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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF THE G-PROTEIN COUPLED RECEPTOR APJ AND ITS LIGAND APELIN IN EMBRYONIC STEM CELLS: A NOVEL PATHWAY REGULATING MAMMALIAN CARDIOMYOGENESIS

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ABSTRACT

The commitment of mesodermal precursors to a cardiac fate is one of the first events in embryogenesis. It results from inductive interactions occurring during gastrulation in a temporally and spatially regulated manner. Embryonic Stem Cells (ESCs) are a powerful tool to study the early events of cardiac lineage specification in mammals; in particular, several cardiac cell populations have been generated from differentiating ESCs and studies of gene expression profiles indicate that their development in culture recapitulates cardiogenesis in the early embryo. Thus, ESCs represent a valid model to elucidate the origin and the molecular identity of cardiovascular progenitor populations as well as to identify key regulators of cardiac specification in mammals for which the molecular control is largely unknown.

Cripto/ALK4/Smad2 signaling is a key pathway required for the correct cardiac myogenesis, acting in the early phases of ESC differentiation.

Here we report the identification of the Angiotensin II Type Receptor-Like I (AGTRL-1/APJ/*msr1*) and its ligand Apelin as previously unrecognized downstream targets of Cripto/Smad2 signaling both *in vivo* and in ESCs.

Gain of function experiments show that APJ suppresses neuronal differentiation, which spontaneously occurs in *cripto*$^{-/-}$ ESCs, and restore the cardiac program, activating the expression of genes pivotal for cardiac specification and terminal differentiation. Furthermore, loss-of-function experiments reveal, for the first time to our knowledge, a central role for APJ/Apelin signaling in the gene regulatory cascade promoting ESC cardiac specification and differentiation.

Most remarkably, we show that Apelin promotes cardiomyogenesis via activation of pERK/p70S6 through coupling to a Go/Gi protein. Together our data point for a previously undescribed functional link between Cripto/Smad2 and APJ/Apelin in the signaling pathways that govern mesoderm patterning and cardiac specification in mammals.

Finally, to further investigate the role of APJ/Apelin signaling in the control of ESC differentiation and, in particular, in the specification of cardiovascular progenitors, we have generated an inducible ESC line, which allow overexpression of APJ in a time-specific manner, by using the tetracycline (Tet)-regulated transactivator system. Our data reveal that APJ overexpression, in an early time window of wild-type ESC differentiation, promotes the expression of markers of the cardiovascular lineage and markedly increases the number of cardiomyocytes.

1 INTRODUCTION

The heart is the first organ to be formed during mammalian development; indeed, during gastrulation mesodermal cells are committed to a cardiogenic fate and migrate into the anterolateral region of the embryo (Garry and Olson, 2006). These processes are temporally and spatially regulated through inductive interactions and instructive signaling that determine the specification of a cardiac fate. The heart is a complex structure composed by several types of cells: cardiac and smooth muscle, valvular, pacemaker and endothelial cells, each of them with specific roles. Indeed, *in vivo*, embryonic cardiogenesis requires the concurrence of a set of multipotent cardiac progenitors, capable to give rise to most of the terminally differentiated cell types that will form the adult organ. Although several progenitor populations have been identified (Martin-Puig et al., 2008) very little is known about the molecular identity of these progenitors and the molecular mechanisms that control cardiac lineage induction during early stages of mammalian development. In the adult heart, cardiomyocytes became mature and well organized and their proliferative potential rapidly declines; thus the intrinsic cardiac regeneration in mammals is very poor (Ahuja et al., 2007). Based on these considerations, understanding the molecular complexity that controls the early events of cardiac commitment and differentiation is of great interest, opening new possibilities for regenerative therapies. In this context, Embryonic Stem Cells (ESCs) represent a powerful tool to elucidate the origin and the molecular identity of the progenitor populations and to identify key factors, signaling molecules and downstream effectors that lead to cardiac cell commitment and specification.

1.1 The Embryonic Stem Cells as model system to study mammalian cardiomyogenesis

ESCs are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman, 1981) (Martin, 1981). They are characterized by two key features that distinguish them from other cell types: 1) can be maintained and expanded in culture for extended period of time as a pure population of undifferentiated cells, retaining normal karyotypes; 2) are pluripotent, meaning that they can generate all the cell types of an organism, i.e. the derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. Pluripotent cells cannot make extra-embryonic tissues (Keller, 1995) (Smith, 2001) (Fig. 1). The pluripotency of mouse ESCs was experimentally proven by their ability to contribute to all tissues of adult mice, including the germline, following their injection into host blastocyst (Bradley et al., 1984).

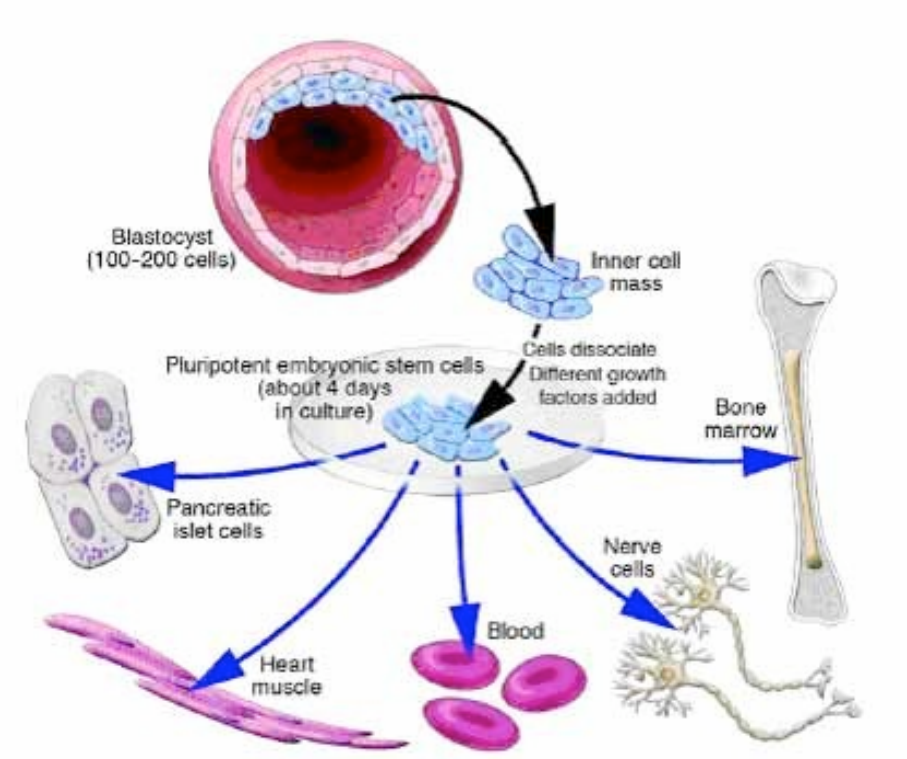

Fig. 1: Derivation of ESCs and their pluripotency.
ESCs are derived from the inner cell mass of blastocyst stage embryos and expanded in culture, giving rise to all the cells deriving from the three germ layers.

These two characteristics make ESCs unique, thus representing an attractive tool for modern biology and regenerative medicine. ESCs can be induced to differentiate through the formation of multicellular, tridimensional structures, named embryoid bodies (EBs) that resembles post-implantation embryos. To date, the study of *in vitro* ESC differentiation have led to the identification of several proteins and growth factors, which act as key molecules in the network of genes regulating the early stages of mouse embryo development. Remarkably, understanding the molecular mechanisms of early embryogenesis is also crucial to get insight into molecules/mechanisms active in pathological conditions, like tumorigenesis or tissue regeneration, in which the same mechanisms are involved. Finally, a great interest for these pluripotent cells derives from the opportunity to use ESCs for regenerative therapies of several degenerative diseases, like Parkinson and Alzheimer, for which no effective therapy is currently available. Many studies are focused on the possibility to generate specific protocols to obtain pure population of differentiated cells that can be transplantated into the damaged tissue, without the formation of teratomas. The establishment of human embryonic stem cells (Thomson et al., 1998) and the recent discovery of induced pluripotent stem cells (iPS) (Takahashi and Yamanaka, 2006) open new possibilities for research and cell replacement therapy.

ESCs are maintained and propagated in the undifferentiated state by co-culture with a feeder layer of mouse embryonic fibroblasts (MEFs), inactivated in the cell cycle and in the presence of leukemia inhibitory factor (LIF), a pivotal molecule in the maintenance of pluripotency. LIF functions through gp130 activation of the intracellular effector STAT3 (Nichols et al., 1998) (Matsuda et al., 1999). In combination with LIF, ESC pluripotency is modulated by the transforming growth factor β (TGF-β) superfamily members, such as Bmps (Wu and Hill, 2009). The effect of Bmp4 on ESCs is mediated by the activation of Smad1 and the subsequent expression of Id factors (Ying et al., 2003). In addition to these signaling pathways, ESC pluripotency is regulated through a transcriptional network core that includes Oct3/4 (Nichols et al., 1998), Sox2 (Avilion et al., 2003) and Nanog (Chambers et al., 2003) (Fig. 2).

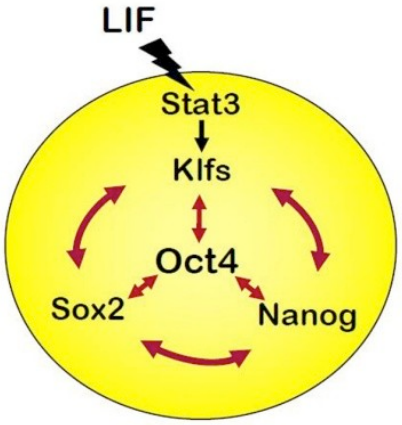

Fig. 2: Schematic representation of the core gene regulatory of pluripotency.
ESC pluripotency is regulated by several signaling pathways, such as LIF/STAT3 and intrinsic transcription factors core, like Oct3/4, Sox2 and Nanog (Smith, 2010).

When cultured in the absence of LIF and feeder cells, the ESCs differentiate and, under appropriate culture conditions, they generate progenies deriving from the three germ layers: mesoderm, endoderm and ectoderm (Keller, 2005) (Smith, 2001). Several protocols have been designed to obtain specific population of differentiated cells. In general, ESCs can be induced to differentiate either through the formation of aggregates, called Embryoid Bodies (EBs), which resemble the mammalian embryos, or in monolayer, in the presence or absence of growth factors and/or stromal cells. EBs formation, by hanging drop culture, is the principal and most efficient method used to differentiate ESCs into cardiomyocytes (Boheler et al., 2002). Following this protocol, cardiac specific proteins, such as ion channels, receptors and structural proteins are expressed in a developmental continuum, which closely recapitulates the developmental patter of early cardiogenesis. Indeed, in the early stages of cardiac differentiation, ESCs appear as round-shaped small EBs and start to lose the expression of pluripotency markers. Later on, when differentiation proceeds, EBs become elongated with well-developed

myofibrils and sarcomeres, typically of cardiomyocytes (Westfall et al., 1997). During this process a characteristic sequence of mesoderm and cardiac specific transcription factors and structural proteins are expressed. *Brachyury*, a marker of early mesoderm induction, is transiently expressed, peaking at day 4. Subsequently, EBs, committed to the cardiac lineage, start to express markers of cardiac precursors like *mesp1* and *2*, *nkx2.5*, and *tbx5*. At terminal differentiation stage markers of mature and functional cardiomyocytes are expressed like α and β *myosin heavy chain* (*αMHC*) *atrial natriuretic factor* (*ANF*), *ventricle-specific myosin light chain* (*mlc2v*) and *Troponin-T* (Fig. 3). Several signaling molecules have also been identified, which are essential for cardiac specification and differentiation in cardiomyogenesis, such as Wnt/β-catenin, TGF-β, BMP and Cripto (Martin-Puig et al., 2008) (Minchiotti, 2005).

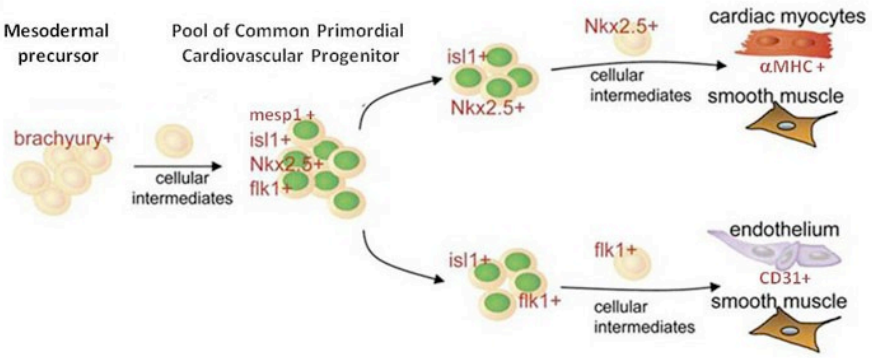

Fig. 3: ESC cardiac differentiation.
During *in vitro* differentiation mesodermal precursors, expressing *brachyury*, are committed towards cardiac/endothelial lineage. These primordial cardiovascular progenitors express several markers like *mesp1*, *isl1*, *nkx2.5* and *flk1*. Later on, when differentiation proceeds, progenitors give rise to either cardiomyocytes, expressing αMHC and endothelial cells that are CD31+ (modified from Moretti et al., 2006).

1.2 The EGF-CFC Cripto

Cripto, also known as Teratocarcinoma-derived growth factor-1 (TDGF-1) is the founder member of the EGF-CFC family, a group of extracellular proteins highly conserved during vertebrates evolution (Ciccodicola et al., 1989). This family includes: human Cripto-1 (CR-1/TDGF-1) and cryptic, mouse cripto-1 (Cr-1/tdgf-1) and cryptic, chicken cripto (c-cripto), Xenopus FRL-1 and Zebrafish one-eyed pinhead (oep). The EGF-CFC proteins play crucial roles during early vertebrate development, such as anteroposterior (A-P) axis formation and left-right specification. In particular, Cripto is required during gastrulation for mesoderm specification and cardiac differentiation. Furthermore, Cripto is involved in several processes related not only to embryo development, but also to cellular proliferation and transformation and tumor progression (Minchiotti, 2005).

The EGF-CFC proteins share some structural characteristics: a NH_2–terminal signal peptide, a modified Epidermal Growth Factor (EGF) like domain, a conserved cystein-rich motif (CFC) and an hydrophobic COOH-terminus containing the consensus sequences for glycosylphosphatidylinositol (GPI) binding to the membrane and cleavage (Ciccodicola et al., 1989) (Dono et al., 1993). Indeed, the EGF-CFC proteins, initially described as secreted molecules, are extracellular membrane proteins anchored through the GPI moiety (Minchiotti et al., 2000). Removing the COOH-terminal stretch of residues where GPI linkage occurs, generates soluble forms of biological active mouse and human Cripto (Minchiotti et al., 2001). All the members of the EGF-CFC proteins, except for Oep in Zebrafish, are glycoproteins that contain a single N-glycosilation site and potential O-glycosilation sites (Fig. 4).

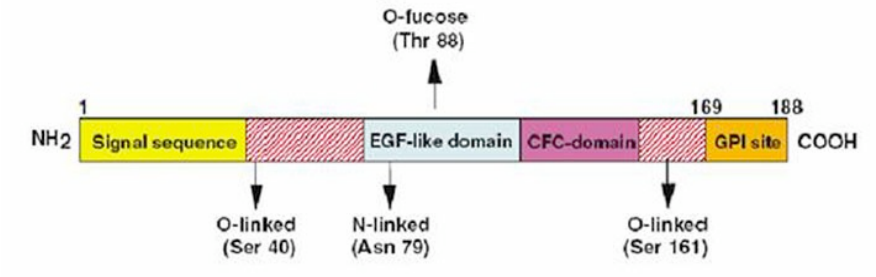

Fig. 4: Schematic representation of the domains structure of the EGF-CFC proteins.
Yellow: signal peptide; cyan: pseudo EGF motif; violet: CFC motif; orange: hydrophobic C-terminus with the GPI anchorage to the membrane and cleavage (Saloman et al., 2000).

Cripto is a multifunctional protein involved in the activation of several signaling pathways both in development and tumorigenesis (Strizzi et al., 2005). During early embryogenesis Cripto acts as a coreceptor for transforming growth factor-beta (TGF-β) family proteins such as Nodal and GDF1-3 (Chen et al., 2006) (Tanaka et al., 2007). Cripto is required to recruit Nodal to an Activin type I (ALK4 or ALK7) and Activin type II serine/threonine kinase receptor (ActRIIB) complex (Reissmann et al., 2001), which, once activated, phosporylates and activates the intracellular effectors Smad2 and/or 3. Smad2/3, together with Smad4, enters the nucleus and activate the target genes (Reissmann et al., 2001) (Yeo and Whitman, 2001) (Fig. 5).

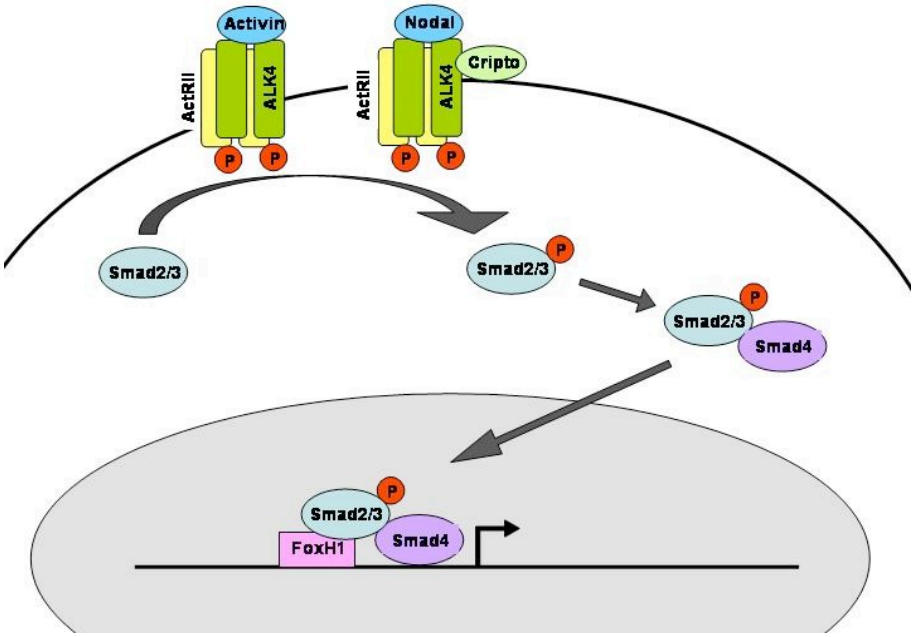

Fig. 5: Nodal/Cripto/ALK4/Smad2 signaling pathway.
Cripto acts as a coreceptor for Nodal/ALK4 to activate the downstream effector Smad2 (Hou et al., 2010).

Besides its role as coreceptor for Nodal, Cripto can mediate signaling of other TGF-β ligands, such as Activin, Xenopus Vg-1 and its ortholog in mouse GDF-1 (Adkins et al., 2003). Similar to Nodal, Vg-1 and GDF-1 can bind to ALK4/ActRIIB receptor complex only in the presence of EGF-CFC coreceptors (Strizzi et al., 2005). More recently, it has been shown that Cripto can block Activin signaling by binding to Activin, through its CFC domain, and blocking the assembly of ALK4 receptor complex (Adkins et al., 2003) (Gray et al., 2003) (Harrison et al., 2005); thus leading to hypothesizing that Cripto could promote tumorigenesis by inhibiting the tumor suppressor activity of Activin (Shen, 2003).

