Fibroblast Growth Factor Receptor-1 Is Essential for In Vitro Cardiomyocyte Development

Patrizia Dell’Era, Roberto Ronca, Laura Coco, Stefania Nicoli, Marco Metra, Marco Presta

Abstract—Fibroblast growth factor (FGF)/FGF receptor (FGFR) signaling plays a crucial role in mesoderm formation and patterning. Heartless mutant studies in Drosophila suggest that FGFR1, among the different FGFRs, may play a role in cardiogenesis. However, fgfr1−/− mice die during gastrulation before heart formation. To establish the contribution of FGFR1 in cardiac development, we investigated the capacity of murine fgfr1+/− and fgfr1−/− embryonic stem (ES) cells to differentiate to cardiomyocytes in vitro. Clusters of pulsating cardiomyocytes were observed in >90% of 3-dimensional embryoid bodies (EBs) originated from fgfr1−/− ES cells at day 9 to 10 of differentiation. In contrast, 10% or less of fgfr1−/− EBs showed beating foci at day 16. Accordingly, fgfr1−/− EBs were characterized by impaired expression of early cardiac transcription factors Nkx2.5 and d-Hand and of late structural cardiac genes myosin heavy chain (MHC)-α, MHC-β, and ventricular myosin light chain. Homozygous fgfr1 mutation resulted also in alterations of the expression of mesoderm-related early genes, including nodal, BMP2, BMP4, T(brachyury), and sonic hedgehog. Nevertheless, fgfr1+/− and fgfr1−/− EBs similarly express cardiogenic precursor, endothelial, hematopoietic, and skeletal muscle markers, indicating that fgfr1-null mutation exerts a selective effect on cardiomyocyte development in differentiating ES cells. Accordingly, inhibitors of FGFR signaling, including the FGFR1 tyrosine kinase inhibitor SU 5402, the MEK1/2 inhibitor U0126, and the protein kinase C inhibitor GF109 all prevented cardiomyocyte differentiation in fgfr1−/− EBs without affecting the expression of the hematopoietic/endothelial marker flk-1. In conclusion, the data point to a nonredundant role for FGFR1-mediated signaling in cardiomyocyte development. (Circ Res. 2003;93:414-420.)

Key Words: fibroblast growth factor receptor • cardiomyocytes • embryonic stem cells

Fibroblast growth factor receptors (FGFRs) belong to the subclass IV of membrane-spanning tyrosine kinase (TK) receptors.1,2 Four fgfr genes have been identified.3 FGFR1–4 proteins share common structural features4 and interact with the members of the FGF family composed of at least 23 polypeptides.5 The FGF/FGFR system has been implicated in a variety of physiological and pathological conditions, including embryonic development, tissue growth and remodeling, inflammation, tumor growth, and vascularization.3

After ligand binding and receptor dimerization, a number of tyrosine autophosphorylation sites have been identified in FGFR1: Y463 is responsible for Crk binding; Y653/654 are critical for TK activity; and Y766 is responsible for phospholipase C-γ (PLC-γ) and Shb binding.6,7 Activated PLC-γ hydrolyzes membrane phospholipids, generating inositol 1,4,5-trisphosphate and diacylglycerol that activates certain protein kinase C (PKC) isoforms.8,9 Also, FGFR1 activation leads to FRS2 phosphorylation,10 followed by Grb2 and Shp-2 interactions that are required for activation of the Ras/Raf-1/MEK/ERK pathway cascade.11

FGF/FGFR signaling plays important functions in mesoderm formation and development.13 Accordingly, fgfr1−/− mice die during gastrulation, displaying defective mesoderm patterning with reduction in the amount of paraxial mesoderm and lack of somite formation.14,15 Studies on chimeric embryos using FGFR1-deficient embryonic stem (ES) cells revealed a early defect in the mesodermal and endodermal cell movement through the primitive streak, followed by deficiencies in contributing to anterior mesoderm, including heart tissue.16,17 Moreover, FGFR1 has been implicated in embryonic anterior-posterior axis patterning.18

