Published in final edited form as:

Exp Cell Res. 2013 August 15; 319(14): 2152–2165. doi:10.1016/j.yexcr.2013.05.022.

Cardiomyocyte FGF signaling is required for Cx43 phosphorylation and cardiac gap junction maintenance

Takashi Sakurai^{a,*}, Mariko Tsuchida^b, Paul D. Lampe^c, and Masahiro Murakami^a

^aCardiovascular Research Center, Section of Cardiovascular Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06511, USA

^bDentistry and Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Okayama, Japan

^cTranslational Research Program, Fred Hutchinson Cancer Center, Seattle, WA 98109, USA

Abstract

Cardiac remodeling resulting from impairment of myocardial integrity leads to heart failure, through still incompletely understood mechanisms. The fibroblast growth factor (FGF) system has been implicated in tissue maintenance, but its role in the adult heart is not well defined. We hypothesized that the FGF system plays a role in the maintenance of cardiac homeostasis, and the impairment of cardiomyocyte FGF signaling leads to pathological cardiac remodeling.

We showed that FGF signaling is required for connexin 43 (Cx43) localization at cell–cell contacts in isolated cardiomyocytes and COS7 cells. Lack of FGF signaling led to decreased Cx43 phosphorylation at serines 325/328/330 (S325/328/330), sites known to be important for assembly of gap junctions. Cx43 instability induced by FGF inhibition was restored by the Cx43 S325/328/330 phospho-mimetic mutant, suggesting FGF-dependent phosphorylation of these sites. Consistent with these *in vitro* findings, cardiomyocyte-specific inhibition of FGF signaling in adult mice demonstrated mislocalization of Cx43 at intercalated discs, whereas localization of N-cadherin and desmoplakin was not affected. This led to premature death resulting from impaired cardiac remodeling.

We conclude that cardiomyocyte FGF signaling is essential for cardiomyocyte homeostasis through phosphorylation of Cx43 at S325/328/330 residues which are important for the maintenance of gap junction.

Keywords

Fibroblast growth factor; Heart failure; Cardiac remodeling; Gap junction; Connexin43; Phosphorylation

© 2013 Elsevier Inc. All rights reserved.

*Correspondence to: Section of Cardiovascular Medicine, Department of Internal Medicine Yale University School of Medicine 300 George Street, Rm-794, New Haven, Connecticut 06511, USA Phone: +1-203-737-4603 Fax: +1 203 785 7567. takashi.sakurai@yale.edu, takashi_sakurai@gakushikai.jp. .

Appendix A. Supplementary information Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.yexcr.2013.05.022>.

Disclosures None declared.

Introduction

Heart failure is a final common result of different forms of cardiovascular disease, including pressure and volume overload, myocardial ischemia and various forms of cardiomyopathy. While our understanding of molecular mechanisms driving cardiac hypertrophy and heart failure progression is rapidly expanding, very little is known about factors responsible for the maintenance of the normal myocardial architecture in the healthy heart.

Gap junctions, formed from the connexin family of proteins, couple neighboring cardiomyocytes and synchronize heart contraction. Connexin43 (Cx43) is the major isoform expressed in mammalian heart [1], and is found in most parts of the heart. Connexin40 is mainly expressed in the cardiac atria and in the ventricular conduction system [2], whereas Connexin45 is expressed in embryonic stages and in parts of the ventricular conduction system [3]. Recently, Cx43 has been identified to localize not only at cell–cell contacts, but also in the mitochondria [4] of cardiomyocytes.

The fibroblast growth factor signaling system is one of the most diverse growth factor families in vertebrates, controlling a wide variety of physiological and pathological processes [5,6]. In mice and humans, twenty-two ligands, four tyrosine kinase high-affinity receptors and co-receptors comprise the fibroblast growth factor (FGF) signaling system. Activation of FGF receptors (FGFRs) causes phosphorylation of FGFR substrate 2 (FRS2) which serves as an adapter protein for the assembly of a signaling complex with growth factor receptor-bound protein 2 (GRB2), GRB2-associated-binding protein 1 (GAB1) and Src-homology 2 domain-containing phosphatase 2 (SHP2). This results in activation of downstream signaling pathways including mitogen-activated protein kinases (MAPK) pathways through activation of Ras, the Akt pathway via GAB1 mediated recruitment of phosphatidylinositol 3-kinase (PI3K), and the protein kinase C (PKC) pathway via activation of phospholipase C- (PLC-) and production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃) [7]. Besides its mitogenic effect, FGF2 is widely recognized as a cell survival/tissue protective factor in part due to its anti-apoptotic effect resulting from Akt activation [8,9]. The FGF system produces diverse biological responses that include proliferation, migration, growth arrest, differentiation and cell death in a highly context-dependent manner [10]. This suggests that FGF has a regulatory role in many biological processes including tissue maintenance.

In the cardiovascular system, FGFs are known to mediate various biological processes such as angiogenesis, cardioprotection and embryonic heart development [11–14], but the precise roles played by the FGF system in the adult heart are not well understood. Gene disruption of *Fgfr1* or *Fgfr2* causes embryonic lethality at the gastrulation and implantation stage of mouse embryonic development, respectively, which limits further studies on adult pathophysiology [15]. FGF knockouts, on the other hand, fail to show overt cardiovascular phenotypes, due to the functional redundancy of the ligand system [16].

A series of studies by the Kardami laboratory demonstrated that FGF2, particularly the low molecular weight isoform of FGF2, regulates Cx43 and cardiac function in a variety of ways [13,17–20]. FGF2 has been shown to have beneficial effects when it is administered or overexpressed in the experimental setting of myocardial infarction and ischemia–reperfusion injury [21]. Although the precise mechanism of FGF-mediated cardioprotection is not fully understood, Cx43 has been thought to be one of the effectors of FGF signaling. Several studies have linked FGF signaling with increased PKC activity [22], including a report involving protection of cardiac tissue from hypoxia [23].

Using an endothelium-specific gene expression system, we have recently demonstrated that inhibition of FGF signaling using a FGFR1 dominant negative (FGFR1DN) construct in the

adult mouse vasculature leads to impairment of vascular integrity, resulting from disassembly of endothelial cell–cell junctions [24]. This study demonstrated the essential role of endothelial FGF signaling in vascular maintenance. Based on this finding, we hypothesized that FGF plays a regulatory role in tissue homeostasis and decided to investigate FGF's role in the regulation of junctional structures in the adult heart. In this study, using both *in vitro* and *in vivo* approaches, we found that FGF is required for the maintenance of cardiomyocyte cell–cell junctions and myocardial integrity. Specifically, a lack of FGF signaling in neonatal rat myocytes led to impairment of gap junction formation as detected by decreased Cx43 phosphorylation at serines 325/328/330. The importance of FGF signaling was demonstrated in the adult mouse heart from FGFR1DN mice as progressive cardiac remodeling accompanied by Cx43 lateralization, ultimately resulted in the development of heart failure and premature death. Therefore, we conclude that cardiomyocyte FGF signaling mediates Cx43 S325/328/330 phosphorylation, thereby stabilizing gap junctions and playing a role in the maintenance of the normal cardiac function. Our study revealed the molecular mechanism of FGF-mediated cardioprotection and demonstrated that physiological maintenance of cardiac homeostasis is an active process that requires ongoing FGF signaling in the heart.

