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# Embryonic Stem Cells as a Model System to Elucidate Early Events in Cardiac Specification and Determination

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## 1. Introduction

Cardiac development is a highly ordered process that involves several steps whose features are conserved from insects to vertebrates. In the mouse, two distinct mesodermal heart fields with a common origin contribute to heart development in a temporally and spatially specific manner (Buckingham *et al.* 2005). Heart progenitor cells located in the primitive streak migrate anteriorly and spread laterally to form the two paired primary heart fields (FHFs). Mesodermal precursors for heart-forming cells express initially Brachyury T, a T-box transcription factor, and lately, at the precardiac stage, the mesoderm posterior 1 (Mesp1) marker (Solloway and Harvey 2003). Later in development, cardiac precursors coalesce into the linear heart tube and ultimately give rise to the left ventricle of the mature four-chambered mammalian heart. The secondary heart field (SHF) is instead derived from cells of the pharyngeal and the splanchnic mesoderm that will migrate into the developing heart and give rise to the right ventricle, the outflow tract, and portions of the inflow tract (Chien *et al.* 2008). FHF and SHF are characterized by the expression of specific genes: the T-box protein *tbx5* and the first wave of Nkx2.5 appear to be restricted to derivatives of the FHF (Bruneau *et al.* 2001; Zaffran *et al.* 2004; Buckingham *et al.* 2005), whereas SHF is marked by the expression of the LIM domain homeobox gene *isl1*, or the second wave of Nkx2.5 (Kelly *et al.* 2001; Cai *et al.* 2003). Finally cardiomyocyte progenitor cells that contribute to the atrial and ventricular myocardium have been identified also within the epicardium, and are marked by the expression of either Wt1 or Tbx18 (Cai *et al.* 2008; Zhou *et al.* 2008).
Instructions for cardiac commitment derive from neighboring embryonic tissues (Abu-Issa and Kirby 2007). Cell commitment occurs in two stages: specification and determination. While a cell is specified if it differentiates toward the definitive lineage in a neutral environment, it is determined if it differentiates in an antagonistic environment. Cardiac specification factors include activin or Transforming Growth Factor (TGF)-β while bone

morphogenetic proteins (BMPs) and FGFs are considered as determination factors (Ladd *et al.* 1998). These inductive signals, summarized in Fig. 1, are released in a precisely timed and spatially regulated fashion (Zaffran and Frasch 2002). Generally, endoderm-derived signals act as inducers of cardiac mesoderm formation, while ectoderm secretes inhibitory factors.
Pro-cardiogenic molecules comprise specification factors, determination factors, which includes Wnt/b-catenin, TGF-ß family, Bone Morphogenetic Proteins (BMPs) and Cripto, Sonic Hedgehog, Crescent (Harvey 2002; Foley and Mercola 2004).

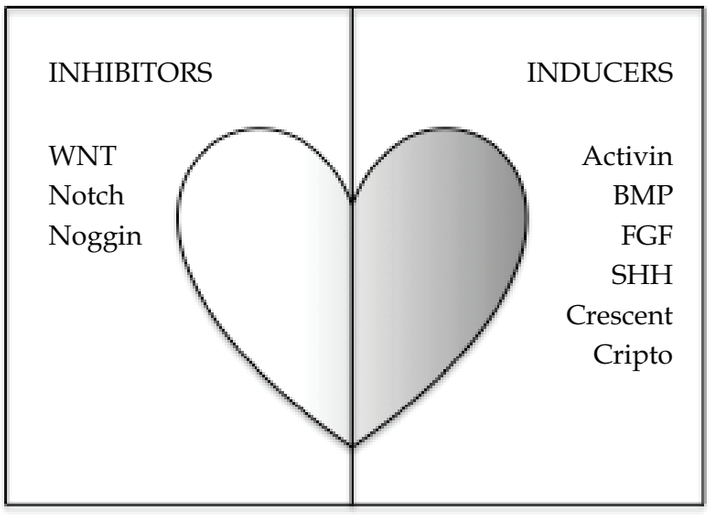



Fig. 1. Schematic diagram of cardiogenic inhibitors and inducers

At variance, myocardial differentiation is inhibited by Notch and by canonical Wnt signal through β-catenin (for a complete overview of the cardiac developmental process see (Kirby 2007). However, the scenario of the heart inducer morphogens is still incomplete, and several hints may come from *in vitro* study of the embryonic stem cell (ESC) differentiation process.
Since ESCs possess the ability to give rise to all cell lineages (Nishikawa *et al.* 2007), they represent a powerful approach to elucidate the origin and the molecular identity of the cardiovascular progenitor populations. Indeed, all the cells belonging to the cardiovascular lineage have been generated in the embryoid bodies (EBs) formed during *in vitro* ESCs differentiation protocol and gene expression analysis suggest that their development in culture recapitulates cardiogenesis in the early embryo (Boheler *et al.* 2002).
ESCs are a promising tool in cellular therapy for the repair of injured myocardium. Heart failure represents a major cause of death and hospitalization in Western countries. Because of the limited capacity of mammalian heart to regenerate, the lonely therapy for CM loss is cardiac transplantation. However, the possibility to replace damaged heart tissue with cells capable of *in situ* differentiation and of myocardial integration is very attractive. Indeed the identification, isolation, and characterization of murine ESC (mESC)-derived cardiac progenitor cells (CPCs) on the basis of Brachyury/Flk1 (Kattman *et al.* 2006), Isl1/Flk1/Nkx2-5 (Moretti *et al.* 2006), cKit/Nkx2-5 (Wu *et al.* 2006), or Nkx2-5 (Christoforou *et al.* 2008) expression, has been recently reported by several groups. These cells represent a promising source for heart repair because of their restricted capacity to differentiate into cardiac muscle, smooth muscle, and vascular endothelium (Kattman *et al.* 2006; Moretti *et al.* 2006; Wu *et al.* 2006; Christoforou *et al.* 2008). Indeed, when injected in infarcted area of