The heart is the first organ to be formed in vertebrate embryo development. Precardiac mesoderm cells become allocated at or shortly after gastrulation, leading to the formation of a single beating linear heart tube that undergoes right-ward looping followed by segmentation and growth of cardiac chambers.19 Commitment to a cardiac fate results from inductive interactions during gastrulation. In amphibian and avian embryos, the endoderm adjacent to the mesodermal cardiac precursors is the source of instructive signaling capable of specifying a cardiac fate.20–22 In this context, FGF family members, in cooperation with members of the transforming growth factor-β family, play a pivotal role.20,22–25
FGF/FGFR signaling drives cardiac differentiation also in Drosophila where the cardiac counterpart is the contractile dorsal vessel. Fly cardiac progenitor cells are characterized by the expression of tinman, a transcription factor of the NK homeobox protein family.26 Mesoderm spreading depends on the expression of heartless, homologous to vertebrate fgfr1.27,28 Heartless mutant embryos, like tinman mutants, do not develop the dorsal vessel.27,29 Finally, FGFRs are present in rodent embryonic heart30,31 and fgf8 expression is required for induction and patterning of myocardial precursors in zebrafish.32

Taken together, several studies point to the importance of FGF/FGFR signaling in heart development. In particular, heartless mutant studies in Drosophila suggest that FGFR1 may play a nonredundant role in cardiogenesis. Accordingly, previous analysis on mouse chimeras showed deficiencies of fgfr1−/− ES cells in contributing to heart tissue.33 However, the early embryonic lethality observed in fgfr1−/− mice occurs before a stage in which the role of FGFR1 in murine cardiogenesis can be evaluated.

Pluripotent ES cells differentiate into a variety of cell lineages in vitro after aggregation into 3-dimensional structures termed embryoid bodies (EBs).33 EBs originate a variety of specialized cell types, including cardiomyocytes that are manifested by the appearance of spontaneously contracting foci.34 Thus, ES cell differentiation in EBs can be used to study the impact of specific gene inactivation during cardiomyocyte development also for those loss of function mutations resulting in early embryonic lethality.35 On this basis, to investigate the contribution of FGFR1 in murine cardiomyocyte development, we studied the differentiation of EBs originating from murine fgfr1−/− ES cells.

The data demonstrate the absence of contracting cardiomyocyte foci and of specific cardiac gene transcripts in differentiating fgfr1−/− EBs, pointing to a crucial role for FGFR1 in heart development.

Materials and Methods

ES Cell Culture and Differentiation

Murine 11-22 fgfr1+/−, 23-46 fgfr1+/−, and 23-18 fgfr1−/− ES cells15 were adapted to grow without feeder cells and maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 20% fetal bovine serum (Hyclone), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM L-β-mercaptoethanol, 2 mM L-glutamine, and 1000 U/mL LIF (ESGRO, Chemicon).

Differentiation experiments were performed in parallel on 11-22 fgfr1+/− and 23-46 fgfr1−/− cells. At T0 of differentiation, exponentially growing ES cells were resuspended in LIF-deprived (EB) medium and cultured in 30 μL hanging drops (400 cells) for 2 days to allow cell aggregation. Then, aggregates were transferred onto 0.7% agarose-coated dishes and grown for 5 days in EB medium. At day 7, EBs were transferred into 24-well tissue culture plates, one aggregate per well, and allowed to adhere. Aggregates were monitored for the appearance of spontaneously contracting foci during the following 9–10 days. When indicated, EBs were immunostained for cardiac α-myosin heavy chain expression (see expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org).