Materials and methods

Antibodies and reagents

Antibodies against the following antigens were obtained: total Cx43 (Sigma, C6219), unphosphorylated isoforms of Cx43 (Fred Hutchinson Cancer Research Center, Cx43CT1) [25], N-cadherin (R&D systems, AF6426), hemagglutinin (HA) (Covance, MMS-101P), -tubulin (Sigma, T7816) and phosphorylated isoforms of Cx43 at S368 (Cx43 pS368) (Cell Signaling Technology, 3511), S325/328/330 (pS325/328/330) and S365 (pS365)–preparation and specificity of the latter two antibodies has been previously described [26,27].

Preparation of neonatal rat cardiomyocytes

Primary neonatal rat cardiomyocytes were isolated from hearts of 1–2 day-old Sprague-Dawley rats using the cardiomyocyte isolation system (Worthington biochemical corporation, Lakewood, New Jersey, USA) according to instructions. In brief, the hearts were dissected from anesthetized one day-old rat pups and were minced into small pieces on ice. The tissues were digested with Worthington trypsin overnight at 4 °C. After adding trypsin inhibitor, the tissues were further digested with Worthington purified collagenase at 37 °C for 30 min. Isolated cardiomyocytes were filtered through a cell strainer, partially purified by pre-plating and then plated onto cell culture dishes or glass bottom dishes covered with 0.1% bovine gelatin (Sigma). The next day the medium was changed to Dulbecco's modified Eagle's medium (DMEM)/minimum essential medium (MEM) containing 5% fetal bovine serum (FBS) and 0.1 mM 5-bromo-2'-deoxyuridine (BrdU) and the medium was changed every 3 days with DMEM/MEM containing 5% FBS.

Adenovirus transduction

Isolated primary neonatal rat cardiomyocytes or COS7 cells were plated onto 6 cm gelatin-coated dishes or gelatin-coated microscope slides, grown for 3 days in culture and adenovirus was added in medium at an MOI of 0.5, 2, 5 and 10 PFU/cell for 6 h. The day after adenovirus transduction, the cells were lysed for western blotting analysis or fixed for immunocytochemistry. Under these conditions, approximately 90% of cells were typically infected based upon green fluorescent protein (GFP) expression as determined by fluorescence microscopy. The adenoviral form of dominant-negative FGFR1 (Ad-FGFR1DN) is the same construct used for transgenic mouse generation and has been proven

to efficiently inhibit FGF signaling of multiple FGFR isoforms [28,29]. We checked the cell density before and after adenovirus treatment and confirmed that the number of cells were essentially the same after adenovirus treatment.

Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in PIPES lysis buffer with NP-40 (Boston BioProducts, Ashland, MA) containing cOmplete Mini protease inhibitors (Roche) and PhosSTOP phosphatase inhibitors (Roche). The suspensions were rocked in the cold room for 30 min to lyse cells. The lysates were centrifuged and the supernatants were mixed with sodium dodecyl sulfate (SDS) sample buffer (Boston BioProducts). Total cell lysates were boiled for 5 min and subjected to SDS polyacrylamide gel electrophoresis (PAGE) using 4–15% Criterion TGX gels or 4–15% Mini-PROTEAN TGX gels at 200 V constant voltage for about 30 min. The separated protein was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) at 150 V constant voltage for 15 min. Membranes were blocked with 2.5% skim milk in Tris buffered saline with 0.05% Tween 20 (TBS-T), incubated with primary antibody overnight at 4 °C or for 1 h at room temperature, followed by secondary antibody. Detection was conducted using SuperSignal West Pico or West Femto chemiluminescent substrate (Thermo Fisher Scientific) and G-box (SynGene, Cambridge, England).

For the blotting with the Cx43 pS325/328/330 and pS365 antibodies, protein was transferred to nitrocellulose membranes (Biorad) and the membranes were blocked with 2.5% skim milk in TBS without Tween 20. pS325/328/330 and pS365 were detected with the Li-Cor Biosciences Odyssey infrared imaging system and associated software as previously indicated [27].

Immunofluorescence

Cells were washed with PBS, fixed with 2% paraformaldehyde (PFA) in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS containing 2% PFA at room temperature for 5 min and blocked with 3% bovine serum albumin (BSA) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature for 30 min. Cells were washed with PBS and incubated with diluted primary antibody at 4 °C overnight, washed three times with PBS and incubated with diluted Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Then the slides were washed three times with PBS and mounted using Fluor-Gel (Electron Microscopy Sciences, Hartfield, PA). Images were taken on Zeiss 510 laser scanning confocal microscope or a PerkinElmer spinning disk microscopy confocal system.

Dye transfer

Dye transfer assay was performed according to previously published methods [30–33] with slight modifications. One 3.5 cm dish of COS-7 cells (transformed African green monkey kidney fibroblast cells, American Type Culture Collection, Rockville, MD) was transduced with Ad-FGFR1DN at an MOI of 0.5–10 PFU/cell or with Ad-GFP at an MOI of 5 PFU/cell. After incubation with the virus for 6 hours, cells were trypsinized and re-plated into two 3.5 cm dishes. 24 h after adenovirus transduction, one of the 3.5 cm dishes was labeled with 0.4 μ M calcein AM (Invitrogen), a membrane-permeant dye, which is intracellularly cleaved to membrane-impermeant calcein, and the other dish was labeled with 2 μ M CM-DiI (chloromethylbenzamido derivatives of 1, 1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine) (Invitrogen), a lipophilic dye or VE-cadherin-TagRFPT, for 30 min in a 5% CO₂ incubator at 37 °C [33]. After washing twice with PBS, the two populations of cells were each trypsinized and treated with defined trypsin inhibitor (Invitrogen). The cells were mixed together, plated on a fibronectin-coated 3.5 cm glass

bottom dish and placed in a 5% CO₂ incubator at 37 °C for 2 h. The digital fluorescent images of calcein and red fluorescence were captured by a PerkinElmer UltraView VoX spinning disk confocal system with a Nikon eclipse Ti inverted microscope. The percentages of cells that transferred dye were determined by dividing the number of cells labeled by red fluorescence that contained calcein (*i.e.*, transfers) by the number of cell interfaces between calcein-loaded and cells labeled by red fluorescence (*i.e.*, total).

Time-lapse live cell imaging

Time-lapse live cell imaging was performed with a PerkinElmer UltraView VoX spinning disk confocal system with a Nikon eclipse Ti inverted microscope and a Prior NanoScanZ 250 µm motorized stage, enclosed by temperature controlled chamber with a CO₂ environment system (IVS-3001) (In Vivo Scientific, LLC, St. Louis, MO). Isolated neonatal rat cardiomyocytes were spread onto 3.5 cm glass bottom dishes and transduced with a lentiviral vector containing Cx43-WT-EGFP or Cx43-S325/328/330E-EGFP 3 days after isolation. Three days after lentivirus transduction, cells were transduced with Ad-FGFR1DN at an MOI of 5 PFU/cell. The time when lentiviral solution was added into 3.5 cm dishes was defined as time 0 and time-lapse images were acquired with a 40× air NA 0.9 objective lens every 5 min for 6 h by using a chamber heater and warmed humidified 5% CO₂ and were played back as movie at 10 frames per second.

Generation of αMHC-FGFR1DN mice

To drive FGFR1DN expression specifically in adult myocytes, we employed a Tet-Off system using an α -myosin heavy chain (α -MHC) promoter. In this system, withdrawal of doxycycline from the diet efficiently induces transgene expression [34]. A transgenic line carrying the FGFR1DN gene under the control of tetracycline responsive element (TRE) has been crossed with the p MHC-tTA mouse line, generating double transgenics (α -MHC-FGFR1DN mice) which allows tetracycline sensitive, cardiomyocyte-specific FGFR1DN expression. These mice were then subjected to gene induction by doxycycline withdrawal at the age of 10 weeks. Littermates carrying a single transgene were subjected to the same doxycycline regimen and used as controls. All animal experiments are performed accordingly to the protocol approved by the institutional animal care and use committee of Yale University.

