Fibroblast growth factor receptor-1 phosphorylation requirement for cardiomyocyte differentiation in murine embryonic stem cells

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Abstract

Fibroblast growth factor receptor-1 (Fgfr1) gene knockout impairs cardiac and haematopoietic development in murine embryonic stem cells (mESC). In FGFR1, tyrosine residues Y653 and Y654 are responsible for its tyrosine kinase (TK) activity whereas phosphorylated Y463 and Y766 represent docking sites for intracellular substrates. Aim of this study was the characterization of FGFR1 signalling requirements necessary for cardiomyocyte differentiation in mESC. To this purpose, fgfr1−/− mESC were infected with lentiviral vectors harbouring human wild-type hFGFR1 or the Y653/654F, Y463F and Y766F hFGFR1 mutants. The resulting embryonic stem (ES) cell lines were differentiated as embryoid bodies (EBs) and beating foci formation was evaluated. In order to appraise the presence of cells belonging to cardiovascular and haematopoietic lineages, specific markers were analysed by quantitative PCR, whole mount in situ hybridization and immunofluorescence. Transduction with TKY hFGFR1 or the TKY Y766F-hFGFR1 mutant rescued cardiomyocyte beating foci formation in fgfr1−/− EBs whereas the TK− Y653/654F-hFGFR1 mutant and the TK− Y463F-hFGFR1 mutant were both ineffective. Analysis of the expression of early and late cardiac markers in differentiating EBs confirmed these observations. At variance with cardiomyocyte differentiation, all the transduced TK− FGFR1 forms were able to rescue haematopoietic differentiation in EBs originated by infected fgfr1−/− mESC, only the TK− Y653/654F-hFGFR1 mutant being ineffective. In keeping with these observations, treatment with different signalling pathway inhibitors indicates that protein kinase C and ERK activation are essential for cardiomyocyte but not for haematopoietic differentiation in EBs generated by fgfr1−/− mESC. In conclusion, our results suggest that, although FGFR1 kinase activity is necessary for both cardiac and haematopoietic lineage maturation in mESC, phosphorylation of Y463 in the intracellular domain of the receptor is a specific requirement for cardiomyocyte differentiation.

Keywords: FGFR • embryonic stem cells • cardiomyocyte

Introduction

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 rightward looping followed by segmentation and growth of cardiac chambers [1]. Cardiac development involves several stages: early mesodermal differentiation, generation of cardiovascular common progenitors that can differentiate in cardiomyocytes, smooth muscle cells and endothelial cells [2–4], cardiac lineage commitment of cardiac progenitor (CP) cells and maturation of functional cardiomyocytes [5]. The classical view of cardiogenesis has been recently modified by the demonstration that two distinct mesodermal heart fields with a common origin contribute to heart development in a temporally and spatially specific manner [6]. The expression of tbx5, a T-box transcription factor that is initially expressed throughout the cardiac crescent, appears lately to be restricted to derivatives of the first/primary heart field (FHF) [6–8], whereas the progenitors of the secondary heart field (SHF) are characterized by the expression of fgf10 and of the LIM domain homeobox gene isl1 [9, 10]. Both FHF and SHF progenitors express instead the homeodomain transcription factor nkrx2.5 [6, 11].
Most of these recent developmental findings involved the use of animal models paralleled by in vitro differentiation studies on murine embryonic stem cells (mESC). mESC originate from the inner cell mass of the blastocyst and are capable of cell renewal and differentiation after in vitro aggregation into three-dimensional structures termed embryoid bodies (EBs) [12, 13]. Cardiac development in mESC differentiation cultures is well established and is easily detected by the appearance of areas of contracting cells that display characteristics of mature cardiomyocytes [14]. All of the cardiac cell types have been generated from differentiating EBs, and gene expression analyses suggest that their development in culture recapitulates cardiogenesis in the early embryo [15, 16]. Indeed, CP cells originated from both FHF and SHF, characterized by the common expression of nkx2.5 and by the selective expression of tbx5 and fgf10/ist1, have been isolated from differentiating ESC populations [2, 3].

