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Restraint of Fgf8 signaling by retinoic acid signaling is required for proper heart and forelimb formation

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ABSTRACT

Cardiomelic or heart–hand syndromes include congenital defects affecting both the forelimb and heart, suggesting a hypothesis where similar signals may coordinate their development. In support of this hypothesis, we have recently defined a mechanism by which retinoic acid (RA) signaling acts on the forelimb progenitors to indirectly restrict cardiac cell number. However, we still do not have a complete understanding of the mechanisms downstream of RA signaling that allow for the coordinated development of these structures. Here, we test the hypothesis that appropriate Fgf signaling in the cardiac progenitor field downstream of RA signaling is required for the coordinated development of the heart and forelimb. Consistent with this hypothesis, we find that increasing Fgf signaling can autonomously increase cardiac cell number and non-autonomously inhibit forelimb formation over the same time period that embryos are sensitive to loss of RA signaling. Furthermore, we find that Fgf8a, which is expressed in the cardiac progenitors, is expanded into the posterior in RA signaling-deficient zebrafish embryos. Reducing Fgf8a function in RA signaling-deficient embryos is able to rescue both heart and forelimb development. Together, these results are the first to directly support the hypothesis that RA signaling is required shortly after gastrulation in the forelimb field to temper Fgf8a signaling in the cardiac field, thus coordinating the development of the heart and forelimb.

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Introduction

Proper development of an organism requires the simultaneous formation of multiple organs. In humans, many developmental syndromes include both congenital heart and forelimb defects, which has led to the proposal that development of these two organs may be coordinated (Wilson, 1998). A correlation between heart and forelimb defects is also observed in zebrafish, chick and mouse embryos, implying the coordinated development of these organs in certain contexts is a conserved feature in vertebrates (Bruneau et al., 2001; Garrity et al., 2002; Grandel et al., 2002; Heine et al., 1985; Niederreither et al., 1999; Wilson, 1998). Recent lineage tracing studies in zebrafish support a close physical association of these two progenitors fields during early vertebrate development, suggesting that the same signals may influence the fates of these two progenitors (Keegan et al., 2005; Waxman et al., 2008). However, we still do not have a clear understanding of the mechanisms that may coordinate forelimb and heart development in vertebrates.

Retinoic acid (RA) signaling is required for proper heart and forelimb development in vertebrates (Grandel et al., 2002; Keegan et al., 2005; Lohnes et al., 1994; Mendelsohn et al., 1994; Niederreither et al., 2001; Niederreither et al., 2002; Waxman et al., 2008). RA signaling-deficient vertebrate embryos lack forelimbs (pectoral fins in fish) coupled with an increase in cardiac cells (Keegan et al., 2005; Niederreither et al., 1999; Waxman et al., 2008). Although the inverse relationship and close physical association of forelimb and heart progenitors initially suggested that RA signaling could be regulating a fate transformation between these progenitors (Keegan et al., 2005; Waxman et al., 2008), instead our analysis in zebrafish supports the hypothesis that there is not a trade-off between cardiac and forelimb progenitor fates (Waxman et al., 2008). Specifically, we found that RA responsive genes are expressed in the forelimb progenitor field adjacent to the cardiac progenitor field. Moreover, *hoxb5b*, a RA responsive gene, was indirectly required to limit cardiac cell number (Waxman et al., 2008). Because loss of *hoxb5b* can only partially recapitulate the RA signaling-deficient phenotype, this suggests other signals must also be involved downstream of RA signaling in coordinating forelimb and cardiac development.

Fgf signaling is a good candidate to be involved in the coordinated development of the heart and forelimb downstream of RA signaling. In mice, loss of RA signaling results in a posterior expansion of cardiac Fgf8 expression, a Fgf10 reporter and Fgf responsive genes in the lateral plate mesoderm (LPM) (Ryckebusch et al., 2008; Sirbu et al., 2008). However, these studies did not determine if the ectopic Fgf signaling in RA signaling-deficient mouse embryos is a simultaneous cause of the heart and forelimb defects or simply a marker of aberrant patterning. In zebrafish, Fgf8a and Fgf responsive genes overlap with cardiac progenitors in the LPM (Reifers et al., 2000; Znosko et al., 2010). Although the Fgf signaling components have not been examined in the LPM of RA