murine heart, CPCs engrafted and differentiated into CMs, as well as contributed to neovascularization, thus improving the cardiac function of treated animals (Christoforou *et al.* 2010).

As stated before, the *in vitro* differentiation process of mESC mostly recapitulates the embryonic development and indeed CMs derived from mESC resemble beating cells of the embryonic heart tube (Fijnvandraat *et al.* 2003). By combining several data, the cardiac differentiation process can be traced relying on the expression of molecular markers (Fig. 2).

The cardiogenic mesoderm will give rise to a cardiovascular precursor (CVP) population characterized by the expression of Flk1, Nkx2.5, Isl1, and c-kit. From these cells cardiomyocyte progenitors, as well as precursors of endothelial and smooth muscle cell lineages, will originate. Cardiomyocyte progenitors, identified by Nkx2.5, Gata4, and Mef2c expression, will afterwards differentiate into functional mature CMs, characterized by the appearance of specific structural proteins, e.g. myosin heavy chain (MHC)-α, -β, and the ventricular myosin Myl2.

Cardiomyocyte development in ESC differentiation cultures is well established and is easily detected by the appearance of areas of spontaneously contracting cells (beating foci) that display characteristics of mature CMs (Sachinidis *et al.* 2003). Beating cells are absent if the genetical manipulation of ESCs abrogates the expression of a gene involved in CM differentiation. The study of knocked-out ESCs has lead to the discovery of several genes, whose contribution to cardiogenesis could not be assessed in mice because of the early embryonic lethality. Indeed, the absence of EphB4 (Wang *et al.* 2004), Cripto-1 (Xu *et al.* 1998), Shp-2 (Qu and Feng 1998), FGFR1 (Dell'Era *et al.* 2003), Sik1 (Romito *et al.* 2010)., JSAP1 (Sato *et al.* 2005), and the overexpression of a constitutively active Rac (Puceat *et al.* 2003), severely impaired the appearance of beating foci. Due to the complexity of events, the blockade of cardiac differentiation can occur either in different step of CM differentiation or in cell lineages that secrete cardiac specification/determination peptides. Indeed, has been recently shown that the absence of CM differentiation in EphB4$^{-/-}$ mESC can be rescued by EphB4$^{+}$, CD31$^{+}$ endothelial cells (Chen *et al.* 2010).

In this review we will focus on two selected membrane receptor systems, FGFR1 and Cripto, both of them involved in cardiac mesoderm formation and patterning at different levels, whose contribution to murine early cardiac development has been established by studying mESC differentiation cultures.

## 2. Cripto

Cripto is the original member of a family of vertebrate signaling molecules, the EGF-CFC family (Ciccodicola *et al.* 1989) which includes human, mouse and chick Cripto; human and mouse Cryptic, Xenopus FRL-1 and Zebrafish OEP (one eyed-pinhead) (Shen and Schier 2000). Initially described as secreted molecules, members of this family are extracellular membrane proteins, anchored to the lipid bilayer through a glycosilphosphatidylinositol (GPI) moiety (Minchiotti *et al.* 2000).

Early studies of *cripto* were focused on its possible role in cell transformation and tumor progression (Salomon *et al.* 1999; Persico *et al.* 2001). *Cripto* expression was first found in human and mouse embryonal carcinoma cells and male teratocarcinomas and was demonstrated to be over-expressed in breast, cervical, ovarian, gastric, lung, colon and pancreatic carcinomas, in contrast to normal tissues where *cripto* expression was invariably absent (Strizzi *et al.* 2005).

In mouse embryos, *cripto* is expressed early in the ICM and the throphoblast cells of the blastocyst (Johnson *et al.* 1994). At 6.5 dpc *cripto* is expressed in the epiblast and at the primitive streak stage in the forming mesoderm. Later on, *cripto* expression is associated with the developing heart structures; its expression is restricted to the myocardium of the developing heart tube at 8.5 dpc and in the outflow region of the heart at 9.5 dpc (Dono *et al.* 1993). This expression pattern suggests that *cripto* may play a role in the early events leading to heart morphogenesis. Mouse embryos deficient for the *cripto* gene die around day 7.5 of embryogenesis due to defects in mesoderm formation and axial organization (Ding *et al.* 1998; Liguori *et al.* 2003). Notably, mouse *cripto* mutants exhibit defects in myocardial development as evidenced by the absence of expression of terminal myocardial differentiation genes such as αMHC and MLC2v (Ding *et al.* 1998; Xu *et al.* 1999a).