Fibroblast growth factor receptors (FGFRs) belong to the subclass IV of membrane-spanning tyrosine kinase (TK) receptors [17]. Four fgf genes have been identified: FGFR1–4 proteins share common structural features and interact with the members of the FGFR family comprising of at least 23 polypeptides [18]. The FGFR system has been implicated in a variety of physiological and pathological conditions, including embryonic development, tissue growth and remodelling, inflammation, tumour growth and vascularization [19, 20]. Following ligand binding and receptor dimerization, a number of tyrosine autophosphorylation sites have been identified in human FGFR1 (hFGFR1); Y653/654 are critical for TK activity [21], Y663 is involved in endothelial cell proliferation by binding to Src homology (SH)2/SH3 domain-containing adaptor protein Crk [22] and phosphorylated Y766 has shown to bind phospholipase C-γ (PLC-γ) in L6 myoblasts, Shb in endothelial cells and Grb14 in MDA-MB-231 human breast cancer cells [23–25]. Also, FGFR1 activation leads to FRS2 phosphorylation [26] followed by Grb2 and Shp2 interactions [27], FRS2, Crk and Shb binding to FGFR1 affect the classical Ras/Raf-1/MEK/ERK/Jun proliferation pathway activated by TK receptors, while PLC-γ activates protein kinase G (PKG) [28], whose role in cardiomyocyte differentiation has been demonstrated [29]. FGF/FGFR signalling plays important functions in mesoderm formation and development [30]. Accordingly, fgf1–/– mice die during gastrulation, displaying defective mesoderm pattern with reduction in the amount of paraxial mesoderm and lack of somite formation [31, 32]. Studies on chimeric embryos using FGFR1-deficient mESC revealed an early defect in the mesoderm and endodermal cell movement through the primitive streak, followed by deficiencies in contributing to anterior mesoderm, including heart tissue [33, 34]. The pivotal contribution of FGF signalling in heart formation has been demonstrated in different animal models: in C. intestinalis, FGF signalling delineates the cardiac progenitor field [35]; in Drosophila, mesoderm spreading depends upon the expression of heartless, homologous to vertebrate fgf1, and the heartless mutant is characterized by the absence of the heart [36, 37]; in chicken, FGF signalling activated by FGF8 contributes to the heart-inducing properties of the endoderm [38]; in zebrafish, induction and differentiation of the heart requires FGF8 [39]; in mice, Fgf8+/– mutants show complex cardiac defects [40].

FGFR1 has been implicated in cardiac development also during murine EB differentiation. Indeed, analysis of the in vitro differentiation process of fgfr1–/– mESC following EB formation led us to determine a non-redundant role for FGFR1 in cardiomyocyte development [41]. Moreover, the FGFR1 TK inhibitor SU 5402 [42], the MEK1/2 inhibitor U0126 [43] and the classical/novel PKC inhibitor GF109203X [44] were all able to hamper beating foci formation in EBs originated by fgfr1–/– mESC, further implicating the FGFR1 TK activity, ERK and PKC in cardiomyocyte differentiation.

Here, in order to further define the requirements for FGFR1 signalling in cardiomyogenesis, fgfr1–/– mESC were transduced via a lentiviral vector system with hFGFR1 or with hFGFR1 forms mutated in different tyrosine autophosphorylation sites. Analysis of the differentiative capacity of EBs originated by the different mESC populations demonstrates the non-redundant role for the tyrosine autophosphorylation site Y493 in cardiomyocyte development. Y493 phosphorylation appears instead to be dispensable for haematopoietic differentiation, thus indicating that distinct FGFR1-dependent signalling pathways are required for cardiomyocyte and haematopoietic differentiation in mESC.