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signaling-deficient zebrafish embryos, these embryos do have a posterior expansion of cardiac progenitor markers, such as *nkx2.5* and *gata4* (Waxman et al., 2008). While it has yet to be demonstrated in mice, increasing Fgf signaling during early somitogenesis in zebrafish embryos results in a modest expansion of cardiac differentiation markers and loss of the forelimbs, thus phenocopying RA signaling-deficient embryos (Marques et al., 2008). Building on this observation, a recent study hinted that in zebrafish RA signaling may be required to repress Fgf signaling in forelimb initiation (Zhao et al., 2009), though the underlying nature of this relationship of Fgf to RA signaling was not explored in zebrafish. Therefore, synthesizing the available mouse and zebrafish data (Ryckebusch et al., 2008; Sirbu et al., 2008; Waxman et al., 2008; Zhao et al., 2009), a model is suggested where RA signaling in the forelimb progenitor field is required to restrict Fgf signaling in the adjacent cardiac progenitor field, in order to allow for the proper development of both these organs. Despite this attractive model, it is derived from data in multiple studies and has therefore not yet been rigorously tested.

Here, we directly tested the hypothesis that RA signaling is required to restrict Fgf signaling in the cardiac progenitor field allowing for the proper development of both the heart and forelimb in zebrafish. We first show that increased Fgf signaling can promote cardiomyocyte (CM) specification and inhibit forelimb formation over a developmental period that parallels sensitivity to loss of RA signaling (Waxman et al., 2008), thus confirming and extending previous observations (Marques et al., 2008). We go on to demonstrate that increasing Fgf signaling causes a posterior expansion of cardiac progenitor markers. We then demonstrate that loss of RA signaling results in a posterior expansion of *fgf8a* and Fgf signaling responsive genes. Importantly, we find that reduction of Fgf8a signaling, through injection of sub-optimal doses of *fgf8a* morpholinos (MOs), can simultaneously rescue heart and forelimb formation in RA signaling-deficient embryos. Finally, using cell transplantation experiments, we find that Fgf signaling acts cell autonomously to promote cardiac cell specification, but non-autonomously to restrict forelimb specification. Together, these results are the first to demonstrate that proper signaling of Fgf8a downstream of RA signaling is responsible for balancing autonomous and non-autonomous interactions between the cardiac and forelimb progenitor fields. Therefore, building on our previous model of RA signaling in the LPM (Waxman et al., 2008), we propose a feedback inhibition model in which RA signaling promotes the forelimb field and restrains Fgf8a signaling, which promotes the adjacent cardiac field, to coordinate the development of the heart and forelimb.

Materials and methods

Zebrafish husbandry and transgenic lines used

Zebrafish were maintained and embryos were collected and raised under standard laboratory conditions (Westerfield, 1995). *Tg(hsp70:ca-fgfr1)^{pd3}* (Marques et al., 2008) and *Tg(−5.1myl7:nDsRed)^{f2}* (Mably et al., 2003) lines were used.

Heat-shock experiments and genotyping

Heterozygous transgenic *Tg(hsp70:ca-fgfr1)^{pd3}* adults were crossed with wild-type sibling adults or homozygous *Tg(−5.1myl7:nDsRed)^{f2}* adults. The resulting embryos were raised at 28.5 °C until heat-shock was performed at the designated stages by raising the temperature to 37 °C for 30 min in a Biorad C1000 or S1000 PCR machine. Following heat-shocks, transgenic embryos were manually sorted using morphology. Affected transgenic embryos have slightly elongated bodies and enlarged heads. The genotypes of heat-shocked wild-type sibling and affected transgenic embryos were confirmed using PCR with primers that recognize the transgene. The sequence (5′-tccccgacgaggtgtttattc-3′) for the forward primer to the *hsp70* promoter was reported previously (Connors et al., 2006). The sequence for the reverse primer that recognizes the 5′ end of *Xenopus fgfr1* is 5′-atgtgaccaaagtggacggttcgg-3′.

Zebrafish embryo MO injections

Zebrafish embryos were injected at the one cell stage with a 2 ng mixture (1 ng each) or 6 ng mixture (3 ng each) of *fgf8a* MOs. Sequences for the *fgf8a* MOs (*fgf8E2I2* and *fgf8E3I3*) were reported previously and were shown to cause phenotypes consistent with *fgf8a* loss of function (Draper et al., 2001). Embryos injected with a 2 ng *fgf8a* MO mixture appeared overtly wild-type or exhibited a hypomorphic *fgf8a* loss-of-function phenotype relative to those injected with a 6 ng MO (3 ng each) mixture, which had phenotypes consistent with stronger loss of *fgf8a* function (Supplemental Fig. 1).