Cripto is also involved in non TGF-β signaling pathways. Indeed, it can activate the ras/raf/mitogen-activated protein kinase (MAPK) and PI3K/AKT pathway in mouse and human mammary epithelial cells. Activation of these two pathways is independent of Nodal and ALK4 and occurs through a direct binding of Cripto to Glypican-1, a membrane-associated heparan sulfate proteoglycan (HSPG) that function as coreceptor for several growth factors (Bianco et al., 2003). Binding of Cripto to Glypican-1 activates the cytoplasmatic tyrosin kinase c-Src triggering the activation of MAPK and AKT signaling pathways (Bianco et al., 2003). Since MAPK and AKT signaling pathways have been involved in regulating cell proliferation and survival, Cripto might be involved in the pathogenesis of cancer through an inappropriate activation of these pathways.

While growing evidence indicates a key role of Cripto in embryo development and ESC differentiation (Paragraph 3), its role in adult life is still controversial. Indeed, *cripto* is expressed at low levels in several adult organs such as spleen, testis, heart, lung and brain under physiological conditions (Dono et al., 1993). However, no function has been described in any normal tissue except for the mammary gland development, where *cripto* expression increases during pregnancy and lactation (Bianco et al., 2001). On the contrary, the expression of *cripto* strongly increases under pathological conditions, like tumorigenesis and increasing evidence points for a role of *cripto* in cell transformation and tumor progression (Salomon et al., 1999) (Persico et al., 2001). *Cripto* is expressed in human and mouse Embryonal carcinoma (EC) cells as well as in male teratocarcinomas. Cripto shows several oncogene-like characteristics, such as the ability to induce cellular transformation, proliferation and migration. Interestingly, Cripto is able to induce Epithelial-Mesenchymal Transition (EMT) (Strizzi et al., 2004), a process characterized by a dramatic change in the phenotype of epithelial cells from a well differentiated and polarized cell to a mesenchymal cell able to migrate in the tissue (Thiery, 2003), which is common to both tumor progression and embryo development.

1.3 Cripto in embryo development and ESC differentiation

Cripto is expressed during early mouse embryogenesis in the inner cell mass and the trophoblast cells of the mouse blastocyst (Johnson et al., 1994). In gastrulating embryos Cripto is expressed in the epiblast and in the primitive streak (Dono et al., 1993) (Ding et al., 1998). Later on, Cripto expression is associated with the developing heart structure. At 8.5 day post coitum (dpc) Cripto is expressed in the myocardium of the developing heart tube and in the outflow region of the heart at 9.5 dpc (Dono et al., 1993). After 10.5 dpc Cripto expression is not detected (Dono et al., 1993). This expression profile correlates with a fundamental role for *cripto* in the early events of development associated with mesoderm formation and cardiac specification. Indeed, mouse embryos deficient for *cripto* gene die early, around day 7.5 of gestation, for severe defects of gastrulation and mesoderm formation (Ding et al., 1998) (Liguori et al., 2003) (Fig. 6). Indeed, *cripto* mutant embryos show deficit of myocardial development and the absence of the expression of the cardiac structural genes *αMHC* and *mlc2v* (Ding et al., 1998) (Xu et al., 1998). These embryos consist mostly of anterior neuroectoderm and lack posterior structures (Ding et al., 1998) (Liguori et al., 2008).
The early lethality of *cripto*$^{-/-}$ mouse embryos precludes the possibility to study its role in later stages of embryogenesis, like cardiac development. In this light ESCs have been a powerful model system to investigate the functional role of *cripto* in this process. Indeed, by using EBs derived from *cripto*$^{-/-}$ ESCs, it has been demonstrated that *cripto* acts early during ESC differentiation playing a key role in the induction of pre-cardiac mesoderm and cardiac differentiation (Dono et al., 1993).

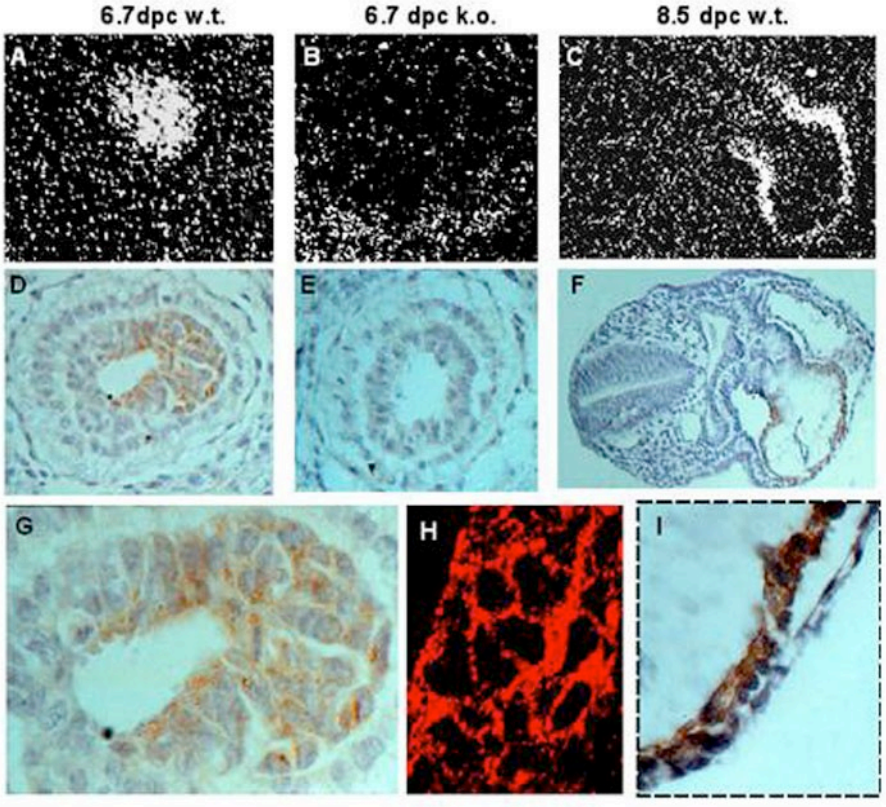

Fig. 6: Expression profile of Cripto in the early stages of mouse embryo development.
In situ hybridization (A, B, C) and Immunoistochemistry (D, E, F, G and I) on mouse embryo sections reveal the expression of Cripto in the mesoderm of 6.7 dpc embryos and in the developing heart of 8.5 dpc embryos. Panel B and E indicate the absence of Cripto in 6.7 dpc *cripto*$^{-/-}$ mouse embryos (Minchiotti et al., 2000).

In fact, EBs derived from *cripto*$^{-/-}$ ESC fail to differentiate into contractile cardiomyocytes (Xu et al., 1998) and spontaneously differentiate into neurons (Parisi et al., 2003), thus suggesting that neuroectodermal differentiation is the preferred default pathway that occurs in the absence of *cripto*. Interestingly, the cardiac phenotype can be fully rescued by the re-expression, in mutant cells, of both the GPI-anchored and the soluble form of *cripto* (Parisi et al., 2003). A structure function analysis performed using different deletion mutant derivatives of *cripto* cDNA, showed that the EGF-CFC is the minimal functional domain capable to restore cardiac differentiation of *cripto*$^{-/-}$ ESCs (Parisi et al., 2003). Notably, kinetic experiments indicate that Cripto is required in a precise time window during differentiation to specify the cardiac lineage. In fact, the addition of recombinant Cripto protein to *cripto*$^{-/-}$ ESCs between day 0 and 2 of differentiation is able to fully rescue the cardiac phenotype. On the contrary, the addition of the protein at later time points (3-6 days) blocks the ability of mutant cells to acquire the cardiac phenotype, increasing their ability to shift to a neural fate (Fig. 7). This suggests that different timing of Cripto signaling induces different fates in ESCs. Besides the

timing, other important issues for the correct specification of cardiac lineage are the strength and the duration of Cripto signaling. These functional data are supported by the expression profile of *cripto* indicating that it is already expressed in undifferentiated ESCs, picks at day 4 of cardiac differentiation while, later on, when mature cardiomyocytes start to appear, its expression is absent (Parisi et al., 2003).

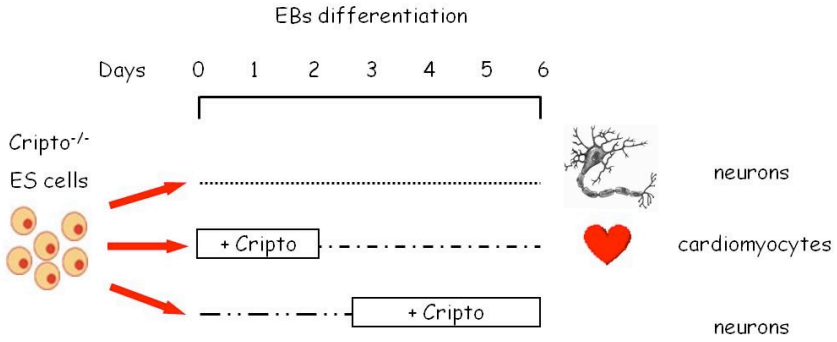

Fig. 7: Different timing of Nodal-dependent Cripto signaling induces different cell fates.
Cardiomyocytes versus neural differentiation of *cripto*$^{-/-}$ EBs. Addition of Cripto protein during 0-2 day interval of differentiation results in cardiomyocytes induction. Addition at later time points (3-6 days) fails to specify the cardiac lineage and results in neuronal differentiation (modified from Minchiotti, 2005).

These data thus indicate that Cripto is a key molecule required in the early phases of differentiation for the commitment of cardiac lineage. Stimulation of *cripto*$^{-/-}$ ESCs with recombinant Cripto protein activates the intracellular effector Smad2 that has been recently associated with the early events that control mammalian cardiac induction (Kitamura et al., 2007). Thus, Cripto signals through Nodal/ALK-4/Smad2 pathway to promote cardiac differentiation and impair neurogenesis of ESCs.
Interestingly, *cripto*$^{-/-}$ ESCs generate dopaminergic neurons and, once grafted into the brain of animal model of Parkinson disease, they are able to restore their behavior in the absence of teratomas formation, thus indicating that Cripto may be a good target for future ESC–based therapeutic approach in Parkinson disease (Parish et al., 2005) (Lonardo et al., 2010).
Although the Nodal/Cripto/Smad2 signaling pathway has been relatively well described, little is known about the mechanisms of action and the downstream effectors in mammalian cardiomyogenesis. Combining microarray technology to ESC cardiac differentiation, we have identified two genes, the G-protein-coupled receptor Angiotensin type- I like receptor (AGTRL-1/APJ) and its ligand Apelin, as downstream targets of Cripto in cardiomyogenesis.

1.4 The APJ/ Apelin system

APJ is a seven transmembrane receptor associated with G-proteins, identified and characterized in 1993 (O'Dowd et al., 1993). It shows a high sequence

homology (30%) with angiotensin II type 1 receptor (AT-1), although it 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 amminoacids and its biological activity resides in the C-terminus (apelin-36 and apelin-13) (Tatemoto et al., 1998).
Early studies demonstrated that Apelin and its receptor APJ are abundantly expressed in several adult organs like the heart, the central nervous system (CNS) and the lungs (Medhurst et al., 2003) (Kawamata et al., 2001). 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 as in fluid homeostasis and the regulation of vascular tone (Kleinz and Davenport, 2005). More recently, several studies indicate that APJ/Apelin are associated with the cardiovascular system and its embryonic development (Falcao-Pires and Leite-Moreira, 2005). Indeed, they are expressed in the endothelial cells and vascular smooth muscle cells playing important roles in the regulation of cardiac contraction (Kleinz et al., 2005).
One of the major transduction pathways activated by Apelin depends on the interaction with a Gi-protein coupled to the APJ receptor and the subsequent interaction with the protein Kinase C (PKC) (Masri et al., 2004). PKC activation results in the Na^{+}-H^{+} (NHE) exchanger phosphorylation, which promotes the activation of Na^{+}-Ca^{2+} (NCX) exchanger, the entry of Ca^{2+} into the cell and the contraction. In addition to the inhibition of adenyl cyclase pathway, Apelin activates the phosphorylation of the intracellular kinase p70S6K through a pertussis toxin (PTX) sensitive G protein (Masri et al., 2002). This activation depends on two mechanisms, which are either ERK or PI3K/AKT-dependent (Masri et al., 2004). The latter is responsible for the phosphorylation and activation of endothelial nitric oxide (eNOS) synthase, which mediates Apelin vasoactive effects (Kleinz and Davenport, 2005).
APJ/Apelin system is involved not only in the regulation of adult functions, but also in the early stages of cardiovascular development, as indicated by more recent evidence in the literature. Most remarkably, growing evidence point for a key role of APJ and Apelin 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, both gain of function and loss of function of APJ/Apelin signaling results in a reduction in cardiomyocytes numbers and abnormal cardiac morphology (Scott et al., 2007) (Zeng et al., 2007). Nevertheless their function in mammalian cardiomyogenesis was still unproven.

2 AIM OF THE PROJECT

Given the complexity of the molecular mechanisms controlling early events of cardiac commitment and differentiation in mammals, ESCs and/or induced pluripotent stem cells (iPSCs) provide an ideal paradigm for the *in vitro* study of cardiomyogenesis, facilitating the identification and characterization of genes/mechanisms implicated in this process. Recently, the possibility of reprogramming adult somatic cells into pluripotent stem cells (iPSCs) has generated a renewed interest into stem cell research and promises to overcome several key issues, including the ethical concerns of using human embryonic stem cells. However, the successful use of stem cells in programs of drug discovery and/or regenerative medicine is still limited by the lack of methods and technologies able to optimize both the identification/purification of stem cells and the identification of molecules able to modulate (induction/inhibition) specific differentiation pathways.

To date, little is known about the regulatory mechanisms, including signals of differentiation and growth factors, which specify or promote the cardiogenic phenotype. Recent data point out a key/indispensable role for Nodal/Cripto-dependent Smad2 activation in ESC cardiomyogenesis; however little is known about the mechanisms of action of Cripto/Smad2 signaling and the molecular identity of its downstream effectors in mammalian cardiomyogenesis.

The aim of my PhD project was to identify and characterize the genes acting downstream of *cripto* in ESC differentiation, which may represent novel molecules involved in cardiac lineage specification and differentiation in mammals.

Interestingly, growing evidence indicate that many of the molecules and mechanisms that regulate growth of the embryonic heart are reactivated in the adult heart in response to stress signals that provoke cardiac enlargement and heart failure. Thus, understanding the mechanisms involved in mammalian cardiomyogenesis promises to provide insights both into molecular basis for pathogenesis of the adult heart and to reveal novel therapeutic targets and strategies for the treatment of cardiovascular diseases.

3 MATHERIALS AND METHODS

3.1 ESC propagation and maintenance

The ESC lines RI, *cripto*$^{-/-}$ DE7 (Xu et al., 1998) and E14 were used throughout this study. Wild-type and *cripto*$^{-/-}$ ES cells were maintained in the undifferentiated state by culture on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layer, according to standard protocols. E14 were propagated in culture without feeder layer support. The culture medium consists of high glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 15% fetal bovin serum (DEFINED, Euroclone), 0,1mM β-mercaptoethanol (Sigma- Aldrich), 1mM sodium pyruvate (GIBCO), 1x non essential amminoacids (GIBCO), 2mM glutamine (GIBCO), 100U/ml penicillin/ streptomycin (GIBCO), and 10^3 U/ml Leukemia Inhibitory Factor (LIF) (Euroclone). Tetracycline and puromycin were purchased from Sigma-Aldrich and used at concentration of 1ug/ml and 1.5ug/ml, respectively.
ES cells were routinely passaged every 2 days, and the medium was changed every day.

3.2 ESC *in vitro* differentiation

For *in vitro* differentiation to cardiomyocytes ESCs were cultivated in embryoid bodies (EBs) as described (Parisi et al., 2003), the protocol is schematized in Figure 8.

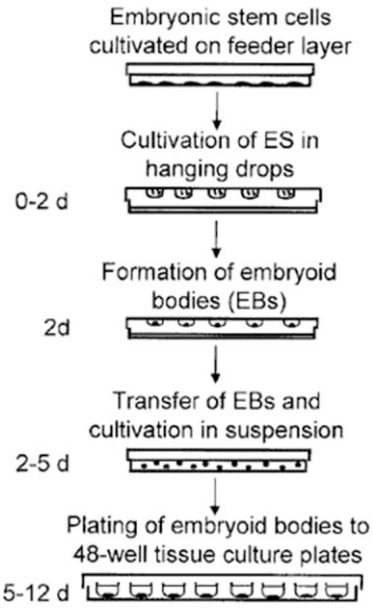

Fig. 8: Schematic representation of the hanging drop method used to differentiate ESCs toward cardiomyocytes (Parisi et al., 2003).

Briefly, ESCs were removed from feeder layer and LIF to allow their differentiation through EBs in hanging drop (400 cells/ drop) placed on the lids of tissue culture dishes for two days. After further three days of culture in bacteriological Petri dishes, the five day-old EBs were plated on gelatin coated 48-well plates for morphological analysis, on 60m and 100mm tissue culture plates for Western Blotting, Immunofluorescence, FACS and RT-PCR analysis. Rhythmic beating of the EBs, indicating cardiac muscle differentiation, was monitored starting from day 7 of differentiation using phase microscopy (Leica).

3.3 ESC transfection and plasmids

Murine APJ/msr1 cDNA (kindly provided by Dr. Y Audigier) was sub-cloned into the pEF1α vector (Clonetech) to generate an APJ/msr1-V5 tagged fusion protein and then cloned into the Pallino βA vector (Parisi et al., 2003) for expression in ESCs.
Sh-RNAs vectors (pGIPZ lentiviral and pLKO vectors) were purchased from OpenBiosystem and used accordingly to manufacturer's instructions. Undifferentiated ESCs ($4x10^6$ cells) were transfected using Nucleofector Technology (AMAXA), following the manufacturer instructions and subjected to puromycin selection for ten days for clone isolation and selection.

3.4 Western Blotting and inhibitors

Two day-old EBs were treated with SB-431542 (10uM) for 24 hours and then lysated for RNA extraction. Four day-old EBs were starved for 16 hours and the treated with the following inhibitors: U0126 (10uM) for two hours; PTX (100ng/ml) overnight.
Undifferentiated ESCs and EBs, collected at different time points during differentiation, were lysated in NP-40 lysis buffer or in 2x laemmli buffer. Protein extracts were quantified by Biorad assay and Coomassie staining and resolved on (8- 12%) SDS- polyacrylamide gel electrophoresis (PAGE). Gels were transferred to a PVDF membrane (Amersham), blocked with 5% milk or 5% BSA in Tris-buffered saline tween (TBST) buffer and incubated with primary antibody overnight. After washing 3 times with TBST buffer, blots were incubated with secondary antibody in 5% milk-TBST buffer for 1 hour. Then blots were washed 3 times with TBST and once with TBS buffer before colorimetric revelation with ECL substrate (PIERCE), following manufacture instructions. The Antibodies used are the following: anti-βIII-tubulin (1:400; Sigma-Aldrich), anti-V5 (1:1000; Invitrogen), anti-Gapdh (1:10000; AbCam), anti-pERK (1:1000), anti-ERK (1:500), anti pAKT (1:1000), anti-AKT (1:1000), anti-pP70S6K Thr389 and anti-pP70S6K Thr421/Ser424 (1:500), anti-p70S6K (1:1000), anti-Smad2 (1:500) (Cell Signaling). Apelin-13 peptide was purchased from Sigma-Aldrich and used at concentration of 10uM.