Statistical analysis

Data were expressed as mean±SD. Comparison between groups were performed with the Tukey post hoc honestly significance difference (HSD) using one-way ANOVA method. Results were considered significant at $P < 0.01$ or 0.05 as described in each figure captions.

Results

Inhibition of FGF signaling in cardiac myocytes affects Cx43 localization at cell–cell contacts

To study the role of FGF signaling in cardiac myocytes *in vitro*, we employed a truncated form of FGFR1 (dominant-negative FGFR1, FGFR1DN), which is capable of inhibiting signal transduction from multiple isoforms of FGFRs by ligand trapping and heterodimerization. Introduction of FGFR1DN in cells results in total disruption of FGF signaling [28,35]. FGF2 facilitates intercellular communication by upregulating Cx43 expression in the heart [18]. Furthermore, in the setting of ischemia reperfusion injury, exogenous FGF2 plays a cytoprotective role by increasing S262 and S368 phosphorylation levels of Cx43 and preventing mislocalization of Cx43 from intercalated discs [19]. Based

on these findings, we chose to examine the effect of FGF signaling inhibition on Cx43 and other junctional protein functions.

Neonatal rat cardiomyocytes are an established model that has been used to study cardiac regulation via a variety of molecular pathways. We employed a standard method to isolate neonatal rat cardiomyocytes. In general, the cardiomyocyte content of our cultures was greater than 90%. When we checked areas where the proportion of cardiomyocytes was lower, neonatal rat cardiomyocytes (α-actinin positive) only formed Cx43 containing gap junctions at cell–cell contacts with each other (Fig. 1a, arrow), and not with neighboring α-actinin negative cells, non-cardiomyocytes (Fig. 1a, arrowheads). Inhibition of FGF signaling by Ad-FGFR1DN in cardiomyocytes resulted in a decrease in Cx43 at cell–cell contacts (Fig. 1b and c). Co-staining with other junctional proteins revealed that inhibition of FGF signaling decreased the localization of Cx43 from cell–cell contacts in a viral dose-dependent manner (Fig. 2a and b), but did not have much effect on the localization of ZO-1 and N-cadherin at cell–cell contacts (Fig. 2a and c). These results demonstrated that FGF signaling in cardiomyocytes controls Cx43 localization and is required for Cx43 retention at cell–cell junctions.

FGF signaling is required for Cx43 phosphorylation

Cx43 phosphorylation, occurring at multiple sites in its C-terminal, cytoplasmic domain, is known to regulate Cx43 function and gap junction assembly [36]. In cardiac myocytes, Cx43 phosphorylation at specific sites is implicated in its localization at intercalated discs [37]. To understand the mechanism of FGF-mediated Cx43 function at junctions, we examined the effect of FGF signaling depletion on Cx43 phosphorylation in neonatal rat cardiomyocytes. In contrast to Cx43 in heart tissue, rat neonatal cardiomyocytes in culture have much more P0, the fastest migrating isoform(s) of Cx43 which comigrates with the unphosphorylated form of Cx43, than P2, the phosphorylated form of Cx43, even under basal conditions. Western analysis showed that FGF inhibition increased the band detected with the Cx43 CT1 antibody (Fig. 3a). The Cx43 CT1 antibody binds primarily to P0 band [25]. Western analysis using the total Cx43 antibody was consistent with the results obtained with the Cx43 CT1 antibody, showing an increase in the P0 band in cells transduced with Ad-FGFR1DN. The P1 band detected by the total Cx43 antibody also increased according to Ad-FGFR1DN MOI. In contrast, the phosphorylated form of Cx43 (P2 band) decreased in these cells, further indicating FGF inhibition reduces specific Cx43 phosphorylation levels (Fig. 3a and b).

To identify specific phosphorylation sites regulated by FGF signaling, we performed western analysis using a panel of phospho-specific Cx43 antibodies. We observed that Cx43 phosphorylation at S368 (pS368, migrates predominately at the P0 band) increased, phosphorylation at S365 was low (pS365 isoform migrates at the P1 band) with no apparent change and phosphorylation at S325/328/330 (pS325/328/330 migrates at the P2 band) decreased in an Ad-FGFR1DN dose-dependent manner (Fig. 3c and d). Positive and negative changes in P0 and P2 levels, respectively, in response to inhibition of FGF signaling suggest that pS368 and pS325/328/330 levels are decreased and increased, respectively, by FGF signaling. While S368 phosphorylation of Cx43 correlates with reduced gap junction assembly, phosphorylation of S325, S328, S330 and S365 enhance Cx43 assembly into gap junctions [26,27,31,36,38]. FGF inhibition caused increased phosphorylation of S368 (Fig. 3c), consistent with our finding in immunocytochemistry showing mislocalization of Cx43 at cell–cell junctions (Figs. 1 and 2). The decreased phosphorylation levels of S325/328/330 Cx43 in Ad-FGFR1DN cells suggests that Cx43 assembly is impaired in the absence of FGF signaling. Together, these data indicate that FGF signaling is capable of increasing S325/328/330 phosphorylation and thus promotes Cx43 assembly and retention at cell–cell contacts.

Gap junction communication is decreased in cells lacking FGF signaling

We observed a similar mislocalization of Cx43 from cell–cell junction and increased Cx43 expression after Ad-FGFR1DN transduction in COS7 cells as we saw with rat neonatal cardiomyocytes (compare Fig. 1 with Supplemental Fig. 1). In terms of connexins, COS7 cells have been previously reported to express only Cx43 [39]. Since COS7 cells could be more readily assayed via our fluorescent dye transfer assay, we chose them to measure the functional consequences of FGF inhibition on gap junction conductance. Dye transfer assay was performed with calcein, as the transferable dye, and exogenously expressed VE-cadherin-TagRFPT, to mark the recipient cells. Two populations of cells, stained with calcein or expressing VE-cadherin-TagRFPT, respectively, were mixed together and plated on a fibronectin-coated plate. The transfer of calcein to red fluorescence positive cells, which is a measure of gap junction conductivity, was visualized with confocal microscopy (Fig. 4a). The number of cells positive for both calcein and red fluorescence was significantly reduced by introduction of Ad-FGFR1DN in a dose-dependent manner (Fig. 4a and b), clearly indicating that FGF inhibition impairs the communication between cells by gap junctions.

Impaired gap junction stability in Ad-FGFR1DN cells is rescued by S325/328/330E Cx43.

Our findings indicated that FGF signaling in cardiomyocytes led to increased S325/328/330 phosphorylation and reduced Cx43 at cell–cell contacts. To establish a causal linkage of FGF signaling and Cx43 phosphorylation, we asked whether the phenotype generated by FGF inhibition could be reversed by phosphomimetic mutation of Cx43 corresponding residues. To measure Cx43 stability at cell–cell contacts, we employed live cell imaging of Cx43 using spinning disk confocal microscopy, which allows real-time, serial imaging, fast image acquisition and minimum light toxicity to cells. After each experiment, we were able to confirm the viability of cardiomyocytes by their spontaneous beating. In cardiac myocytes transduced with Ad-FGFR1DN, the level of gap junction plaques formed by wild-type Cx43 (Cx43-WT-EGFP), as measured by fluorescence intensity, decayed to the 50% level in about 3 h (Fig. 5 and Supplemental Movie 1). However, the S325/328/330E Cx43 mutant (Cx43-3SEEGFP), phosphomimetic mutant of Cx43, showed stable gap junctional structures over the same time course (Fig. 5 and Supplemental Movie 2), indicating that S325/328/330E Cx43 is insensitive to FGF inhibition and is capable of rescuing FGFR1DN-induced Cx43 instability.

