The Aplnr GPCR regulates myocardial progenitor development via a novel cell-non-autonomous, \( G_{\alpha_{i/o}} \) protein-independent pathway

Sivini Paskaradevan\(^1,2 \) and Ian C. Scott\(^1,2,3,\ast \)

\(^1\)Program in Developmental and Stem Cell Biology, The Hospital for Sick Children, 555 University Avenue, Toronto, ON M5G 1X8, Canada
\(^2\)Department of Molecular Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada
\(^3\)Heart & Stroke/Richard Lewar Centre of Excellence, 150 College Street, Toronto, ON M5S 3E2, Canada

*Author for correspondence (ian.scott@sickkids.ca)

Biology Open 1, 275–285
doi: 10.1242/bio.2012380

Summary

Myocardial progenitor development involves the migration of cells to the anterior lateral plate mesoderm (ALPM) where they are exposed to the necessary signals for heart development to proceed. Whether the arrival of cells to this location is sufficient, or whether earlier signaling events are required, for progenitor development is poorly understood. Here we demonstrate that in the absence of Aplnr signaling, cells fail to migrate to the heart-forming region of the ALPM. Our work uncovers a previously uncharacterized cell-non-autonomous function for Aplnr signaling in cardiac development. Furthermore, we show that both the single known Aplnr ligand, Apelin, and the canonical \( G_{\alpha_{i/o}} \) proteins that signal downstream of Aplnr are dispensable for Aplnr function in the context of myocardial progenitor development. This novel Aplnr signal can be substituted for by activation of Gata5/Smarc3 in myocardial progenitors, suggesting a novel mechanism for Aplnr signaling in the establishment of a niche required for the proper migration/development of myocardial progenitor cells.

© 2012. Published by The Company of Biologists Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (http://creativecommons.org/licenses/by-nc-sa/3.0).

Key words: Myocardial Progenitors, Apelin Receptor, Gastrulation, Zebrafish

Introduction

A key step to organogenesis is the proper recruitment and differentiation of progenitors that form the mature cell types needed for organ development and function. Cardiovascular progenitor cells (CPCs) are the building blocks of the heart, and have the potential to form cardiomyocytes, endocardium and smooth muscle cells (Kattman et al., 2006; Moretti et al., 2006). CPCs are among the first to migrate during gastrulation, ultimately reaching bilateral regions of the anterior lateral plate mesoderm (ALPM) (Wu et al., 2008). Fate mapping studies in multiple vertebrate models have shown that CPCs arise from a fixed location in the pregastrulation embryo (reviewed in (Evans et al., 2010)). In zebrafish, cells fated to become cardiomyocytes arise from bilateral regions within the first few tiers of cells at the embryonic margin of the 4–6 hours post-fertilization (hpf) embryo, displaced 60–140° relative to the dorsal side of the embryo (Keegan et al., 2004; Stainier et al., 1993). Localization of cells to this “heart field” region of the pregastrula embryo can instruct later myocardial fate, as donor cells transplanted to this area can form cardiomyocytes in vivo (Lee et al., 1994). Upon arrival at the ALPM, multiple signals lead to initiation of cardiogenesis (reviewed in (Evans et al., 2010)), with expression of \( \text{Nkx2.5} \), encoding an NK-class homeodomain transcription factor, being the earliest marker of myocardial progenitors in zebrafish and other vertebrates (Chen and Fishman, 1996; Komuro and Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994).

It is apparent that myocardial progenitor cells arise from a specific location in the embryo and migrate during gastrulation to the ALPM, where they receive signals necessary for differentiation into cardiomyocytes. However, it remains to be determined whether cells fated to form the myocardium require additional signals during gastrulation to effectuate proper heart development, or if it is sufficient that cells reach the ALPM. Explant experiments, primarily done in the chick embryo, have yielded conflicting results on this question (reviewed in (Yutzey and Kirby, 2002)). Whereas some studies argue that cells exist in the early gastrula that are capable of forming cardiomyocytes regardless of later location in the embryo (Auda-Boucher et al., 2000; Lopez-Sanchez et al., 2009), others suggest that ultimate localization to the ALPM is sufficient for cardiac differentiation (Inagaki et al., 1993; Tam et al., 1997). A lack of mutants that specifically affect early myocardial progenitor development, and markers to isolate and characterize these cells, has hindered progress on this key question of early heart development. Members of the \( \text{Mesp} \) basic helix-loop-helix transcription factor family are essential for cardiogenesis in mouse and \( \text{Ciona intestinalis} \), and can promote myocardial differentiation in embryonic stem cells (reviewed in (Bondue and Blanpain, 2010)). \( \text{Mesp} \) function in mice is required for proper migration of cardiac progenitors during gastrulation. Perturbations in BMP, FGF, Wnt (both canonical and non-canonical), and Nodal signaling prior to and during gastrulation lead to later deficits in development of \( \text{Nkx2.5} \)-expressing myocardial progenitors.
(Reifers et al., 2000; Reiter et al., 2001; Ueno et al., 2007). However, as embryos exhibit gross developmental defects when these pathways are manipulated, it is difficult to uncouple function in myocardial progenitor development from general patterning defects. Nevertheless, these results suggest that CPC potential and behaviour is influenced during gastrulation.