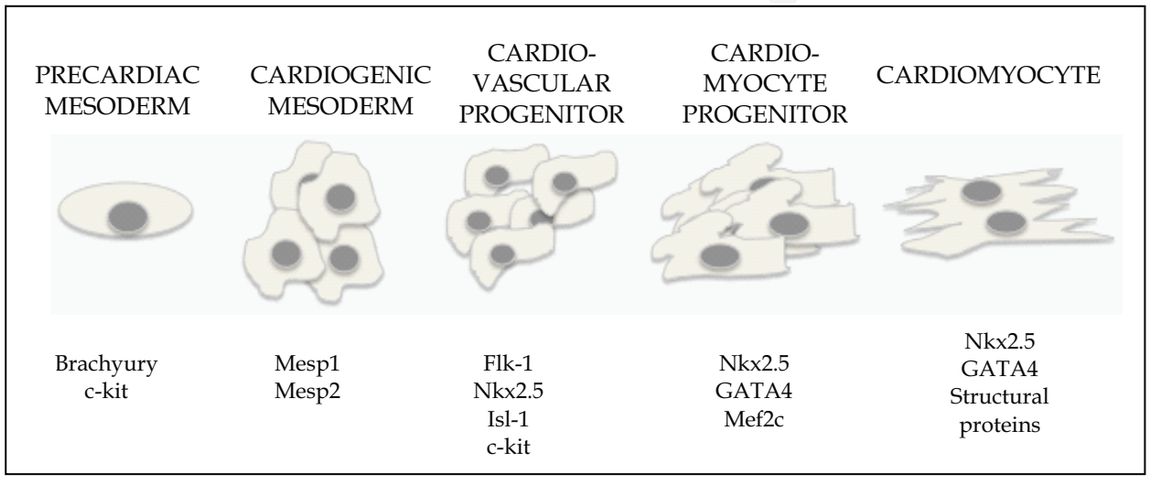



Fig. 2. Schematic illustration of cardiomyocyte development. Adapted from Chen et al., 2008

Given the early lethality of *cripto*-/- mice, ESCs have been a powerful tool to shed light into the functional role of *cripto* in mammalian cardiomiogenesis. Indeed, by using EBs derived from Cripto$^{-/-}$ ES cells, it has been demonstrated that genetic ablation of *cripto* block cardiac differentiation (Xu *et al.* 1998) and that forced expression of wild-type *cripto* fully rescues the cardiac phenotype, thus providing experimental evidence of a functional role of this gene in mammalian cardiomyogenesis. Interestingly, a structure function analysis using different deletion mutant derivatives of *cripto* cDNAs showed that i) a secreted form of Cripto, which lacks the C-terminus, is capable of retaining its biological activity and efficiently induces cardiogenesis and ii) the EGF-CFC domain represents the minimal functional domain, which is sufficient per sè to restore cardiac differentiation of Cripto$^{-/-}$ ES cells (Parisi *et al.* 2003). Worth noting, the EGF-like domain, which was previously shown to be mitogenic on mammary cell lines (Salomon *et al.* 1999), although essential, it is not sufficient per sè to promote cardiac differentiation, suggesting that there may be divergent Cripto signaling pathways depending both on different domains of the protein and/or on specific cell types, an issue that still remains undefined.

Another important issue was the timing of initiation, the strength and duration of Cripto signaling in the commitment of ESCs to a cardiac fate, which was experimentally addressed by using a recombinant secreted Cripto protein. Infact, kinetic experiments performed by adding recombinant Cripto to the culture medium of Cripto$^{-/-}$ ES cells indicate that Cripto is required in a precise moment during differentiation after which it fails to specify the cardiac

lineage. Moreover, a transient presence of Cripto protein is inefficient and a sustained Cripto signaling is strictly required to promote cardiogenesis; thus providing evidence that timing and strength of the signaling are critical parameters for correct specification and differentiation of the cardiac lineage (Parisi *et al.* 2003).

Both the functional data and the expression profile of Cripto, which is expressed early during cardiac differentiation of ESCs (i.e., day 0-4) while is absent at stages where contracting cardiomyocites appear, indicate that Cripto is required for cardiac commitment of ESCs rather than for terminal differentiation of cardiomyocytes in culture.

Intriguingly, disruption of *cripto* leads to spontaneous neuronal differentiation of ESCs in the presence of serum, and in the absence of either specific inducers or defined culture conditions. Again, kinetic experiments indicate that the timing of Cripto signaling required for priming ESCs to cardiomyocytes resembles the competence window those cells to acquire a neural fate (Parisi *et al.* 2003). Indeed, addition of effective doses of Cripto protein to Cripto$^{-/-}$ ESCs in the 0-2 day interval of differentiation rescues the cardiac phenotype and results in a dramatic inhibition of neural differentiation. Conversely, addition of recombinant Cripto at later time points (i.e 3-6 day interval) results in progressive impairment of cardiac differentiation, and in increased competence of the cells to acquire a neural fate; thus suggesting that different timing of Cripto signaling induces different fates in ESCs.