Cardiomyocyte FGF signaling controls Cx43 expression and localization in the mouse heart

To validate the results obtained from *in vitro* studies and further evaluate the effect of FGF inhibition in the heart, we generated a mouse transgenic line that lacks FGF signaling in adult cardiac myocytes. FGFR signaling inhibition in adult myocytes is achieved by a Tet-Off system using an MHC promoter driving the FGFR1DN construct. In this system withdrawal of doxycycline from the diet efficiently induces transgene expression [34]. A transgenic line carrying the FGFR1DN gene under the control of TRE crossed with the p MHC-tTA driver mouse line, generated double transgenics (MHC-FGFR1DN) that allow doxycycline sensitive, cardiomyocytespecific FGFR1DN expression. These mice were then subjected to gene induction by doxycycline withdrawal at 10 weeks of age. Littermates carrying a single transgene (p MHC-tTA) were subjected to the same doxycycline regimen and used as controls.

To evaluate the mechanism of heart failure development in MHC-FGFR1DN mice, we performed a series of experiments using mice which had not yet shown overt clinical symptoms. It has been shown that N-cadherin can physically interact with FGFR and modify

FGF signaling in specific biological contexts such as cancer metastasis [40–42]. We examined the localization and expression of several junction proteins which are normally present at intercalated discs. In hearts lacking FGF signaling, Cx43 is essentially absent from intercalated discs, but the distribution of cadherin and desmoplakin was not altered (Fig. 6a). Cx43^{+/-}-heterozygous mice are viable and even mice lacking Cx43 expression in the heart can survive 2 months [43], so apparently on a little Cx43 is needed for survival. Intriguingly, transgene expression was not uniform in the cardiomyocytes of MHC-FGFR1DN mouse hearts (Fig. 6a). A closer examination revealed that the partial colocalization of Cx43 and N-cadherin at intercalated discs in control hearts was completely disrupted in hearts expressing FGFR1DN (Fig. 6b). Cx43 did not localize at intercalated discs and showed distribution in the lateral membranes (lateralization), implying normal Cx43 function at gap junctions was impaired. Moreover, we have found that at 30 weeks of age, MHC-FGFR1DN mouse hearts showed downregulation of Cx43 and slightly decreased levels of N-cadherin (Fig. 6c). The phosphorylated forms of Cx43 (upper bands), which presumably are expressed mainly at the intercalated discs, were markedly reduced in the heart with high FGFR1DN expression (Fig. 6c, #6194). These data indicate that basal FGF signaling is important for the maintenance of normal cardiac architecture, and the loss of FGF signaling leads to junctional and pathological abnormalities.

The activation of MHC-FGFR1DN transgene expression at 10 weeks of age led to premature death starting at 40 weeks of age (Fig. 7a). The hearts isolated from MHC-FGFR1DN mice showed strong and restricted expression of β -galactosidase expression driven by the bidirectional promoter [24] (Fig. 7b). In MHC-FGFR1DN mice, hearts were enlarged and the left ventricular cavity was noticeably dilated, showing typical morphological changes seen in the end stage heart failure (Fig. 7c). Some of the MHC-FGFR1DN mice showed clinical symptoms characteristic of advanced heart failure such as decreased body weight, tachypnea and pleural effusion. We also noticed that in the FGFR1DN heart, the formation of mural thrombus was observed in the enlarged left ventricle (Fig. 7c, arrow). Ratios of heart-to-body weight of MHC-FGFR1DN mice were significantly increased at 60 weeks old compared with the control group, further suggesting the presence of cardiac hypertrophy associated with heart failure in MHC-FGFR1DN mice (Fig. 7d). Moreover, histological analysis demonstrated moderate myocyte loss with fibrotic changes and fat deposition in the left ventricle of MHC-FGFR1DN mice (Fig. 7e). These data indicate that basal FGF signaling in cardiac myocytes is essential for the maintenance of cardiac homeostasis, and disruption of FGF signaling ultimately leads to the development of heart failure.

Discussion

In the current study, we found that suppression of FGF signaling in cardiomyocytes led to: (1) loss of Cx43 from myocyte cell–cell contacts, (2) decreased Cx43 S325/328/330 phosphorylation, and (3) impaired cardiac remodeling progressing to heart failure. These findings link FGF signaling to Cx43 phosphorylation at S325/328/330 and Cx43 assembly and localization. Moreover, our *in vivo* results revealed a role for FGF signaling in the maintenance of the normal cardiac architecture, suggesting a possible link between Cx43 function and cardiac remodeling.

Loss of Cx43 from cell–cell contacts was caused by Ad-FGFR1DN treatment in both rat neonatal cardiomyocytes (Figs. 1 and 2) and COS7 cells (Supplemental Fig. 1), but other junction proteins, ZO-1 and N-cadherin, were not appreciably affected. Expression of total Cx43 initially increased upon Ad-FGFR1DN treatment in a dose-dependent manner (Fig. 3). FGF inhibition impaired gap junctional communication between cells (Fig. 4 and Supplemental Fig. 2). Phosphorylation of Cx43 at S325/328/330 was also decreased by Ad-

FGFR1DN treatment in a dose-dependent manner (Fig. 3), and over-expression of phosphomimetic mutation of Cx43 at S325/328/330 in neonatal rat cardiomyocytes reversed FGFR1DN-induced Cx43 instability at cell–cell contacts (Fig. 5). We conclude that FGF signaling is essential for Cx43 homeostasis at least partially through phosphorylation of Cx43 S325/328/330 residues which are important for the maintenance of Cx43 at cell–cell contacts.

To study the role of FGF signaling in cardiac myocytes *in vitro*, we employed FGFRDN1. FGFR1DN is a cytoplasmic truncated form of FGFR1 able to bind all FGF isoforms and heterodimerize with all FGFRs as a dominant-negative construct, thereby suppressing overall FGF signaling [28,29,35]. Because of the redundancy of FGF system (22 FGF isoforms in mice and human) it is very difficult to identify which FGF isoform is most important for FGF signaling.

A substantial body of evidence indicates beneficial effects of FGF2 administration or transgenic overexpression in the experimental setting of myocardial infarction and ischemia–reperfusion injury [21]. The short term effect has been associated with the direct cytoprotective effect of FGF, whereas the long-term effect has been attributed to the angiogenic potential of FGF2 [44–47]. Although the precise mechanism of FGF-mediated cardioprotection is not fully understood, Cx43 has been thought to be one of the effectors of FGF signaling. It has been shown that PKC, activated by FGF, mediates the cardioprotective effect by regulating gap junction stability via increasing Cx43 phosphorylation [13,19]. The previous studies also showed that FGF administration is capable of inducing Cx43 phosphorylation on S262 and S368 in the ischemic heart. Interestingly, in the adult normal heart, Cx43 is highly phosphorylated with a very small fraction of P0 Cx43 being detectable. Ischemia causes overall Cx43 dephosphorylation, which can be blocked by FGF2 supplementation [13,19]. In this study, we used *in vitro* rat neonatal cardiomyocyte isolation system and *in vivo* Tet-Off system with an MHC promoter driving the FGFR1DN construct. We found that, in normoxic heart, FGF inhibition results in Cx43 loss of gap junctions due to decreased Cx43 S325/328/330 phosphorylation, indicating that basal FGF signaling is required for Cx43 homeostasis and function in addition to the role in cardiac ischemia.