Our group has previously described a zebrafish mutant, grinch (grn), in which there is a striking and specific deficit in myocardial progenitors (Scott et al., 2007). Characterization of the mutant showed that there was a decrease in, or in the most severe cases a loss of, nkx2.5-expressing cells. The grn mutation was mapped to the gene encoding Angiotensin II receptor-like 1b (aplnrb, apj, msr1, agtrl1b; referred to as aplnrb in this study), a G protein-coupled receptor (GPCR). Both loss of aplnrb and overexpression of apelin (which encodes the only known Aplnr ligand (Tatemoto et al., 1998)) during early gastrulation resulted in a heartless phenotype in zebrafish (Scott et al., 2007; Zeng et al., 2007). Aplnr shares functional properties with chemokine receptors (Zou et al., 2000), and has been shown to promote angiogenesis in several contexts (Cox et al., 2006; Kasai et al., 2004; Sorli et al., 2007). The loss of heart following Apelin overexpression therefore suggested a chemotactic role for Aplnr/Apelin signaling to guide migration of myocardial progenitor cells to the ALPM during gastrulation. Both apelin overexpression and morpholino-mediated knockdown has been shown to affect migration of cells during gastrulation (Scott et al., 2007; Zeng et al., 2007). However, knockdown of apelin did not fully recapitulate the grn/aplnrb heartless phenotype. This suggested that Aplnr may not simply act via Apelin signaling in cardiac progenitor development, and left as an open question the mechanism of the aplnrb heartless phenotype.

In this study we carried out a detailed analysis of the aplnrb loss-of-function phenotype to elucidate the mechanism through which Aplnr signaling regulates vertebrate heart development. We find defects in a specific region of the aplnrb mutant ALPM, coincident with the site of cardiac development. By tracking cells as they migrate from the heart field region of the early embryo, we find that these cells fail to reach the ALPM in the absence of aplnrb function due to a defect in the initiation of migration, resulting in the complete absence of cells in the heart-forming region. Unexpectedly, via transplantation analysis we find that Aplnr function is required non-autonomously, in cells not destined to form cardiomyocytes, for proper myocardial progenitor development. This occurs independently of classic heterotrimeric G-protein signaling downstream of the GPCR Aplnr. Finally, initial work suggests the activation of the cardiac chromatin remodeling complex, cBAF, in cardiac progenitors as a consequence of Aplnr signaling. This study therefore identifies a novel, non-autonomous function for Aplnr signaling to support proper myocardial progenitor development. Interestingly, this signal may provide a niche for proper CPC development and migration.

Results
Cells from the pregastrula heart field fail to reach the ALPM in aplnra/b morphant embryos
Our previous analysis suggested defects in ALPM formation in aplnrb morphants (morpholino-injected embryos) and mutants (Scott et al., 2007). To further characterize ALPM development, expression of additional Aplnr markers was examined in aplnrb mutants. RNA in situ hybridization analysis demonstrated that, along with loss of nkx2.5 expression, there is a decrease in expression of spi1 and the more posterior domains of fli1 and tal1 in the ALPM, marking myeloid and presumed endocardial progenitors, respectively (supplementary material Fig. S1). This decrease in gene expression in aplnrb mutants may reflect a failure of cells to reach the APLM during development. Alternatively, cells may reach the ALPM but fail to differentiate into the proper cell types. To distinguish between these two possibilities, we performed lineage tracing to determine whether cells from the lateral embryonic margin migrate to the ALPM. For these experiments, the photoconvertible protein KikGR was employed. KikGR normally fluoresces with spectral characteristics similar to EGFP, however after exposure to UV light undergoes a permanent change in its fluorophore such that it fluoresces as a red fluorescent protein (Hatta et al., 2006). To obtain a more complete loss of Aplnr activity, we employed morpholinos (MOs) to inhibit aplnrb along with its paralog aplnra (Tucker et al., 2007). We have previously found that co-injection of these MOs results in a robust heartless phenotype and recapitulates loss of gene expression observed in the ALPM of aplnrb mutants (Scott et al., 2007; supplementary material Fig. S2). One-cell stage wildtype and aplnra/aplnrb MO-injected embryos were co-injected with mRNA encoding KikGR. At 6hpf, a cluster of about 50 cells at the lateral embryonic margin (displaced 90° from the shield) was photoconverted via exposure to UV light (Fig. 1B). Embryos were subsequently scored at 12–14hpf (6–10 somite stage) for the presence of photoconverted cells that had migrated to the heart-forming region of the ALPM (Fig. 1A). In 48% of wildtype embryos, cells from the lateral margin were found as a continuous stripe throughout the lateral plate mesoderm, beginning at the ALPM and extending posterior to the level of the anterior somites (Fig. 1D,D’). Photoconversion of lateral margin cells in aplnra/b morphant embryos similarly often resulted in a lateral stripe of cells being evident on one side of the embryo. However, we found a striking and significant difference (p=0.001) between wildtype and aplnra/b morphant embryos in the presence of photoconverted cells in the heart-forming region of the ALPM. In aplnra/b morphants, cells were rarely evident in this area (brackets in Fig. 1E’ compared to wildtype in Fig. 1D’). In some cases, cells were found both anterior and posterior to this area but were missing specifically form the heart-forming region (arrowhead in Fig. 1F’). In the few instances that cells were found in the ALPM, their organization into a stripe was highly disrupted (supplementary material Fig. S3). Contribution of photoconverted cells to the heart-forming region of the ALPM was thus markedly reduced in morphant embryos (27/57 in wildtype versus 3/104 in aplnra/b morphants, Fig. 1C). These data demonstrate that the deficit in myocardial progenitor development in aplnra/b morphants reflects the failure of cells from the lateral embryonic margin to reach the ALPM.