3.5 Immunofluorescence

Monoclonal anti-V5 (1:1000; Invitrogen), anti-βIII-tubulin (1:400; Sigma-Aldrich) and MF20 (1:50; obtained from the Developmental Studies of Hybridoma Bank, University of Iowa) were used. Both ESCs and EBs were fixed for 15 minutes at room temperature in 4% paraformaldehyde (PFA; anti-βIII-tubulin and anti-V5 antibody). EBs were fixed for 30 minutes, in methanol:acetone (7:3; MF20 antibody) at 4°C. Cells were rehydrated in 1x PBS and permeabilized with 0,1% Triton; then blocked with 10% normal goat serum, 1% BSA in 1x PBS for 30 minutes and incubated with primary antibodies overnight at 4° C.
After 3 times washings in 1x PBS, cells were incubated with secondary antibodies: goat anti-mouse FITC (1:35; DAKO) or anti-mouse Red Alexa Fluo 594 (1:400; Invitrogen) for 30 min at room temperature in 1% BSA, 1x PBS. Finally, cells were washed and mounted with VectaShield medium with DAPI (Vector Laboratories). Labeling was visualized by fluorescent illumination using an inverted microscope (Leica DMi 6000).

3.6 Fluorescence Activated Cell Sorting (FACS)

Eight day-old EBs were dissociated into single cells suspension using Trypsin-EDTA solution 10x (SIGMA). $2x10^6$ cells were then permeabilized with 0,05% saponin/PBS 1x buffer for 20 minutes on ice. Cells were washed with 15% FBS/DMEM and stained with anti-myosin heavy chain antibody (MF-20, 1:50) for 1 hour. Following washes with 15% FBS/DMEM cells were incubated with anti-mouse FITC (1:100; Jackson) secondary antibody. After 30 minutes cells were washed with 15% FBS/DMEM and resuspended in 500ul of 15% FBS/DMEM and analyzed on the FACS Instrument (FACS Aria, Becton Dickinson). Data were analyzed using Diva software.

3.7 RNA preparation and RT-PCR

Total RNAs from both undifferentiated ESCs and EBs collected during differentiation, were isolated using RNeasy mini kit (Qiagen), following manufacturer instructions. Total RNAs were reverse transcribed using QuantiTect Reverse Transcription kit (Qiagen) following manufacturer instructions. Quantitative Real time PCR was performed using SYBR Green PCR master mix (EuroClone). The primers used are described in Table 1.

gene	primer (sense)	primer (antisense)
Apj	CTATGGGGCTGACAACCAGT	GGCAAAGTCACCACAAAGGT
Apelin	GATTGCACGTGTAGGGAGGT	ATTCGAACAGATGCCAAAGG
Brachyury	GAACCTCGGATTCACATCGT	TTCTTTGGCATCAAGGAAGG
Mesp1	GCGACATGCTGGCTCTTCTA	TGGTATCACTGCCGCCTCTTCC
Nkx2.5	CAGTGGAGCTGGACAAAGCC	TAGCGACGGTTGTGGAACCA
Gata4	CTGTCATCTCACTATGGGCA	CCAAGTCCGAGCAGGAATTT
Tbx5	GGAGCCTGATTCCAAAGACA	TTCAGCCACAGTTCACGTTC
Mef2c	ACCCCAATCTTCTGCCACTG	TCAGACCGCCTGTGTTACCTG
Mlc2v	AAAGAGGCTCCAGGTCCAAT	CCTCTCTGCTTGTGTGGTCA
aMHC	TGAAAACGGAAAGACGGTGA	TCCTTGAGGTTGTACAGCACA
NeuroD	GAGGAATTCGCCCACGC	GGTCATGTTTCCACTTCCTGTTG
NFM	CACATCACGGTAGAGCGCAA	CGCCGTGGAGATGTCTGTCT
aFP	CCACGTTAGATTCCTCCCAGTGCGT	CATACTTGTTAGAGAGTTCCGTCTC
Flk1	GGCGGTGGTGACAGTATCTT	GAGGCGATGAATGGTGATCT
Pecam1	TGCTCTCGAAGCCCAGTATT	TGTGAATGTTGCTGGGTCAT
VE-cadherin	CAATGACAACTTCCCCGTCT	CGTTTGGGGTCTGTCTCAAT
tTAUP/Hygro	GCATCAAGTCGCTAAAGAAGAAAG	GAGTGCTGGGGCGTCGGTTTCC

Table 1: Marker genes used for Real-Time PCR analysis

3.8 In situ hybridization (WISH)

Embryos were dissected in PBS and fixed in 4% paraformaldehyde in PBS at 4°C for 2-16 hours, washed in PBT (0.1% Tween 20 in PBS) and WISH was performed as previously described (Liguori et al., 2003).

4 RESULTS

4.1 APJ and Apelin are downstream targets of Cripto in ESCs

Cripto/Nodal/Smad2 is one of the key signaling pathways, which regulates cardiac specification in mammals. Indeed, several lines of evidence show a key role for Cripto/Nodal in the temporal and spatial regulation of Smad2 pathway that is fundamental for early induction of cardiac lineage and subsequent cardiac differentiation (Kitamura et al., 2007). In searching for genes that might act in concert with Cripto to promote cardiac differentiation of ESCs, we were attracted to the G-protein-coupled receptor Angiotensin type- I like receptor (AGTRL-1/APJ) and its ligand Apelin, both upregulated in *cripto*$^{-/-}$ embryoid bodies (EBs) exposed to recombinant Cripto protein for 24hrs in a microarray analysis. Although APJ and Apelin have been recently described as important regulators of cardiovascular function both in Xenopus (Inui et al., 2006) (Cox et al., 2006) and in Zebrafish (Scott et al., 2007) (Zeng et al., 2007) their role in mammalian cardiogenesis was still unproven.
We thus compared the expression profile of *apj* and *apelin* in wild-type and *cripto*$^{-/-}$ ESCs during cardiac differentiation, using the hanging drop method. Real-Time PCR showed that *apj* and *apelin* expression was strongly reduced in *cripto*$^{-/-}$ ESCs, while both genes were markedly upregulated in wild-type ESCs (Fig. 9A-B).

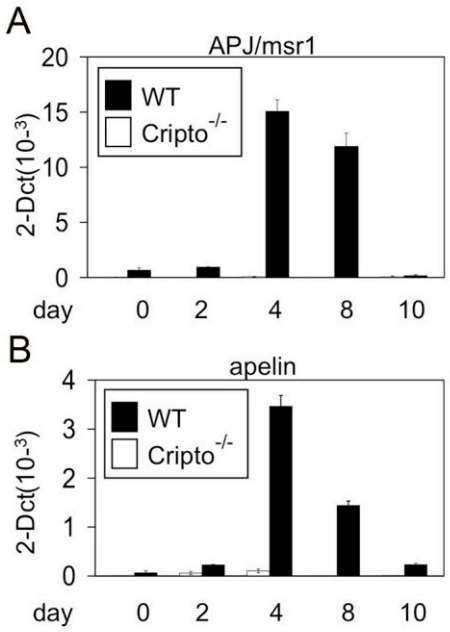

Fig. 9: Expression profile of *apj* and *apelin* in ESC cardiac differentiation.
Expression of *apj* (A) and *apelin* (B) in wild-type and *cripto*$^{-/-}$ ESCs by Real-Time PCR; mRNA was normalized to *gapdh* expression. Data are means +/- SE, n=3.

We further analyzed the expression profile of *apj* and *apelin*, *in vivo*, on mouse embryos at 6.7 day post coitum (dpc). To our knowledge, no previous studies have described the expression profile of *apelin* in early mouse embryogenesis and the only expression of *apj* transcripts was from the late headfold stages (E8) (Devic et al., 1999). We performed whole-mount *in situ* hybridization on gastrulating wild-type and *cripto*$^{-/-}$ embryos. Expression of both *apj* and *apelin* genes correlates with that of *cripto* at this developmental stage (Fig. 10A). While *apelin* mRNA identifies the developing primitive streak, *apj* is expressed both in the primitive streak and in the adjacent mesoderm, similar to *cripto* (Fig. 10A). In accordance with the results obtained in ESCs, *apj* and *apelin* expression was almost undetectable in the mesoderm of *cripto*$^{-/-}$ embryos.

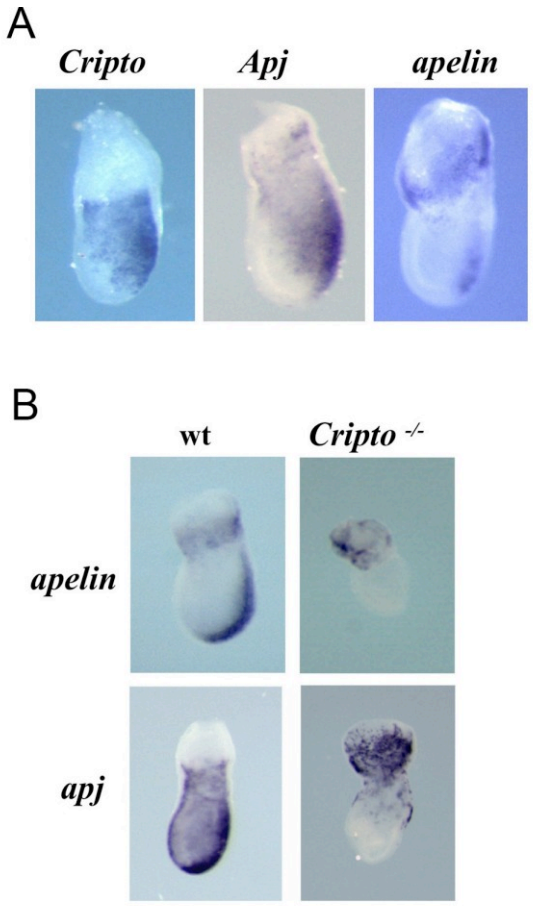

Fig. 10: Expression of *apj*, *apelin* and *cripto* in gastrulating embryos.
Whole mount *in situ* hybridization of *apj*, *apelin* and *cripto* in wild-type embryos at 6.7 dpc (A); and *apj* and *apelin* in *cripto*$^{-/-}$ embryos at 7 dpc (B).

Remarkably, expression of both genes persisted in the extraembryonic tissue of *cripto*$^{-/-}$ embryos, thus indicating that *apj* and *apelin* expression in the embryonic mesoderm is *cripto*-dependent, whereas, in the extraembryonic tissue is *cripto*-independent (Fig. 10B).
The expression profile of *apj* and *apelin* both in ESC differentiation and embryonic development therefore support the idea that *apj* and *apelin* may represent early response genes of Cripto signaling.

4.2 Cripto regulates *apj* and *apelin* expression through early activation of Smad2

Suppression of early Cripto/Smad2 activation results in the inhibition of cardiomyogenesis and the induction of neuronal differentiation (Kitamura et al., 2007; Parisi et al., 2003).
Based on these considerations, we proposed that the expression of the putative downstream targets of *cripto*, *apj* and *apelin*, might be regulated through the early activation of Smad2. To verify this hypothesis we treated two day–old wild-type EBs with SB-431542, a specific inhibitor of ALK-4, -5, -7 - dependent Smad2 activation (Inman et al., 2002), using DMSO as negative control. After 24 hours, total RNAs were collected and the expression of *apj* and *apelin* was analyzed by Real-Time PCR. Cripto has been recently described as a direct target of Smad4 (Mancino et al., 2008); accordingly, expression of *cripto* was reduced in SB-431542 treated EBs (Fig. 11A). Most remarkably, both *apj* and *apelin* expression was also dramatically reduced, suggesting that *cripto* regulates the expression of these genes via ALK4/Smad2 signaling (Fig.11A). On the contrary, the expression of the neuroectodermal marker NeuroD1 was upregulated, thus supporting the idea that Smad2 inhibition enhances neuroectodermal differentiation in ESCs (Fig. 11B) (Kitamura et al., 2007).

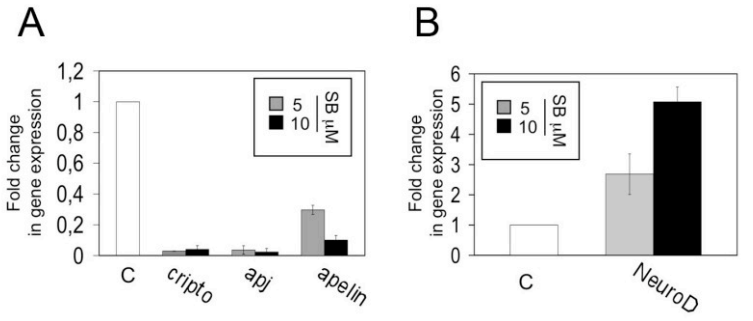

Fig. 11: Effect of Smad2 inhibition on *apj*, *apelin* and *cripto* expression.
Two day–old wild-type EBs were treated for 24hrs with increasing amounts of SB-431542 (SB) or DMSO (vehicle); expression of *apj*, *apelin*, *cripto* (A) and *neuroD* (B) were analyzed by Real Time-PCR. mRNA expression was normalized to *gapdh* and presented as fold change in gene expression relative to the control (DMSO); data are mean ± SE, n=3.

To date, no other targets of SB-431542 have been reported (Inman et al., 2002); however, since SB-431542 inhibits different ALKs receptors, namely ALK-4, -5 and -7, we decided to support our data using shRNAs to target Smad2 expression. To this end, Smad2–silenced ESCs were generated by using vectors, which targeted different, non-overlapping Smad2 mRNA sequences. Following puromycin selection, several ESC clones were isolated and the silencing efficiency was evaluated by Western Blot (Fig. 12A). Two independent clones were selected in which endogenous Smad2 expression was suppressed, compared to control ESCs transfected with a non-targeting shRNA. Moreover, another ESC clone, in which Smad2 expression was not affected, was used as an additional control.
ESC clones were induced to differentiate and the expression of *apj*, *apelin* and *cripto* was evaluated. As expected and in line with results obtained with the chemical inhibitor SB-431542, we observed a strong reduction of *apj*, *apelin* and *cripto* expression in Smad2-silenced EBs compared to controls (Fig. 12B).

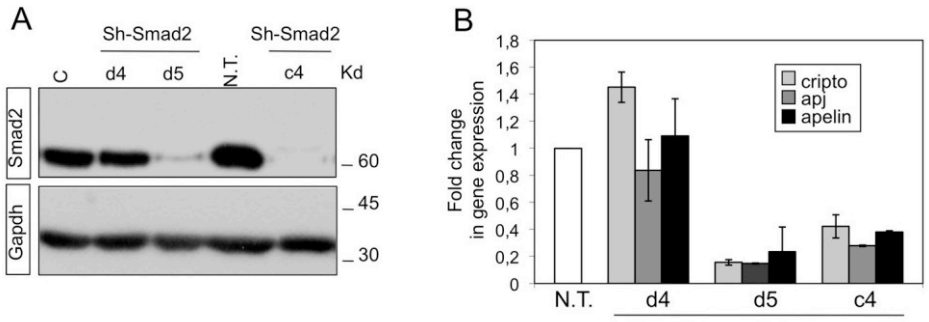

Fig. 12: *apj* and *apelin* expression is downregulated in Smad2-silenced ESCs.
Verification of Smad2 expression in independent Smad2–silenced ES clones at protein level, as shown by western blot using anti-Smad2 antibody. Anti-Gapdh antibody was used as loading control (A). Effect of Smad2 silencing on *apj*, *apelin* and *cripto* expression assessed by Real-Time PCR on 4 day–old EBs derived from the Smad2–silenced ESC clones and controls. mRNA expression was normalized to *gapdh* expression and presented as fold change in gene expression relative to the N.T. control. Data are mean ± SE, n=3.

Together our data indicate that early activation of Smad2 is critical for the induction of *cripto, apj* and *apelin* expression and the consequent inhibition of the neuronal fate.

4.3 APJ overexpression redirects the neural fate of *cripto*$^{-/-}$ ESCs and promotes the cardiac lineage

4.3.1 Generation of ESC clones overexpressing APJ

The expression profile, both *in vivo* and in ESCs, as well as the signaling pathway analysis, point for a correlation between *cripto* and *apj*/*apelin* during cardiac specification and differentiation. To get insight into the functional link

between these signaling pathways we performed gain-of-function experiments in *cripto*$^{-/-}$ ESCs.
Given the essential role of Cripto in redirecting the neuronal fate and promoting cardiomyogenesis in ESCs (Parisi et al., 2003) we evaluated whether forced overexpression of APJ might compensate for the lack of Cripto signaling in cardiomyogenesis. We thus generated a recombinant vector carrying *apj* cDNA fused, at C-terminus, with a V5 epitope, under a constitutive promoter. APJ/V5 overexpression was verified by Western Blot analysis in three independent clones (Fig. 13A) and its membrane localization was confirmed by immunofluorescence analysis, using an anti-V5 antibody (Fig. 13B).

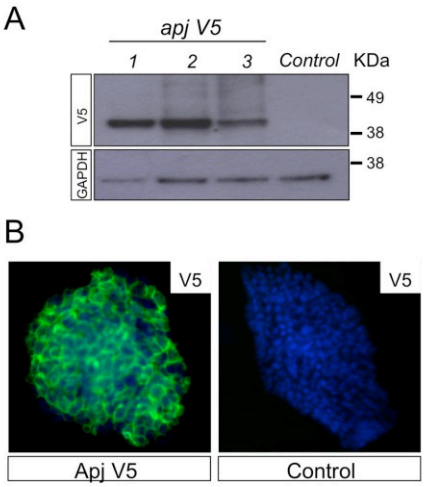

Fig. 13: Functionality of APJ overexpression construct in *cripto*$^{-/-}$ ESCs.
Verification of APJ/V5 overexpression, as shown by western-blot using anti-V5 antibody. Anti Gapdh antibody were used as loading control (A). Immunofluorescence analysis of undifferentiated ESCs showing membrane localization of APJ-V5 (B).

Furthermore, we confirmed, by Real-Time PCR, that the levels of *apj* expression were comparable to that of wild type ESCs (Fig. 14A). Worth noting, APJ overexpression did not alter the expression of *nanog*, a pluripotency marker of ESCs, meaning that it does not affect the undifferentiated state of the cells (Fig. 14B).

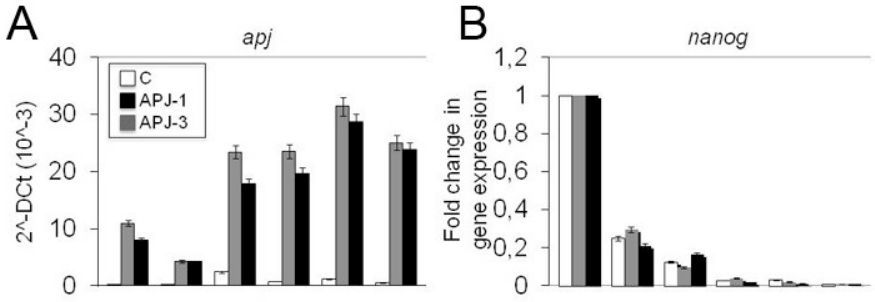

Fig. 14: Time course-expression of *apj* in *cripto*$^{-/-}$ ESCs and APJ-V5 *cripto*$^{-/-}$ ESCs.
Constitutive expression of *apj* during ESC differentiation analyzed by Real Time-PCR (A). expression of the pluripotency marker *nanog* in APJ/V5 clones (B). mRNA expression was normalized to *gapdh*; data are mean ± SE, n=3.