Cx43 is a major component of the gap junction in the left ventricle, and cardiac myocytes are extensively interconnected with clusters of Cx43-containing gap junctions. Previous studies have demonstrated that the loss of mechanical coupling of cardiac myocytes causes Cx43 downregulation and abnormalities that have been linked to a various forms of diseased myocardium, and the loss of Cx43 contributes to the formation of the arrhythmia substrate in the ventricle [48–50]. Although the loss of electrical coupling does not seem to directly cause cardiac structural abnormalities, Cx43 is implicated in cytoprotection in many tissues including the heart and brain through its capability to control mitochondrial functions. Cx43 appears to modulate mitochondrial function in the brain and thus influence cell survival [51]. Therefore, Cx43 appears to have the capacity to mediate a cytoprotective response in a gap junction independent manner [20]. Moreover, a loss of function study in mice shows that Cx43 is required for embryonic heart development, suggesting that the lack of Cx43 *per se* can cause a deleterious effect in the heart [52].

Gap junctions are organized together with two other types of adhesive junctions at intercalated discs: adherens junctions and desmosomes. Cadherins are calcium-dependent transmembrane adhesion proteins that are engaged in homophilic binding and adherens junction formation [53]. N-cadherin is the only cadherin expressed and localized at intercalated discs in the myocardium, playing a critical role in the maintenance of structural integrity of the heart [54]. The FGF system is involved in the N-cadherin function through

direct interaction of FGFR and N-cadherin [41,42]. These findings initially led us to speculate that N-cadherin is the FGF target in the heart; however, the immunolocalization results we obtained were not consistent with this hypothesis. Although a slight decrease of N-cadherin levels in the MHC-FGFR1DN mouse heart was detected (Fig. 6c), N-cadherin localization in the heart was not overtly affected (Fig. 6b).

Cx43 phosphorylation has been correlated with changes in gap junction assembly, stability and channel properties and been shown to be regulated by several different kinase systems [36,37]. While we showed that FGF signaling is required for Cx43 phosphorylation and stability, certain aspects of this regulation remain elusive. Our data demonstrated FGF signaling increases Cx43 phosphorylation at S325/328/330 and its inhibition increases S368, however, the precise mechanism of action has not been elucidated. In a previous study, casein kinase 1 (CK1), particularly the δ isoform, interacted with and could phosphorylate Cx43 *in vitro* at S325/328/330 and a CK1 inhibitor reduced the phosphorylation level at these sites and gap junction assembly in cultured cells [55]. Furthermore, phosphorylation at S325/328/330 was reduced in cardiac tissue upon hypoxia [26]. Here, we showed depletion of FGF signaling leads to Cx43 S368 phosphorylation, which has been previously shown to lead to a reduction in gap junction conductance [38,56]. PKC, especially the PKC δ isoform, has been shown to phosphorylate Cx43 at S368 and mutation of the site eliminates the PKC-mediated change [18,56,57]. Several studies have linked FGF signaling with increased PKC activity [22], including a report involving protection of cardiac tissue from hypoxia [23]. We are not aware of any activation dependent antibodies for CK1 and its mode of regulation is not easily exploited to make the linkage between FGF and CK1. CSNK1D^{-/-} homozygous mice die within days of birth [58,59], and cardiac-specific CSNK1D conditional knockout mice are not currently available to us. The activity of CK1 δ has been shown to be negatively regulated by phosphorylation of its C-terminal domain [60]. We speculate FGF signaling could facilitate proteolytic processing of CK1 δ into a truncated constitutively active form.

In this study, we have clearly demonstrated that FGF depletion *in vivo* ultimately leads to congestive heart failure. Cx43 abnormality is a widespread phenotype of the diseased heart and is generally perceived as an end stage event. Although our study suggests that the pathological cardiac remodeling is mediated by Cx43 regulation, the FGF family is involved in a wide variety of biological processes and the nature of FGF action is pleiotropic. Therefore, we recognize that there is a chance that the inhibition of FGF signaling could promote cardiac pathology in parallel with impairment of Cx43 function. As cardiac-specific inactivation of Cx43 in mice leads to increased propensity for arrhythmogenesis, we are not able to exclude the possibility that MHC-FGFR1DN mice die because of conductance abnormality caused by Cx43 dysfunction [43]. We have tried to evaluate arrhythmogenicity of these mice using an electrocardiography monitoring system; however, due to slow disease development and heterogeneous Cx43 depletion, we were not able to detect significant arrhythmias in the MHC-FGFR1DN mouse group. Interestingly, heterogeneous remodeling of gap junction, which we consistently observed in the myocardium of MHC-FGFR1DN mice along with highly variable nature of transgene expression (Fig. 6a and b), has been implicated in ventricular dysfunction [61].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Robert Ross (University of California, San Diego), Frank Giordano and Michael Simons (Yale University School of Medicine) for helpful discussions and suggestions; Karen Moodie (Dartmouth Medical School), Kanako Yuasa and Eriko Yasutomi (Okayama University School of Medicine) for technical support.

Source of funding This work is supported by American Heart Association Scientist Development Grant 10SDG4170137 and grant GM55632 from the National Institutes of Health.

Abbreviations

FGF	fibroblast growth factor
Cx43	connexin43
S	serine
MOI	multiplicity of infection
PFU	plaque forming units
Ad	adenovirus
WT	wild type
NA	numerical aperture
SD	standard deviation
HA	hemagglutinin
DAPI	4 ,6-diamidino-2-phenylindole

REFERENCES

- [1]. Beyer EC, Paul DL, Goodenough DA, et al. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* 1987; 105:2621–2629. [PubMed: 2826492]
- [2]. Delorme B, Dahl E, Jarry-Guichard T, Marics I, Briand JP, Willecke K, Gros D, Theveniaud-Ruissy M, et al. Developmental regulation of connexin 40 gene expression in mouse heart correlates with the differentiation of the conduction system. *Dev. Dyn.* 1995; 204:358–371. [PubMed: 8601030]
- [3]. Coppen SR, Kodama I, Boyett MR, Dobrzynski H, Takagishi Y, Honjo H, Yeh HI, Severs NJ, et al. Connexin45, a major connexin of the rabbit sinoatrial node, is co-expressed with connexin43 in a restricted zone at the nodal-crista terminalis border. *J. Histochem. Cytochem.* 1999; 47:907–918. [PubMed: 10375379]
- [4]. Boengler K, Dodoni G, Rodriguez-Sinovas A, Cabestrero A, Ruiz-Meana M, Gres P, Konietzka I, Lopez-Iglesias C, Garcia-Dorado D, Di Lisa F, Heusch G, Schulz R, et al. Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. *Cardiovasc. Res.* 2005; 67:234–244. [PubMed: 15919068]
- [5]. Beenken A, Mohammadi M, et al. The FGF family: biology, pathophysiology and therapy. *Nat. Rev. Drug Discovery.* 2009; 8:235–253.
- [6]. Powers CJ, McLeskey SW, Wellstein A, et al. Fibroblast growth factors, their receptors and signaling. *Endocr.-Relat. Cancer.* 2000; 7:165–197. [PubMed: 11021964]
- [7]. Eswarakumar VP, Lax I, Schlessinger J, et al. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* 2005; 16:139–149. [PubMed: 15863030]
- [8]. Chen CH, Poucher SM, Lu J, Henry PD, et al. Fibroblast growth factor 2: from laboratory evidence to clinical application. *Curr. Vasc. Pharmacol.* 2004; 2:33–43. [PubMed: 15320831]
- [9]. Alzheimer C, Werner S, et al. Fibroblast growth factors and neuroprotection. *Adv. Exp. Med. Biol.* 2002; 513:335–351. [PubMed: 12575827]