Materials and methods

ES cell culture

Murine ESC [32, 41] were adapted to grow without feeder cells and maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 20% foetal bovine serum (HyClone, South Logan, UT, USA), 0.1 mmol/l non-essential amino acids, 1 mmol/l sodium pyruvate, 0.1 mmol/l β-mercaptoethanol, 2 mmol/l L-glutamine and 1000 U/ml LIF (ESGRO, Millipore, Milan, Italy). At T0 of differentiation, exponentially growing mESC 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 0.5% agarose-coated dishes and grown for 5 days in LIF-deprived EB medium. At day 7, EBs were transferred into 24-well tissue culture plates and allowed to adhere. Aggregates were monitored for the appearance of spontaneously contracting foci during the following days.

RNA extraction, semi-quantitative and quantitative RT-PCR analysis

Total RNA was extracted from mESC as described [45]. Contaminating DNA was digested using DNAse, following indications reported in RNeasy® Micro Handbook (Qiagen, Milan, Italy). Two μg of total RNA were retro-transcribed with MMLV reverse transcriptase (Invitrogen, Milan, Italy) using random hexamers in a final 20 μl volume. For semi-quantitative PCR, 2 μl of the retrotranscribed RNA were subjected to polymerase chain reaction (PCR) using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma, Milan, Italy). Oligonucleotide primers and PCR conditions were described previously [41]. The data were confirmed by analysing RNA extracted from two or more independent differentiation experiments. Quantitative PCR was performed with a Biorad iCycler IQ™ Real-Time PCR Detection System...
using a qRTM SYBR Green Supremix (Biorad, Milan, Italy) according to manufacturer’s instructions. The qPCR-specific primers (final concentration 400 nM) were as follows: nkh2.5 forward (for) primer: 5’-ACAGGCTGACAGTCTCTGTTCTCC-3’; reverse (rev) primer: 5’-CTGTCATGTCACCATCTTCTGTCGTTCCGTTG-3’; tubulin for: 5’-TTGGATCCGCAAGGCCTGGAAT-3’; reverse (rev) primer: 5’-GCGTTCGACGATGACAGTCCG-3’. 

**Vector production and transduction**

Human FGFR1, Y653/654F-hFGFR1, Y463F-hFGFR1 and Y766F-hFGFR1 cDNAs [47] were independently cloned in the transfer vector pRRL-SIN-PPT-hPGK-GFP-WPRE by replacing green fluorescent protein (GFP) gene [48]. Viral particles were produced, purified by ultracentrifugation and used to infect murine fgfr1–/– mESC following EBs formation had shown that FGFR1 is essential for normal cardiac development [49]. As a control experiment, fgfr1–/– EBs (data not shown).

**Binding assay and immunoblot analysis**

125I-FGF2 binding assay was described previously [45]. Briefly, human recombinant FGF2 was labelled with 125I (37 GBq/ml; GE Healthcare Life Sciences, Milan, Italy) using Iodogen (Pierce, Rockford, IL, USA) at a specific activity of 1800 cpm/fmol. mESC were seeded on 10% SDS-PAGE and transferred to Immobilon-P (Millipore, Billerica, MA, USA) with methanol 100% and stored at –20°C until hybridization. EBs were rehydrated and rinsed twice in PBS, 0.1% Tween® 20 (PBT), then digested with proteinase K (100 μg/ml in PBT) for 15 min. at room temperature, followed by incubation in 4% PFA in PBS for 20 min. EBs were subsequently rinsed twice in PBT for 5 min. and pre-hybridized at 65°C in hybridization mix (HM: 50% formamide, 5 × SSC, 10 mM citric acid pH 6, 0.1% Tween® 20, 50 μg/ml heparin, 50 μg/ml tRNA) for 2 hrs. EBs were then incubated overnight at 65°C in HM containing 1 μg/ml of denatured riboprobe. On the second day, EBs were sequentially washed in 2× SSC containing 75%, 50%, and 25% of hybridization wash (50% formamide, 5 × SSC, 10 mM citric acid pH 6, 0.1% Tween® 20) at 65°C for 15 min. each, followed by three washes with 0.2 × SSC at 65°C for 30 min. EBs were then rinsed at room temperature with increasing concentrations of PBT (25%, 50% and 75%, respectively, 10 min. each) in 0.2 × SSC, incubated for 3 hrs in blocking buffer (BB: 2% sheep serum, 2 mg/ml BSA in PBT), and immunodecorated overnight at 4°C in BB containing 10,000 alkaline phosphate-coupled anti-digoxigenin antibody (Roche Diagnostics). On the following day, EBs were extensively washed with PBT and the reaction was developed in staining solution [100 mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween® 20, 500 μM Tetramisole, 0.5% NBT and 0.5% BCIP (Roche Diagnostics)] following manufacturer’s instruction. Hybridized EBs were post-fixed for 20 min. in 4% PFA in PBS and subsequently dehydrated and included in paraffin. Seven μm sections were cut, mounted with DPX (Fluka, Milan, Italy), observed and photographed under a Zeiss Axioshot2 stereomicroscope.

