002). Together, these findings suggest that the coronary artery anomalies in the Cx43α1 knockout mouse cannot be solely dependent on neural crest perturbations, but rather may arise from the combined effects of perturbations involving the neural crest and proepicardial cells.

Neural crest and proepicardial cells and the regulation of coronary artery development

Given that neural crest and proepicardial cells are both found at the base of the heart where the coronary artery stems form by tunneling through the base of the aorta, perhaps signaling mediated through interactions between the neural crest and proepicardial cells may play a role in modulating the initial patterning of coronary arteries. The altered motility of the proepicardial and neural crest cells in the Cx43α1 knockout mouse potentially could lead to an excess of proepicardial cells and a reduction in neural crest cells. Such an imbalance between the two cell populations may alter cell signaling that is important in the patterning of coronary arteries. This signaling could involve gap junction-mediated communication between crest and proepicardial cells, or paracrine signaling not requiring direct cell-cell contact. It is also possible that the

Table 3. Analysis of cell proliferation and density of proepicardial organ explants from wild-type and Cx43α1 knockout mouse embryos

Genotype	Proliferation rate (mean±s.d.)	Cell density (per mm^2) (mean±s.d.)
Cx43α1$^{+/+}$	0.283±0.067 (*n*=6)	987±210 (*n*=5)
Cx43α1$^{+/-}$	0.347±0.102* (*n*=6)	1031±144† (*n*=5)
Cx43α1$^{-/-}$	0.344±0.075* (*n*=7)	906±162‡ (*n*=6)

Values in brackets are numbers of explants analyzed. Analysis by ANOVA showed *P<0.01 and $^{\dagger}P$<0.05 when compared with wild type; $^{\ddagger}P$<0.01 when compared with heterozygous.

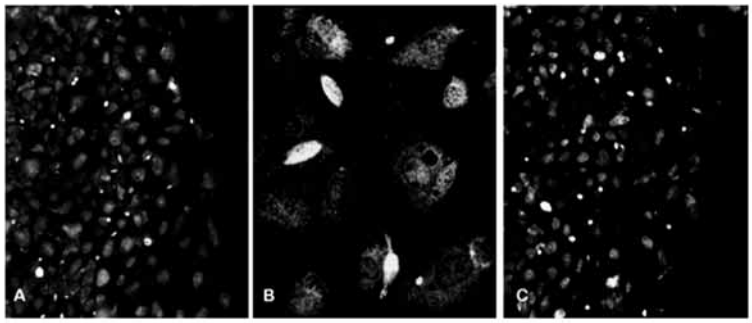

Fig. 10. Epicardial cell differentiation into smooth muscle-like cells. Epicardial cells are obtained from an E11.5 mouse heart explanted in culture on a collagen coated glass coverslip. After 5 days, small round or spindle-shaped like calponin positive cells are scattered between unstained epicardial cells from a wild-type heart (A). This is shown at higher magnification in B. These same cell types were also seen in the Cx43α1 knockout heart explants (C).

role of neural crest may be mediated via secondary interactions involving the myocardium or endocardium. Various hypotheses have been put forward regarding the role of neural crest cells in coronary artery development, including ensuring the survival of persisting coronary arteries by laying down the cardiac ganglia at the base of the heart (Waldo et al., 1994). Another possibility is that neural crest cells involved in peripheral innervation in the heart may provide signals that can modulate proepicardial cell deployment and thus help pattern coronary artery development. Regardless of the precise mechanism of proepicardial and neural crest interactions, our findings suggest that normal coronary artery development may be dependent on a precise balance of cardiac neural crest versus proepicardial cells, not merely whether neural crest or proepicardial cells are present in the heart.

Infundibular pouches in the Cx43α1 knockout mouse heart

We also investigated formation of the pouches in the Cx43α1 knockout mouse heart. Such pouches are not observed in neural crest-ablated embryos, and have never been seen in transgenic mouse models with Cx43α1 perturbations targeted to neural crest cells. We observed that these pouches are lined by a tissue deficient in the expression of cardiomyocyte markers. Although the precise identity of cells in this tissue is not known, we have previously shown that it expresses smooth muscle α-actin (Huang et al., 1998b; Lo and Wessels, 1999), which normally is turned off in the working myocardium at this stage of development. This would suggest that the pouch comprises cells that are in early stages of vascular smooth muscle cell differentiation. Consistent with this possibility, we observed a subendocardial layer of cells expressing vascular smooth muscle markers. However, subjacent to these smooth muscle-like cells, we also detected the presence of fibroblast cells as indicated by heavy trichrome staining. These observations suggest that formation of the pouches may involve the proepicardial cells, which are progenitors of all vascular smooth muscle and fibroblast cells in the heart. Thus, the enhanced rate of proepicardial cell migration and proliferation potentially could result in an excess of proepicardial cells at the base of the heart, thereby playing a role in pouch formation.