Portions of the ALPM are absent in aplnra/b morphants
To determine whether cells are present in the heart-forming region of the ALPM in aplnra/b morphants, we sectioned cardiomyocyte-specific myl7:EGFP (Huang et al., 2003) transgenic embryos at 20hpf and imaged cross-sections stained for filamentous Actin. Given the failure of lateral margin cells to reach the heart-forming region of the ALPM following loss of Aplnr signaling, it is possible that cells from other regions of the early embryo migrate to this area in their place. At 20hpf, the bilateral fields of cardiac mesoderm are folded epithelial sheets...
that have begun their migration to the midline (Trinh and Stainier, 2004). Sections were selected for analysis based on the absence of head structures (such as brain ventricles) and the notochord to demarcate anterior and posterior ALPM boundaries, respectively. In wildtype embryos the expected folded epithelial sheet and cardiac-specific myl7:EGFP signal was observed (Fig. 1G). In contrast, there was a marked absence of cells in the ALPM of aplnr/b morphants (Fig. 1H). These data suggest that in aplnr/b morphants and aplnr/b mutants, there are no cells in the region of the ALPM that typically forms cardiac tissue.

**Lateral margin cells exhibit early migration defects**

To explore further the migration defect seen in the absence of Apnr signaling, we performed time-course analysis of wildtype and aplnr/b morphant embryos. Cells were photoconverted as described above and embryos were imaged over time from shield stage (6hpf) to bud stage (10hpf), when gastrulation movements have been largely completed (Warga and Kimmel, 1990). Upon gross observation we saw the typical migration pattern in wildtype embryos with a streak of cells moving toward the animal pole (arrow in Fig. 2A,B). In aplnr/b morphant embryos, we found the leading cells of the migrating group to be absent (arrow in Fig. 2D,E). Importantly, cells in the leading streak are those that will form the ALPM (Sepich et al., 2005; Yin et al., 2009) and are found in the appropriate location in wildtype embryos (arrowhead in Fig. 2C) but not in aplnr/b morphants (arrowhead in Fig. 2F) at bud stage. We next examined this in more detail to determine when defects in migration are first apparent in aplnr/b morphants. By imaging embryos at higher magnification and at more frequent time intervals, we found that labeled cells exhibited a delay in the initiation of migration toward the animal pole in aplnr/b morphants. In contrast to wildtype embryos in which migration is well underway by 70% epiboly (Fig. 2G), cells in aplnr/b morphant embryos do not start migrating until roughly 85% epiboly (Fig. 2J; supplementary material Fig. S4), a lag of 1.0 to 1.5 hours. Our data therefore suggest that cells from the lateral embryonic margin in aplnr/b morphants fail to reach the ALPM due to a defect or delay in the initiation of migration towards the animal pole of the embryo.

**Aplnr signaling functions cell-non-autonomously in the development of cardiomyocytes**

We next performed transplantation analysis to determine whether Aplnr signaling functions cell-autonomously or cell-non-autonomously in the development of cardiomyocytes. It has been suggested that Aplnr signaling modulates a chemotactic cue that attracts cardiac progenitors to the heart-forming region of the ALPM (Scott et al., 2007; Zeng et al., 2007). If this were the case, a cell-autonomous role for aplnr/b would be expected. While we have previously shown a decreased ability of aplnr/b morphant cells to contribute to the heart (Scott et al., 2007), a possible non-autonomous role for this gene was not evaluated in these studies.

Cells from wildtype or aplnr/b MO-injected donor embryos harboring a myl7:EGFP transgene were transplanted into the
embryonic margin of wildtype or aplnra/b morphant host embryos. Transplant embryos were then scored at 48hpf for EGFP-positive cells, which would indicate donor cells that have formed cardiomyocytes. In control (wildtype donor to wildtype host) transplants, differentiated cardiomyocytes were evident in 19% of host embryos, in agreement with previously published work (Scott et al., 2007; Thomas et al., 2008; Fig. 3A,E). As expected, aplnra/b morphant cells had a greatly reduced capacity to differentiate into cardiomyocytes when placed in morphant host embryos (1% of transplants were EGFP+, Fig. 3B,E), recapitulating the aplnra/b phenotype. Surprisingly, wildtype donor cells were rarely able to differentiate into cardiomyocytes in aplnra/b morphant hosts (2% of transplants were EGFP+, n=190, Fig. 3C,E), whereas aplnra/b morphants cells had the capacity to differentiate into cardiomyocytes in wildtype hosts, albeit to a lesser extent than wildtype donor cells (13% of transplants were EGFP+, n=190; Fig. 3D,E). Our results demonstrate an unappreciated cell-non-autonomous role for Apnlr signaling in cardiomyocyte development.

We next wished to confirm that the non-autonomous function seen in aplnra/b morphants is specific to Aplnr activity and not secondary to a host embryo heartless phenotype. We utilized gata5/6 morphants, in which myocardial differentiation is fully compromised (Holtzinger and Evans, 2007). When transplants were performed as described above we found that Gata5/6 activity is required cell-autonomously in cardiomyocyte development. Wildtype cells were able to contribute to heart in 18% of wildtype and 11% of gata5/6 morphant hosts, whereas gata5/6 morphant cells were unable to contribute to the heart in wildtype embryos (0/83 hosts) (data not shown). These data confirm that the non-autonomous function seen in aplnra/b morphants is specific to the loss of Apnlr activity, and not a general consequence of an absence of host cardiac progenitors.

