Molecular Medicine

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). APJ is a G protein-coupled receptor that has been reported to be involved in cardiac development. It has been postulated that APJ/apelin signals through the Gi/o protein, leading to activation of mitogen-activated protein kinase/p70S6 through coupling to a Go/Gi protein.

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

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.109.201186

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
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. 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. 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 API/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 precursors of cardiovascular function both in Xenopus and in Zebrafish, yet a role for these proteins in mammalian cardiomyogenesis 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). Whole-mount in situ hybridization on gastrulating wild-type and Cripto−/− embryos revealed that the expression of both Apj and apelin were detectable specifically in the posterior mesoderm and posterior–posterior axis formation; however, Cripto−/− embryos showed that Apj and apelin expression was undetectable in Cripto−/− embryos, whereas both genes were markedly upregulated in wild-type ESCs (Figure 1A).

**Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AGTRL-1</td>
<td>angiotensin type I–like receptor</td>
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<td>ALK</td>
<td>activin receptor–like kinase</td>
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<tr>
<td>APJ</td>
<td>apelin receptor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal–regulated kinase</td>
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<tr>
<td>EB</td>
<td>embryoid body</td>
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<td>ESC</td>
<td>embryonic stem cell</td>
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<tr>
<td>GDF</td>
<td>growth/differentiation factor</td>
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<td>MAPK</td>
<td>mitogen–activated protein kinase</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<tr>
<td>PTX</td>
<td>pertussis toxin</td>
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<tr>
<td>QRT-PCR</td>
<td>quantitative RT-PCR</td>
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<td>shRNA</td>
<td>short hairpin RNA</td>
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**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.

Short hairpin (sh)RNAs vectors (pGPZIP 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.

**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 API (also known as angiotensin type I–like receptor [AGTRL-1]) and its ligand apelin. API and apelin have been recently described as important regulators of cardiovascular function both in Xenopus and in Zebrafish; yet a role for these proteins in mammalian cardiomyogenesis 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). 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−/−
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 activation29 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 Smad430; accordingly, SB-431542 affected Cripto expression as well. By contrast, 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 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 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).

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.
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 panmesodermal 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. 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). 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

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**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 βIII-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).

**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).
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 ESCs did not further enhance cardiac differentiation, as revealed by expression of 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. 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). 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
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
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.\(^\text{18}\) 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,\(^\text{31,32}\) 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.\(^\text{31,35,36}\)

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.\(^\text{25,37}\)

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.\(^\text{2,38}\)

In line with our findings, apelin-null mice do not show any early embryonic patterning defects,\(^\text{37,39}\) nor have Apj and apelin-null mice been reported to have congenital cardiac defects.\(^\text{39,40}\) 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.\(^\text{2}\)

Remarkably, although recent experiments in zebrafish showed a critical role for APJ/apelin pathway in myocardial cell specification and heart development,\(^\text{24,25}\) 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.\(^\text{33}\) 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\(^\text{33,41}\); 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,\(^\text{8,13}\) Cripto overexpression has been associated with tumor initiation and progression, including tumor angiogenesis\(^\text{9,42}\); however, the molecular mechanism is still not clear. Intriguingly, apelin has recently attracted considerable interest as potent inducer of tumor angiogenesis.\(^\text{43–45}\) 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.

**Acknowledgments**

We thank Y. Audigier for the kind gift of APJ plasmid.

**Sources of Funding**

This work was supported by grants from the Associazione Italiana Ricerca sul Cancro (to G.M.).

**Disclosures**

None.