- [10]. Dailey L, Ambrosetti D, Mansukhani A, Basilico C, et al. Mechanisms underlying differential responses to FGF signaling. *Cytokine Growth Factor Rev.* 2005; 16:233–247. [PubMed: 15863038]
- [11]. Presta M, Dell’Era P, Mitola S, Moroni E, Ronca R, Rusnati M, et al. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev.* 2005; 16:159–178. [PubMed: 15863032]
- [12]. Murakami M, Simons M, et al. Fibroblast growth factor regulation of neovascularization. *Curr. Opin. Hematol.* 2008; 15:215–220. [PubMed: 18391788]
- [13]. Kardami E, Detillieux K, Ma X, Jiang Z, Santiago JJ, Jimenez SK, Cattini PA, et al. Fibroblast growth factor-2 and cardioprotection. *Heart Fail. Rev.* 2007; 12:267–277. [PubMed: 17516168]
- [14]. Mima T, Ueno H, Fischman DA, Williams LT, Mikawa T, et al. Fibroblast growth factor receptor is required for *in vivo* cardiac myocyte proliferation at early embryonic stages of heart development. *Proc. Nat. Acad. Sci. U.S.A.* 1995; 92:467–471.
- [15]. Itoh N, Ornitz DM, et al. Evolution of the Fgf and Fgfr gene families. *Trends Genet.* 2004; 20:563–569. [PubMed: 15475116]
- [16]. Miller DL, Ortega S, Bashayan O, Basch R, Basilico C, et al. Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Mol. Cell. Biol.* 2000; 20:2260–2268. [PubMed: 10688672]
- [17]. Doble BW, Kardami E, et al. Basic fibroblast growth factor stimulates connexin-43 expression and intercellular communication of cardiac fibroblasts. *Mol. Cell. Biochem.* 1995; 143:81–87. [PubMed: 7776963]
- [18]. Doble BW, Ping P, Kardami E, et al. The epsilon subtype of protein kinase C is required for cardiomyocyte connexin-43 phosphorylation. *Circ. Res.* 2000; 86:293–301. [PubMed: 10679481]
- [19]. Srisakuldee W, Jeyaraman MM, Nickel BE, Tanguy S, Jiang ZS, Kardami E, et al. Phosphorylation of connexin-43 at serine 262 promotes a cardiac injury-resistant state. *Cardiovasc. Res.* 2009; 83:672–681. [PubMed: 19423616]
- [20]. Jeyaraman MM, Srisakuldee W, Nickel BE, Kardami E. Connexin43 phosphorylation and cytoprotection in the heart. *Biochim. Biophys. Acta.* 2012; 1818:2009–2013. [PubMed: 21763271]
- [21]. Liao S, Bodmer J, Pietras D, Azhar M, Doetschman T, Jel J. Schultz, et al. Biological functions of the low and high molecular weight protein isoforms of fibroblast growth factor-2 in cardiovascular development and disease. *Dev. Dyn.* 2009; 238:249–264. [PubMed: 18773489]
- [22]. Lu HC, Swindell EC, Sierralta WD, Eichele G, Thaller C, et al. Evidence for a role of protein kinase C in FGF signal transduction in the developing chick limb bud. *Development.* 2001; 128:2451–2460. [PubMed: 11493562]
- [23]. Wang J, Nachtigal MW, Kardami E, Cattini PA, et al. FGF-2 protects cardiomyocytes from doxorubicin damage via protein kinase C-dependent effects on efflux transporters. *Cardiovasc. Res.* 2013; 98:56–63. [PubMed: 23341575]
- [24]. Murakami M, Nguyen LT, Zhuang ZW, Moodie KL, Carmeliet P, Stan RV, Simons M, et al. The FGF system has a key role in regulating vascular integrity. *J. Clin. Invest.* 2008; 118:3355–3366. [PubMed: 18776942]
- [25]. Sosinsky GE, Solan JL, Gaietta GM, Ngan L, Lee GJ, Mackey MR, Lampe PD, et al. The C-terminus of connexin43 adopts different conformations in the Golgi and gap junction as detected with structure-specific antibodies. *Biochem. J.* 2007; 408:375–385. [PubMed: 17714073]
- [26]. Lampe PD, Cooper CD, King TJ, Burt JM, et al. Analysis of Connexin43 phosphorylated at S325, S328 and S330 in normoxic and ischemic heart. *J. Cell Sci.* 2006; 119:3435–3442. [PubMed: 16882687]
- [27]. Solan JL, Marquez-Rosado L, Sorgen PL, Thornton PJ, Gafken PR, Lampe PD, et al. Phosphorylation at S365 is a gatekeeper event that changes the structure of Cx43 and prevents down-regulation by PKC. *J. Cell Biol.* 2007; 179:1301–1309. [PubMed: 18086922]
- [28]. Ueno H, Gunn M, Dell K, Tseng A Jr. Williams L, et al. A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. *J. Biol. Chem.* 1992; 267:1470–1476. [PubMed: 1309784]

- [29]. Murakami M, Nguyen LT, Hatanaka K, Schachterle W, Chen PY, Zhuang ZW, Black BL, Simons M, et al. FGF-dependent regulation of VEGF receptor 2 expression in mice. *J. Clin. Invest.* 2011; 121:2668–2678. [PubMed: 21633168]
- [30]. Lampe PD, et al. Analyzing phorbol ester effects on gap junctional communication: a dramatic inhibition of assembly. *J. Cell Biol.* 1994; 127:1895–1905. [PubMed: 7806568]
- [31]. Solan JL, Fry MD, TenBroek EM, Lampe PD, et al. Connexin43 phosphorylation at S368 is acute during S and G2/M and in response to protein kinase C activation. *J. Cell Sci.* 2003; 116:2203–2211. [PubMed: 12697837]
- [32]. Lampe PD, Nguyen BP, Gil S, Usui M, Olerud J, Takada Y, Carter WG, et al. Cellular interaction of integrin $\alpha 3 \beta 1$ with laminin 5 promotes gap junctional communication. *J. Cell Biol.* 1998; 143:1735–1747. [PubMed: 9852164]
- [33]. Chen SC, Kennedy BK, Lampe PD, et al. Phosphorylation of connexin43 on S279/282 may contribute to laminopathy-associated conduction defects. *Exp. Cell Res.* 2013; 319:888–896. [PubMed: 23261543]
- [34]. Tirziu D, Simons M, et al. Endothelium-driven myocardial growth or nitric oxide at the crossroads. *Trends Cardiovasc. Med.* 2008; 18:299–305. [PubMed: 19345317]
- [35]. Li Y, Basilico C, Mansukhani A, et al. Cell transformation by fibroblast growth factors can be suppressed by truncated fibroblast growth factor receptors. *Mol. Cell. Biol.* 1994; 14:7660–7669. [PubMed: 7935480]
- [36]. Solan JL, Lampe PD, et al. Connexin43 phosphorylation: structural changes and biological effects. *Biochem. J.* 2009; 419:261–272. [PubMed: 19309313]
- [37]. Marquez-Rosado L, Solan JL, Dunn CA, Norris RP, Lampe PD. Connexin43 phosphorylation in brain, cardiac, endothelial and epithelial tissues. *Biochim. Biophys. Acta.* 2012; 1818:1985–1992. [PubMed: 21819962]
- [38]. Lampe PD, TenBroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF, et al. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J. Cell Biol.* 2000; 149:1503–1512. [PubMed: 10871288]
- [39]. George CH, Kendall JM, Campbell AK, Evans WH, et al. Connexin-aequorin chimeras report cytoplasmic calcium environments along trafficking pathways leading to gap junction biogenesis in living COS-7 cells. *J. Biol. Chem.* 1998; 273:29822–29829. [PubMed: 9792698]
- [40]. Murakami M, Elfenbein A, Simons M, et al. Non-canonical fibroblast growth factor signalling in angiogenesis. *Cardiovasc. Res.* 2008; 78:223–231. [PubMed: 18056763]
- [41]. Suyama K, Shapiro I, Guttman M, Hazan RB, et al. A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell.* 2002; 2:301–314. [PubMed: 12398894]
- [42]. Sanchez-Heras E, Howell FV, Williams G, Doherty P, et al. The fibroblast growth factor receptor acid box is essential for interactions with N-cadherin and all of the major isoforms of neural cell adhesion molecule. *J. Biol. Chem.* 2006; 281:35208–35216. [PubMed: 17005551]
- [43]. Gutstein DE, Morley GE, Tamaddon H, Vaidya D, Schneider MD, Chen J, Chien KR, Stuhlmann H, Fishman GI, et al. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. *Circ. Res.* 2001; 88:333–339. [PubMed: 11179202]
- [44]. Detillieux KA, Cattini PA, Kardami E, et al. Beyond angiogenesis: the cardioprotective potential of fibroblast growth factor-2. *Can. J. Physiol. Pharmacol.* 2004; 82:1044–1052. [PubMed: 15644945]
- [45]. Horrigan MC, MacIsaac AI, Nicolini FA, Vince DG, Lee P, Ellis SG, Topol EJ, et al. Reduction in myocardial infarct size by basic fibroblast growth factor after temporary coronary occlusion in a canine model. *Circulation.* 1996; 94:1927–1933. [PubMed: 8873670]
- [46]. Sheikh F, Sontag DP, Fandrich RR, Kardami E, Cattini PA, et al. Overexpression of FGF-2 increases cardiac myocyte viability after injury in isolated mouse hearts. *Am. J. Physiol. Heart Circ. Physiol.* 2001; 280:H1039–H1050. [PubMed: 11179045]
- [47]. House SL, Bolte C, Zhou M, Doetschman T, Klevitsky R, Newman G, Jel J. Schultz, et al. Cardiac-specific overexpression of fibroblast growth factor-2 protects against myocardial dysfunction and infarction in a murine model of low-flow ischemia. *Circulation.* 2003; 108:3140–3148. [PubMed: 14656920]