Role of Apelin in Apnlr signaling during cardiac progenitor development

With our data revealing a non-autonomous role for Apnlr in cardiomyocyte development, we decided to revisit the role of the Apelin ligand in this process. While injection of aplnra/b MO leads to embryos lacking cardiac tissue, injection of apelin MO fails to recapitulate this heartless phenotype, with only a reduced heart size evident in the most severe cases (Zeng et al., 2007), Fig. 4E). A trivial explanation for this discrepancy could be that the apelin MOs used do not fully inhibit Apelin function. To determine if this may be the case, we injected apelin MO into hsp:apelin transgenic embryos and compared the phenotype of the embryos with or without the induction of apelin over-expression. Heat-shock of hsp:apelin embryos prior to 6hpf greatly increased apelin expression compared to wildtype siblings (Fig. 4A). As we have previously found, hsp:apelin embryos heat-shocked at 4hpf resulted in a heartless phenotype (Scott et al., 2007; Fig. 4D). Injection of apelin MO resulted in a slight reduction in the size of the heart (Fig. 4E). Interestingly, when hsp:apelin transgenic embryos injected with apelin MO were heat-shocked, they re-capitulated the apelin morphant phenotype (small heart) rather than the apelin over-expression (heartless) phenotype (Fig. 4F). As the hsp:apelin transgene expresses, by RNA in situ hybridization, greatly elevated levels of apelin, this strongly suggested that the amount of apelin MO injected was sufficient to suppress the endogenous apelin transcript levels. Therefore, in wildtype embryos injection of apelin MO likely results in a complete knockdown of endogenous Apelin, and lends further support to the model that Apelin may not be the functional ligand for Apnlr signaling during myocardial progenitor development.
Aplnr signaling functions cell-non-autonomously in the development of cardiomyocytes. Transplantation of wildtype (WT) or aplnra/b MO-injected (±MO) myl7:EGFP donor embryos to the margin WT or aplnra/b MO-injected host embryos was carried out at 4hpf. (A–D) Representative host embryos at 2dpf following transplantation; lateral view, anterior to left. (C) Wildtype cells are unable to contribute to the heart in aplnra/b morphant host embryos, whereas aplnra/b morphant cells are able to contribute to the heart in wildtype hosts (D). (E) Graph of percentage of host embryos with contribution from myl7:EGFP donor cells. N=5, n=236 for wildtype donors and hosts; N=4, n=130 for aplnra/b morphant donors and hosts; N=4, n=190 for wildtype donors and aplnra/b morphant hosts; N=3, n=190 for aplnra/b morphant donors and wildtype hosts. Views in (A–D) are lateral views, with anterior to the left. Scale bar represents 200μm (A).

Fig. 3. Aplnr signaling functions cell-non-autonomously in the development of cardiomyocytes. Transplantation of wildtype (WT) or aplnra/b MO-injected (±MO) myl7:EGFP donor embryos to the margin WT or aplnra/b MO-injected host embryos was carried out at 4hpf. (A–D) Representative host embryos at 2dpf following transplantation; lateral view, anterior to left. (C) Wildtype cells are unable to contribute to the heart in aplnra/b morphant host embryos, whereas aplnra/b morphant cells are able to contribute to the heart in wildtype hosts (D). (E) Graph of percentage of host embryos with contribution from myl7:EGFP donor cells. N=5, n=236 for wildtype donors and hosts; N=4, n=130 for aplnra/b morphant donors and hosts; N=4, n=190 for wildtype donors and aplnra/b morphant hosts; N=3, n=190 for aplnra/b morphant donors and wildtype hosts. Views in (A–D) are lateral views, with anterior to the left. Scale bar represents 200μm (A).
and acts by uncoupling $\alpha_{i/o}$ proteins, and presumably their $\beta_7$ subunits, from associated GPCRs (Codina et al., 1983). We first injected RNA encoding PTX at the 1-cell stage to globally inhibit $\alpha_{i/o}$-mediated signaling. Global over-expression of PTX recapitulated the aplnra/b morphant embryo heartless phenotype (Fig. 5B,C). However, $\alpha_{i/o}$ proteins both act downstream of multiple GPCRs and have been shown to regulate aspects of Hedgehog and Wnt signaling (Ogden et al., 2008; Slusarski et al., 1997). We could therefore not conclude from these results that the loss of cardiac tissue following PTX overexpression was specifically due to inhibition of $\alpha_{i/o}$-mediated Aplnr signaling.

To further examine whether $\alpha_{i/o}$ proteins are the major effectors of the Aplnr signaling pathway, we performed transplant analyses as described above using wildtype and PTX over-expressing embryos as both donors and hosts. In control experiments where PTX RNA-injected donor cells and host embryos were used, we observed no EGFP+ donor cells (n=80). Transplantation of PTX over-expressing cells to wildtype host embryos revealed a minor cell-autonomous role for $\alpha_{i/o}$-mediated signaling in cardiac differentiation, with a reduction in transplant embryos with EGFP-positive cells being observed (EGFP+ cells in 13% of hosts, n=216, Fig. 5D,F). Surprisingly, and in contrast to aplnra/b morphant transplant experiments, we found that wildtype cells were able to differentiate into cardiomyocytes after being transplanted into PTX over-expressing host embryos (EGFP+ cells in 12% of hosts, n=158, Fig. 5E,F). These data suggest that the cell-non-autonomous effect of Aplnr signaling in cardiomyocyte development is independent of, or at least not fully dependent upon, signaling through $\alpha_{i/o}$ proteins. In addition, we found that wildtype cells were able to contribute to heart in host embryos in which $\beta_7$ subunit activity was inhibited by injection of RNA encoding the C-terminal fragment of GRK2 (Koch et al., 1994).