**References**


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Circ Res. 2009;105:231-238; originally published online July 2, 2009;
doi: 10.1161/CIRCRESAHA.109.201186

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/105/3/231

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/07/02/CIRCRESAHA.109.201186.DC1
Online Results

To provide more direct evidence that Cripto/Smad2 signaling activated expression of Apj and/or apelin genes, we assessed the expression of both genes in the absence of Smad2. To this end, Smad2–silenced ESCs were generated, by using vectors, which targeted different, non-overlapping Smad2 mRNA sequences. Following puromycin selection, two independent clones were isolated, in which endogenous Smad2 expression was suppressed (c4 and d5), compared to either wild-type ESCs or cells transfected with vector expressing non-targeting shRNA, used as controls (Online Figure I,A). In line with results obtained with the chemical inhibitor of ALKs SB-431542, expression of Apj, Apelin and Cripto was downregulated in 4 day-old EBs derived from the Smad2-silenced (c4 and d5) but not control clones (Online Figure I,B).

Furthermore, we evaluated whether Apj and apelin might be induced shortly after the induction of Smad signaling. To this end, we added increasing amount of recombinant Nodal to undifferentiated ESCs and verified that Nodal was able to induce Smad2 phosphorylation, by western blot analysis (Online Figure II,A). We thus compared the expression of Apj and apelin in ESCs that were either left untreated (control) or treated with recombinant Nodal for three hours, by Q-RT PCR. Interestingly, neither apj nor apelin expression was induced (Online Figure II,B, right panel). On the contrary, in line with previous findings showing that Cripto is a direct target of Smad4, expression of Cripto was significantly upregulated (Online Figure II,B, left panel). Although we cannot completely rule out the possibility of a different effect of Smad signaling on apj/apelin expression in Embryoid Bodies, our results suggest that Apj and apelin are not direct targets of Smad2/4 in ESCs.

Online Figures

Online Figure I. APj/apelin expression is downregulated in Smad2-silenced ESCs (A) Verification of Smad2 expression in independent Smad2 –silenced ES clones at protein level, as shown by western-blot using anti-Smad2 antibodies. Anti-Gapdh antibodies were used as loading control. (B) Effect of Smad2 silencing on Apj, apelin and Cripto expression, as assessed by QRT-PCR on 4 day –old EBs derived from the Smad2 – silenced ESC clones and controls (panel A). mRNA expression was normalized to gapdhh expression and presented as fold change in gene expression relative to the N.T. control. Data are mean ± SE, n≥3.

Online Figure II. Cripto but not APJ and apelin are induced shortly after Smad2 induction in undifferentiated ESCs. (A) Nodal induces Smad2 phosphorylation in ESCs. ESCs were serum starved for 2 hours and treated with increasing amount of recombinant Nodal (R&D) for 20min or left untreated as control (-). Wild-type EBs were also used as positive control (C). Smad2 activation was detected by Western blot analysis using anti-phospho-Smad2 antibody. Levels of total Smad2 and Cripto were also compared. (B) Effect of Smad2 induction on Apj, apelin and Cripto expression. ESCs were serum starved for 2 hours and then treated with Nodal (50nM) for three hours or left untreated as control (-). Expression of Cripto, Apj and Apelin was analysed by QRT-PCR. 4 day –old wild-type EBs were used as control for Apj and apelin expression (right panels). mRNA expression was normalized to gapdh expression; data are mean ± SE, n≥3.

Online Figure III. Apelin redirects the neural fate of Cripto⁻ ESCs and promotes APJ-dependent cardiomyogenesis. (A) Time course-expression of Apj and apelin in Cripto⁻ ESCs and APJ-V5 Cripto⁻ ESCs, showing that Apelin expression is rescued in APJ-V5
Cripto⁻/⁻ ESCs. (B) 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. (C) 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.

Online Figure IV. APJ silencing by shRNAs impairs cardiomyogenesis. (A) Verification of APJ expression in three independent APJ –silenced (Sh-apj 1a, 1b and 1c) 4 day-old EBs by QPCR-RT; mRNA expression was normalized to gapdh expression. Data are mean ± SE, n≥3. Effect of APJ silencing on cardiac differentiation by Apj shRNAs as shown by the prevalence of beating EBs (B) and immunostaining with anti-αMHC antibodies (MF20; C).
Online Figure I
Online Figure II
Online Figure IV
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