4.3.2 APJ redirects the neural fate of *cripto*$^{-/-}$ ESCs

To evaluate whether APJ overexpression might compensate for the lack of Cripto in ESCs, both control and APJ/V5 overexpressing *cripto*$^{-/-}$ ESCs were induced to differentiate toward cardiomyocytes. As expected, *cripto*$^{-/-}$ derived EBs gave rise to a population of cells with a neuron-like morphology. Notably, this characteristic morphology was neither observed in wild type nor in APJ/V5-*cripto*$^{-/-}$ -derived EBs. These data suggested that APJ overexpression likely prevented neuronal differentiation of *cripto*$^{-/-}$ ESCs. To directly address this issue, we performed immunofluorescence analysis using anti-βIII tubulin antibody, which recognizes the neuron-specific form of class III β-tubulin. To semi-quantify the effect of APJ on *cripto*$^{-/-}$ ESCs we arbitrarily defined 4 grades of neuronal differentiation. The absence of neurons defined Grade 0; while the full neuronal differentiation, meaning the presence of a dense network of βIII-tubulin-positive cells is classified as Grade 3 of differentiation. The intermediate phenotypes that are the presence of few isolated neurons or areas of βIII-tubulin-positive cells were defined as Grade 1 and Grade 2, respectively (Fig. 15A). Results clearly showed that APJ overexpression blocked neuronal differentiation of *cripto*$^{-/-}$ ESCs. Indeed, most, if not all, APJ/V5 *cripto*$^{-/-}$ EBs analyzed showed grade 0 and 1 of neuronal differentiation, with few βIII-tubulin-positive cells (Fig. 15B). Accordingly with the morphological analysis, βIII -tubulin protein was reduced, as shown by Western Blot (Fig. 15C). Notably, one out of the three clones showed βIII -tubulin level comparable to control cells, which was likely due to an atypically high percentage of Grade1 neural differentiation. Furthermore, we investigated the mRNA expression pattern of APJ/V5 *cripto*$^{-/-}$ EBs. We analyzed the expression of the neuroectodermal marker of the early stages of differentiation, *neuroD*. As expected, we found a down-regulated expression of *neuroD* in APJ/V5 *cripto*$^{-/-}$ EBs compared to control (Fig. 15D).
Taken together these data indicate that APJ overexpression is able to prevent neuronal differentiation of *cripto*$^{-/-}$ ESCs.

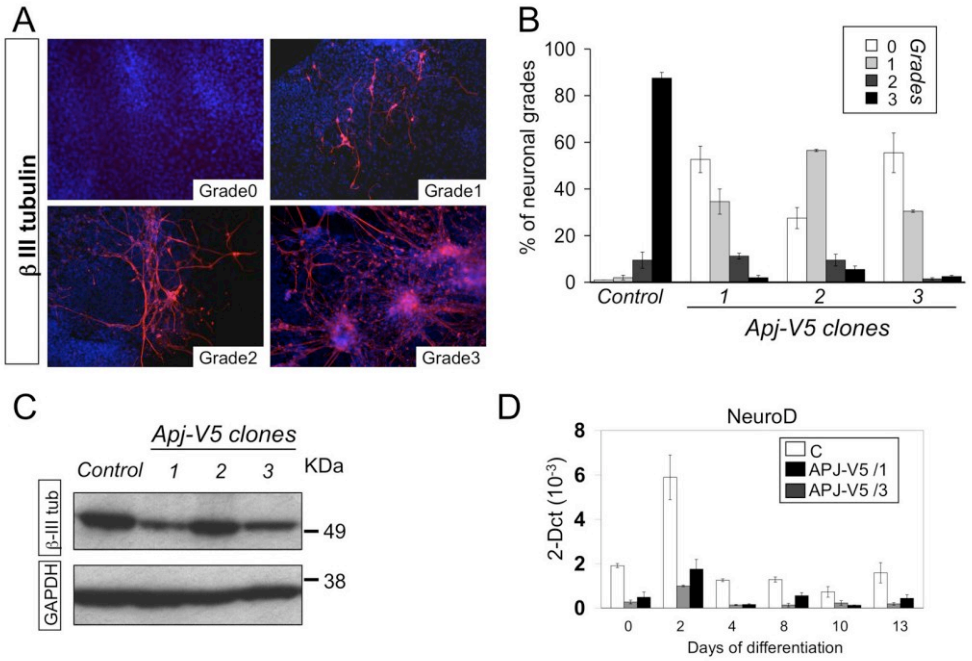

Fig. 15: APJ overexpression redirects the neural fate of *cripto*$^{-/-}$ ESCs.
Grades of neuronal differentiation of *cripto*$^{-/-}$ EBs, as arbitrarily defined. Nuclei were visualized by DAPI counterstaining. Magnification 20x (A). Immunofluorescence analysis performed on 13 day–old EBs and distribution of neuronal grades, as indicated in A. Data are mean ± SE; numbers of EBs scored/clone ~60; n=3 (B). Expression of β-III tubulin, shown by western blot on 13 day-old EBs. Anti Gapdh antibody was used as loading control (C). Expression of neuronal marker *neuroD* in control and APJ-V5 *cripto*$^{-/-}$ EBs (clones 1-3), by Real-Time PCR. mRNA expression was normalized to *gapdh* expression. Data are mean ± SE, n=3(D).

4.3.3 APJ specifies the cardiac lineage of ESCs in the absence of *cripto*

Given the ability of APJ to redirect the neural differentiation of *cripto*$^{-/-}$ ESCs, we asked whether APJ was able to restore the cardiac genetic program in the absence of Cripto.
To this end, both control and APJ overexpressing *cripto*$^{-/-}$ ESCs were allowed to differentiate toward cardiomyocytes and the expression profile of selected markers was evaluated by Real-Time PCR. Notably, APJ overexpression resulted in the induction of the pan-mesodermal marker *T/bra* as well as the earliest cardiac marker *mesp1* (Fig.16). *Mesp1* functions subsequent to *brachyury* as the earliest molecular marker of cardiac precursors and a key regulator of the commitment of mesodermal cells towards the cardiac lineage (Kitajima et al., 2000) (David et al., 2008). According to these observations, we found that other important cardiac transcription factors that are expressed subsequently to *brachyury* and *mesp1* activation, such as *mef2C*, *nkx2.5*, *tbx5* and *gata4* are all induced in APJ/V5 *cripto*$^{-/-}$ EBs (Fig.16). Furthermore, the cardiac structural gene *mlc2v* resulted also expressed upon APJ overexpression (Fig.16). Most remarkably, the expression profile of the cardiac markers

correlated with that of wild-type ESCs. Interestingly, the extraembryonic endoderm marker *afp* was also induced, suggesting that APJ induced the mesendodermal program (Fig. 16).

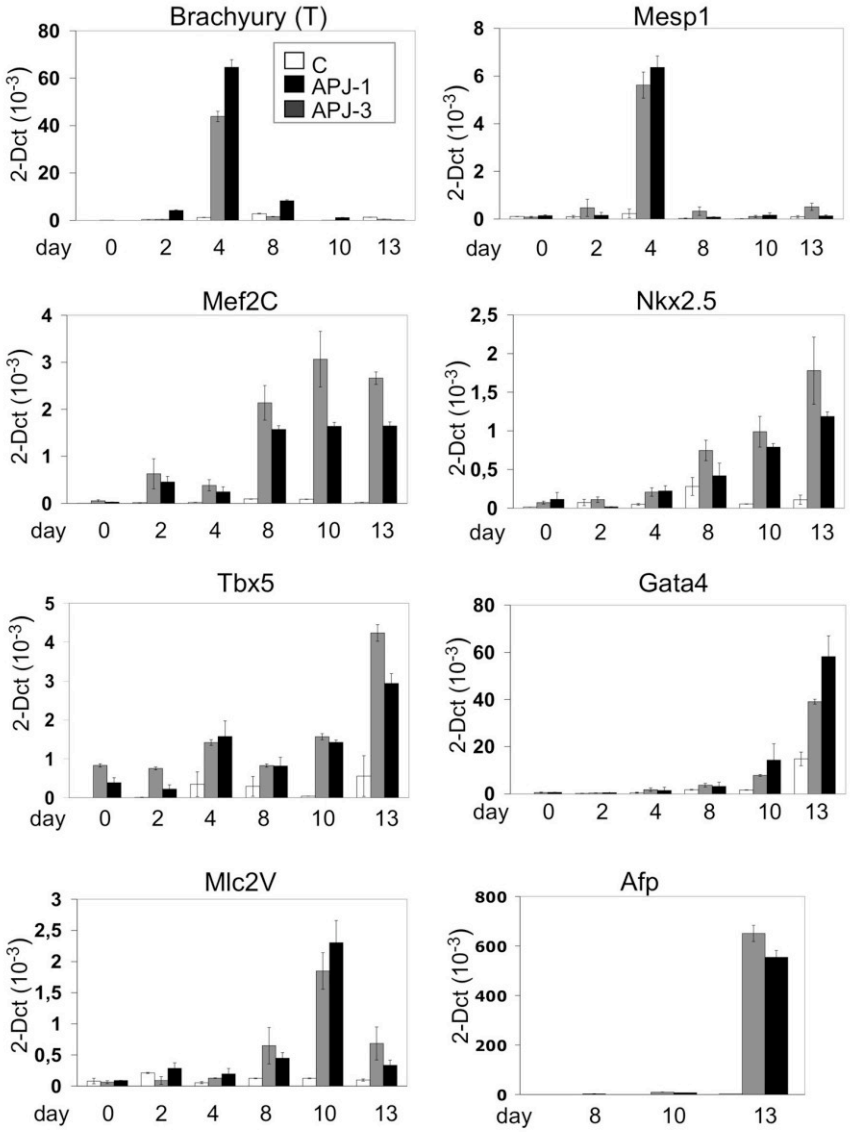

Fig. 16: APJ specifies embryonic stem cells toward the cardiac lineage.
Induction of mesendodermal program and cardiac myogenesis, as shown by Real-Time PCR. *cripto*$^{-/-}$ APJ-V5 clones 1 and 3 were used for the analysis; mRNA expression was normalized to *gapdh* expression; data are mean ± SE, n=3.

Although the cardiogenic program seems to be restored upon APJ overexpression we did not observe the formation of beating cardiomyocytes, even after long-term culture, and αMHC positive cells were absent in APJ/V5 ESC–derived EBs. The absence of beating phenotype might have several

reasons. First of all, the APJ ligand Apelin might represent the major limiting factor. However we observed that *apelin* expression increased upon APJ overexpression and persisted throughout ESC differentiation (Fig. 17). Nevertheless, the transient nature of Apelin accumulation in wild-type EBs (Fig. 9B) suggested that it might be required in a specific moment and in a defined step of cardiac induction.

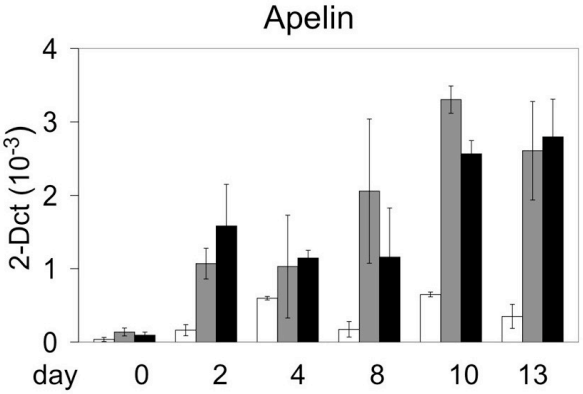

Fig. 17: Time course-expression of *apelin* in *cripto*$^{-/-}$ ESCs and APJ-V5 *cripto*$^{-/-}$ ESCs.
Apelin expression is rescued in APJ-V5 *cripto*$^{-/-}$ ESCs, as shown by Real-Time PCR. mRNA expression was normalized to *gapdh* expression; data are mean ± SE, n=3.

We thus went on to verify whether addition of Apelin protein might improve the effect of APJ overexpression on cardiomyogenesis or redirect the neural phenotype of *cripto*$^{-/-}$ ESCs. We thus added Apelin to APJ/V5 ESC–derived EBs, every 24 hrs, between day 2 and 4, which reflects the peak of endogenous *apelin* expression. Interestingly, we found that the expression of the cardiac structural gene *mlc2v* was significantly increased, although in the absence of beating EBs. Interestingly, Apelin treatment of *cripto*$^{-/-}$ EBs reduced the expression of the neuronal marker *neuroD* suggesting that it antagonizes neuronal differentiation (Fig. 18A). Apelin activity in *cripto*$^{-/-}$ EBs was likely due to residual APJ expression; although, we cannot completely rule out the possibility that Apelin still induced its biological effect independent of APJ receptor.
Notably, addition of Apelin to wild-type EBs, in the presence of *cripto* and thus in a more physiological contest, did not further enhance cardiac differentiation, as revealed by expression of *mlc2v* and *αMHC*, which was comparable in control and Apelin-treated EBs (Fig. 18B).

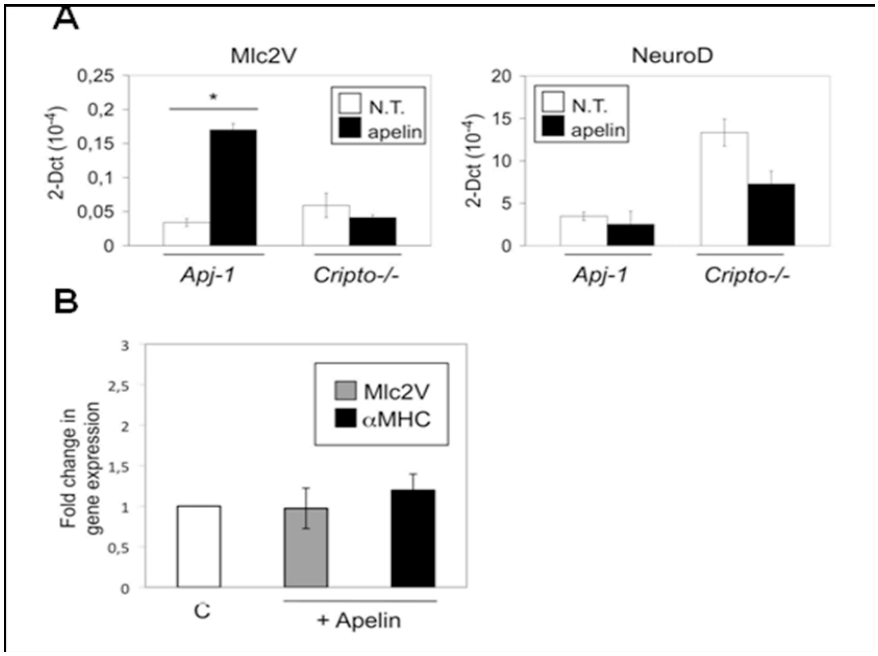

Fig. 18: Apelin redirects the neural fate of *cripto*$^{-/-}$ ESCs and promotes cardiomyogenesis.
Expression of cardiac and neuronal markers (*mlc2V* and *neuroD*) in 13 day-old EBs, following Apelin treatment. *cripto*$^{-/-}$ or APJ-V5 (APJ-1) *cripto*$^{-/-}$ EBs were treated with Apelin (10μM) every 24hrs, during the 2-4 day interval of differentiation. mRNA expression was normalized to *gapdh* expression. Data are mean ± SE, n≥3. * P <0.05. (A) Expression of *αMHC* and *mlc2V* in 13 day-old wild type EBs treated with Apelin (10μM) every 24hrs during the 3-4 day-interval or left untreated as control. mRNA expression was normalized to *gapdh* expression and presented as fold change in gene expression relative to the control (untreated EBs). Data are mean ± SE, n=2.

4.4 APJ and Apelin are key molecules for cardiac commitment and differentiation

Gain of function experiments in *cripto*$^{-/-}$ ESCs indicate a crucial role for APJ/Apelin in cooperating with Cripto for cardiac induction. To further assess the role of *apj* and *apelin* in cardiomyogenesis we performed silencing experiments. We thus used two different shRNAs, which targeted different, non-overlapping *apelin* sequence. Following puromycin selection we isolated several independent ESC clones to minimize effects of clone-to-clone variation and we evaluated the efficacy of gene silencing, by Real-Time PCR Following puromycin selection, independent ESC clones for each shRNA, were isolated, with a silencing efficacy in the range of 80 to 95% (Fig. 19A). Interestingly, we observed a dramatic suppression of cardiac differentiation, with a strong reduction of the percentage of beating EBs in the *apelin* silenced ESC clones compared to the control (Fig. 19B).

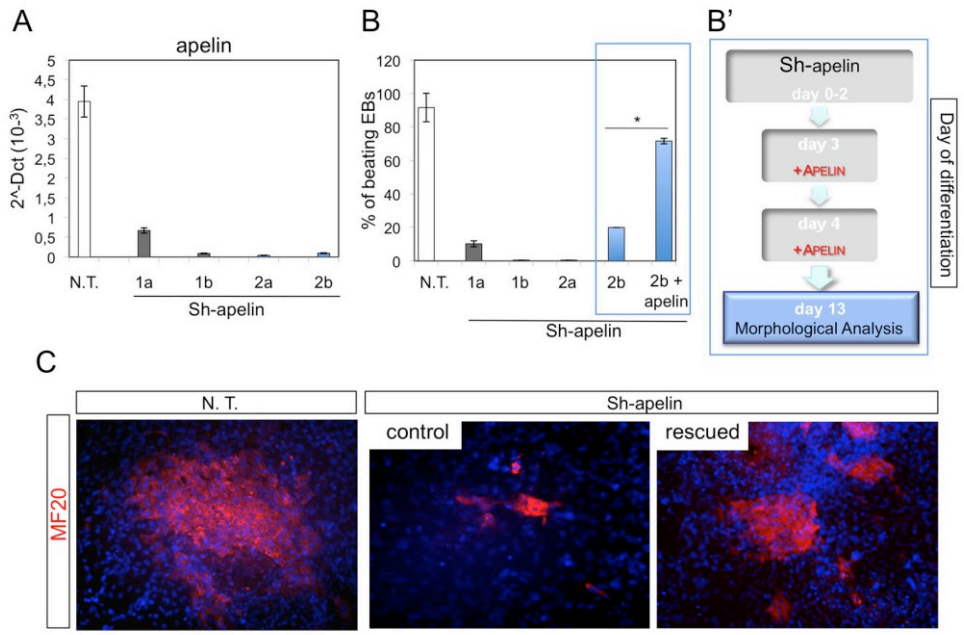

Fig. 19: A pivotal role for *apelin* in cardiac differentiation of ESCs.
Suppression of *apelin* expression by shRNAs (A). Two independent ESCs for each shRNA vector (Sh1, 2) are shown (Sh1a, b; Sh2a, b); Non Targeting control vector (NT). Suppression of cardiac differentiation by *apelin* shRNAs in 13 day-old EBs; (B) and immunostaining with anti-αMHC antibody (MF20; C). Addition of recombinant Apelin to EBs in the 3-4 day interval, described in B', rescues the cardiac phenotype, as shown by the prevalence of beating EBs (B) and MF20 immunostaining (C). Numbers of EBs scored/condition, n~ 50.