In situ hybridization

In situ hybridization (ISH) was performed essentially as previously reported (Oxtoby and Jowett, 1993). All probes were reported previously: *myl7* (ZDB-GENE-991019-3), *amhc* (ZDB-GENE-031112-1), *vmhc* (ZDB-GENE-991123-5), *nkx2.5* (ZDB-GENE-980526-321), *hey2* (ZDB-GENE-000526-1), *tal1* (ZDB-GENE-980526-501), *etv2* (ZDB-GENE-050622-14), *tbx5a* (ZDB-GENE-991124-7), *gata4* (ZDB-GENE-980526-476), *ntla* (ZDB-GENE-980526-437), *fgf3* (ZDB-GENE-980526-178), *fgf8a* (ZDB-GENE-990415-72), *dusp6* (ZDB-GENE-030613-1), *etv5b* (ZDB-GENE-991228-4), *pea3* (ZDB-GENE-990415-71), *spry4* (ZDB-GENE-010803-2), *hand2* (ZDB-GENE-000511-1), and *fgfrl1b* (ZDB-GENE-050201-3).

Area measurements

Areas of total cells expressing *myl7*, *vmhc*, and *amhc* were measured as previously reported (Waxman et al., 2008). Briefly, ImageJ was used to measure the areas of expression of the markers of interest. Because slight variation can occur between ISH experiments, only samples from individual experiments were compared to each other. Measurements were made using arbitrary units. When comparing area measurements, Student's *t*-test was used to determine if the differences between conditions from the individual experiments was statistically significant ($p<0.05$).

Overlap of cardiac progenitor markers and notochord

To measure the overlap of cardiac progenitor markers and the notochord, double single color ISH was performed with *gata4* and *ntla* or *nkx2.5* and *ntla* probes. Embryos were flat mounted after removing the yolk and photographed using a Zeiss M2Bio. ImageJ was used to measure the distance from the anterior tip of the notochord to the posterior end of LPM staining at the 8 somite (s) stage. Because slight variation can occur between ISH experiments, only samples from individual experiments were compared to each other. Measurements were made using arbitrary units. When comparing distance measurements, Student's *t*-test was used to determine if there was a statistically significant difference ($p<0.05$) between measurements of the different conditions.

Cell counting and imaging of zebrafish hearts

CMs were counted as previously described (Waxman et al., 2008). Briefly, heterozygous transgenic *Tg(hsp70:ca-fgfr1)^{pd3}* adults were crossed with homozygous *Tg(−5.1myl7:nDsRed)^{f2}* adults. After selection of *Tg(hsp70:ca-fgfr1)^{pd3}* transgenic and sibling control embryos, the embryos were harvested at 48 hpf when the nuclear DsRed is easily visible in nuclei of CMs. Embryos were then fixed in 1% formaldehyde in 1× PBS for 1 h followed by washes in 1× PBS/0.2% saponin. The embryos were then blocked in 10% sheep serum (Jackson ImmunoResearch)/1× PBS/0.2% saponin and incubated in α-DsRed (Clontech) and S46

(Stainier and Fishman, 1992) antibody. Embryos were gently compressed under a cover slip and the hearts were photographed with a Zeiss M2Bio. Adobe Photoshop was used to count the number of cardiac cells in each chamber. Because slight variation in cell number can occur between different clutches of embryos, only counts from individual experiments were compared to each other. When comparing cell number data, Student's *t*-test was used to determine if there was a statistically significant difference ($p<0.05$) between cell counts of the different conditions.

4-Diethylaminobenzaldehyde (DEAB) treatments

DEAB (Sigma) treatments were performed essentially as previously described (Waxman et al., 2008). Embryos were treated beginning at 40% epiboly with concentrations of DEAB (2.5–5 μM) that cause heart, hindbrain, and loss of forelimb phenotypes, resembling those caused by genetic reduction of RA signaling in zebrafish *neckless/aldh1a2 (nls)* mutants (Grandel et al., 2002). Treatments performed on dechorionated embryos and embryos with their chorions produced no difference in phenotype.