**Immunostaining**

EBs were grown from day 7 of differentiation in LabTekTM Chamber Slide™ System (Nunc, Rochester, NY, USA). At day 10 of differentiation, they were fixed overnight at 4°C in zinc fix (0.1 M Tris HCl, pH 7.5, 3 mM calcium acetate, 23 mM zinc acetate and 37 mM zinc chloride). After blocking unspecific binding in 3% BSA for 1 hr at room temperature, EBs were incubated overnight at 4°C with primary mouse anti-troponin T monoclonal antibody (Abcam, Cambridge, UK). The following day, EBs were washed with PBT and the reaction was developed in staining solution [100 mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween® 20, 500 μM Tetramisole, 0.5% NBT and 0.5% BCIP (Roche Diagnostics)] following manufacturer’s instruction. Hybridized EBs were post-fixed for 20 min. in 4% PFA in PBS and subsequently dehydrated and included in paraffin. Seven μm sections were cut, mounted with DPX (Fluka, Milan, Italy), observed and photographed under a Zeiss Axioshot2 stereomicroscope.

**Whole mount in situ hybridization (WISH)**

Total RNA from fgfr1–/– EBs at day 10 of differentiation was reverse transcribed to cDNA and used as template for PCR reactions using the following oligonucleotide primers: myd2 for: 5’-GGCAAGAAGGGGATAGGAGG; rev: 5’-CTCTGTGGTTGGCGGCTAGCT; chdh for: 5’-TTTGAAATCAAATGCGA-CACTGA; rev: 5’-TCTGTCATGTCACCATCTTCTGTCGTTCCGTTG. Fragments were subcloned into pCR®II-TOPO® vector (Invitrogen). The plasmids were linearized and used as template for RNA synthesis with T7 or SP6 polymerase for antisense and sense control probes in the presence of digoxigenin-11-UTP by using DIG RNA labelling kit (Roche Diagnostics, Milan, Italy).

At day 10 of differentiation, EBs were fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), dehydrated with methanol 100% and stored at –20°C until hybridization. Fixed EBs were rehydrated and rinsed twice in PBS, 0.1% Tween® 20 (PBT), then digested with proteinase K (10 μg/ml in PBT) for 15 min. at room temperature, followed by incubation in 4% PFA in PBS for 20 min. EBs were subsequently rinsed twice in PBT for 5 min. and pre-hybridized at 65°C in a hybridization mix (HM: 50% formamide, 5 × SSC, 10 mM citric acid pH 6, 0.1% Tween® 20, 50 μg/ml heparin, 50 μg/ml tRNA) for 2 hrs. EBs were then incubated overnight at 65°C in HM containing 1 μg/ml of denatured riboprobe. On the second day, EBs were sequentially washed in 2× SSC containing 75%, 50%, and 25% of hybridization wash (50% formamide, 5 × SSC, 10 mM citric acid pH 6, 0.1% Tween® 20) at 65°C for 15 min. each, followed by three washes with 0.2 × SSC at 65°C for 30 min. EBs were then rinsed at room temperature with increasing concentrations of PBT (25%, 50% and 75%, respectively, 10 min. each) in 0.2 × SSC, incubated for 3 hrs in blocking buffer (BB: 2% sheep serum, 2 mg/ml BSA in PBT), and immunodecorated overnight at 4°C in BB containing 1:10,000 alkaline phosphate-coupled anti-digoxigenin antibody (Roche Diagnostics). On the following day, EBs were extensively washed with PBT and the reaction was developed in staining solution [100 mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween® 20, 500 μM Tetramisole, 0.5% NBT and 0.5% BCIP (Roche Diagnostics)] following manufacturer’s instruction. Hybridized EBs were post-fixed for 20 min. in 4% PFA in PBS and subsequently dehydrated and included in paraffin. Seven μm sections were cut, mounted with DPX (Fluka, Milan, Italy), observed and photographed under a Zeiss Axioshot2 stereomicroscope.