In these experiments, EGFP+ cells were evident in 12% of hosts (n=75; data not shown), further supporting a $\alpha_{i/o}$ protein-independent mechanism for Aplnr signaling.

Given the differences in the autonomy of Aplnr and $\alpha_{i/o}$ function, we next sectioned myl7:EGFP embryos over-expressing PTX at 20hpf to examine the ALPM. Unlike what was observed in aplnra/b morphants, cells were present in the ALPM at the level of the heart-forming region in embryos injected with PTX RNA. However, these cells were unable to differentiate into cardiomyocytes, as noted by the absence of EGFP+ cells in myl7:EGFP transgenic embryos (arrow in Fig. 5H). Therefore, while global inhibition of $\alpha_{i/o}$ protein signaling blocks differentiation of cells into cardiomyocytes, the autonomy and ultimate cause of this phenotype differs from that seen following aplnra/b knockdown. Taken together, these data suggest that Aplnr signaling acts via a mechanism independent of $\alpha_{i/o}$ proteins in cardiac progenitor development (Fig. 6D).

Aplnr signaling functions upstream of the cardiac BAF complex

Recent work published by our lab demonstrated that overexpression of gata5 and smarcd3b, two factors in a cardiac-specific chromatin remodeling complex (cBAF) effectively directed the migration of non-cardiogenic cells to the ALPM and their subsequent contribution to various

Fig. 5. Aplnr activity is independent of $\alpha_{i/o}$ proteins in cardiomyocyte development. (A–C) Bright field images of wildtype (WT), aplnra/b morphant, and PTX overexpressing myl7:EGFP embryos at 2dpf. (A’–C’) Fluorescent images of embryos in (A–C), (A’–C’) are overlays of (A–C) and (A’–C’). Overexpression of PTX (C) recapitulates the aplnra/b morphant (B) cardiac phenotype. (D–F) Transplantation of myl7:EGFP donor cells from WT or PTX overexpressing cells to WT or PTX overexpressing host embryos at 4hpf was carried out. Hosts were subsequently scored at 2dpf for EGFP+ve donor cells in the heart. (D–E) PTX overexpressing cells are able to contribute to the heart in wildtype embryos (D), and wildtype cells are able to contribute to the heart in PTX overexpressing host embryos (E), suggesting that $\alpha_{i/o}$ proteins are not downstream of Aplnr signaling in cardiomyocyte development. (F) Graph showing percentage of host embryos with contribution from myl7:EGFP donor cells in transplants performed. N=5, n=236 for wildtype donors and hosts; N=2, n=80 for PTX O/E donors and hosts; N=3, n=216 for PTX O/E donors and wildtype hosts. O/E=overexpression. (G,H) Cross sections through the ALPM of 20hpf myl7:EGFP wildtype and PTX overexpressing embryos. The ALPM is evident at the level of the heart-forming region following PTX addition (arrow in H) but cells fail to differentiate into cardiomyocytes as noted by the absence of EGFP+ve cells. (A–E) are lateral views, with anterior to the left. Scale bars represent 200μm (A and D), 17μm (G) and 22μm (H).
cardiovascular lineages (Lou et al., 2011). As both Aplnr signaling and the cBAF complex influence the migration of cells to the heart-forming region of the ALPM, we investigated the relationship between these two activities. When gata5 and smarcd3b were overexpressed in aplnra/b morphant embryos in a myl7:EGFP transgenic background, we were able to rescue the aplnra/b heartless phenotype as demonstrated through the presence of EGFP+ cardiomyocytes (Fig. 6B). Additionally, when cells from myl7:EGFP embryos overexpressing gata5 and smarcd3b were transplanted into aplnra/b morphant host embryos, the cells were able to differentiate into cardiomyocytes in a cell autonomous manner (Fig. 6C), with GFP+ cells in 18% (19/107) of host embryos. Taken together, these data suggest that the non-autonomous effects of Aplnr signaling may lead to the activation of the cBAF complex, which acts cell-autonomously (Lou et al., 2011) to guide the migration of cells to the heart-forming region of the zebrafish embryo (Fig. 6D).

**Discussion**

In previous studies of Aplnr signaling in zebrafish, a role for the migration of cells during gastrulation was inferred based on analysis of knockdown and global over-expression of Apelin (Scott et al., 2007; Zeng et al., 2007). Here, we have conducted a detailed analysis of ALPM development in the absence of Aplnr signaling. Our data demonstrate a role for Aplnrb signaling in the migration of cells from the lateral embryonic margin to the heart-forming region of the embryo, likely through the activity of cBAF (Fig. 6D). In the absence of Aplnrb signaling, a discrete domain of the ALPM, which overlaps with the site of nkd2.5 expression, is devoid of cells. This effect on a confined region of the ALPM likely explains the cardiac specificity of the aplnrb mutant phenotype. Interestingly, we report here an unexpected cell-non-autonomous function for Aplnr signaling in heart development. Further, we show here that Aplnrb function in the context of cardiac progenitor development occurs via a Gαi/o protein-independent mechanism that is not dependent on the canonical Apelin ligand. The previous chemotactic model of Aplnr function, in which it guides migrating cardiac progenitors to the ALPM via response to an Apelin gradient, therefore clearly must be revised.