Cell transplantation experiments

Cell transplantation experiments were performed similarly to previously described (Waxman et al., 2008). Donor embryos were injected with 100 pg *ifgfr* mRNA (Pownall et al., 2003) along with 1% fluorescein dextran (Invitrogen) at the one cell stage. The inducible Fgfr1 (iFgfr) has been used previously along with the dimerization agent AP20187 (ARIAD Pharmaceuticals) in *Xenopus* and zebrafish embryos to induce Fgf signaling (Londin et al., 2007; Pownall et al., 2003), which we confirmed (Supplemental Fig. 2). At the sphere stage, 10–20 cells from the donor embryos were transplanted to the margin of wild-type host embryos. A portion of the host embryos were then treated at the tailbud stage (TB) for 2 h with the dimerization agent AP20187 to induce Fgf signaling in the donor cells. Untreated control and treated embryos were then raised until 52 hpf when the embryos were fixed overnight in 4% formaldehyde in PBS. Embryos were then washed in PBS/0.1%Tween, dehydrated with methanol and re-hydrated in PBS/0.1%Tween and the fluorescein dextran was detected with an alkaline phosphatase conjugated α-fluorescein antibody (Roche). Embryos were scored for frequency of contribution to the forelimb mesenchyme, atrial and ventricular cells (Supplemental Fig. 3; Waxman et al., 2008). To determine if there was a significant difference ($p<0.05$) between the different contribution frequencies found in control and treated host embryos, we employed a normal approximation of the chi-square test.

Results

Increased Fgf signaling at the end of gastrulation results in loss of forelimb

Recently, it was shown that increasing Fgf signaling at the 8s stage increases CM cell number along with concomitant loss of forelimb (Marques et al., 2008). However, with respect to heart and forelimb development, it was not determined if increasing Fgf signaling can mimic loss of RA signaling only at the 8s stage or if increasing Fgf signaling can mimic loss of RA signaling over a longer developmental period (Grandel et al., 2002; Grandel and Brand, 2010; Waxman et al., 2008). To distinguish between these possibilities, the *Tg(hsp70:ca-fgfr1)^{pd3}* line was used to increase Fgf signaling at the tailbud (TB; the end of gastrulation), 8s and 14–16s stages. Increasing Fgf signaling resulted in complete loss of forelimbs at the TB stage, >50% of embryos with loss of forelimbs at the 8s stage, and minimal effect on forelimb development at 14–16s (Fig. 1A–C). Embryos treated with DEAB at the same developmental stages showed a similar trend in forelimb loss (Fig. 1D), confirming the results of previous studies (Grandel et al., 2002; Grandel and Brand, 2010). Therefore, these results suggest that forelimb development is sensitive to increased Fgf signaling over a similar time period as loss of RA signaling.