**Results and discussion**

**Transduction of hFGFR1 in murine fgfr1–/– mESC**

Analysis of the in vitro differentiation process of fgfr1–/– and fgfr1–/– mESC following EBs formation had shown that FGFR1 is...
Phosphorylation was evaluated by incubation with anti-phospho-ERK 1/2 antibody and processing for Western blot analysis. ERK1/2 phosphorylation was directly lysed in reducing sample buffer. Samples were loaded on 10% SDS-PAGE and processed for Western blot analysis. ERK1/2 phosphorylation was judged by incubation of the same membrane with anti-ERK2 antibody (sc-7383, Santa Cruz Biotechnology Inc.), while uniform loading was assessed from fgfr1 populations. 

The mean of at least four independent experiments was judged by incubation of the same membrane with anti-ERK2 antibody (sc-7383, Santa Cruz Biotechnology Inc.), while uniform loading was judged by incubation of the same membrane with anti-Erk2 antibody (sc-1647, Santa Cruz Biotechnology Inc.).

Cardiomyocyte differentiation in TK⁺ and TK⁻ hFGFR1 transduced fgfr1⁻/⁻ mESC

At day 7 of differentiation, EBs derived from the different mESC populations were allowed to adhere to the substratum and monitored during the following days for the appearance of spontaneously contracting foci (Fig. 2A). As anticipated, a high percentage of fgfr1⁻/⁻ EBs generates pulsating cardiomyocytes 1 day after attachment whereas fgfr1⁻/⁻ EBs show an increase in high-affinity 125I-FGF2 binding when compared with control cells (Fig. 1B), indicating that both TK⁺ and TK⁻-transduced receptors are exposed on the cell surface. It must be pointed out that a certain amount of high-affinity 125I-FGF2 binding was observed also in fgfr1⁻/⁻ mESC, probably due to the ability of FGF2 to bind other FGFRs [52]. Finally, to assess the signalling capacity of the transduced receptors, undifferentiated ES cell populations were stimulated with exogenous recombinant FGF2 and ERK12 phosphorylation was evaluated by Western blotting. As shown in Fig. 1C, significant levels of activated ERK12 were observed in FGF2-stimulated fgfr1⁻/⁻ mESC and in fgfr1⁻/⁻ mESC transduced with the TK⁻ hFGFR1, but not in non-infected fgfr1⁻/⁻ mESC and in fgfr1⁻/⁻ mESC transduced with the TK⁺ hFGFR1 and TK⁻ Y653/654F-hFGFR1 mutant. On this basis, we assessed the capacity of TK⁺ hFGFR1 and TK⁻ Y653/654F-hFGFR1 to rescue cardiomyocyte differentiation in the mESC fgfr1 knockout background.