It remains unclear why, despite the broad expression of aplnrb in gastrulating mesendoderm (Scott et al., 2007; Zeng et al., 2007), migration of cardiac progenitors (and presumably their neighbours in the ALPM) is specifically affected in aplnrb mutants. Cells contributing to cranial vasculature, circulating blood, pectoral fin mesenchyme and pharyngeal pouches have been shown to arise from overlapping regions of the ALPM (Keegan et al., 2004). However, the pectoral fin and cranial vasculature form normally in aplnrb mutants (this study and Scott et al., 2007)) Thus Aplnrb function appears to be necessary for specific cell populations that are found in a mixed progenitor pool at the lateral embryonic margin. Our photoconversion analysis demonstrated that cells from the lateral embryonic margin fail to migrate to the ALPM in the absence of Aplnr signaling. This may be due to a delay in the initiation of migration of “leading edge” mesendoderm towards the animal pole (“anterior” of the embryo). While we did observe the eventual migration of cells toward the animal pole in aplnra/b morphants, the nature and fate of these cells remains unclear. They may represent myocardial progenitors that have initiated migration at a later time than in a wildtype embryo. Alternatively, they may be more posterior LPM progenitors that are initiating migration at the correct time. In this latter scenario, myocardial progenitors may have never been specified and/or failed to gastrulate, instead accumulating at the embryonic margin or moving passively with neighboring cells. From this perspective it is interesting to note that myocardial progenitors are among the first cell types to involute during vertebrate gastrulation (Garcia-Martinez and Schoenwolf, 1993; Parameswaran and Tam, 1995). The timing of this event may be essential for later heart development by allowing migration to the ALPM or rendering myocardial
progenitors competent to initiate cardiogenesis. In this regard, the ability of Gata5/Smarcd3 to rescue myocardial differentiation in aplnrb/a morphants is instructive (see below).

Many signals affecting cardiac progenitor migration to the midline, after the onset of nck2.5 expression, have been described, with perturbations resulting in cardiac bifida (2 hearts, (Chen et al., 1996; Saga et al., 1999)). In the case of Apnlr signaling, mis-regulation of migration appears specific to a subset of gastrulating cells: those destined to reach the heart-forming region. This result is intriguing, as the molecular mechanisms regulating the migration of cardiac progenitors to the ALPM are poorly understood. Wnt3a/Wnt5a signaling has been shown to affect the path of cardiac progenitor migration in chick embryos, but not to affect cardiac progenitor specification (Sweetman et al., 2008; Yue et al., 2008). The non-canonical Wnt/planar cell polarity (PCP) signaling pathway regulates convergence and extension of the gastrulating vertebrate embryo (reviewed in (Roszko et al., 2009)). However, while perturbation of PCP leads to cardiac morphological defects (Phillips et al., 2007), even severe zebrafish PCP mutants, such as MZtri mutants (where maternal and zygotic vangl2 are mutated) form a heart (data not shown). Accumulation of cells at the embryonic margin may also suggest failed regulation of cellular properties such as cell-shape changes or regulation of adhesion molecules, critical regulatory mechanisms for gastrulation (Speirs et al., 2010; Yin et al., 2009). Early patterning in the zebrafish embryo by a dorsoventral (DV) gradient of Bmp signaling has been shown regulate a gradient of Cadherin-based cell adhesion, in turn resulting in differential migratory behaviours for subsets of mesodermal populations along the DV axis (von der Hardt et al., 2007).

In the mouse, Mesp1 and Mesp2 are required for migration of cranio-cardiac mesoderm. In Mesp1/2 mutants, presumptive cardiac progenitors fail to migrate from the primitive streak and as a consequence accumulate there, resulting in heartless embryos (Kitajima et al., 2000). However, we have not observed changes in mespab expression in aplnrb mutant embryos (data not shown).

The non-autonomous function of aplnrb in cardiac progenitor migration was unexpected, and provides further insight into the mechanism of Apnlr function. In general, autonomous versus non-autonomous roles of Apnlr signaling have not been closely examined. In the context of angiogenesis, the receptor is expressed in endothelial cells, suggesting a cell-autonomous mechanism of action (Cox et al., 2006; Del Toro et al., 2010), however this has not been tested formally. The temporal requirement for Apnlr signaling in early heart development is not known, however use of a hsp:apelin transgene has shown that apelin overexpression prior to (but not after) 6hpf results in a heartless phenotype (Scott et al., 2007). We therefore favour at present a non-autonomous “niche” function for Apnlr signaling. The ultimate output of this signal is likely modulation of cell-cell contacts or ECM composition that results in an alteration of cardiac progenitor migration. It is interesting to note that a role for Apnlr/Apelin signaling in embryonic stem (ES) cell differentiation to cardiomyocytes was recently described (D’Aniello et al., 2009). In this study, Apnlr and Apelin were shown, via a PTX-sensitive mechanism, to act downstream of Nodal signaling to prevent neural differentiation at the expense of cardiomyocytes. It is unclear if this ES cell mechanism is fully conserved in vivo: in ES cells a Gαi/o signal is used, mediated by the Apelin ligand, which we do not observe in the context of zebrafish heart development. Further, the autonomy of Apnlr function in ES cells was not examined.