RNA Extraction and Semiquantitative RT-PCR Analysis

Total RNA was extracted from ES cells as described.36 Two μg of total RNA were retrotranscribed with Ready-To-Go You-Prime First Strand Beads (Amersham Biosciences), using a 18 mer oligo-dT primer in a 20-μL volume. For each transcript, 2 μL of the retrotranscribed RNA were subjected to polymerase chain reaction using Pre-Aliquoted ReadyMix PCR Master Mix (Abgene) under the following conditions: 94°C (30 seconds), 60°C (30 seconds) for all transcripts with the exception of fgfr1 (72°C) and Oct4 (64°C), and 72°C (1 minute). Oligonucleotide primers and the number of PCR cycles are listed in the online Table (online data supplement). In order to compare multiple gene expression, several independent RT reactions were mixed together before PCR. The data were then confirmed by analyzing the RNA extracted from two or more independent differentiation experiments. Titration analysis of PCR amplification of tubulin and Nkx2.5 mRNAs confirmed that the adopted conditions allowed a semiquantitative analysis of gene expression (online Figure in the online data supplement).

Results

FGF and FGFR Expression During In Vitro Differentiation of fgfr1+/− and fgfr1−/− ES Cells

The 11-22 fgfr1+/− ES cell line was obtained by homologous recombination, replacing fgfr1 exon 4 with PGKneo. The two related fgfr1+/− ES cell lines were then generated by targeting the remaining wild-type allele with pFGFR1hyg.15 Fgfr1+/− mice are normal in terms of growth, morphology, health, and fertility.15 Accordingly, the in vitro differentiation process of fgfr1+/− EBs resembles that of wild-type EBs with a similar expression of several markers belonging to cellular lineages derived from all the three embryonic layers (see below and data not shown). On this basis, cardiomyocyte differentiation was compared in fgfr1+/− versus fgfr1−/− ES cells throughout the present study. Fgfr1+/− and fgfr1−/− ES cells are morphologically indistinguishable, have similar rates of growth in vitro, and can originate muscle cells when injected in nude mice to form teratomas.15

Preliminarily, we evaluated the expression of FGFRs and FGFR ligands implicated in early development during in vitro differentiation of fgfr1+/− and fgfr1−/− EBs (Figure 1). In fgfr1+/− EBs, all FGFRs showed a maximal expression between days 7 and 10 of differentiation. In contrast, together with the anticipated lack of fgfr1 expression, no fgfr4 transcripts were detectable in fgfr1−/− EBs that showed also a delayed expression of fgfr2 and fgfr3. Moreover, differenti-
ating fgfr1+/− EBs were characterized by the rapid disappearance of fgf4 expression that was paralleled by the transient expression of fgf10 between days 7 and 10 and by the late increase in fgf8 transcript levels. Homozygous fgfr1 mutation resulted instead in sustained fgf4 expression and lack of fgf10 upregulation in differentiating EBs that also showed an earlier increase in fgf8 expression (Figure 1).

Contractile Cardiomyocyte Foci in fgfr1+/− and fgfr1−/− EBs
At day 7 of differentiation, fgfr1+/− and fgfr1−/− EBs were allowed to adhere and monitored during the following 13 days for the appearance of clusters of spontaneously pulsating cardiomyocytes. Fgfr1+/− and fgfr1−/− ES cell aggregates showed a similar capacity to attach to the substrate. More than 50% of fgfr1+/− EBs generated contracting foci one day after attachment. The percentage of beating foci increased up to 90% at day 9–10 to decrease slowly after day 16 (Figure 2A). In contrast, fgfr1−/− EBs showed a dramatic decrease in the capacity to generate contractile foci, with no more than 10% of EBs showing beating areas after 16 days of differentiation. Moreover, the few contractile fgfr1−/− EBs never showed more than one beating focus per EB, whereas two or more foci were constantly observed in the majority of heterozygous EBs. Also, the beating frequency was significantly reduced in fgfr1−/− EBs compared with fgfr1+/− EBs (47±6 and 99±9 bpm, respectively).