Fig. 1 Characterization of fgfr1⁻/⁻ mESC transduced with TK⁺ or TK⁻ hFGFR1. Fgfr1⁻/⁻ mESC were infected with a lentiviral vector carrying either TK⁺ (wt) or TK⁻ Y653/654F-hFGFR1 cDNAs. (A) Total RNA was extracted from fgfr1⁻/⁻ (+/-), fgfr1⁻/⁻ (-/-), and the infected mESC populations v-wt-hFGFR1 (TK⁺), and v-Y653/654F-hFGFR1 (TK⁻). Equivalent amounts of cDNA were amplified by PCR using the indicated primers. The tubulin gene was used for normalization. (B) fgfr1⁻/⁻ (+/-), fgfr1⁻/⁻ (-/-), v-wt-hFGFR1 (TK⁺) and v-Y653/654F-hFGFR1 (TK⁻) mESC were seeded at 250,000 cells/cm² and incubated for 2 hr at 4°C with 125I-FGF2. At the end of the incubation, cells were washed and radioactivity bound to high-affinity sites was evaluated. Data represent the mean of at least four independent experiments ± S.D. (C) fgfr1⁻/⁻ (+/-), fgfr1⁻/⁻ (-/-), v-wt-hFGFR1 (TK⁺) and v-Y653/654F-hFGFR1 (TK⁻) mESC were seeded at 200,000 cells/cm² and starved in 1% FCS overnight. Then, cells were stimulated with FGF2 (100 ng/ml) for 15 min. and directly lysed in reducing sample buffer. Samples were loaded on 10% SDS-PAGE and processed for Western blot analysis. ERK12 phosphorylation was evaluated by incubation with anti-phospho-ERK12 antibody (sc-7383, Santa Cruz Biotechnology Inc.), while uniform loading was judged by incubation of the same membrane with anti-Erk2 antibody (sc-1647, Santa Cruz Biotechnology Inc.).
Previous observations had shown that differentiating fgfr1^{+/−} EBs do not express the transcription factor nkx2.5, a marker of cardiac progenitors of both FHF and SHF [5, 41]. On this basis, to assess the effect of transduced TK^{+/+} hFGFR1 and TK^{−/−} hFGFR1 on the block of cardiomyocyte differentiation in fgfr1^{−/−} mESC, total RNA was extracted from differentiating EBs and analysed for the expression of nkx2.5 and fgf10 by quantitative PCR (qPCR), using the relative undifferentiated state as reference sample. As shown in Fig. 2B, nkx2.5 is strongly up-regulated in fgfr1^{+/−} EBs from day 3 of differentiation, whereas its expression remains at very low levels in fgfr1^{−/−} EBs until day 9. Transduction of TK^{+} hFGFR1 in fgfr1^{−/−} EBs restores the up-regulation of nkx2.5 expression to levels similar to those observed in fgfr1^{+/−} EBs, while transduction of the TK^{−} hFGFR1 mutant is ineffective. Similar results were obtained for fgf10 expression: again, a significant up-regulation of fgf10 expression was observed in EBs generated by fgfr1^{+/−} and v-wt-hFGFR1 mESC but not by v-Y653/654F-hFGFR1 mESC (Fig. 2B).

We had previously demonstrated that the expression of the cardiogenic mesodermal marker met2c is not affected in differentiating fgfr1 knockout mESC [41]. Since Met2c cooperates with Nkx2.5 and other cardiac transcription factors in inducing downstream effectors [53], we hypothesized that FGFR1 may mediate cardiomyocyte differentiation by activating Nkx2.5 in Mef2c^{+} cardiogenic mesodermal cells. The present data confirm the non-redundant role of FGFR1 in nkx2.5 up-regulation and cardiomyocyte differentiation. However, in all our studies, the expression analysis of cardiac markers in differentiating mESC has been performed on the total RNA extracted from the whole EB. Considering that the expression of these markers is not always restricted to a single cell population, only the isolation of the different cardiomyocyte progenitor populations (reviewed in [5]) will allow the precise
identification of the step(s) in the cardiomyocyte differentiation pathway characterized by a non-redundant role for FGFR1. For instance, the FHF and SHF markers Tbx5 and Isl1, whose expression is not limited to heart tissue [54, 55], show similar up-regulation kinetics in fgfr1/H11001, fgfr1/H11002–, and the infected v-wt-hFGFR1 and v-Y653/654F-hFGFR1 mESC populations (data not shown).