### 2.1 Cripto/Smad2 signaling pathway in cardiomyogenesis

Cripto is involved in the modulation of several signaling pathways in development and tumorigenesis (Strizzi *et al.* 2005). Genetic studies and cell-based assays provide evidence for a role of Cripto and, more generally of the EGF-CFC factors, in the activation of the TGFβ-family member Nodal or related ligands GDF1 and -3 (Chen *et al.* 2006; Tanaka *et al.* 2007) through Activin type IB (ALK-4 and Alk7) and Activin type IIB serine/threonine kinase (ActRIIB) receptors (Reissmann *et al.* 2001). Upon receptor activation, the intracellular kinase domain of the type I receptor phosphorylates Smad2 and/or Smad3, which form a hexameric complex with the common Smad4, and translocate into the nucleus to regulate gene expression in conjunction with other transcription factors, such as FoxH1 (Massague and Chen 2000; Adkins *et al.* 2003; Gray *et al.* 2003; Harrison *et al.* 2005).

Notably, acute stimulation of Cripto$^{-/-}$ ESCs with recombinant Cripto protein rapidly induces Smad2 phosphorylation; thus although competent in activating Smad2, transient stimulation with Cripto is insufficient to achieve proper terminal cardiac differentiation, again highlighting the importance of Cripto signalling duration for cardiomyogenesis (Parisi *et al.* 2003).

Intracellular activation of Smad2 upon stimulation with Cripto, requires assembly of an active activin type I (ALK4) and type II receptor complex. In fact, forced overexpression of the activated forms of ALK-4 receptor are able to compensate for the lack of Cripto in cardiac differentiation. Moreover, loss-of-function experiments performed using Nodal antagonist, Cerberus-S, provide direct evidence that the TGF-β family member Nodal is required to support Cripto-regulated cardiac induction and differentiation in ES cells. Infact, addition of Cerberus Short protein, which specifically blocks Nodal by direct binding to the ligand (Piccolo *et al.* 1999) results in a strong inhibition of Cripto activity in ESCs (Parisi *et al.* 2003).

Besides the above mentioned data, several other line of evidence support the idea that temporal and spatial regulation of the Smad pathway is important for normal cardiac

development from initial cardiomyocyte differentiation to terminal cardiac morphogenesis in pluripotent cells. In fact, data on P19 cells indicate that the Smad pathway is indispensable for normal cardiomyocyte differentiation (Monzen *et al.* 2001). Moreover, more recent data pointed for a key role of Nodal/Cripto for the early activation of Smad2, which was indispensable for mesendodermal induction and the subsequent cardiac differentiation of ESCs (Kitamura *et al.* 2007).

### 2.2 Downstream targets of Cripto signaling in cardiomyogenesis

It is now well accepted that Cripto/Smad2 is one of the key signaling pathway which regulates cardiac specification in mammals; however, little is yet known about the mechanisms of action and the identity of the factors downstream of this pathway in mammalian cardiomyogenesis. Very recently, two genes, the Angiotensin Type-I Like Receptor (AGTRL-1/APJ/*msr1*) and its ligand apelin, have been identified as previously undescribed downstream targets of Cripto-Smad2 pathway in cardiogenesis (D'Aniello *et al.* 2009).

Apj was identified and characterized in 1993 as a seven transmembrane receptor associated with G-proteins (O'Dowd *et al.* 1993); it shows a high sequence homology (30%) with angiotensin II type 1 receptor (AT-1), although APJ does not bind angiotensin II (AngII). Apj was kept "orphan" until 1998 when Tatemoto et al. identified Apelin as its selective endogenous ligand. Apelin is a prepropeptide of 77 aminoacids but its biological activity resides in the C-terminus (apelin-36 and apelin-13) (Tatemoto *et al.* 1998).

In the adult, Apelin and Apj are abundantly expressed in the heart, the central nervous system (CNS) and the lungs (Kawamata *et al.* 2001; Medhurst *et al.* 2003). The wide distribution of Apj and apelin in several organs correlates with multifunctional activities, such as the regulation of gastrointestinal and immune functions, the modulation of the hypothalamus-hypophysis axis activity and the regulation of vascular tone, cardiac contractile function and fluid balance (Kleinz and Davenport 2005).

Most remarkably, growing evidence indicate that apelin and APJ play an important role in cardiac development both in *Xenopus* (Inui *et al.* 2006; Cox *et al.* 2006) and in Zebrafish (Scott *et al.* 2007; Zeng *et al.* 2007). Indeed, under or over expression of apelin-APJ signalling results in a reduction in cardiomyocyte numbers and abnormal cardiac morphology (Scott *et al.* 2007; Zeng *et al.* 2007).