Accordingly, immunofluorescence analysis showed few and isolated, if any, α-MHC immunoreactive cells. Morphological observation was also supported by molecular analysis, showing a dramatic down-regulation of *α-MHC* expression (Fig. 19C). The expression profile of *apelin* suggested that it acts in a precise and early time window of cardiac differentiation. To directly address this issue we performed rescue experiments on *apelin* silenced clones. Apelin protein was added directly to shRNA derived EBs, during the 3-4 day interval, which reflects the time window of *apelin* expression (Fig. 19B'). After 24 hours of treatment we allowed EBs to continue the differentiation and analyzed the efficiency of cardiomyocyte formation. Notably, the addition of Apelin was able to rescue the cardiac phenotype, thus providing the first direct evidence that Apelin is required in an early window for priming differentiation of ESCs to a cardiac fate (Fig. 19B-C). Because neuronal differentiation occurs in *cripto*$^{-/-}$ ESCs, we asked whether the same phenotype could be observed in *apelin*-silenced clones. We thus analyzed the expression of βIII tubulin protein by both immunofluorescence and western blotting. As expected, βIII tubulin accumulated in *cripto*$^{-/-}$ EBs; whereas it was undetectable in EBs derived from ESCs expressing either non-targeting shRNA or *apelin*–shRNAs (Fig. 20A); thus indicating that *apelin* was not able to redirect the neural fate of ESCs. We

thus went on to analyze at which step of cardiomyogenesis *apelin* is required and examined the expression of different markers, by Real-Time PCR. Expression of the pan-mesodermal marker *brachyury* was not affected in *apelin*-silenced clones. On the contrary the earliest cardiac marker *mesp1*, as well as the cardiac genes, *nkx2.5* and *αMHC* were all strongly down-regulated in *apelin*-silenced ESCs compared to control (Fig. 20B). These findings suggest a key role for *apelin* in cardiac commitment, acting after *brachyury* induction and immediately upstream of *mesp1*.

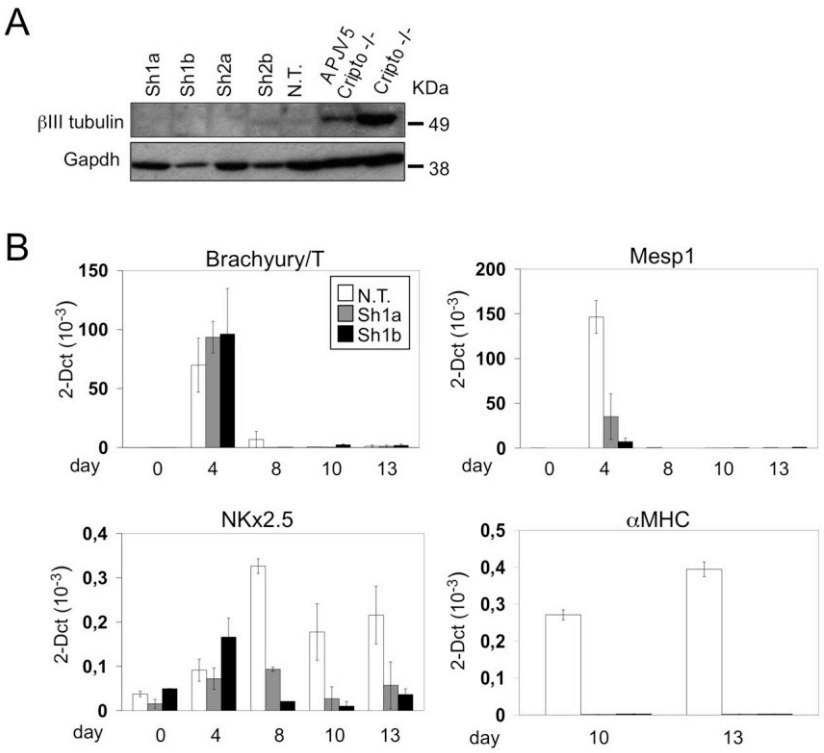

Fig. 20: *apelin* knock out selectively impairs cardiac specification and does not induce neuronal differentiation of ESCs.
Expression of βIII tubulin on 13 day-old EBs (clones Sh1a, b; Sh2a, b). Non Targeting (NT) control EBs. *cripto*$^{-/-}$ and APJ-V5 *cripto*$^{-/-}$ are positive controls. Anti-Gapdh antibody were used as loading control (A). Real Time-PCR showing that *apelin* acts downstream of mesoderm formation (*brachyury/T*) but upstream of cardiac specification (*mesp1*). N.T., Sh1a and Sh1b ESCs were used. mRNA expression was normalized to *gapdh* expression; data are mean ± SE, n=2.

To further assess the role of APJ/Apelin signaling in ESC differentiation we evaluated the effect of APJ silencing in cardiac differentiation. Following the same experimental approach used for *apelin*, we generated three independent ESC clones in which *apj* expression was silenced (Fig. 21A).
In line with our hypothesis, the downregulation of *apj* reduced the percentage of beating EBs (Fig. 21B), as confirmed by immunofluorescence analysis, showing few α-MHC immunoreactive cells in *apj*–silenced EBs compared to control (Fig. 21C).

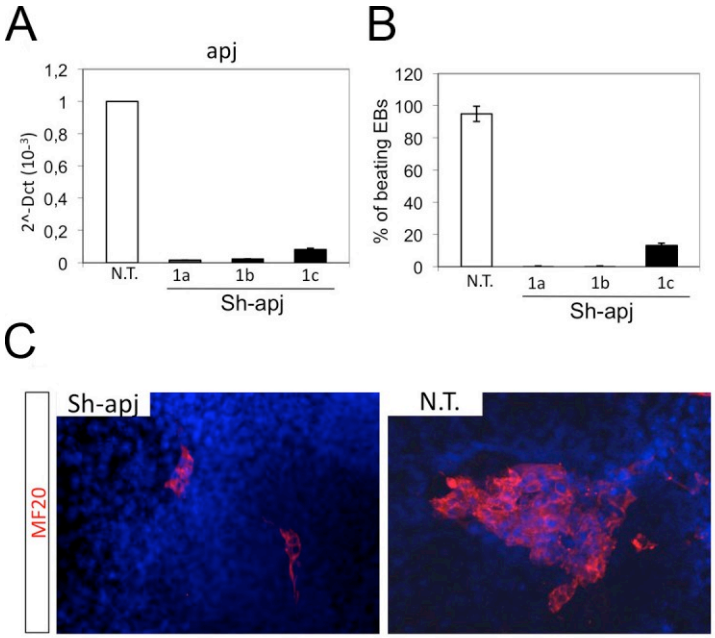

Fig. 21: *apj* silencing by shRNAs impairs cardiomyogenesis.
Verification of *apj* expression in three independent *apj*–silenced (Sh-apj 1a, 1b and 1c) 4 day-old EBs by Real Time-PCR; mRNA expression was normalized to *gapdh* expression. Data are mean ± SE, n=3 (A). Effect of *apj* silencing on cardiac differentiation by *apj* shRNAs as shown by the prevalence of beating EBs (B) and immunostaining with anti-αMHC antibody (MF20; C).

All together these data indicated that APJ/Apelin signaling is pivotal in promoting cardiac specification and terminal differentiation of ESCs and that silencing of both the ligand, *apelin* and the receptor, *apj* in ESCs resulted in a dramatic suppression of cardiac differentiation.

4.5 Functional dissection of signaling pathways involved in APJ/Apelin–dependent cardiomyogenesis

Evidence obtained in different cell types, such as HUVEC and 293T, indicate that Apelin signals through a Gi/o protein coupled to APJ receptor, resulting in the phosphorylation of an intracellular kinase, p70s6K, which occurs on different aminoacidic residues (Masri et al., 2002). The phosphorylation of specific amminoacids depends on the activation of ERKs and/or AKT signaling pathways (Masri et al., 2004). Based on these data, we asked whether the same mechanisms might be involved in APJ/Apelin–dependent ESC cardiac differentiation.
We thus first evaluated whether addition of Apelin induced MAPK and AKT signaling pathways in 4 day–old *apelin*-silenced EBs, a time window which reflects *apj* expression. *apelin*-silenced EBs were starved for 24 hrs before addition of Apelin to the culture medium. Interestingly, phosphorylation of ERK but not AKT was transiently induced by Apelin (Fig. 22A). Accordingly, we showed that p70s6K was phosporylated at residues T421 and S424,

common to ERK and AKT signaling pathway, but not at residue T389, which is specifically activated by AKT (Fig. 22A). These data suggest that Apelin preferentially activates ERK signaling pathway in mammalian cardiomyogenesis.

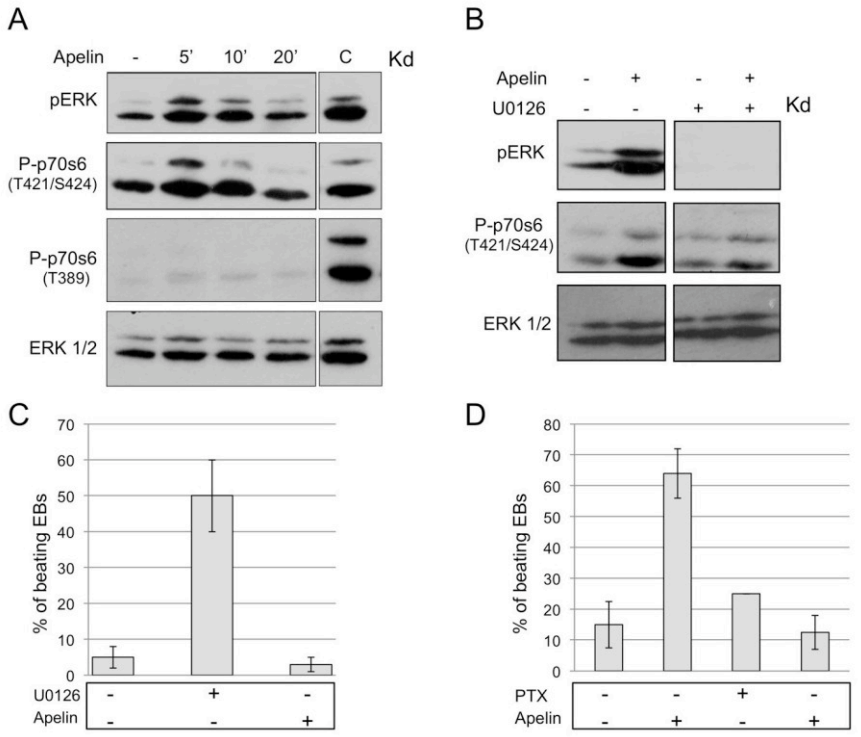

Fig. 22: Apelin promotes cardiomyogenesis through MAPK/p70SK and via a PTX-sensitive G protein.
Serum starved 4 day–old *apelin*–silenced EBs treated with Apelin (10 mM) for the indicated time or left untreated as control (NT). Western Blot were probed with anti–phospho ERK, anti-phospho p70SK (T421/S424 and T389) and anti-ERK antibodies. C indicates positive controls (A). Serum starved 4 day–old *apelin*–silenced EBs treated with UO126 (10mM) for two hours, followed by incubation with Apelin (10 mM) for 5'. Western Blots were probed with anti–phospho ERK, anti-phospho p70SK (T421/S424) and anti-ERK Abs(B). Three day–old EBs treated with U0126 for two hours, followed by incubation with Apelin. U0126 was added twice, every 4 hours, to Apelin–treated EBs. DMSO was added to control EBs (C). Three day–old *apelin*–silenced EBs treated over night with PTX, followed by incubation with Apelin (D). The prevalence of beating EBs was measured on 13 day-old EBs (C, D).

To gain further insight into the functional correlation between Apelin-dependent activation of ERK and cardiomyogenesis we used *apelin* silenced ESC clones, in which impaired cardiomyogenesis can be rescued by Apelin treatment and the rescue assay previously described (Fig. 19B'). We thus used 4 day-old *apelin*–silenced EBs, treated or not with U0126, a specific inhibitors of ERK signaling pathway, before addition of Apelin. As expected, Apelin rescued cardiac differentiation in DMSO–treated control EBs but not in the U0126–treated EBs (Fig. 22B-C), thus indicating that Apelin promotes cardiomyogenesis through the activation of MAPK signaling pathway. Finally, we verified whether APJ acted through a Gi/o protein in ESC cardiac

differentiation. We thus pre-treated three day-old *apelin*-silenced EBs with Pertussis Toxin (PTX), a specific inhibitor of Gi/o protein. PTX treatment fully abrogated the ability of Apelin to rescue cardiomyogenesis (Fig. 22D), thus providing the first direct evidence that the MAPK is activated by Apelin through coupling to a Go/Gi-protein, which contributes to mammalian cardiomyogenesis.

4.6 Regulated *apj* overexpression in an early time window of ESC differentiation enhances cardiac and vascular lineage specification

In order to get insight into the role of APJ/Apelin signaling pathway in controlling ESC differentiation, we generated an inducible cell line, which allow APJ overexpression in a time-specific manner, by using the tetracycline (Tet)-regulated transactivator system. To this end we took advantage of an ESC line (EB3) previously generated, in which the locus Rosa26 was modified with the inducible Tet-OFF expression unit cassette and the hygromycin resistance followed by an IRES-GFP (Cobellis et al., 2005) (Fig.23A).

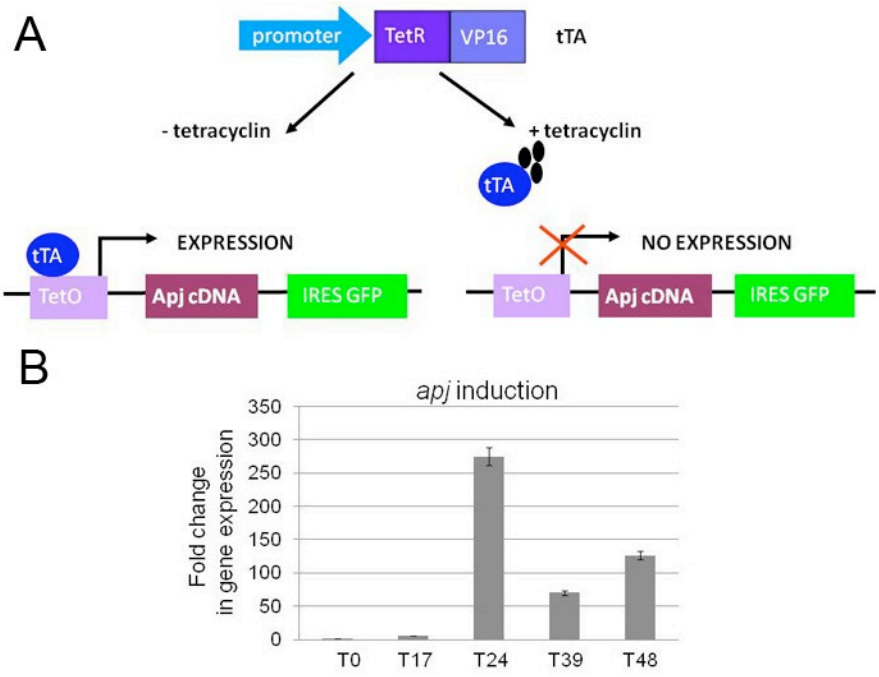

Fig. 23: Generation of TET OFF-*apj* construct.
An inducible ESC line was generated to modulate *apj* overexpression in wild-type ESCs based on Tet OFF system. *Apj* cDNA is followed by an IRES-GFP (A). Verification of *apj* induction in one representative clone by Real Time-PCR. mRNA expression was normalized to *gapdh* expression and presented as fold change in gene expression relative to the control (ESCs + tetracycline). Data are mean ± SE, n=3 (B).

We thus cloned the *apj* cDNA into the exchange vector carrying the short Flippase Recognition Target (FRT) sites for the Flippase (FLP) -mediated exchange into the Rosa26 locus and the puromycin resistance. Following transfection with the recombinant vector, the EB3 cells were subjected to both puromycin and hygromycin selection and ESC clones, which were puromycin but not hygromycin resistant, were subjected to further analysis. We first confirmed the correct recombination of the target gene in the Rosa26 locus by PCR analysis on genomic DNA. To assess the functionality of the system, we then performed a time course experiment on three independent clones and verified the induction of *apj* at different times after tetracycline removal. Figure 23B shows the levels of *apj* induction in one representative clone, indicating that *apj* expression reached the maximum level at 24 hours after tetracycline removal. We thus went on to study the effect of *apj* overexpression in ESC differentiation, starting with the evaluation of the effect of *apj* overexpression in an early time window of differentiation in which mesodermal precursors are committed to the cardiovascular lineage.

Several lines of evidence both *in vivo* and in ESCs, indicate that cardiac and vascular lineages arise from a common progenitor during early stages of development (Kattman et al., 2006) (Kattman et al., 2007). Worth noting, growing evidence indicate that APJ and Apelin are associated not only with cardiac development but also with the vascular system in embryonic development (Inui et al., 2006), tumor angiogenesis (Sorli et al., 2007) and in the regulation of blood vessel diameter (Kidoya et al., 2008).

Based on these considerations, we hypothesized that APJ/Apelin signaling could be required not only for cardiogenesis but also for vascular development. To this end, we choose the 0-4 day interval to induce *apj* overexpression, which anticipates the physiological window of *apj* expression. We first verified that *apj* induction occurs in two day-old EBs (48 hour after Tetracycline removal) by Real-Time PCR and FACS analysis (Fig. 24A-B).

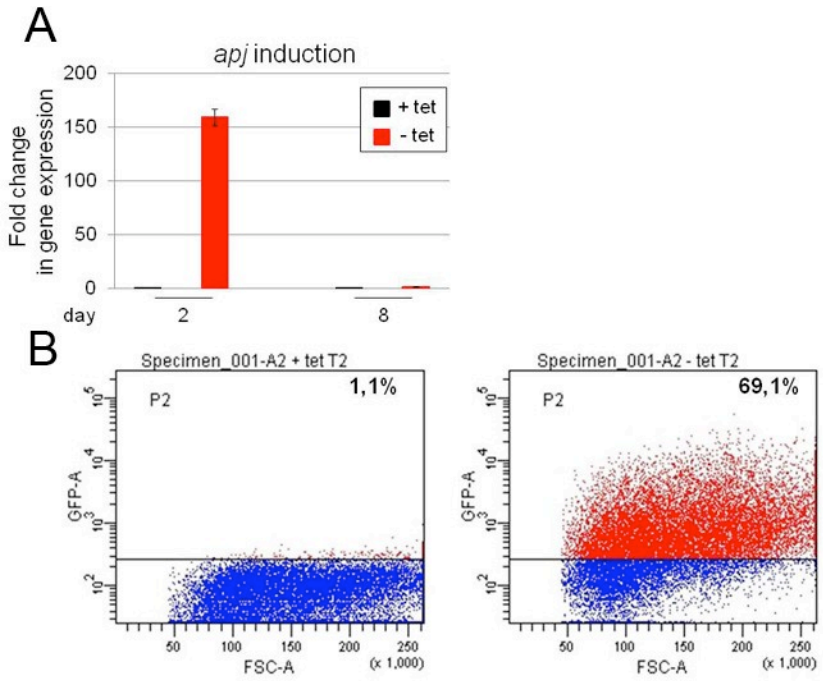

Fig. 24: Functionality of TET OFF-*apj* construct in wild-type ESC differentiation.
apj expression was induced during ESC differentiation, in day 0-4 interval, by removing tetracycline from culture medium. *apj* induction was verified in two day-old EBs (48 hour after tetracycline removal) and the TET OFF was monitored in eight day-old EBs by Real-Time PCR. mRNA expression was normalized to *gapdh* expression. Data are mean ± SE, n=3(A). GFP induction was measured by FACS analysis in the presence or in the absence of tetracycline in two day-old EBs (B).

To assess the effect of *apj* overexpression on terminal cardiac differentiation, we quantified the numbers of cardiomyocytes in ten day-old EBs cultured either in the presence or in the absence of tetracycline, during the 0-4 day interval. Interestingly, the number of cardiomyocytes increased three-fold upon *apj* overexpression, as shown by FACS analysis using αMHC antibody (Fig. 25A-B). Worth noting, these results were confirmed in two independent clones (Fig. 25A). FACS data were also supported by molecular analysis, showing an increase of αMHC expression (Fig. 26).