Numerous studies, both in vitro and in vivo, have described roles for Apnlr signaling in adult cardiovascular function (Ashley et al., 2005; Chandrasekaran et al., 2008; Charo et al., 2009; Szokodi et al., 2002). In these studies, a prerequisite role for Apelin, the only known Apnlr ligand (Tatemoto et al., 1998) in Apnlr function has been assumed. In vitro, PTX-sensitive signaling pathways downstream of Apnlr are activated by Apelin administration (Masri et al., 2002). Indeed, many in vivo studies of Apnlr function have relied on addition of Apelin ligand as a proxy for pathway activation. We were surprised to find that Apnlr signaling apparently acts independently of Apelin during early cardiac development in zebrafish. However, this result is consistent with aplnrb and apelin gene expression patterns, as apelin transcripts are not evident until 10hpf (Scott et al., 2007; Zeng et al., 2007), by which time cardiac progenitors have largely reached their target location in the ALPM. Obviously, our negative MO results for apelin with respect to heart formation cannot alone absolutely prove that Apnlr is acting independently of Apelin. It is interesting to note, however, that analysis of Apelin and Apnlr mutant mice has in some cases noted discrepancies in what would be expected to be identical phenotypes (Charo et al., 2009; Ishida et al., 2004; Kuba et al., 2007). Notably, murine Apnr -/- mutants exhibited cardiac developmental defects while Apelin -/- mutants did not. This led the authors to suggest that the Apnlr may act in an Apelin-independent manner in some contexts (Charo et al., 2009). Future evaluation of endogenous Apnlr function should therefore be careful to consider not only ectopic addition of Apelin, but also loss of Apnlr function.

Interestingly, our results strongly argue for a Gαi/o-dependent mechanism of Apnlr activity in the context of cardiac progenitor migration to the ALPM. While both loss of aplnrb and overexpression of PTX result in heartless embryos, this appears to be due to distinct mechanisms. Apnlr was absolutely required in a non-autonomous fashion for heart development, whereas PTX did not show strict autonomous or non-autonomous functions. Further, embryos lacking Apnlr signaling did not contain cells in the heart-forming region of the ALPM, suggesting a failure of cells to reach this location. In contrast, inhibition of Gαi/o signaling via PTX did not affect the migration of cells to the ALPM. Instead, PTX-treated embryos had cells in the ALPM that could not initiate myocardial differentiation. An effect on cardiomyocyte development following global inhibition of Gαi/o proteins is not unexpected. Gαi/o proteins have been shown to be effectors of Hh signaling through their coupling to Smoothened (Riobo et al., 2006) and it was recently shown in zebrafish that Hh signaling functions cell-autonomously in the development of cardiomyocytes (Thomas et al., 2008). Gαi/o proteins also act downstream of Fzd1 in the Wnt/Ca2+ pathway, and are implicated in patterning of the zebrafish embryo, likely through a cell-non-autonomous mechanism (Slusarski et al., 1997). G-protein independent signaling by GPCRs has become appreciated as a critical component of many signaling events (Shenoy et al., 2006). In the absence of a G-protein mediated (and perhaps Apelin/Apnlr-mediated) signal, Apnlr may be functioning in a number of ways. Signaling may be via recruitment of β-arrestin, and may occur following receptor internalization (Shenoy et al., 2006). Apnlr may
dimerize with other GPCRs, affecting their response to ligands, nature or strength of downstream signaling pathways (Han et al., 2009; Maurice et al., 2010; Monnier et al., 2011). Finally, signaling may occur via Aplnr ligand(s) that remain to be discovered. Recent work suggests that the hypotensive effects of Apelin/Aplnr signaling in the vasculature may occur independently of Gα13o-mediated cAMP inhibition (Iurrioz et al., 2010). The mechanisms through which Apelin/Aplnr signaling regulates many described aspects of development, homeostasis and disease (Barnes et al., 2010; Carpene et al., 2007; Quazi et al., 2009) therefore requires further investigation.

Due to the specific involvement of both Aplnr and cBAF activity in the migration of myocardial progenitor cells to the ALPM, and subsequent differentiation into cardiomyocytes, we investigated the relationship between the two. Interestingly, our results suggest that cBAF functions downstream of Aplnr signaling, in a cell-autonomous manner, to mediate the migration of cells to the heart-field region of the developing embryo. The extracellular factor(s) activated downstream of Aplnr is yet to be determined. However, in our hands activation of signaling pathways critical for early heart development (Fgf, Nodal, Bmp, canonical Wnt, and Shh) were not sufficient to rescue the aplnra/b morphant phenotype (results not shown). The relationship between Aplnr signaling and cBAF function requires future study. However, it is tempting to speculate that the Aplnr signaling may allow for formation of an active form of a Gata5 associated with Smarcd3-containing BAF complex. As neither the effectors of Aplnr signaling in cardiac progenitor migration nor the regulators of cBAF activity have been elucidated, the link between the two is an important connection that requires further investigation.

In summary, our work shows that Aplnr signaling regulates the migration of cardiac progenitor cells from the lateral embryonic margin to the ALPM (Fig. 6D), a step essential for cardiomyocyte development. The non-autonomous nature of Aplnr signaling in this context suggests that Aplnr supports a niche that cardiac progenitors require for their proper differentiation or migration. As a key early step in cardiac progenitor development has been postulated to involve changes in migratory behaviour (Christian et al., 2008; Tam et al., 1997), Aplnr signals may be instructive or permissive for this event. Inducers and effectors of this pathway remain to be elucidated, and their identification will be critical to understanding the earliest events of cardiac progenitor development. Further, this will likely aid future work in the differentiation and growth of cardiomyocyte populations from various stem cell populations for cell therapy, preclinical drug screening and disease mechanism study applications.