Notably, the expression of Apj and apelin i) correlates with that of *cripto* both in ESC differentiation and in gastrulating embryos and ii) is regulated by Cripto/Smad2 pathway in ESCs. Indeed, Apj and apelin expression is dramatically reduced in the absence of *cripto* (D'Aniello *et al.* 2009) both in ESC cardiac differentiation and *in vivo*. Most remarkably, APJ overexpression is capable of redirecting the neuronal fate of *cripto* knockout ESCs, restoring mesendodermal patterning and the cardiogenic program, although it fails to induce beating EBs. Finally, both apelin and Apj silencing blocks cardiac differentiation, thus pointing out a central role of APJ/Apelin in the gene regulatory cascade promoting cardiac specification and differentiation in ESCs (D'Aniello *et al.* 2009).

One of the major transduction pathways activated by Apelin depends on the interaction with a $G_i$-protein coupled to the Apj receptor with the subsequent interaction with the protein Kinase C (PKC) (Masri *et al.* 2004). Morever, apelin activates the phosphorylation of the intracellular kinase p70S6K through a pertussis toxin (PTX) sensitive G protein (Masri *et al.* 2002). Data on primary endothelial cells indicate that stimulation of p70S6K by Apelin depends on two mechanisms, which are either ERK or PI3K/Akt-dependent (Masri *et al.*

2004). In line with these findings, Apelin induces mammalian cardiomyogenesis via a PTX-sensitive GTP binding protein associated to Apj receptor, through the activation of ERK-dependent p70S6K signaling pathway (D'Aniello *et al.* 2009).

## 3. FGFR1

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 (Powers *et al.* 2000; Presta *et al.* 2005). FGFR1 is one of the four member of the FGFR family, whose amino acid sequence is highly conserved between members and throughout evolution (Itoh and Ornitz 2004). FGFRs differ from one another in their ligand affinities and tissue distribution. A full-length representative protein consists of an extracellular region, composed of three immunoglobulin-like domains (D1-D3), a single hydrophobic transmembrane region and an intracellular tyrosine kinase (TK) domain (Beenken and Mohammadi 2009). The ligand binding site for FGFs is located in the D2-D3 domains and the linker that connects them (Plotnikov *et al.* 2000), whereas the D1 domain is involved in receptor autoinhibition (Olsen *et al.* 2004). Ligand specificity is achieved primarily through splicing events in which the alternative exons IIIb and IIIc encode the carboxyl terminal portion of the third Ig-like loop. Indeed, alternative splicing of FGFR1 results in isoforms FGFR1-IIIb and FGFR1-IIIc with distinct FGF binding characteristics: FGFR1-IIIb binds efficiently to FGF1, FGF3 and FGF 10, whereas FGFR1-IIIc binds to FGF1, FGF2, FGF4, FGF6, FGF8 and FGF9 (Ornitz *et al.* 1996). A variety of other alternative spliced receptor molecules have been described, including the β isoforms that lack the first Ig-like domain whereas the α isoforms identify the full-length receptors (Wang *et al.* 1995).

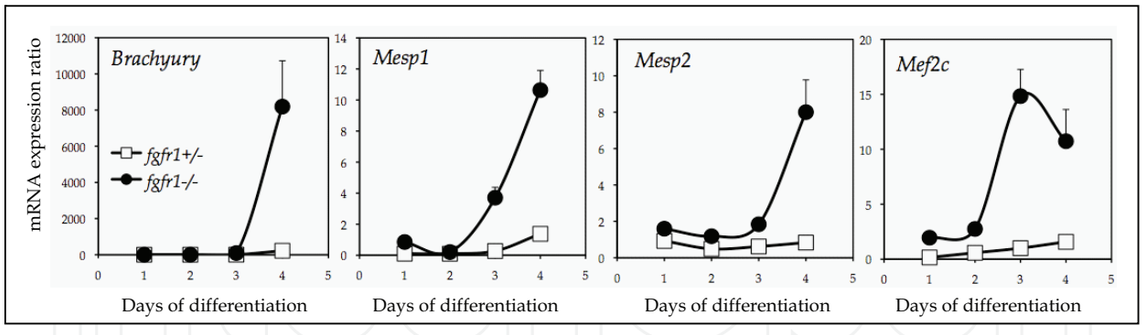



Fig. 3. Mesodermal marker expression by qPCR analysis in *fgfr1*$^{+/-}$ and *fgfr1*$^{-/-}$ EBs

FGFR1 signaling plays important functions in mesoderm formation and development (Xu *et al.* 1999b). Heterozygous animals develop normally but *fgfr1*$^{-/-}$ mice dye during gastrulation, displaying defective mesoderm patterning with reduction in the amount of paraxial mesoderm and lack of somite formation (Deng *et al.* 1994; Yamaguchi *et al.* 1994). Studies on chimeric embryos using FGFR1-deficient mESC revealed an early defect in the mesodermal and endodermal cell movement through the primitive streak, followed by deficiencies in contributing to anterior mesoderm, including heart tissue (Ciruna *et al.* 1997; Deng *et al.* 1997). We analyzed early mesodermal marker expression during the first days of mESC differentiation and indeed, *Brachyury, Mesp1, Mesp2,* and *Mef2c* genes are strongly

upregulated in *fgfr1*$^{-/-}$ compared to *fgfr1*$^{+/-}$ mESC (Fig. 3). Although we still don't know the significance of this upregulation, we can confirm a bias in mesodermal lineage development also in the mESC differentiation model.