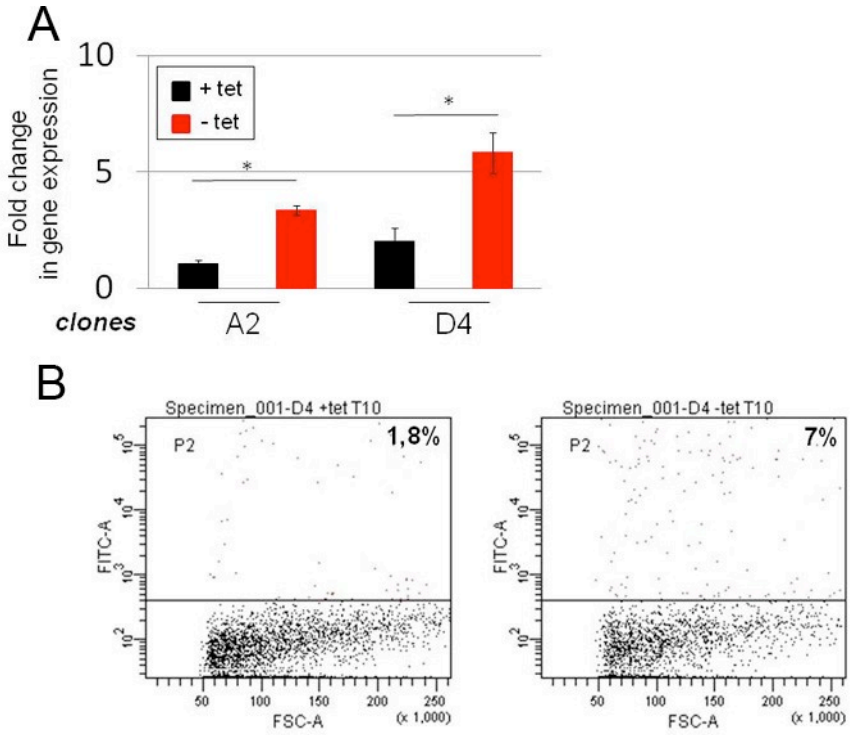

Fig. 25: *apj* overexpression in an early time window of ESC differentiation increases the number of cardiomyocytes.
apj-induced ESCs, in day 0-4 interval, were allowed to differentiate into cardiomyocytes. The number of cardiomyocytes was quantified in ten day-old EBs, cultured either in the presence or in the absence of tetracycline, by FACS analysis, using anti-αMHC antibody (B). The number of cardiomyocytes increased three-fold upon *apj* overexpression in two independent clones (A-B). Data are mean ± SE, n=3, $p<0,05$.

Remarkably, expression profile analysis indicated that *brachyury* is not significantly altered in *apj* induced ESCs; by contrast, expression of *mesp1* and the subsequent cardiac markers *mef2c* and *αMHC* were increased upon *apj* induction (Fig. 26). Interestingly, we found that the expression of some endothelial markers, such as *flk1*, *PECAM1*, *Tie1* and *2* and *VE-cadherin* were also strongly induced, suggesting that forced APJ signaling in an early time window promoted both cardiac and vascular lineage specification and differentiation (Fig. 26). Furthermore the expression of the endodermal marker *αfp* was not affected upon *apj* overexpression, suggesting that the effect of *apj* is specific for the mesodermal progenitors committed to the cardiovascular fate. Finally, we collected RNAs samples from both *apj* induced ESCs and *apj* induced EBs, during day 0-4 interval, to perform microarray experiment. This strategy would allow us to identify novel genes activated by APJ/Apelin signaling in mammalian cardiogenesis and to clarify the complex network of molecules involved in the cardiovascular lineage specification.

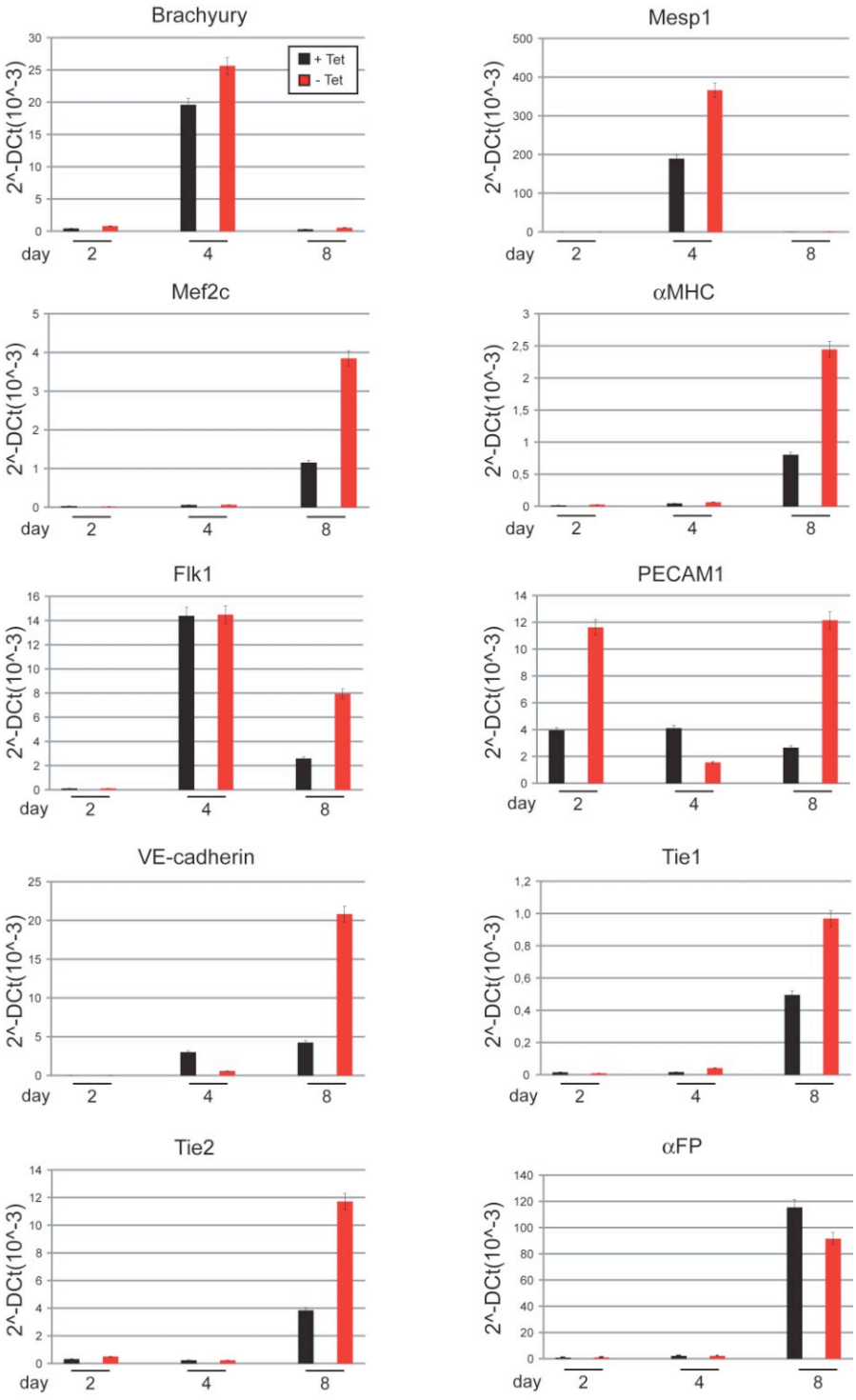

Fig. 26: Forced *apj* expression in an early time window promoted both cardiac and vascular lineage specification and differentiation.
Induction of cardiovascular program, as shown by Real-Time PCR. *apj*-induced ES clone A2, in the presence or absence of tetracycline, was used for the analysis. mRNA expression was normalized to *gapdh* expression.

5 DISCUSSION

Cardiac development is a highly ordered process in which cardiovascular progenitor cells are specified from early mesoderm. Multipotent cardiovascular progenitors (MCPs) generate soon after gastrulation and give rise to all different cardiac cell types, including cardiomyocytes, endothelial cells and smooth muscle cells which contribute to heart development in a temporally and spatially specific manner (Garry and Olson, 2006) (Moretti et al., 2006) (Buckingham et al., 2005). To date, the study of the molecular effectors involved in cardiac specification and differentiation is still incomplete; thus, dissecting the extracellular signals and the intracellular effectors controlling the early steps of mammalian cardiomyogenesis is of great interest. Recent studies provide evidence that different MCPs, which are specified during embryonic development, are also generated during ESC differentiation (Murry and Keller, 2008). Thus, ESCs represent a very attractive model for the identification of several molecular signals implicated in cardiovascular cell fate specification (Martin-Puig et al., 2008).

Cripto signaling is one of the key inducer of cardiac development and its role in the events leading to heart development has been characterized through the study of knock out ESCs, because of the early lethality of mouse models. Indeed, Cripto/Smad2 is an indispensable signaling pathway required in the early stages of cardiogenesis for the commitment of progenitors towards cardiac fate and their terminal differentiation. Although the contribution of this pathway to the specification of cardiac lineage is well established, the identity of the molecular downstream targets of Cripto/Smad2 is still unknown.

Our findings led to the identification of APJ and Apelin as novel molecules, which act downstream of Cripto in the early phase of mesoderm patterning and cardiac specification in mammals (D'Aniello et al., 2009). The characterization of their expression profile both in ESC differentiation and in gastrulating embryos show that *apj* and *apelin* expression correlate with that of *cripto*. Notably, using both chemical approach to inhibit ALKs receptors and shRNAs to target directly Smad2, we demonstrate that *apj* and *apelin* expression is regulated by Cripto/Smad2 pathway (D'Aniello et al., 2009). Of note and in accordance with recent findings showing that Cripto is a direct target of Smad4 (Mancino et al., 2008), *cripto* expression is inhibited by SB-431542 treatment and in Smad2–silenced EBs. Furthermore, it has been recently shown that the early activation of Smad2 is required for the mesendodermal induction and cardiac commitment of ESCs. Indeed, inhibition of this pathway in the early stages, impaired cardiomyogenesis and induced neuroectodermal lineage (Kitamura et al., 2007).

Our findings provide evidence that APJ and Apelin are effectors of Cripto/Smad2 pathway. Accordingly, gain of function experiments show that APJ redirects the neural fate of *cripto*$^{-/-}$ ESCs and restores the mesendodermal and the cardiogenic program. *Mesp1* expression, which is considered as the earliest cardiac transcription factor required for cardiac morphogenesis (David

et al., 2008) (Kitajima et al., 2000) increases upon APJ overexpression. Several lines of evidence indicate that *mesp1* acts as a master regulator of multipotent cardiovascular progenitors (MPC) specification during ESC differentiation (Bondue et al., 2008; Kitajima et al., 2000; Lindsley et al., 2008). Accordingly, APJ is able to restore the genetic cardiac program, in the absence of *cripto*, following also the correct timing of cardiac gene expression (D'Aniello et al., 2009). However it fails to induce beating EBs in *cripto*$^{-/-}$ ESCs. We suggest several reasons to explain the incomplete rescue of cardiomyogenesis. First of all, the ligand Apelin might represent the major limiting factor, given that we overexpressed the receptor APJ but not the ligand. However, we observed that *apelin* is induced in APJ/V5 *cripto*$^{-/-}$ EBs, suggesting that APJ and Apelin interact through a positive regulatory loop. However, while *apelin* is transiently expressed in wild-type ESC differentiation, as we previously showed, it accumulates throughout differentiation in APJ/V5 *cripto*$^{-/-}$ EBs. Moreover, further addition of Apelin peptide to APJ/V5 *cripto*$^{-/-}$ EBs, in the time window of its endogenous expression, does not induce the beating phenotype. Most remarkably, the gain of function approach used in these experiments do not allow the modulation of receptor signaling either in terms of timing or signal strength, thus we can only measure the effect of constitutive but not transient activation of APJ signaling.

In line with this hypothesis, it has been demonstrated that Apelin acts as a gradient for migration of mesodermal cells fated to myocardial lineage. Indeed, both excess and deficit of APJ/Apelin in Zebrafish embryos impair gastrulation and block myocardial differentiation, leading to defective migration of heart precursors and loss of critical inductive differentiation signals (Quertermous, 2007; Zeng et al., 2007).

In line with our findings that suggest a functional role of APJ/Apelin pathway in mammalian cardiomyogenesis, we provide the first evidence that both *apj* and *apelin* are crucial for ESC cardiac differentiation; though, unlike *cripto*, *apelin* is not able to redirect the neural fate of ESCs (D'Aniello et al., 2009). Interestingly, expression of the cardiac transcription factor *mesp1* but not of the mesodermal marker *brachyury* was reduced in *apelin*–silenced EBs, indicating that *apelin* acts preferentially for mesoderm patterning more than mesoderm formation. A similar function has been previously described for the HMG-box transcription factor *Sox17*, which is involved in the molecular events subsequent to mesoderm formation yet before *mesp1* induction in ESC differentiation (Liu et al., 2007).

Interestingly, neither *apelin* nor *apj* null mice show early embryonic patterning and congenital cardiac defects (Kuba et al., 2007) (Ishida et al., 2004) (Quertermous, 2007), while they develop progressive impairment of cardiac contractility associated with the age or in response to pressure overload (Kuba et al., 2007). Possibly, cardiac differentiation was insufficiently examined; however, it is now becoming evident that factors contributing to early cardiac development are highly redundant. Indeed, it has been recently hypothesized that in the embryo, cardiac myogenesis might depend on a more complex

network of signals and mediators than in ESC differentiation. Thus, embryonic development might be less vulnerable than EBs to loss of genes of early cardiomyogenesis (Liu et al., 2007).
In line with this idea, growing evidence indicate that APJ/Apelin signaling has a crucial role in the events leading to cardiovascular development. Indeed, recent data in Zebrafish showed a critical role for APJ/Apelin pathway in myocardial cell specification and migration (Scott et al., 2007; Zeng et al., 2007). Our study provides the first evidence that APJ/Apelin are required for mammalian cardiogenesis, shedding light also on the molecular mechanisms involved in APJ/Apelin-induced cardiomyogenesis that, up to now, was still unproven. Indeed, our data show that APJ/Apelin induce cardiac differentiation through phosphorylation of p70S6K, which depends on the activation ERK signaling. Indeed, we showed that blocking ERK signaling by the specific inhibitor U0126 abrogates the phosphorylation of p70S6K and also prevents Apelin–induced cardiomyogenesis; thus providing the first evidence that, in mammalian cardiomyogenesis, Apelin activates ERK-dependent but not AKT–dependent p70S6K cascade. Furthermore we proved that Apelin induces cardiomyogenesis through a PTX-sensitive GTP binding protein associated to APJ receptor (D'Aniello et al., 2009). Previous studies in primary endothelial cells, indicated that Apelin induces the phosphorylation of p70S6K with different pattern of phosphorylation. These patterns are associated with two transduction cascades: AKT and ERK pathways (Masri et al., 2004). Moreover several studies indicate that Apelin has a key role in endothelial cell proliferation dependent on p70s6K pathway activation (Eyries et al., 2008) (Masri et al., 2004); it would be of great interest to establish whether Apelin might have a mitogenic effect also on cardiovascular progenitors, activating the same mechanism.
To further assess the role of APJ/Apelin in the specification of cardiovascular progenitors we used a gain of function approach and evaluated the effect of *apj* overexpression in an early time point during ESC differentiation. Indeed, using an inducible system we were able to overcame the limits of a constitutive expression and to analyze the effect of *apj* overexpression in a more physiological contest. Our data indicate that the regulated overexpression of *apj* in the 0-4 day time window, increases the number of terminally differentiated cardiomyocytes. Moreover, forced *apj* expression also promotes vascular lineage specification and differentiation, thus activating the cardiovascular program. Interestingly, several studies point out a strong connection between cardiac and vascular lineage, indicating that these population segregate from a common progenitor (Kattman et al., 2006) (Kattman et al., 2007). Our data suggest that APJ/Apelin signaling might be required in the early stages of ESC differentiation not only for the correct specification of cardiac but also the vascular lineage: thus leading to hypothesize that it could promote the expansion of a common cardiovascular progenitor.

Finally, given the crucial role of Cripto not only in embryo development and ESC differentiation (Minchiotti, 2005; Schier, 2003) but also in tumor progression and angiogenesis (Bianco et al., 2005; Shen, 2003; Strizzi et al., 2005) it would be of great importance to verify whether Cripto activity in tumor angiogenesis correlates with the activation of the APJ/apelin system. In line with this hypothesis, Apelin has been recently involved in tumor angiogenesis as a potent inducer (Cox et al., 2006; Kidoya et al., 2008; Sorli et al., 2007).

Our study points toward the understanding of the biological complexity of mammalian cardiogenesis. The characterization of APJ/Apelin as novel regulators of cardiac lineage specification will help understanding not only the mechanisms governing heart development but also the etiology of cardiac diseases. Moreover, our data might be helpful to open the way toward new approaches for improving cardiomyocyte differentiation and the development of cell-based therapies.

6 CONCLUSIONS

Cardiovascular disease is the leading cause of death in the western world. Thus, repairing the damaged tissue with a source of cells able to proliferate and differentiate into cardiomyocytes provides a very attractive therapeutic option to restore cardiac function. Pluripotent stem cells, which are capable of differentiating into the three most important components of the heart (smooth muscle cells, endothelial cells and cardiomyocytes) represent an attractive source both for cell replacement therapy and for modeling cardiovascular disease. However, the fascinating possibility of stem cell-based therapy require a detailed understanding of the molecular mechanisms that drive the commitment of the cardiovascular progenitors.

Our study provides important insights into the molecular mechanisms controlling cardiomyogenesis in mammals, revealing a previously undescribed functional link between APJ/Apelin and Cripto signaling in redirecting the neural fate of ESCs and promoting cardiogenesis. Furthermore, we demonstrated that APJ/Apelin are key molecules involved in ESC cardiac differentiation, residing in a crucial step of cardiomyogenesis. Indeed, we place APJ/Apelin signaling in a specific step of the cardiac cascade, acting upstream of *mesp1* induction for the specification of multipotent cardiac progenitors, through the activation of p70s6K signaling pathway. Moreover, our data point for a critical role of APJ in promoting the specification/differentiation of the cardiovascular lineage, likely acting on the common cardiovascular progenitor.

In conclusion our findings contribute to the general understanding of the molecular complexity of mammalian cardiogenesis and lay the groundwork for further study of the APJ/Apelin target genes, using genome-wide transcriptional approach, to eventually provide a more comprehensive analysis of the molecular mechanisms controlling mammalian cardiomyogenesis.

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LIST OF PUBLICATIONS

1. **D'Aniello C**[*], Lonardo E, Iaconis S, Guardiola O, Liguoro AM, Liguori GL, Autiero M, Carmeliet P, Minchiotti G.
G Protein-Coupled Receptor APJ and Its Ligand Apelin Act Downstream of Cripto to Specify Embryonic Stem Cells Toward the Cardiac Lineage Through Extracellular Signal-Regulated Kinase/p70S6 Kinase Signaling Pathway
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2. Gabriella Minchiotti, **Cristina D'Aniello**[*], Roberto Ronca, Laura Gualandi and Patrizia Dell'Era
Embryonic Stem Cells as a Model System to Elucidate Early Events in Cardiac Specification and Determination
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G Protein–Coupled Receptor APJ and Its Ligand Apelin Act Downstream of Cripto to Specify Embryonic Stem Cells Toward the Cardiac Lineage Through Extracellular Signal-Regulated Kinase/p70S6 Kinase Signaling Pathway

Cristina D'Aniello, Enza Lonardo, Salvatore Iaconis, Ombretta Guardiola, Anna Maria Liguoro, Giovanna L. Liguori, Monica Autiero, Peter Carmeliet, Gabriella Minchiotti

Rationale: Pluripotent stem cells represent a powerful model system to study the early steps of cardiac specification for which the molecular control is largely unknown. The EGF-CFC (epidermal growth factor–Cripto/FRL-1/Cryptic) Cripto protein is essential for cardiac myogenesis in embryonic stem cells (ESCs).