Similar results were obtained with EBs originated from 100 to 800 cells per aggregate or when aggregates were allowed to attach to various substrata, including gelatin, type I collagen, laminin, vitronectin, fibronectin, or Matrigel. Also, changes in the pH of the cell culture medium (from 6.7 to 8.7), treatment with the cardiomyocyte-inducing compound 5-azacytidine, induction of hypoxia-like conditions after treatment with CoCl2 did not induce the appearance of contractile areas in fgfr1−/− ES cells (data not shown). Thus, fgfr1 loss of function dramatically affects the capacity to originate the formation of contractile cardiomyocyte foci in differentiating EBs in a variety of experimental conditions. Similar results were obtained for the 23-18 fgfr1−/− ES cell line, further supporting these conclusions (data not shown).

Expression of Cardiac Markers in fgfr1+/− and fgfr1−/− EBs

ES-derived cardiomyocytes express cardiac gene products during EB differentiation in a developmentally controlled manner that closely recapitulates the developmental pattern of early cardiogenesis. To gain insights on the developmental alterations responsible for the lack of contractile foci in fgfr1−/− EBs, we evaluated the expression of early and late myocardial genes during EB differentiation (Figure 2B). Between days 7 and 10, fgfr1−/− EBs showed a peak of expression of the early cardiac gene Nkx2.5, homologous to the Drosophila gene tinman, and of the cardiac transcription factors d-Hand, e-Hand, GATA-4, and Irx4. This was followed by the upregulation of genes encoding for the myocardial structural proteins myosin heavy chain (MHC)-α, MHC-β, and ventricular myosin light chain (MLC-2V). In contrast, homozygous EBs were characterized by the lack of Nkx2.5 expression, by delayed d-Hand, e-Hand, GATA-4, and Irx4 expression, and by a prolonged Irx4 upregulation (Figure 2B). This was paralleled by the absence of MHC-α, MHC-β, and MLC-2V transcripts throughout the whole experimental period. Accordingly, no MHC-α immunoreactivity was observed in fgfr1−/− EBs (Figure 2C).

Expression of Mesoderm-Related Genes in fgfr1+/− and fgfr1−/− EBs

To assess whether the lack of cardiomyocyte formation in fgfr1−/− EBs is related to a more general effect on mesodermal differentiation, we compared differentiating fgfr1+/− and fgfr1−/− ES cells for the expression of genes involved in early stages of mesoderm formation (Figure 3). Oct4, nodal, and cripto1 expression are promptly downregulated in fgfr1−/− EBs after LIF withdrawal. In parallel, the expression of the cardiogenic mesodermal marker Mef2c progressively in-
creases during differentiation. Also, BMP2, BMP4, T(tra), and sonic hedgehog (SHH) expression are upregulated after LIF withdrawal with different kinetics. Homozygous fgfr1 mutation did not hamper the capacity of ES cells to undergo differentiation (as shown by Oct4 downregulation) and to originate cardiogenic precursors (as shown by Mef2c induction), although an apparent general delay in the modulation of some mesoderm-related genes was observed. Indeed, fgfr1<sup>+/−</sup> EBs were characterized by the following: (1) the persistent expression of nodal; (2) the delayed and/or prolonged expression of BMP2 and BMP4; (3) the strong and persistent upregulation of T(tra); and (4) the reduced increase in SHH expression.

Despite these differences in the expression of early mesodermal markers, both fgfr1<sup>+/−</sup> and fgfr1<sup>−/−</sup> EBs showed similar expression patterns for genes characterizing different mesodermal-derived lineages (Figure 3). Indeed, the hematopoietic/endothelial markers vascular endothelial growth factor receptor flk-1, CD34, and c-kit, and the later endothelial [CD31 and vascular endothelial cadherin (ve-cad)] and hematopoietic (β-major) markers were similarly expressed in the two differentiating ES cell lines. Also, both cell lines express the skeletal muscle markers myogenin and myoD at day 16 of differentiation (data not shown).