The pivotal contribution of FGF signaling in heart formation has been demonstrated in different animal models: in *C. intestinalis*, FGF signaling delineates the cardiac progenitor field (Davidson 2007); in *Drosophila*, mesoderm spreading depends upon the expression of *heartless,* homologous to vertebrate *fgfr1* (Beiman *et al.* 1996; Gisselbrecht *et al.* 1996), and *heartless* mutant is characterized by the absence of the heart (Frasch 1995; Beiman *et al.* 1996); in chicken, FGF signaling activated by FGF8 contributes to the heart-inducing properties of the endoderm (Alsan and Schultheiss 2002); in zebrafish, induction and differentiation of the heart requires FGF8 (Reifers *et al.* 2000); in mice, *Fgf8*$^{neo/-}$ mutants show complex cardiac defects (Abu-Issa *et al.* 2002).

FGFR1 has been implicated in cardiac development also during mESC differentiation. When *fgfr1*$^{-/-}$ mESC were differentiated with the "hanging drop" protocol, no beating foci were seen in EB cultures within the first fourteen days, whereas contracting areas were observed microscopically in more than 90% of the heterozygous EBs at day 8th. To verify the morphological data, total RNA was extracted at different time point of differentiation, and then subjected to retrotranscription, and semiquantitative polymerase chain reaction (PCR) for cardiac marker expression. In parallel, EBs were fixed, paraffin included, cut in 7μM sections, and analyzed by immunofluorescence for the presence of the structural protein MHC-α. Both methods confirmed the presence of cardiac markers lonely in *fgfr1*$^{+/-}$ mESC, thus indicating that the beating areas indeed correspond to CMs, whose differentiation depends upon FGFR1 expression (Dell'Era *et al.* 2003).

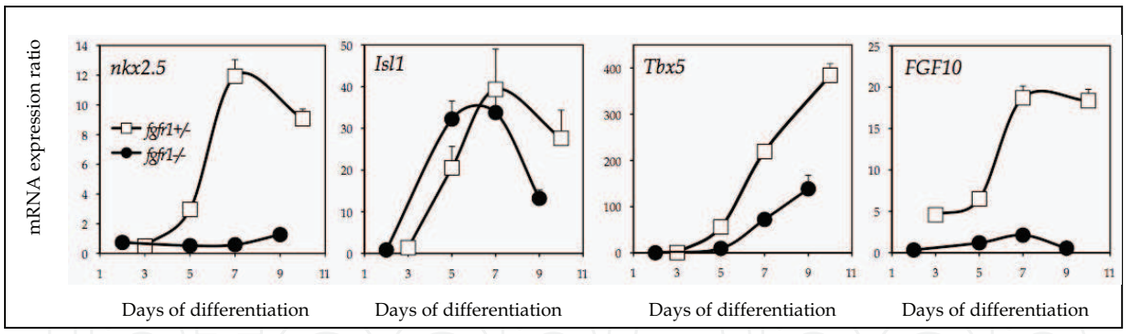



Fig. 4. Heart field marker expression by qPCR analysis in *fgfr1*$^{+/-}$ and *fgfr1*$^{-/-}$ EBs

Nkx2.5 is considered the earliest cardiac transcription factor because of its expression in cardiovascular precursors and the mRNA analysis of *fgfr1*$^{-/-}$ EBs showed that the absence of FGFR1 does not allow Nkx2.5 upregulation (Dell'Era *et al.* 2003; Ronca *et al.* 2009). This result suggest that *fgfr1*$^{-/-}$ EBs cannot make the transition to develop cardiac lineage from cardiogenic mesoderm, and indeed, Mesp1, Mesp2, and Mef2c accumulation suggests a flooding of mesodermal precursors. As mentioned, Nkx2.5$^{+}$ CVP can give rise to CMs, endothelial, and smooth muscle cells (Chen *et al.* 2008). However, CD31 and α-smooth muscle actin immunostaining of EBs showed that differentiation of endothelial and smooth muscle cells is not affected by the lack of FGFR1 (Magnusson *et al.* 2004). Indeed, the vascular plexus in *fgfr1*$^{-/-}$ EBs is more abundant to that observed in heterozygous EBs, thus suggesting that the cells that cannot become CMs are forced toward parallel lineages, such

as endothelial cells. FGFR1 was first isolated by a endothelial cell cDNA library due to its homology with the tyrosine kinase receptor Fms (Dionne *et al.* 1990), and both the prototype FGFs (FGF1 and FGF2) are considered as angiogenic growth factors (Presta *et al.* 2005). Then, it was really surprising to realize that the receptor was not really involved in endothelial development. However, as suggested by immunostaining of EBs with an antibody specific for the activated receptor, FGFR-1 is phosphorylated in a subpopulation of proliferating endothelial cells (Magnusson *et al.* 2005), thus confirming its role in endothelial cell proliferation rather than differentiation.