Consistent with this model is our observation that the FC transgenic mice never exhibit pouches, but have outflow obstruction like that of the Cx43α1 knockout mice. Previous studies of neural crest cells from FC transgenic embryos have shown reductions in dye coupling and migration rate to levels like that of the Cx43α1 knockout mouse. By contrast, FC transgenic proepicardial cells showed no change in dye coupling, nor in the rate of cell migration or proliferation. These observations suggest that Cx43α1 deficiency in the proepicardial cells is necessary for pouch formation.

Nevertheless, it is likely that other cells or tissues are involved in pouch formation. This is indicated by the fact that although predominantly only homozygous knockout animals have pouches, proepicardial cells from both the heterozygous and homozygous Cx43α1 knockout mice showed similar cell migration and proliferation alterations. Our finding that distal coronary artery branches were directly continuous with the pouches in some Cx43α1 knockout animals suggests that coronary artery anomalies have a role in pouch formation. We hypothesize that the infundibular pouches, like the coronary artery anomalies, may arise from perturbations involving both the cardiac neural crest and proepicardial cells. We note that pouch formation probably does not arise from the perturbation of endothelial cells, as cardiac malformations were not observed when tie2 promoter Cre was used to target the deletion of a floxed Cx43α1 allele in endothelial cells (Theis et al., 2001).

Cx43α1 gap junctions and the modulation of cell motility

The present study together with our previous work suggests that Cx43α1 gap junctions play an important role in modulating the motility of two migratory cell populations essential for cardiovascular development, the neural crest and proepicardial cells. In contrast to neural crest cells, which showed a decrease in the apparent rate of cell migration in the absence of Cx43α1 function, the opposite was observed for the Cx43α1-deficient proepicardial cells. The proepicardial cells

Table 4. Analysis of gap junction coupling, cell migration and proliferation of cultured proepicardial cells from FC transgenic mouse embryos

	Wild type	Transgenic
Dye coupling		
Number of injections/number of explants	20/4	29/6
Number of coupled cells (mean±s.d.)	4.85±1.66	4.83±1.63*
Migration		
Number of tracings/number of explants	46/9	60/11
Directionality (mean±s.d.)	0.581±0.113	0.558±0.335*
Speed (μm/minute) (mean±s.d.)	0.515±0.102	0.537±0.130*
Proliferation		
Number of explants	3	9
Proliferation rate (mean±s.d.)	0.524±0.103	0.484±0.104*

Using Student's *t*-test, *P>0.05 when compared with wild-type littermates.

also showed increased cell proliferation, which was not observed in the Cx43α1-deficient cardiac neural crest cells. These differences perhaps reflect different signal transduction pathways functioning in neural crest versus the proepicardial cells, or the differential permeability of Cx43α1 gap junction channels to different signaling molecules active in these cell signaling pathways. In the proepicardial cells, although dye coupling was found to decrease in line with the loss of one versus both Cx43α1 alleles, cell migration and cell proliferation were identically altered in the heterozygous and homozygous knockout proepicardial cells. These observations raise the possibility that the role of Cx43α1 in cell motility may not be mediated simply by its channel-forming capacity, a possibility that is also suggested by our recent study of neural crest cell motility in other knockout mouse models (Xu et al., 2002).

Cx43α1 and congenital coronary arterial anomalies

The infundibular pouches observed in the Cx43α1 knockout mice share many features in common with congenital aneurysm of an aortic sinus of Valsalva in humans (Boutefeu et al., 1978; Barragry et al., 1988). Multiple other coronary anomalies observed in the Cx43α1 knockout mice also have been described in humans. For example, a high anterior origin of the right coronary artery (as seen in 8048, 8462, 8463 and 8065 in Fig. 3) is observed in 2% to 6% of individuals undergoing routine coronary angiography. Anomalous origin of the left coronary artery from the pulmonary artery (similar to Fig. 1E-H) is associated with increased morbidity and mortality (Levin et al., 1978). Anomalous origin of the right coronary artery from the pulmonary artery also has been observed in humans but is relatively rare. Anomalous origin of the left coronary artery from the right coronary artery or a single right coronary artery, similar to that observed in the Cx43α1 knockout mice (see Fig. 3, mouse 7949), is associated with a high incidence of sudden cardiac death in young individuals (Cheitlin et al., 1974; Liberthson et al., 1979; Roberts, 1986; Kragel and Roberts, 1988). In light of these findings, we propose that Cx43α1 should be evaluated as a candidate gene for mutations that may play a role in human coronary artery anomalies.

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