**Role of FGF/FGFR Signaling in Cardiomyocyte Development in fgfr1<sup>+/−</sup> and fgfr1<sup>−/−</sup> EBs**

To confirm the role of FGFR1 in cardiomyocyte development, fgfr1<sup>+/−</sup> ES cells were treated with inhibitors of FGF/FGFR signaling during differentiation. Both FGFR1 TK inhibitor SU 5402<sup>23</sup> and MEK1/2 inhibitor U0126<sup>52</sup> hamper pulsating cardiomyocytes formation and the upregulation of the cardiac marker MLC-2V in fgfr1<sup>−/−</sup> EBs without affecting hematopoietic/endothelial marker flk-1 expression (Figure 4A). In contrast, the MEK1 inhibitor PD098059<sup>52</sup> does not affect cardiomyocyte differentiation (data not shown and reference<sup>53</sup>). Interestingly, U0126 and PD098059 do not affect BMP2 and BMP4 expression (data not shown).

PKC isozymes regulate the Ras/Raf-1/MEK/ERK cascade at different levels.<sup>54</sup> The classical/novel PKC inhibitor GF109<sup>95</sup> prevented the formation of pulsating foci and MLC-2V upregulation in fgfr1<sup>+/−</sup> EBs without affecting flk-1 expression (Figure 4A). Also, using a nonexhaustive library of Antennapedia carrier-conjugated peptides that selectively inhibit translocation of the different PKC isozymes,<sup>56</sup> we observed a 24-hour delay in beating foci appearance in fgfr1<sup>+/−</sup> EBs maintained in the presence of the selective PKCe antagonist (data not shown).

To further investigate the cross-talk between the PKC and the MEK/ERK signaling pathways in cardiogenesis, we assessed the effect of the PKC activator phorbol ester PMA on cardiomyocyte development in fgfr1<sup>+/−</sup> EBs (Figure 4B). PMA treatment partially rescued cardiomyocyte differentiation in these cells, more than 60% of EBs showing beating areas after 14 days of differentiation. The appearance of beating foci was paralleled by MLC-2V upregulation (Figure 4B, inset). The effect of PMA was abolished by pretreatment with U0126, thus indicating that PKC may act upstream of ERK activation during cardiomyocyte development in EBs. Again, PMA treatment in the absence or in the presence of U0126 had no effect on flk-1 expression (Figure 4B, inset).
Discussion

Experimental evidence on various animal species point to a role for FGF/FGFR signaling in heart development. Homozygous fgfr1-null mice display early growth defects and alterations in mesoderm patterning.14,15 However, the early embryonic lethality observed in these animals did not allow to assess the role of FGFR1 in murine cardiogenesis. In the present study, differentiating EBs from murine fgfr1+/- ES cells fail to form contractile cardiomyocyte foci and do not express early and late cardiac markers. This occurs despite the ability of fgfr1+/- EBs to generate cardiogenic precursors, as evidenced by the upregulation of Mef2c expression after LIF withdrawal. Also, inhibitors of FGF/FGFR signaling, including TK-FGFR1, MEK1/2, and PKC inhibitors, all prevented cardiomyocyte differentiation in phenotypically normal fgfr1+/- EBs. Conversely, the PKC activator PMA partially restored the ability of fgfr1+/- EBs to form contractile foci. Finally, lentivirus-mediated FGFR1 overexpression totally rescues cardiomyocyte formation in fgfr1+/- ES cells (P. Dell’Era, unpublished data, 2003). Taken together the data point to a nonredundant role for FGFR1 in cardiomyocyte differentiation in murine ES. This conclusion extends previous observations on the lack of dorsal vessel formation in early and late cardiac markers. This occurs despite the general differentiation capacity of these cells is not impaired. Indeed, fgfr1-null embryonic derm formation and development. In Xenopus laevis, FGF/FGFR signaling is required for the expression of several early mesodermal markers, including MHC-α, MHC-β, and MLC-2V. In contrast, homozygous ES failed to express Nkx2.5. In Nkx2.5-null mice, the heart tube forms, but morphological defects are observed at the looping stage and several cardiac genes are downregulated.58 Accordingly, fgfr1+/- EBs showed a delayed expression of d-Hand, e-Hand, and GATA-4 genes, and a prolonged Irx4 upregulation. Finally, the absence of MHC-α, MHC-β, and MLC-2V transcripts is in keeping with the lack of contractile cardiomyocyte foci in fgfr1-null ES. Thus, the data indicate that fgfr1 inactivation dramatically affects the expression of several cardiac transcription factors with a consequent impairment on the expression of structural myocardial genes and contractile foci formation. Preliminary data indicate the capacity of recombinant FGF2 to upregulate Nkx2.5 expression in FGFR1-expressing murine endothelial cell cultures (Figure 5). This suggests that Nkx2.5 may represent one of the earliest direct FGF/FGFR targets during heart development and that FGFR1 may mediate cardiomyocyte development in EBs by activating Nkx2.5 expression in Mef2c+ cardiogenic mesodermal cells.