To further characterize differentiating transduced EBs, the expression of structural markers belonging to different lineages of mesodermal origin was evaluated by qPCR and whole mount in situ hybridization (WISH). FGFR1 is implicated in cardiac and haematopoietic development but is dispensable for endothelial cell differentiation in murine EBs [41, 49]. Accordingly, fgfr1/H11001 and TK/H11001 v-wt-hFGFR1 mESC, but not fgfr1/H11002– and TK/H11002 v-Y653/654F-hFGFR1 cells, express the cardiac ventricular myosin light chain (myl2) and the primitive haematopoietic marker CD41 (itga2b) at day 10 of differentiation (Fig. 2C and D). All the cell lines express instead the endothelial markers vcam1 (Fig. 2C) and vascular endothelial cadherin (cdh5) (Fig. 2D) at similar levels.

Thus, viral transduction in a fgfr1 null background of TK+/hFGFR1, but not of the TK– Y653/654F hFGFR1 mutant, is sufficient to rescue the ability of mESC to undergo cardiomyocyte and haematopoietic differentiation.

Role of Y463 and Y766 FGFR1 residues in cardiomyocyte differentiation

Seven autophosphorylation sites have been identified in the cytoplasmic domain of hFGFR1 [21]. Among them, Y463 and Y766 represent docking sites for intracellular molecules. To assess the relative contribution of these residues in transducing signal(s) for cardiomyocyte development, we infected fgfr1+/– and TK+ v-wt-hFGFR1 mESC with lentiviral particles harbouring the cDNA for the TK+ hFGFR1 mutants Y463F or Y766F. Again, the efficient transduction of the receptor mutants was confirmed by RT-PCR (data not shown) and 125I-FGF2 binding assay (Fig. 3A), whereas their TK+ signalling
capacity was judged by FGF2-induced ERK1/2 phosphorylation (data not shown). Next, v-Y463F-hFGFR1 and v-Y766F-hFGFR1 mESC underwent the standard differentiation protocol and the appearance of beating cardiomyocytes was monitored. At day 10 of differentiation, v-Y463F-hFGFR1 EBs showed a dramatic impairment in generating beating foci when compared to v-Y766F-hFGFR1 EBs whose ability was only marginally reduced in respect to v-wt-hFGFR1 EBs (Fig. 3B). In agreement with the microscopic observations, analysis of Y463F-hFGFR1 EBs showed a strong reduction of the cardiac gene transcripts nkx2.5 and myl2 (Fig. 3C and E) and of cardiac structural protein troponin T (Fig. 3F). As expected, no down-regulation was seen in the levels of expression of the endothelial markers vcam1 and cdh5 (Fig. 3D and E).

Because of the involvement of FGFR1 in ES haematopoietic development (see above), v-Y463F-hFGFR1 and v-Y766F-hFGFR1 EBs were also evaluated for the expression of haematopoietic markers (Fig. 3D). In contrast with what observed for cardiac markers, the transcript levels of the primitive integrin itga2b, of the pan tyrosine phosphatase CD45 (ptprc), and of the embryonic beta-like globin gene hbb-bh1 were similar in v-Y463F-hFGFR1 and v-Y766F-hFGFR1 EBs and comparable to those detected in v-wt-hFGFR1 EBs. Thus, our data strongly suggest that Y463 residue in FGFR1 is specifically involved in cardiomyocyte differentiation of mESC. Moreover, this residue, together with residue Y766, is dispensable for FGFR1-mediated haematopoietic development.