Objective: Here, we study the role of apelin and its G protein–coupled receptor, APJ, as downstream targets of Cripto both in vivo and in ESC differentiation.

Methods and Results: Gain-of-function experiments show that APJ suppresses neuronal differentiation and restores the cardiac program in Cripto$^{-/-}$ ESCs. Loss-of-function experiments point for a central role for APJ/apelin in the gene regulatory cascade promoting cardiac specification and differentiation in ESCs. Remarkably, we show for the first time that apelin promotes mammalian cardiomyogenesis via activation of mitogen-activated protein kinase/p70S6 through coupling to a Go/Gi protein.

Conclusions: Together our data provide evidence for a previously unrecognized function of APJ/apelin in the Cripto signaling pathway governing mesoderm patterning and cardiac specification in mammals. (*Circ Res.* 2009;105:231-238.)

Key Words: embryonic stem cells ■ cardiomyogenesis ■ cripto ■ apelin ■ APJ/msr1

The earliest event in cardiogenesis is commitment of mesodermal cells to a cardiogenic fate and their migration into the anterolateral region of the embryo during gastrulation.[1] It is therefore important to understand how mesodermal cells are instructed to assume a cardiac fate to elucidate the molecular mechanisms later in heart development. In mammals, these instructive events are largely unknown. Their identification could provide insights into pathways governing cell lineage specification and differentiation, including transcription factor network and extracellular cues that activate them.[2] In addition, understanding early cardiogenesis is of particular interest because cardiomyocyte loss from damage in mammals is largely irreversible and frequently underlies impaired cardiac function in individuals with heart disease. Although there are still multiple barriers to successful regenerative therapies for cardiac disease using embryonic or adult stem cells, cell-based therapeutic approaches remain a valuable goal, particularly when using strategies that do not cross species barriers.[3,4] In this light, embryonic stem cells (ESCs), which faithfully recapitulate early stages of cardiac cell commitment and differentiation, provide a powerful model for investigating how best to control the earliest events in mammalian cardiomyogenesis and ultimately enhance differentiation efficiency.

Proteins essential for heart induction have been studied extensively in ESCs, which includes Wnt/β-catenin, transforming growth factor-β family, bone morphogenetic proteins and Cripto.[5–7] Cripto is a glycosylphosphatidylinositol-anchored multifunctional protein that is involved in the activation of a complex network of signaling pathways both in development and tumorigenesis.[8,9] Cripto stimulates signaling by the transforming growth factor β-family member Nodal or related ligands growth/differentiation factor (GDF)1 and -3,[10,11] through activin type IB (activin receptor–like kinase [ALK]-4) and activin type IIB serine/threonine kinase receptors.[10,12,13] Besides its well-documented stimulatory effect on the canonical Nodal-GDF1-3/ALK-4/Smad2 pathway, Nodal/ALK-4–independent Cripto activities have also been described.[9,14] Notably, recent data highlight a novel role of Cripto as Activin/transforming growth factor-β antagonist.[15,16]

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Abbreviations and Acronyms	
AGTRL-1	angiotensin type I–like receptor
ALK	activin receptor–like kinase
APJ	apelin receptor
ERK	extracellular signal-regulated kinase
EB	embryoid body
ESC	embryonic stem cell
GDF	growth/differentiation factor
MAPK	mitogen-activated protein kinase
MHC	myosin heavy chain
PTX	pertussis toxin
QRT-PCR	quantitative RT-PCR
shRNA	short hairpin RNA

We have previously showed that Cripto acts through the Nodal/ALK-4/Smad2 pathway to negatively regulate neural differentiation and to permit the entry of ESCs into a cardiac lineage.[17] Accordingly, recent data pointed for a key role of Nodal/Cripto-dependent early activation of Smad2, which was indispensable for mesendodermal induction and the subsequent cardiac differentiation of ESCs.[18] However, little is yet known about the mechanisms of action and the identity of the factors downstream of Cripto/Smad2 in mammalian cardiomyogenesis.

Here, we report the identification of the apelin receptor APJ (also known as angiotensin type I–like receptor [AGTRL-1] and *msr1*) and its ligand apelin as previously unrecognized downstream targets of Cripto and provide evidence that the APJ/apelin pathway redirects the neural fate of ESCs in the absence of Cripto and promotes cardiac differentiation. APJ is a G protein–coupled receptor, and its ligand apelin is a peptide originating from the larger precursor preproapelin molecule[19]; recently, emerging as an important regulator of cardiovascular homeostasis and angiogenesis.[20–23] Remarkably, recent data in Zebrafish provided the first evidence that the APJ/apelin pathway is required, at the onset of gastrulation, to mediate migration of myocardial progenitors to the correct position into the anterior lateral plate mesoderm[24,25]; nevertheless, their function in mammalian cardiomyogenesis is still unproven. Here, we reveal a previously undescribed functional link between Cripto/Smad2 and APJ/apelin in the signaling pathways that redirect the neural fate of ESCs and promote cardiomyogenesis in mammals.

Methods

ESC Differentiation, Western Blot, and Immunofluorescence Analysis

The ESC lines RI and Cripto$^{-/-}$ DE7[17] were used throughout the study. Western blot and immunofluorescence analysis were performed as previously described.[17] Anti–βIII-tubulin (Sigma), anti-V5 (Invitrogen), anti-GAPDH (AbCam), anti–phospho–extracellular signal-regulated kinase (ERK), anti-ERK, and anti–p70S6 kinase (p70S6K) (Cell Signaling) antibodies were used according to the instructions of the manufacturers. Monoclonal anti–βIII-tubulin (1:400; Sigma-Aldrich) and MF20 (1:50; obtained from the Developmental Studies of Hybridoma Bank, University of Iowa) were used as previously described.[17] Apelin (13) peptide was purchased from Sigma-Aldrich.

ESC Transfection and Plasmids

Murine *msr1* cDNA was subcloned into the pEF1a vector (Clontech) to generate an msr1-V5 fusion protein and then cloned into the pallino βA vector for expression in ESCs.[17]

Short hairpin (sh)RNAs vectors (pGIPZ lentiviral, pLKO vectors; OpenBiosystem) were used accordingly to the instructions of the manufacturer. Nucleofector Technology (AMAXA) was used for ESC transfection, according to the instructions of the manufacturer.

RNA Preparation and RT-PCR

Total RNAs were isolated using RNeasy Mini Kit (Qiagen) and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technologies Inc) and random hexamers, according to the instructions of the manufacturer. Quantitative real-time PCR was performed using SYBR Green PCR master mix (EuroClone). The primers used are described in Table I in the Online Data Supplement, available at http://circres.ahajournals.org.

Whole-Mount In Situ Hybridization

Embryos were dissected in PBS and fixed in 4% paraformaldehyde in PBS at 4°C for 2 to 16 hours; whole-mount in situ hybridization was performed as previously described.[26]

Results

Complementary Expression of APJ, Apelin, and Cripto in ESC Cardiac Differentiation and Gastrulating Embryos

In searching for genes that might act in concert with Cripto to promote cardiomyogenesis, we were interested in the G protein–coupled receptor APJ (also known as angiotensin type I–like receptor [AGTRL-1]) and its ligand apelin. APJ and apelin have been recently described as important regulators of cardiovascular function both in *Xenopus*[21] and in Zebrafish,[24,25] yet a role for these proteins in mammalian cardiogenesis is still unproven. We therefore determined whether they were involved in cardiogenesis in concert with Cripto. Notably, both Apj and apelin mRNAs were upregulated in Cripto$^{-/-}$ embryoid bodies (EBs) exposed to recombinant Cripto for 24 hours in a microarray analysis (data not shown). We thus compared the expression profile of both Apj and apelin in wild-type and Cripto$^{-/-}$ ESCs that were allowed to differentiate toward cardiomyocytes. Real-time PCR showed that Apj and apelin expression was reduced in Cripto$^{-/-}$ ESCs, whereas both genes were markedly upregulated in wild-type ESCs (Figure 1A).

To extend our analysis in vivo, we compared the expression profile of Cripto with that of Apj and apelin in mouse embryos. To our knowledge, no previous studies have described apelin/Apj expression in early embryogenesis; ie, before the late head-fold stages (embryonic day 8).[27] Whole-mount in situ hybridization on gastrulating wild-type and Cripto$^{-/-}$ embryos revealed that the expression of both Apj and apelin correlates with that of Cripto (Figure 1B). Apelin mRNA clearly identifies the developing primitive streak; whereas, Apj expression domain is detectable both in the primitive streak and adjacent mesoderm, rather similar to Cripto. Of note, both Apj and apelin mRNA were also detected in the extraembryonic mesoderm (Figure 1B). In line with ESCs results, Apj and apelin expression was almost undetectable in Cripto$^{-/-}$ embryos, specifically in the posterior mesoderm. *Cripto* is essential for both primitive streak and anterior–posterior axis formation; however, Cripto$^{-/-}$

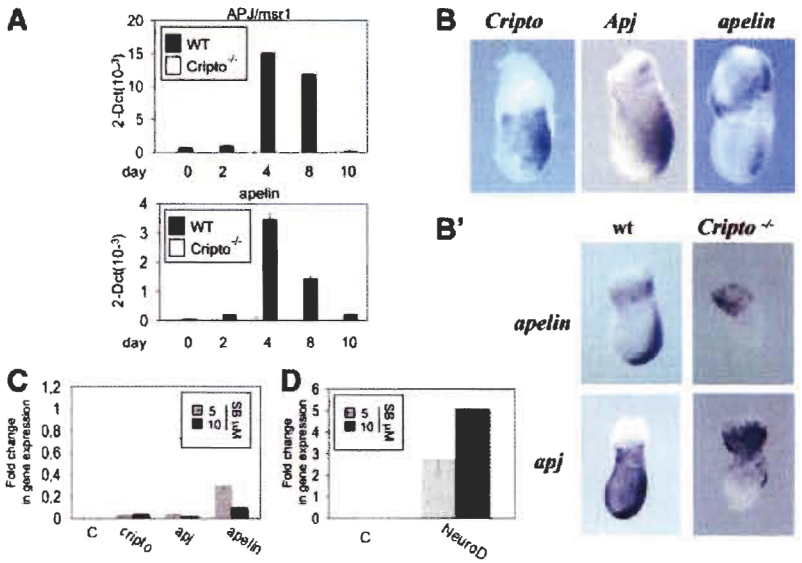

Figure 1. Expression of Apj, apelin, and Cripto in cardiac differentiation and gastrulating embryos. A, Expression of Apj and apelin in wild-type and Cripto$^{-/-}$ ESCs by QRT-PCR; mRNA was normalized to GAPDH expression; data are means±SE (n≥3). B and B′, Whole-mount in situ hybridization of Apj, apelin, and Cripto in wild-type embryos at 6.7 days postconception (B) and Apj and apelin in Cripto$^{-/-}$ embryos at 7 days postconception (B′). C and D, Smad2 inhibition on Apj, apelin, and Cripto expression. Two-day-old wild-type EBs treated (24 hours) with increasing amounts of SB-431542 (SB) or DMSO (vehicle); expression of Apj, apelin, Cripto (C), and NeuroD (D) by QRT-PCR. mRNA was normalized to GAPDH and presented as fold change in gene expression relative to the control (DMSO); data are means±SE (n≥3).

embryos express posterior markers such as Brachyury and form anterior neural structures and extraembryonic mesoderm.[26,28] Remarkably, Apj and apelin expression persisted in the extraembryonic tissue of Cripto$^{-/-}$ embryos, thus indicating that their expression is Cripto-dependent in the embryonic mesoderm and Cripto-independent in the extraembryonic tissue (Figure 1B′).

Early Activation of Smad2 Is Critical for the Expression of Cripto, Apj, and Apelin in Cardiomyogenesis of ESCs

Recent data indicated that early activation of Smad2 was indispensable for mesendodermal induction and subsequent cardiac differentiation of ESCs.[18] We also previously showed that Cripto acts via the Nodal/ALK-4/Smad2 pathway to induce ESC cardiomyogenesis.[17] We thus asked whether early activation of Smad2 induced Apj and/or apelin expression. Two-day-old wild-type EBs were therefore treated with SB-431542, a specific inhibitor of ALK-4–, -5–, -7–dependent Smad2 activation[29] or left untreated as control. SB-431542 reduced both Apj and apelin expression (Figure 1C). Notably, recent data showed that human Cripto is a direct target of Smad4[30]; accordingly, SB-431542 affected Cripto expression as well. By contrast, as previously described, expression of neuroectodermal marker Neurod1 was upregulated (Figure 1D).[18] Comparable results were obtained with Smad2-silenced ESCs (Online Results and Online Figures I and II).

Together, our data indicate that that early activation of Smad2 is critical for induction of Cripto, Apj, and apelin expression and the consequent inhibition of the neuronal fate.

APJ Overexpression Redirects the Neural Fate of Cripto$^{-/-}$ ESCs

Previous data revealed an essential role of Cripto in redirecting the neuronal fate and promoting cardiomyogenesis in ESCs.[17] Therefore, we evaluated whether forced overexpression of Apj might redirect the neural fate of ESCs and thus compensate for the lack of Cripto in cardiomyogenesis. A recombinant vector encoding an APJ/V5-tagged protein was transfected into Cripto$^{-/-}$ ESCs and APJ/V5 overexpression, and its membrane localization was verified in 3 independent clones (Figure 2A and 2B). Following characterization, both control and APJ-overexpressing Cripto$^{-/-}$ ESCs were allowed to differentiate toward cardiomyocytes.[17] As expected, when Cripto$^{-/-}$ EBs were plated onto an adhesive substrate, a population of cells with a neuron-like morphology was observed, which produce a dense neural network. This characteristic morphology was never observed either in wild-type or in Cripto$^{-/-}$ APJ/V5 EBs (data not shown). Notably, overexpression of Apj in Cripto$^{-/-}$ ESCs reached levels comparable to that of WT ESCs, as shown by quantitative RT-PCR (QRT-PCR) (Figure 1A and Online Figure III). These data suggested that Apj overexpression likely prevented neuronal differentiation of Cripto$^{-/-}$ ESCs.

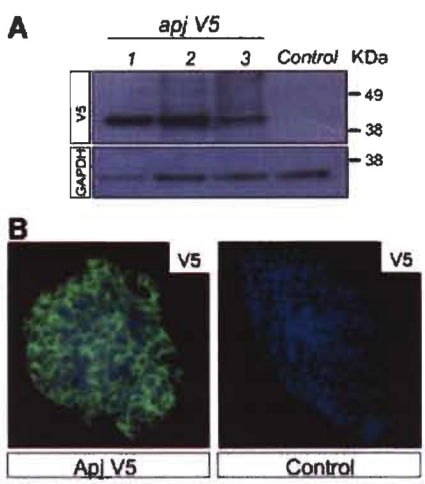

Figure 2. Functionality of APJ overexpression construct in Cripto$^{-/-}$ ESCs. A, Verification of APJ/V5 overexpression by Western blot using anti-V5 antibodies. Anti-GAPDH antibodies were used as loading control. B, Immunofluorescence analysis of undifferentiated ESCs showing membrane localization of APJ-V5.

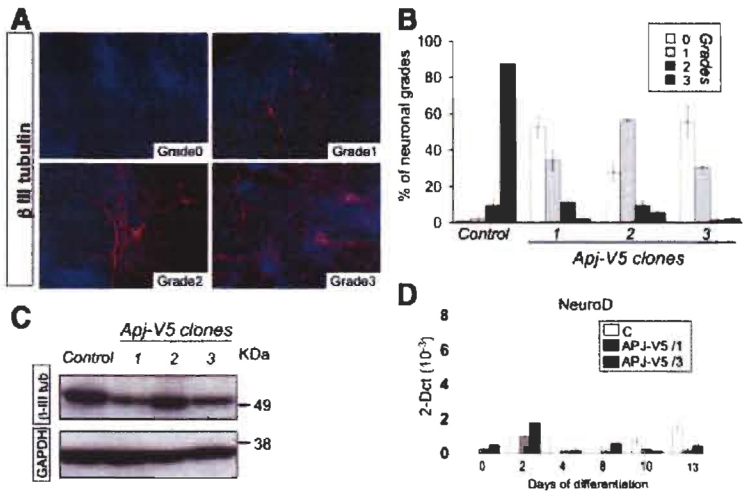

Figure 3. APJ overexpression redirects the neural fate of Cripto$^{-/-}$ ESCs. A, Grades of neuronal differentiation of Cripto$^{-/-}$ EBs, as arbitrarily defined. Nuclei were visualized by DAPI. Magnification, ×20. B, Immunofluorescence analysis on 13-day-old EBs and distribution of neuronal grades, as indicated in A. Data are means±SE; numbers of EBs scored/clone, ≈60 (n≥3). C, Expression of β-III-tubulin, shown by Western blot on 13-day-old EBs. Anti-GAPDH antibodies were used as loading control. D, Expression of neuronal marker NeuroD in Control and APJ-V5 Cripto$^{-/-}$ EBs (clones 1 and 3), by QRT-PCR. mRNA was normalized to GAPDH expression. Data are means±SE (n≥3).

To address this issue directly, immunofluorescence analysis was performed using anti–βIII-tubulin antibodies, which recognize the neuron-specific form of class III β-tubulin. To semiquantify the activity of APJ, we arbitrarily defined 4 grades of neuronal differentiation ranging from the absence of neurons (grade 0) to full neuronal differentiation (grade 3), ie, presence of a dense network of βIII-tubulin–positive cells. The presence of either few isolated neurons or areas of βIII-tubulin–positive cells defined intermediate phenotypes, named grade 1 and grade 2, respectively (Figure 3A). Results clearly showed a negative effect of APJ on neuronal differentiation of Cripto$^{-/-}$ ESCs. Indeed, most, if not all, APJ/V5 Cripto$^{-/-}$ EBs scored showed poor neuronal differentiation (Figure 3B). Accordingly, βIII-tubulin expression was reduced, as shown by Western blot analysis (Figure 3C). Notably, in 1 of 3 clones, βIII-tubulin levels were comparable to control cells, which was likely attributable to an atypically high percentage of grade 1 EBs. Finally, we found downregulated expression of transcripts encoding neuronal transcription factors NeuroD and neurofilament M (Figure 3D and data not shown).

APJ and Apelin Drive ESCs Toward the Cardiac Lineage

Given the ability of APJ to redirect the neural fate of ESCs in the absence of Cripto, we examined whether APJ overexpression might rescue the genetic program of cardiac differentiation in Cripto$^{-/-}$ EBs. To this end, both control and APJ-overexpressing Cripto$^{-/-}$ ESCs were allowed to differentiate toward cardiomyocytes and the expression profile of selected markers was evaluated (Figure 4). Notably, APJ overexpression cells resulted in the induction of the pan-mesodermal marker T/Bra, as well as the earliest cardiac marker Mesp1. Mesp1 functions subsequent to Bra as the earliest molecular marker of cardiac precursors that migrate through the primitive streak and is essential for cardiac myogenesis in committed mesodermal cells.[31,32] Consistent with a restored cardiogenic program in APJ-overexpressing Cripto$^{-/-}$ ESCs, the transcription factor *Mef2C*, *Nkx2.5*, *Tbx5*, and *Gata4*, as well as the cardiac structural gene *Mlc2v* were upregulated, and their expression profile correlates with that of wild-type EBs (Figure 4).[2] Furthermore, upregulation of *Afp* (extraembryonic endoderm marker) also suggested that APJ overexpression induced the mesendodermal program in the absence of Cripto (Figure 4). By contrast, beating EBs did not form, even after long-term culture, and α-myosin heavy chain (αMHC)-positive cells were almost absent (data not shown).