Figure 5. Nkx2.5 upregulation by FGF2 in endothelial cells. Murine endothelial 1G11cells were starved for 24 hours before stimulation with recombinant FGF2 (3.0 pmol/L). At the indicated times, Nkx2.5 and tubulin expression were evaluated by RT-PCR analysis.

Homzygous fgfr1 mutation results in alterations of the expression of the other fgfr genes in EBs with a delayed upregulation of fgfr2 and fgfr3 and the lack of appearance of fgfr4 transcripts. Also, fgf8 upregulation was altered and fgf10 was not expressed in homozygous EBs. Thus, the possibility exists that alterations in the expression of other member(s) of FGF/FGFR signaling consequent to fgfr1 inactivation may affect heart development. Relevant to this point, previous observations had shown that both BMP2 and FGF4 or FGF2 are required to induce a full cardiac differentiation of nonprecardiac mesoderm in the chick embryo.59 Also, fgf8 expression is required for induction and patterning of myocardial precursors in zebrafish.60 However, addition of BMP2 protein alone or together with FGF2 did not restore the capacity of fgfr1+/- EBs to form contractile cardiomyocyte foci in our experimental conditions (data not shown).

FGF/FGFR signaling plays important functions in mesoderm formation and development. In Xenopus laevis, FGF/FGFR signaling is required for the expression of several early mesodermal markers, the induction of posterior and ventral mesoderm, and for proper mesodermal maintenance during gastrulation.60,61 Moreover, fgfr1 and fgfr2 transcripts are detectable in mouse egg cylinder and in the primitive ectoderm.62 At later stages of development, the undifferentiated mesenchyme is a specific domain for fgfr1 expression.62 Also, the nascent mesoderm of homozygous fgfr1 mutant mouse embryos differentiates into diverse mesodermal subtypes, but mesodermal patterning is aberrant.15 Accordingly, the expression of some mesodermal markers, including nodal, T(bra), and SHH, is altered in fgfr1+/- ES cells. Nevertheless, fgfr1-null mutation does not prevent the downregulation of the pluripotent cell marker Oct4, thus indicating that the general differentiation capacity of these cells is not fully impaired. Indeed, fgfr1+/- and fgfr1+/- EBs similarly express endothelial and hematopoietic markers between days 7 and 10 of differentiation and the skeletal muscle markers myogenin and myoD at day 16. This is in keeping with the ability of fgfr1-null embryos to form various mesodermal tissues including allantois, amnion, visceral yolk sac mesoderm, and blood.14,15 Also, fgfr1+/- ES cells originate muscle cells when injected in nude mice to form teratomas15 and residual muscle tissue forms in heartless-null Drosophila embryos.63 In apparent contrast with our observations, a defective in vitro hematopoietic development has been described for fgfr1+/- EBs at early times of differentiation.64
Further experiments are required to elucidate the role of fgfr1 in early endothelial/hematopoietic differentiation. The fgfr1-null mutation appears to exert a rather selective effect on cardiomyocyte development in differentiating EBs. These conclusions are supported by the capacity of various FGF/FGFR signaling inhibitors to prevent cardiomyocyte development in differentiating fgfr1+/− ES cells without affecting the expression of the hematopoietic/endothelial marker flk-1. On the other hand, the capacity of the PKC activator PMA to partially rescue cardiomyocyte differentiation in fgfr1-null EBs points to the lack of an appropriate FGFR1-driven intracellular signaling as a major mechanism responsible for the impaired developmental process. Moreover, the ability of the MEK1/2 inhibitor U0126 to abolish PMA-triggered cardiomyocyte differentiation is in keeping with the cross-talk existing between PKC and the Ras/Raf-1/MEK/ERK signaling pathways, both activated by FGFR1 engagement. Interestingly, the lack of effect of the MEK1 inhibitor PD098059 point to a selective role for MEK2 in cardiomyocyte development. Also, our data implicate the PKCe isozyme in this developmental process, in keeping with its nuclear localization in ES-derived cardiomyocytes. Further experiments are required for a detailed characterization of the cross-talk between PKC/MEK signaling pathways during heart formation.