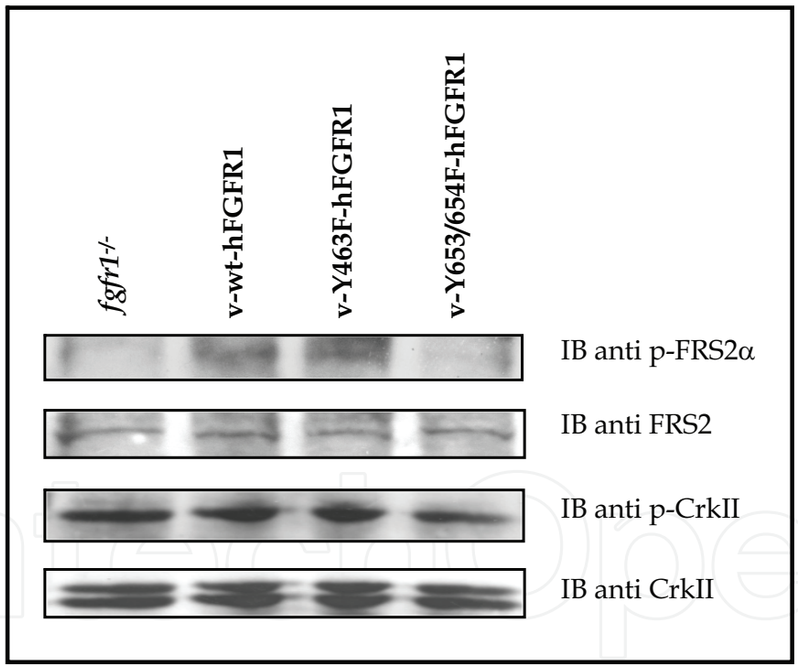
It has previously shown that FGFR-1 is required by epicardium-derived cells for myocardial invasion (Pennisi and Mikawa 2009), and that mature CMs expressing FGFR1 proliferate upon receptor stimulation (Seyed and Dimario 2008). Then, the open question if FGFR1 is expressed by cardiogenic precursors and/or by other mesodermal cells and why is it needed during mESC CM differentiation is still controversial. To add a little piece to the puzzle, we analyzed FHF and SHF markers in *fgfr1*$^{-/-}$ EBs and the results are reported in Fig. 4.

When compared with heterozygous EBs, both *nkx2.5* and *FGF10* genes do not show any upregulation during *fgfr1*$^{-/-}$ mESC differentiation, while a smaller increase can be seen for Tbx5; at variance, the SHF marker Isl1 seem to be comparable between the two populations, thus suggesting that the absence of FGFR1 results in a bias in primary heart field development. It should be pointed out that one of the limitation of the mESC model is that it is impossible to dissect anatomical structures of the EBs; this fact imply that cells anatomically distant can be found in close proximity, thus leading to artificial paracrine stimulations affecting the "normal" development. Taking together, our data suggest that FGFR1, probably present on cardiomyocyte precursor cell surface, may mediate cardiomyocyte differentiation by activating Nkx2.5 in Mef2c-cardiogenic mesodermal cells. Indeed, in other models FGF signaling has been shown to be sufficient to induce cardiac transcription factor expression: FGF8-soaked beads induce *nkx2.5* in chick (Alsan and Schultheiss 2002) and zebrafish (Reifers *et al.* 2000), whereas an ectopic FGF signaling results in a surplus of CMs prior to terminal differentiation (Marques *et al.* 2008).

The interaction of FGFR1 with a ligand leads to a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. Seven tyrosine residues become phosphorylated in human FGFR1 (hFGFR1): Y653/654 are critical for TK activity (Mohammadi *et al.* 1996), Y463 is involved in endothelial cell proliferation by binding to Src homology (SH)2/SH3 domain-containing adaptor protein Crk (Larsson *et al.* 1999), and phosphorylated Y766 has been 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 (Mohammadi *et al.* 1991; Cross *et al.* 2002; Cailliau *et al.* 2005). Also, FGFR1 activation leads to FRS2 phosphorylation (Kouhara *et al.* 1997) followed by Grb2 and Shp-2 interactions (Hadari *et al.* 1998). Frs2, Crk, and Shb binding to FGFR1 affect the classical Ras/Raf-1/MEK/ERK/Jun proliferation pathway activated by TK receptors, while PLCγ1 activates PKC (Hug and Sarre 1993), whose role in CM differentiation has been demonstrated (Zhou *et al.* 2003).