- [48]. Severs NJ, Bruce AF, Dupont E, Rothery S, et al. Remodelling of gap junctions and connexin expression in diseased myocardium. *Cardiovasc. Res.* 2008; 80:9–19. [PubMed: 18519446]
- [49]. Van Norstrand DW, Asimaki A, Rubinos C, Dolmatova E, Srinivas M, Tester DJ, Saffitz JE, Duffy HS, Ackerman MJ, et al. Connexin43 mutation causes heterogeneous gap junction loss and sudden infant death. *Circulation.* 2012; 125:474–481. [PubMed: 22179534]
- [50]. Duffy HS, et al. The molecular mechanisms of gap junction remodeling. *Heart Rhythm.* 2012; 9:1331–1334. [PubMed: 22133632]
- [51]. Azarashvili T, Baburina Y, Grachev D, Krestinina O, Evtodienko Y, Stricker R, Reiser G, et al. Calcium-induced permeability transition in rat brain mitochondria is promoted by carbenoxolone through targeting connexin43. *Am. J. Physiol. Cell Physiol.* 2011; 300:C707–C720. [PubMed: 21148408]
- [52]. Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J, et al. Cardiac malformation in neonatal mice lacking connexin43. *Science.* 1995; 267:1831–1834. [PubMed: 7892609]
- [53]. Gumbiner BM, et al. Regulation of cadherin-mediated adhesion in morphogenesis. *Nat. Rev.* 2005; 6:622–634.
- [54]. Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M, Hynes RO, et al. Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol.* 1997; 181:64–78. [PubMed: 9015265]
- [55]. Cooper CD, Lampe PD, et al. Casein kinase 1 regulates connexin-43 gap junction assembly. *J. Biol. Chem.* 2002; 277:44962–44968. [PubMed: 12270943]
- [56]. Ek-Vitorin JF, King TJ, Heyman NS, Lampe PD, Burt JM, et al. Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. *Circ. Res.* 2006; 98:1498–1505. [PubMed: 16709897]
- [57]. Barnett ME, Madgwick DK, Takemoto DJ, et al. Protein kinase C as a stress sensor. *Cell. Signalling.* 2007; 19:1820–1829. [PubMed: 17629453]
- [58]. Xu Y, Padiath QS, Shapiro RE, Jones CR, Wu SC, Saigoh N, Saigoh K, Ptacek LJ, Fu YH, et al. Functional consequences of a CK1delta mutation causing familial advanced sleep phase syndrome. *Nature.* 2005; 434:640–644. [PubMed: 15800623]
- [59]. Etchegaray JP, Machida KK, Noton E, Constance CM, Dallmann R, Di Napoli MN, DeBruyne JP, Lambert CM, Yu EA, Reppert SM, Weaver DR, et al. Casein kinase 1 delta regulates the pace of the mammalian circadian clock. *Mol. Cell. Biol.* 2009; 29:3853–3866. [PubMed: 19414593]
- [60]. Graves PR, Roach PJ, et al. Role of COOH-terminal phosphorylation in the regulation of casein kinase I delta. *J. Biol. Chem.* 1995; 270:21689–21694. [PubMed: 7665585]
- [61]. Gutstein DE, Morley GE, Vaidya D, Liu F, Chen FL, Stuhlmann H, Fishman GI, et al. Heterogeneous expression of Gap junction channels in the heart leads to conduction defects and ventricular dysfunction. *Circulation.* 2001; 104:1194–1199. [PubMed: 11535579]

Fig. 1.

FGF signaling in neonatal rat cardiomyocytes is required for Cx43 localization at cell–cell contacts. (a) Specific localization of Cx43 at cell–cell contacts between cardiomyocytes. Isolated neonatal rat cardiomyocytes were co-cultured with neonatal rat fibroblasts, and immunostained with anti- α -actin, anti-total Cx43 and anti- α -actinin. Anti- α -actin stains both cardiomyocytes and fibroblasts and anti- α -actinin stains only cardiomyocytes. Cx43 only localized at cell–cell contacts between cardiomyocytes and not at interfaces with neighboring fibroblasts. Scale bar, 20 μ m. (b) Cx43 mislocalization in primary rat cardiomyocytes lacking FGF signaling. Primary rat cardiomyocytes, with or without Ad-FGFR1DN expression, were immunostained with anti-HA, anti-total Cx43 antibody and anti- α -actinin antibody. Inhibition of FGF signaling resulted in decreased Cx43 at cell–cell contacts. Scale bar, 20 μ m. (c) Higher magnification of area indicated by square in b. Arrow shows Cx43 localized at cell–cell contacts and arrowhead shows cell–cell contacts without Cx43 localization. Scale bar, 5 μ m.

Fig. 2.