Materials and Methods

Zebrafish Strains and Embryo Maintenance

Zebrafish embryos were grown at 28°C in embryo medium following standard procedures (Westerfield, 1993). gmr<sup>emo98</sup> mutants, which harbour a mutation in aplnra, as well as tsP53<sup>901</sup>, Tg(mytl7:EGFP)<sup>944</sup> and Tg(hsp-apelin)<sup>902</sup> zebrafish lines have been previously described (Berghmans et al., 2005; Huang et al., 2003; Scott et al., 2007).

Microinjection

MOs used to target translation of aplnra/agtl1a (5′ – cgggtga-ttccgggaggagcgtc – 3′), aplnrb/agtl1b (5′ – cagagaagtttg-ttcgttgg-tacggtgc – 3′), and apelin (5′ – gatcttcacattcactgctctc – 3′) have been previously described (Scott et al., 2007, Zeng et al., 2007) and were purchased from Gene Tools (Oregon, USA). For embryos injected with aplnra/b MO, 1 ng of aplnra and 0.5 ng of aplnrb MO was injected. 8 ng of apelin MO was injected. MO targeting splicing of gata5 (5′ – tgttgaagatcttataggcgtc – 3′) and translation of gata5 (5′ – aaggtgtataccccggtatcaca – 3′) were used as previously described (Peterkin et al., 2003, Trinh et al., 2005), with 2 ng of gata5 MO and 0.5 ng of gata6 MO co-injected into one-cell stage embryos. An 800 bp fragment of the PTX coding sequence was PCR amplified from pSP64-T-Ptx (Hammerschmidt and McMahon, 1998) and subcloned into pcS2+-mynaCterm-GRK2, encoding the C-terminal portion of GRK2 fused to C-terminal palmitoylation and myristoylation sites, was constructed by Stephen Angers (University of Toronto). pcS2+-Gata5 and Smarcd3b mRNA was prepared from linearized plasmids using an Message Machine kit (Applied Biosystems). 100 pg of PTX or 25 pg of mynaCterm-GRK2 mRNA was injected into each one-cell stage embryo. For rescue experiments, 50 pg each of gata5 and smarcd3b mRNA was injected into each one-cell stage embryo.

RNA In Situ Hybridization and Histology

RNA in situ hybridization (ISH) was carried out as previously described (Scott and Thieue, 2008 and Thieue, 2008) using riboprobes specific for apelin, flh, gata1, gata3, nkd2.5, my7, sp1, and tal1. DNA fragments for all probes were amplified by RT-PCR (sequences available upon request). ISH images were taken on a Leica MZ16 microscope at a magnification of 115X. Sectioning of embryos (embedded in 4% low-melt agarose) was performed on a Leica VT1200S Vibratome with a speed of 0.7 mm/s, amplitude of 3.0 mm, and section width of 150 µm. Images were taken on a Zeiss LSM510 confocal microscope.

Photoconversion

The KαGR coding sequence (purchased from MBL International, Japan) was amplified by PCR and subcloned into pcS2+. KαGR mRNA was made using the mMessage mMachine kit (Applied Biosciences) from a linearized pcS2+ KαGR template. 150 pg of KαGR mRNA was injected at the one-cell stage into wildtype embryos either with or without aplnra/b MO. Cells of the lateral embryonic margin (displaced 90° from the shield at 6hpf) were photoconverted on a Zeiss AxioImager M1 by closing the fluorescence diaphragm such that only the cells of interest were visible. This region of the embryo was exposed to UV light (through the DAPI filter) for a period of one minute. Images of embryos were taken on a Zeiss AxiosImager M1 microscope using either the 5X or 10X objective.

Transplantation

Transplantation was carried out as previously described (Scott et al., 2007). Donor and host embryos were injected with appropriate amounts of aplnra/b MO or PTX RNA as specified above. 10–20 cells from my7:EGFP donor embryos were transplanted into the embryonic margin of unlabeled wildtype hosts. Hosts were scored at 48hpf for the presence of donor-derived EGFP+ cardiomyocytes in the heart. Images of embryos were taken on a Leica MZ25FA microscope at a magnification of 175X.

Statistics

For transplantation and photoconversion data, statistics were performed using an unpaired T-test with unequal variances.

Acknowledgements

We thank members of the Scott lab for insightful discussion, suggestions and technical assistance. Thanks to Angela Morley and Sasha Fernando for zebrafish care and facility maintenance. Thanks to Stephane Angers, Jason Berman and Matthias Hammerschmidt for kindly providing pcS2+ mynaC-term GRK2, tsP53<sup>901</sup> fish and pSP64-T-Ptx, respectively. S.P. was supported in part by Restracomp, an award from the Research Training Center at the Hospital for Sick Children. This work was supported in part by a grant from the Canadian Institutes for Health Research to I.C.S. (Funding Reference Number 86663).

References


Apelin and cardiac progenitors

284


Downloaded from http://bio.biologists.org/ by guest on October 23, 2017

Sweetman, D., Wagstaff, L., Cooper, O., Weijer, C. and Munsterberg, A. (2008). The migration of paraxial and lateral plate mesoderm cells emerging from the late primitive streak is controlled by different Wnt signals. BMC Dev. Biol. 8, 63.