Previous observation had shown that the FGFR1 TK inhibitor SU 5402 (Mohammadi *et al.* 1997), the $MEK_{1/2}$ inhibitor U0126 (Favata *et al.* 1998), and the classical/novel protein kinase C (PKC) inhibitor GF109203X (Kuchera *et al.* 1993) were all able to hamper beating foci formation in EBs originated by *fgfr1*$^{+/-}$ mESC (Dell'Era *et al.* 2003). In order to define the requirements for FGFR1 signaling in CM development, we transduced *fgfr1*$^{-/-}$ mESC *via* a lentiviral vector system with the IIIc isoform of either wt receptor (v-wt-hFGFR1), or hFGFR1 mutants in different tyrosine autophosphorylation sites: the tyrosine kinase

defective (TK-) Y653/654F-hFGFR1 mutant, and the two TK+ Y463F-, and Y766F-h-FGFR1. Resulting cell lines were analyzed to confirm that the receptors were correctly exposed on cell surface, by $^{125}$I-FGF2 binding assay and, at least for TK+ receptors, were able to upregulate the downstream signaling molecule $ERK_{1/2}$. Then, we evaluated the presence of cardiomyocyte during the differentiation process by looking microscopically to the appearance of beating foci, and by expression analysis of both early and late cardiac markers. We first observed that transduction of the human receptor molecule fully reconstitutes cardiomyocyte differentiation, while, in agreement with previous data obtained by using pharmacological inhibitors, TK- receptor does not. The analysis of Y463F and Y766F mutant EBs demonstrate that v-Y766F-hFGFR1 ES cells are able to support cardiomyocyte differentiation in a manner undistinguishable from v-wt-hFGFR1 ES cells and *fgfr1*$^{+/-}$ ES cells. In contrast, transduction of *fgfr1*$^{-/-}$ ES cells with the Y463F-hFGFR1 mutant results in a loss of rescue of cardiomyocyte formation, as assessed by the absence of beating foci and early and late cardiomyocyte markers in the corresponding EBs (Ronca *et al*. 2009). At present, the signaling cascade triggered by the autophosphorylation of Y463 in FGFR1 and its cross-talk with PKC- and ERK-mediated signaling during cardiomyocyte differentiation of mESC remains to be elucidated.




*fgfr1*$^{-/-}$ , v-wt-hFGFR1, v-Y463F-hFGFR1, and v-Y653/654F-hFGFR1 ES cells were subjected to standard differentiation protocol. At day 9$^{th}$ of differentiation, cell extracts were analyzed for activated signaling molecules.

Fig. 4. Characterization of FGFR1 signalling during ES differentiation.

Indeed, Western blot analysis of total EB protein extract at day 9 of differentiation does not show any difference in CrkII phosphorylation levels among *fgfr1*$^{-/-}$, v-wt-hFGFR1, v-Y463F-hFGFR1 and v-Y653/654F-hFGFR1 EBs, thus indicating that the overall activation of these signaling molecules in differentiating EBs is not restricted to FGFR1 activity. 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. 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.

## 4. Conclusions

Although ESCs represent a viable source of specific cellular subtypes for drug discovery and transplantation, the successful use of ES-derived donor cells would require the generation of essentially pure cultures of specific cell types. Although further analysis will be required to get insight into the regulatory networks that involve Cripto/APJ and FGFR1 in cardiac differentiation (Figure 5), these membrane receptors may represent valuable targets for the generation of pure cardiomyocyte populations from ESCs.

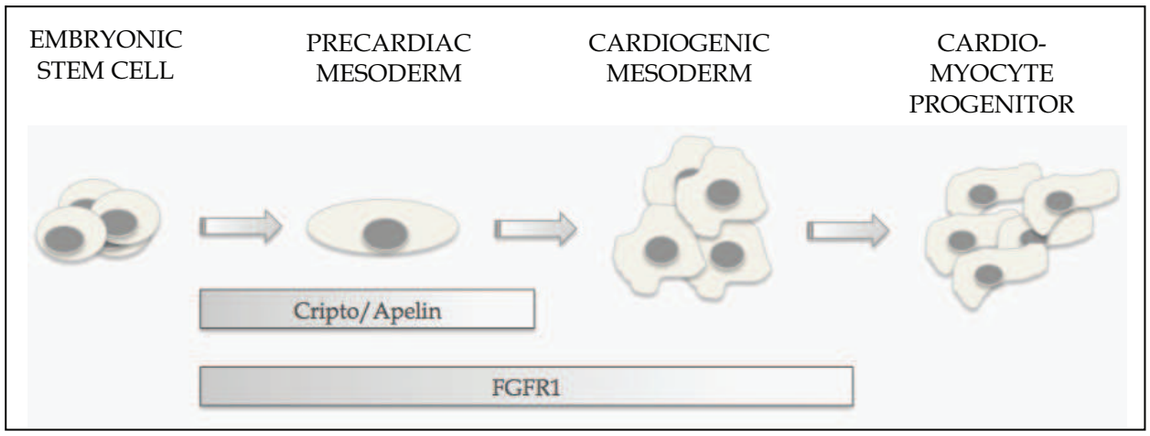



Fig. 5. Cripto/Apelin and FGFR1 influence the early phases of cardiomyocyte differentiation

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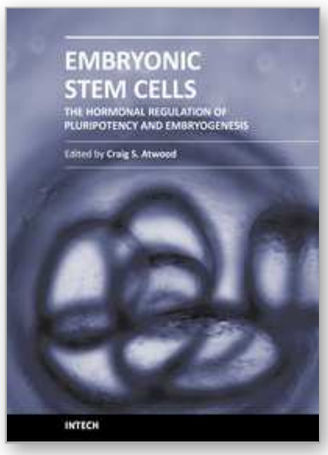


**Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis**

Edited by Prof. Craig Atwood

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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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