We have previously observed that beating cardiomyocyte differentiation in fgfr1−/− EBs can be hampered by ERK1/2 and PKC inhibitors [44]. To get further insights about the signalling cascade that leads to cardiomyocyte differentiation in mESC and to assess the possibility to dissociate cardiac and haematopoietic differentiation at pharmacological level, as suggested by distinct tyrosine autophosphorylation requirements in FGFR1, differentiating fgfr1−/− EBs were treated with different signalling inhibitors and assessed for the expression of lineage-specific markers (Fig. 4). In keeping with previous observations [41], beating foci formation and the expression of the cardiac structural marker myl2 were strongly reduced by 10 μM ERK1/2 inhibitor U0126 [43] or by 2 μM PKC inhibitor GF109203X [44], whereas the expression of the haematopoietic marker itga2b and of the endothelial marker vcam1 were not affected. Moreover, MEK inhibitor PD98059 [56], PLC-γ inhibitor U73122 [57], or p38 inhibitor SB203580 [58], when used at the recommended concentrations of 20 μM [59], 5 μM [60] and 3 μM [61], respectively, did not affect any of the differentiation processes investigated (Fig. 4). No inhibition in beating foci formation was obtained also when the same inhibitors were added at higher non-toxic concentrations, like 50 μM for
PD98059 and 20 μM for both U73122 and SB 203580 (data not shown). Thus, the data confirm the role of ERK1/2 and PKC signalling in cardiomyocyte formation and indicate that different signalling requirements are involved in FGFR1-dependent haematopoietic differentiation.

Phosphorylation of Y463 in FGFR1 leads to the Crk-mediated activation of the Ras/Raf-1/MEK/ERK/Jun pathway in endothelial cells [22], whereas Y766 phosphorylation activates PLCγ in myoblasts and triggers the hydrolysis of phosphatidylinositol that, in turn, stimulates PKC [62]. The ability of the Y766-hFGFR1 mutant to restore cardiomyocyte differentiation in transduced fgfr1−/− mESC and the lack of inhibitory effect of the PLC-γ inhibitor U73122 on the appearance of beating foci in fgfr1−/− EBs rule out a role for PLC-γ in the PKC-dependent pathway involved in cardiomyocyte formation [29]. On the other hand, the ability of the ERK1/2 inhibitor U0126, but not of the MEK inhibitor PD98059, to hamper beating foci formation in fgfr1−/− EBs as well as in fgfr1−/− EBs treated with the PKC activator phorbol mirestate acetate [41] suggests that ERK1/2 activation required for cardiomyocyte differentiation is triggered by a PKC-dependent pathway rather than by the classical Ras/Raf-1/MEK pathway that FGFR1 dimerization activates in NIH3T3 fibroblasts [63]. Indeed, ERK2 can be activated by several PKC isoforms in Swiss 3T3 fibroblasts [29], including the novel PKC γ and ε isoforms involved in cardiomyocyte differentiation [64]. Clearly, the possibility exists that PKC and ERK pathways may play complementary roles at different times and/or in different cell types during EB differentiation.

At present, the signalling cascade triggered by the autophosphorylation of Y463 in FGFR1 and its cross-talk with PKC- and ERK-mediated signalling during cardiomyocyte differentiation of mESC remains to be elucidated. Indeed, Western blot analysis of total EB protein extract at day 9 of differentiation does not show any difference in CrkII and Shp2 phosphorylation levels among fgfr1−/−, v-wt-hFGFR1, v-Y463F-hFGFR1 and v-Y653/654F-hFGFR1 EBs, thus indicating that the overall activation of these signalling molecules in differentiating EBs is not restricted to FGFR1 activity (data not shown). Also, FRS2 appears to be phosphorylated in both v-Y463F-hFGFR1 and v-wt-hFGFR1 EBs but not in TK− v-Y653/654F-hFGFR1 and fgfr1−/− EBs, indicating that its activation depends on the TK activity of FGFR1 but not on Y463 phosphorylation (data not shown). Again, only the isolation of the different cardiomyocyte progenitor populations from these EBs will allow the identification of the FGFR1-dependent signalling pathway(s) involved in cardiomyocyte differentiation in mESC. Nevertheless, our data demonstrate for the first time a specific non-redundant role for residue Y463 of FGFR1 in cardiomyocyte differentiation.

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