In mice, FGFR1-null embryos are developmentally retarded and die during gastrulation (see earlier). FGFR2 mutation results instead in a later embryonic lethality characterized by the lack of a functional placenta and limb buds. FGFR3-deficient mice are normal during gestation and exhibit bone alterations during postnatal development. Finally, FGFR4-null animals are developmentally normal. Thus, the different fgfr genes appear to exert distinct, non-overlapping functions during development. Our results demonstrate a nonredundant role for fgfr1 in cardiomyocyte development in vitro.

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References
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Expanded Materials and Methods

Reagents
SU5402 and bisindolylmaleimide I (GF109) were from Calbiochem; phorbol-12-myristate-13-acetate (PMA) and PD098059 were from Sigma; U0126 was from Promega. Antennapedia-conjugated PKC translocation inhibitors were gifted by D. Mochly-Rosen (Stanford University, CA).

EB immunostaining.
ES cell aggregates at day 10 of differentiation were transferred to microtubes, washed with PBS and fixed with 3% paraformaldehyde for 1 hour at 4°C. After extensive washes with PBS, aggregates were suspended subsequently in 12% saccharose/PBS (1h at 4°C), 15% saccharose/PBS (1h at 4°C), and 18% saccharose/PBS (overnight at 4°C). The day after, the solution was replaced by Tissue-Tek® O.C.T. compound (Miles Scientific) for 2 min. and immediately frozen in liquid nitrogen. The samples were placed in cryostat, and 7 μM thick sections were collected onto pre-coated gelatin glass slides. Then, slides were processed with TSA™ Biotin System (Perkin Elmer Life Science) following manufacturer’s instructions. Monoclonal antibody BA-G5 directed against cardiac α–myosin heavy chain (a gift from S. Schiaffino, University of Padova, Italy) was used at 1:2000 dilution. At the end of the procedure, slides were stained for 10 minutes with a 30 μmol/L solution of 4',6-Diamidino-2-phenylindole (DAPI, Sigma), mounted in 20% Mowiol (Sigma), and observed under a fluorescence microscope.
Table 1. Specific PCR primers utilized in the present study.

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<th>Target gene</th>
<th>Forward PCR oligonucleotide</th>
<th>Reverse PCR oligonucleotide</th>
<th>Nº of cycles</th>
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<td>5'-CACAAACCCCGAAGAAGACA</td>
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Figure 1. Titration analysis of PCR amplification of *tubulin* and *Nkx2.5* mRNAs. Complementary DNAs were synthesized from a dilution series of total cellular RNA (0.0313, 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 μg/20 μl). Samples were extracted from both *fgfr1<sup>+/−</sup>* and *fgfr1<sup>−/−</sup>* ES cells at day 9<sup>th</sup> of differentiation. cDNAs were amplified for the indicated PCR cycles and products were quantified by computerized image analysis of the agarose gel.