Of note, apelin expression increased on APJ overexpression and persisted throughout ESC differentiation (Online Figure III, A). Nevertheless, the transient nature of apelin accumulation in wild-type EBs suggested that its activity

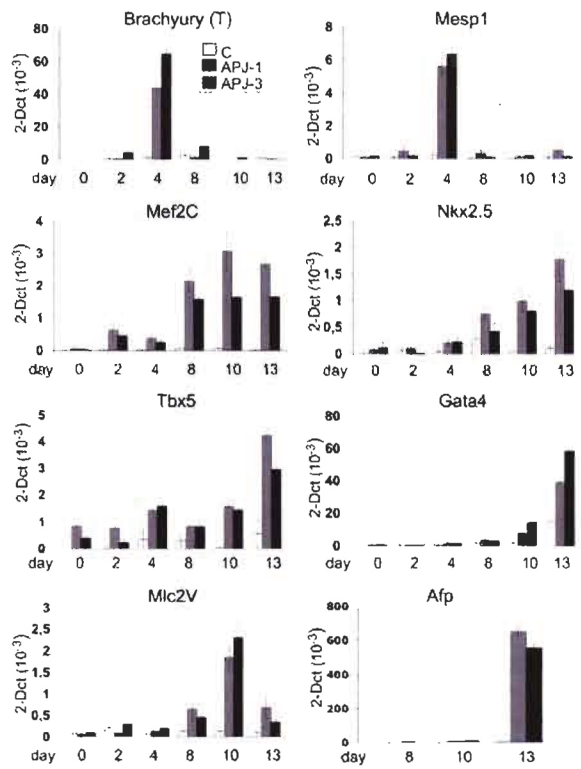

Figure 4. APJ specifies embryonic stem cells toward the cardiac lineage. Induction of mesendodermal program and cardiac myogenesis by QRT-PCR. Cripto$^{-/-}$ APJ-V5 clones 1 and 3 were used for the analysis; mRNA was normalized to *GAPDH* expression; data are means±SE (n=3).

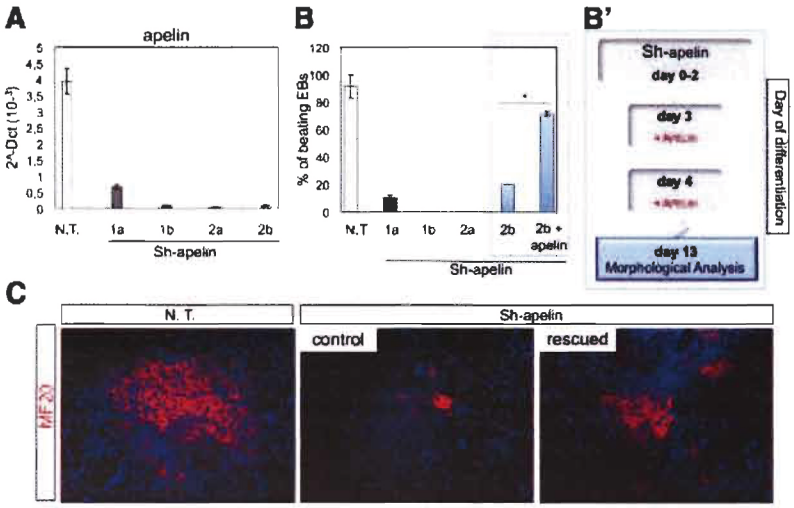

Figure 5. A pivotal role for apelin in cardiac differentiation of ESCs. A, Suppression of apelin expression by shRNAs. Two independent ESCs for each shRNA vector (Sh1, Sh2) are shown (Sh1a, b; Sh2a, b). Nontargeting control vector (NT). B and C, Suppression of cardiac differentiation by apelin shRNAs in 13-day-old EBs; prevalence of beating EBs (B) and immunostaining with anti-αMHC antibodies (MF20) (C). Addition of recombinant apelin to EBs in the 3- to 4-day interval, described in B′, rescues the cardiac phenotype, as shown by the prevalence of beating EBs (B) and MF20 immunostaining (C). Numbers of EBs scored/condition, ≈50.

might be required at a defined step in cardiomyocyte differentiation (Figure 1A). We thus determined whether addition of apelin might redirect the neural fate of Cripto$^{-/-}$ ESCs and/or improve the effect of APJ overexpression on cardiomyogenesis. To this end, apelin was added to the cells every 24 hours during the 2- to 4-day interval of differentiation, which reflected the peak of endogenous apelin expression. Interestingly, expression of the cardiac structural gene Mlc2v significantly increased (Online Figure III, B); however, it did not induce beating EBs. Notably, apelin treatment of Cripto$^{-/-}$ EBs reduced the expression of NeuroD (Online Figure III, B), suggesting that it is antagonizing neuronal differentiation. Apelin activity in Cripto$^{-/-}$ EBs was likely attributable to residual Apj expression; however, we cannot completely rule out the possibility of an APJ-independent apelin activity. Remarkably, addition of apelin to wild-type EBs did not further enhance cardiac differentiation, as revealed by expression Mlc2v and αMHC, which was comparable in control and apelin-treated EBs (Online Figure III, C). Most likely, APJ/apelin signaling becomes saturated in wild-type EBs, and thus addition of apelin cannot further activate the pathway.

To assess the role of APJ/apelin signaling on cardiomyogenesis, silencing experiments were performed, using 2 shRNAs, which targeted nonoverlapping apelin mRNA sequences. To minimize effects of clone-to-clone variation, independent ESC clones for each shRNA were isolated, with a silencing efficacy in the range of 80% to 95% (Figure 5A). These clones showed suppression of cardiac differentiation (Figure 5B), with few and isolated, if any, αMHC immunoreactive cells (Figure 5C). Morphological observation was supported by molecular analysis, showing downregulation of αMHC expression (data not shown and Figure 6B).

The transient nature of apelin expression suggested that its activity might be required at a defined step in cardiomyogenesis. To address this issue, apelin signaling was reconstituted by adding apelin peptide to the EBs (Figure 5B′). This treatment restored cardiac differentiation of apelin-silenced ESCs (Figure 5B and 5C), thus providing the first direct evidence that apelin is required in an early window for priming differentiation of ESCs to a cardiac fate. We then went on to determine whether apelin silencing might be able to promote neuronal differentiation, as in Cripto$^{-/-}$ ESCs. As expected, βIII-tubulin accumulated in Cripto$^{-/-}$ EBs, whereas it was undetectable in EBs derived from ESCs expressing either nontargeting shRNA or apelin shRNAs (Figure 6A), thus indicating that apelin was not able to redirect the neural fate of ESCs.

Next, we examined at which step of cardiac myogenesis apelin functions (Figure 6B). Remarkably, apelin shRNA did not significantly affect expression of Brachyury/T. By contrast, the earliest cardiac marker *Mesp1* and the cardiac genes *Nkx2.5* and *αMHC* were downregulated in apelin silenced compared to control ESCs. Together, these data indicated that APJ/apelin signaling was pivotal in promoting ESC cardiac specification and differentiation. Accordingly, Apj silencing impaired ESC cardiac differentiation (Online Figure IV).

Apelin Promotes Cardiomyogenesis via Mitogen-Activated Protein Kinase/p70S6 Through Coupling to a Pertussis Toxin–Sensitive GTP-Binding Protein

Previous data showed that apelin induced activation of ERKs and AKT and that this resulted in the activation of p70S6K.[33] To gain insight into the molecular basis of APJ/apelin signaling in cardiomyogenesis, we first evaluated whether addition of apelin induced mitogen-activated protein kinase (MAPK) and AKT signaling. Interestingly, phosphorylation ERK but not AKT (data not shown) was transiently induced in 4-day-old EBs treated with apelin (Figure 7A); accordingly, p70 S6K was rapidly phosphorylated at residues T421/S424 but not at T389, which is selectively phosphorylated on AKT activation (Figure 7A).[33] We thus performed the rescue assay described above (Figure 5B and 5B′) in the presence of U0126, a specific MAPK inhibitor (Figure 7B). As expected, apelin rescued cardiac differentiation. Most remarkably, this effect was fully abolished in the presence of U0126 (Figure 7C), thus providing evidence that apelin promotes cardiomyogenesis through the activation of MAPK. Finally, because the apelin receptor is a G protein–coupled receptor, we decided to characterize the G protein that

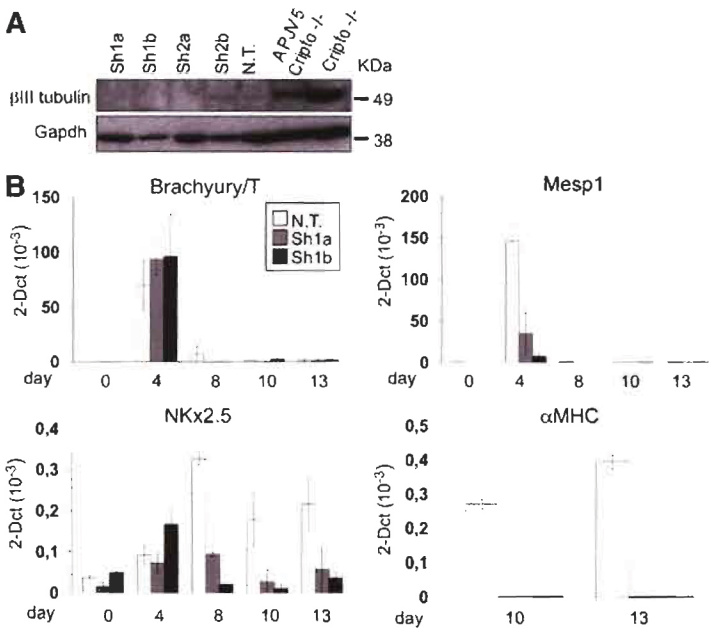

Figure 6. Apelin knockout impairs cardiac specification and does not induce neuronal differentiation of ESCs. A, Expression of βIII-tubulin on 13-day-old EBs (clones Sh1a, b; Sh2a, b). Nontargeting (NT) control EBs. Cripto$^{-/-}$ and APJ-V5 Cripto$^{-/-}$ are positive controls. Anti-GAPDH antibodies were used as loading control. B, QRT-PCR showing that apelin acts downstream of mesoderm formation (Brachyury/T) but upstream of cardiac specification (Mesp1). Nontargeting, Sh1a, and Sh1b ESCs were used. mRNA was normalized to *GAPDH* expression; data are means±SE (n=2).

transduced its activation in cardiomyogenesis. Pretreatment of EBs with pertussis toxin (PTX) fully abrogated the ability of apelin to promote cardiomyogenesis (Figure 7D), thus extending previous findings on APJ overexpressing cells, on the PTX-sensitive activation of ERKs by apelin.[34]

In conclusion, our data provide the first direct evidence that the MAPK is activated by apelin through coupling to a Go/Gi protein, which contributes to mammalian cardiomyogenesis.

Discussion

Dissecting the extracellular signals and their intracellular effectors controlling the early steps of mammalian cardiomyogenesis provide important insight into the mechanisms underlying cardiac fate specification.

Our findings identify APJ and apelin as novel extracellular signals that act downstream of Cripto in the early phase of mesoderm patterning and cardiac specification in mammals. Expression analysis both in ESCs and gastrulating embryos showed that *apj* and apelin expression correlated with that of Cripto. More interestingly, using the chemical inhibitor of ALK receptors SB-431542 and an shRNA-based approach, we provide evidence that expression of both genes is Cripto/Smad2-dependent. In line with our results, it has been

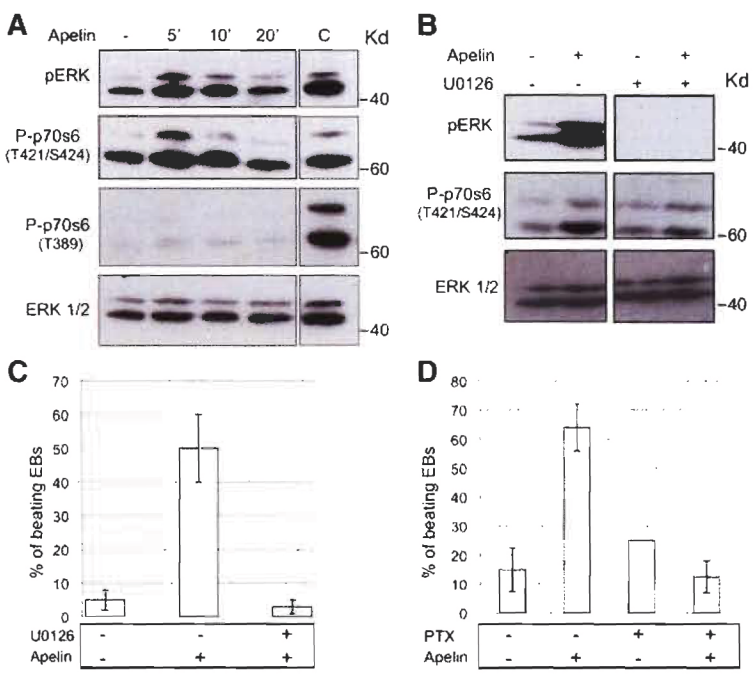

Figure 7. Apelin promotes cardiomyogenesis through MAPK/p70SK and via a PTX-sensitive G protein. A, Serum-starved 4-day-old apelin-silenced EBs treated with apelin (10 μmol/L) or left untreated as control (NT). Western Blot with anti–phospho-ERK, anti–phospho-p70SK, anti-ERK antibodies. The designation C indicates positive controls. B, Serum-starved 4-day-old apelin-silenced EBs treated with UO126 (10 μmol/L) for 2 hours, followed by incubation with apelin (10 μmol/L) for 5 minutes. Western Blots probed with anti–phospho-ERK, anti–phospho-p70SK, and anti-ERK Abs. C, Three-day-old EBs treated with U0126 for 2 hours, followed by apelin incubation. U0126 was added twice, every 4 hours, to apelin-treated EBs. DMSO was added to control EBs. D, Three-day-old apelin-silenced EBs treated overnight with PTX, followed by incubation with apelin. The prevalence of beating EBs was measured on 13-day-old EBs (C and D).

recently shown that early activation of Smad2 is required for mesendodermal induction and patterning in ESCs and that once this pathway is inhibited, cardiomyogenesis is reduced and neuroectodermal induction is augmented.[18] Our data identify Apj and apelin as effectors of Cripto/Smad2 pathway in these circuits. Accordingly, gain-of-function experiments show that APJ redirects the neural fate of Cripto$^{-/-}$ ESCs and restores mesendodermal patterning and the cardiogenic program. Expression of Mesp1, which is the earliest cardiac transcription factor required for cardiac morphogenesis,[31,32] is induced by APJ overexpression. Notably, Mesp1 acts as a key molecular switch in the specification of multipotent cardiovascular progenitors from ESCs, residing at the top of the hierarchy of the cardiovascular transcriptional network.[31,35,36] In line with these findings, APJ overexpression is able to restore the correct timing of cardiac gene expression; however, it fails to induce beating EBs in the absence of Cripto. The incomplete rescue of cardiomyogenesis in APJ-overexpressing Cripto$^{-/-}$ ESCs could have different reasons. First of all, the APJ ligand apelin might represent the major limiting factor. However, apelin is induced in APJ-overexpressing Cripto$^{-/-}$ EBs, suggesting that APJ and apelin interact through a positive regulatory loop. Moreover, further addition of apelin peptide to APJ-overexpressing EBs does not induce the beating phenotype. Remarkably, overexpression experiments do not allow the modulation of receptor signaling either in terms of timing or signal strength; thus, we can only measure the effect of constitutive but not transient activation of APJ signaling. In line with this hypothesis, we show that whereas apelin is transiently expressed in wild-type ESC differentiation, it accumulates throughout differentiation in APJ-overexpressing Cripto$^{-/-}$ ESCs. Interestingly, the introduction of excess of apelin in zebrafish embryos was found to impair gastrulation and block myocardial differentiation. These findings thus suggest that loss of a localized source of apelin did not allow cells fated to form the myocardium to reach their localization.[25,37]

In line with a functional role of APJ/apelin pathway in mammalian cardiomyogenesis, we provide evidence that apelin is crucial for cardiac differentiation in ESCs; however, unlike Cripto, apelin is not able to redirect the neural fate of ESCs. Remarkably, expression of Mesp1 but not Brachyury/T was reduced in apelin-silenced EBs, thus indicating that the action of apelin is preferential for mesoderm patterning more than mesoderm formation. Similarly, both the HMG-box transcription factor Sox17 and Notch signaling have been described as key players in the molecular events, which lead to Mesp1 induction in ESCs and in the embryo, respectively.[2,38]

In line with our findings, apelin-null mice do not show any early embryonic patterning defects,[37,39] nor have Apj and apelin-null mice been reported to have congenital cardiac defects.[39,40] Possibly, either cardiac differentiation was insufficiently examined or there might be genetic redundancy. It has been recently hypothesized that in the embryo, cardiac myogenesis might rely on a more complex ensemble of signals and mediators and be easier to compensate than in ESC differentiation; thus, embryonic development might be less vulnerable than EBs to loss of genes of early cardiomyogenesis.[2]

Remarkably, although recent experiments in zebrafish showed a critical role for APJ/apelin pathway in myocardial cell specification and heart development,[24,25] the molecular mechanism involved was unproven. Our study demonstrates that apelin induces p70S6K phosphorylation through the activation of the ERK cascade in mammalian cardiac differentiation. Most remarkably, we have found that the blockade of ERKs, by the specific inhibitor U0126, not only abrogates the phosphorylation of p70S6K but also prevents apelin-induced cardiomyogenesis. Moreover, we directly prove that there is a PTX-sensitive GTP-binding protein in the transduction cascade leading to apelin-induced cardiomyogenesis.

Previous data in primary endothelial cells showed that stimulation of p70S6K by apelin proceeds through activation of two signaling cascades, implicating AKT and ERK pathways, which are linked to a different pattern of p70S6K phosphorylation.[33] Our data suggest that apelin activates ERK-dependent but not AKT-dependent p70S6K cascade in cardiomyogenesis. p70S6K is implicated in the regulation of cell cycle progression, and several studies have documented its central role in endothelial cell proliferation[33,41]; it is thus tempting to speculate that this mitogenic effect might be extended to cardiac precursors.

Besides its crucial role in embryo development and ESC differentiation,[8,13] Cripto overexpression has been associated with tumor initiation and progression, including tumor angiogenesis[9,42]; however, the molecular mechanism is still not clear. Intriguingly, apelin has recently attracted considerable interest as potent inducer of tumor angiogenesis.[43–45] It would thus be important in the future to determine whether Cripto activity in tumor angiogenesis correlates with the activation of the APJ/apelin system.

Taken together, our data provide new insights into understanding the molecular mechanisms underlying early cardiogenesis in mammals. This might be helpful in improving understanding of the etiology of cardiac disease and lead to ways of enhancing conversion of ESCs to cardiomyocytes, still an important bottleneck to their use in drug discovery and regenerative medicine. Further analysis of the regulatory networks that involve Apj/apelin will be required both in mouse and human ESCs, to reprogram ESCs toward a cardiovascular fate for cell therapy and cardiovascular tissue engineering.

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Disclosures

None.

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