Inhibition of FGF signaling decreases Cx43 localization at cell–cell contacts, but does not significantly affect ZO-1 and N-cadherin. (a) Cx43 mislocalization in primary rat cardiomyocytes lacking FGF signaling. Primary rat cardiomyocytes, infected with Ad-FGFR1DN with different viral doses (MOI 2–10) 3 days after isolation, were immunostained with anti-total Cx43 antibody and anti-N-cadherin antibody. Scale bar, 20 μ m. (b) Quantitative analysis of immunostaining shown in a. The area stained with anti-total Cx43 antibody and anti-N-cadherin antibody were measured with Image J and the ratios were calculated; $n=16$ (control), $n=10$ (Ad-GFP (MOI 5)), $n=10$ (MOI 2), $n=11$ (MOI 5) and $n=13$ (MOI 10). Bars represent means $\pm$ SD. * $P<0.01$ versus NT, by one way ANOVA followed by Tukey HSD. (c) Immunostaining of cardiomyocytes with or without Ad-FGFR1DN treatment by anti-Cx43, anti-ZO-1 and anti-N-cadherin. Inhibition of FGF signaling in neonatal rat cardiomyocytes decreased Cx43 localization at cell–cell contacts, but did not have much affect ZO-1 and N-cadherin. Arrow shows these three proteins localized at cell–cell contacts and arrowhead shows cell–cell contacts without Cx43 localization. Scale bar, 5 μ m.

Fig. 3.

FGF signaling in cardiomyocytes affects Cx43 phosphorylation. Western analysis of total protein extracted from primary rat cardiomyocytes infected with either Ad-GFP (MOI 5) or Ad-FGFR1DN (MOI 0.5-10) with incremental doses of MOI, or left untreated (NT, no treatment) three days after isolation. Samples were separated by SDS-PAGE with 4–15% gel and analyzed by immunoblotting. (a) Effect of FGF signaling inhibition on Cx43 expression in cardiomyocytes. Increased unphosphorylated Cx43 levels in Ad-FGFR1DN transduced cardiomyocytes were observed with Cx43 CT1 antibody. P0, P1 and P2 bands are indicated. (b) Quantitative analysis of Western blot of total Cx43 shown in a. Relative intensity of the P0, P1 and P2 bands normalized with β -tubulin were measured with NIH Image J software ($n=4$). Bars represent means $\pm$ SD. * $P<0.05$ and ** $P<0.01$ versus NT, by one way ANOVA followed by Tukey HSD. (c) Western analysis of FGF regulation of specific Cx43 phosphorylation in cardiomyocytes. Phosphorylated Cx43 levels were evaluated using the anti-phospho Cx43 antibodies described in the materials and methods. (d) Quantitative analysis of Western blot of Cx43 pS325/328/330 shown in b. Relative intensity of Cx43 pS325/328/330 bands normalized with β -tubulin was measured with NIH Image J software ($n=3$). Bars represent means $\pm$ SD. * $P<0.05$ and ** $P<0.01$ versus NT, by one way ANOVA followed by Tukey HSD.

Fig. 4. FGF inhibition affects gap junction communication. (a) Dye transfer is impaired in cells lacking FGF signaling. COS7 cells either with either calcein AM or VE-cadherin-TagRFPT (VEC-TagRFPT) were mixed and plated on a plate. Transfer of gap junction permeant calcein to red VE-cadherin positive cells was visualized using confocal microscopy. Arrows indicate the red fluorescence positive cells that received calcein. Scale bar, 20 μm. (b) The number of red fluorescence positive cells that contained calcein was reduced by introduction of Ad-FGFR1DN. The percentages of cells that transferred dye were determined by dividing the number of red fluorescence positive cells that contained calcein (*i.e.* transfers) by the number of cell interfaces between calcein-loaded and red fluorescence positive cells (*i.e.* total); $n=149$ (NT), $n=101$ (Ad-null, control virus, MOI 5), $n=131$ (MOI 0.5), $n=136$ (MOI 2), $n=92$ (MOI 5) and $n=106$ (MOI 10). Bars represent means $\pm$ SD. * $P<0.01$ versus NT, by one way ANOVA followed by Tukey HSD.

Fig. 5.

Impaired Cx43 stability by FGF inhibition is restored by S325/328/330E Cx43 mutant. Time-lapse imaging of neonatal rat cardiomyocytes. Primary rat cardiomyocytes were transduced with the lentiviral vector containing Cx43-WT-EGFP or Cx43-S325/328/330E(3SE)-EGFP 3 days after isolation and then were transduced with or without Ad-FGFR1DN (MOI 5) 3 days after lentivirus transduction. The time-lapse images were acquired by a spinning disk confocal microscope for 6 h. (a) The gap junction plaques formed by Cx43-WT-EGFP decayed to ~50% of the starting level in about 3 h. Cx43-WT-EGFP localized at cell-cell junction (indicated by arrows). Gap junction plaques formed by Cx43-3SE-EGFP were the most stable. Cx43-3SE-EGFP localized at cell-cell junction is indicated with arrowheads. Scale bar, 20 μm. (b) Quantitative analysis of fluorescent signal shown in a. The area of Cx43-EGFP and Cx43-3SE-EGFP at cell-cell junction were measured with Image J and were normalized to the intensity at 0 h; $n=4$ (Cx43-WT-EGFP (Ad-FGFR1DN-)), $n=3$ (Cx43-3SE-EGFP (Ad-FGFR1DN-)), $n=4$ (Cx43-WT-EGFP (Ad-FGFR1DN+)) and $n=3$ (Cx43-3SE-EGFP (Ad-FGFR1DN+)). Though plaques in Cx43-EGFP (Ad-FGFR1DN-) gradually decreased, there was no significant difference between 0 hour and other time points. Similarly, there was no significant differences between 0 h and other time points for Cx43-3SE-EGFP (Ad-FGFR1DN-) and Cx43-3SE-EGFP (Ad-FGFR1DN+), either. Bars represent means $\pm$ SD. * $P<0.01$, Cx43-WT-EGFP (Ad-FGFR1DN+) vs Cx43-3SE-EGFP (Ad-FGFR1DN+), by one way ANOVA followed by Tukey HSD.

Fig. 6. Inhibition of FGF signaling in cardiomyocytes *in vivo* results in Cx43 lateralization. (a) Immunostaining of left ventricle at 40 weeks. Sections were stained for Cx43, pan-cadherin, or desmoplakin (red), HA (FGFR1DN, white), actin (green) and DAPI (blue). Note Cx43 lateralization (yellow arrows) in the FGFR1DN heart. Scale bar, 20 μ m. (b) Immunostaining of N-cadherin in the left ventricle at 40 weeks. Sections were stained for Cx43 (red), N-cadherin (green) and HA (FGFR1DN, white). Scale bar, 20 μ m. (c) Western blotting of whole tissue lysate of the heart from aMHC-FGFR1DN or control mice (three different mice in each group) at 40 weeks of age.

Fig. 7.

Disruption of cardiomyocyte FGF signaling in adult mice leads to heart failure and premature death. (a) Survival curves of control (A) and aMHC-FGFR1DN (AF) mice ($n=20$ in each group). Transgene expression was induced at the age of 10 weeks (Doxycycline (Dox) off). (b) -galactosidase staining of the heart indicating uniform expression of the transgene in the aMHC-FGFR1DN mouse heart. (c) Gross examination of the heart from control and aMHC-FGFR1DN mouse at 52 weeks. Left panel: whole heart, right panel: cross section at the mid-ventricular level. Note the dilated heart of aMHC-FGFR1DN mouse. The arrow indicates mural thrombus. (d) Ratios of heart-to-body weight for control and FGFR1DN mice at 40 weeks and 60 weeks old [$n=6$ (control, 40 weeks), $n=6$ (FGFR1DN, 40 weeks), $n=6$ (control, 60 weeks), $n=7$ (FGFR1DN, 60 weeks)]. Bars represent means $\pm$ SD. * $P < 0.05$ by t -test, compared with control mice at 60 weeks. (e) Histological analysis of the control and the MHC-FGFR1DN mouse heart at 52 weeks. White squares in the upper panels are enlarged and shown in the lower panels.