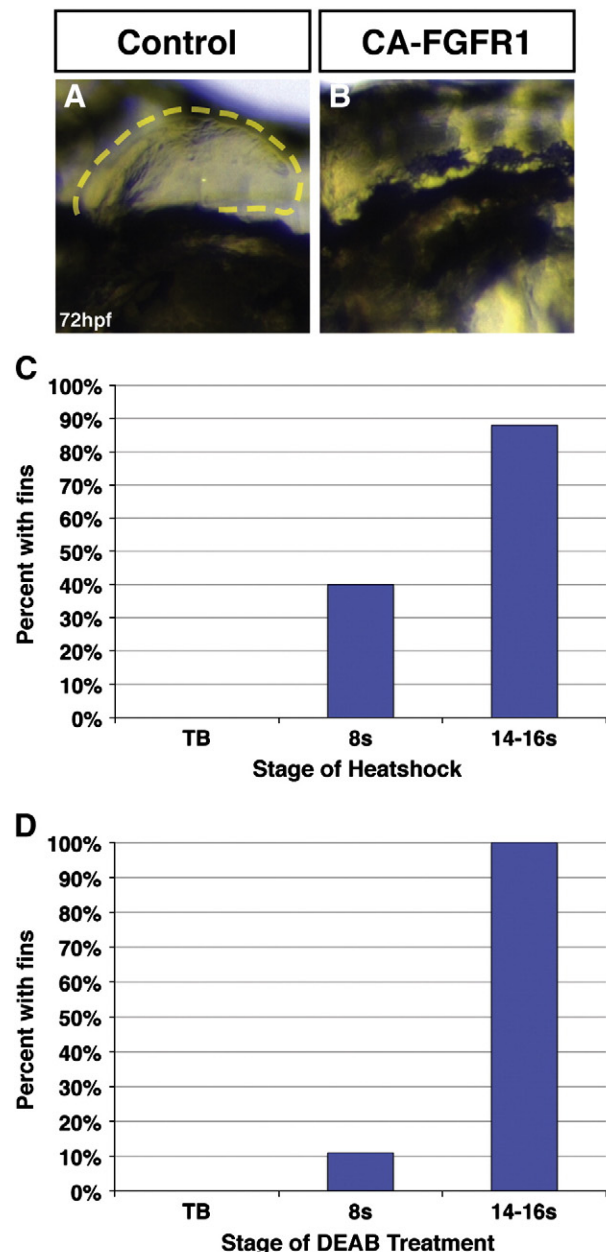

Fig. 1. Increased Fgf signaling in the post-gastrula embryo eliminates forelimbs. (A,B) Representative images from a heat-shocked control sibling embryo and a *Tg(hsp70:ca-fgfr1)^{pd3}* embryo. (A) Heat-shocked control sibling embryos had forelimbs (yellow outline). (B) Forelimbs are absent in *Tg(hsp70:ca-fgfr1)^{pd3}* embryos heat-shocked at the TB stage. (C) Graph indicating the percentage of *Tg(hsp70:ca-fgfr1)^{pd3}* embryos with forelimbs when heat-shocked at the TB (n = 10), 8s (n = 15) or 14–16s (n = 6) stages from a representative experiment. 100% of control sibling embryos heat-shocked at the TB (n = 11), 8s (n = 16) or 14–16s (n = 7) stages had forelimbs. (D) Graph indicating the percentage of DEAB treated embryos with forelimbs when treatments were initiated at the TB (n = 42), 8s (n = 46) and 14–16s (n = 27) stages. 100% (n = 85) of untreated control sibling embryos had forelimbs.

Increased Fgf signaling at the end of gastrulation results in increased cardiac cell number

Because we observed a correlation between the temporal sensitivity of forelimb development to increased Fgf signaling and loss of RA signaling, we next investigated if increased Fgf signaling at the TB and 8s

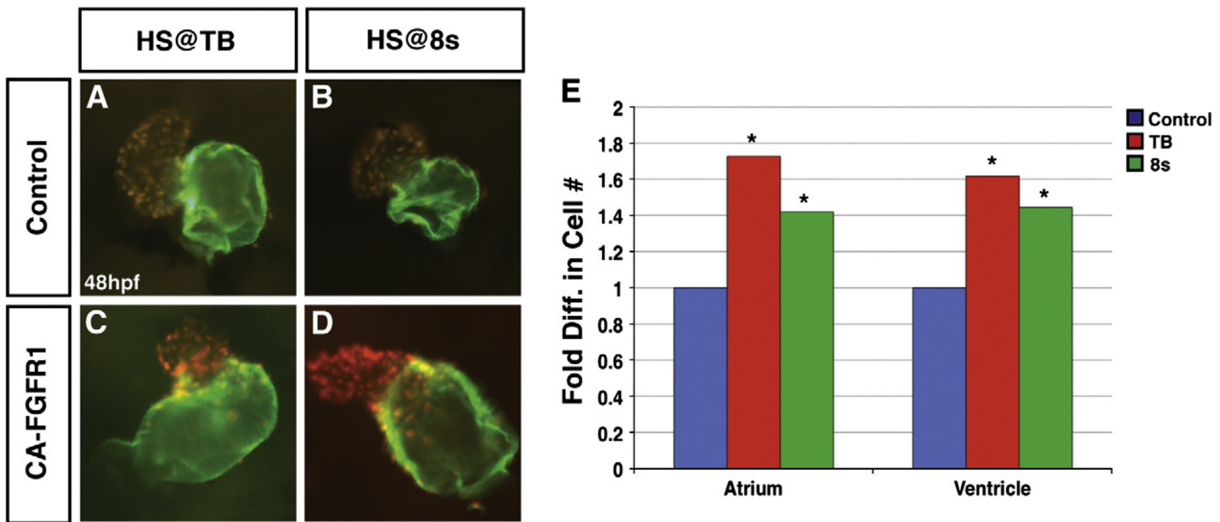

Fig. 2. Increased Fgf signaling in the post-gastrula embryo increases cardiac cell number. (A–D) Images of representative hearts at 48 hpf in heat-shocked control sibling embryos and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos. (A,B) The hearts of heat-shocked control sibling embryos have the typical S-shaped curvature of wild-type embryos. (C,D) Hearts of *Tg(hsp70:ca-fgfr1)*^{pd3} embryos are enlarged and dysmorphic. (E) Graph indicating the fold difference of the mean cell number of heat-shocked control sibling embryos and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos. Increasing Fgf signaling at the TB and 8s stage increases atrial and ventricular cell number, with 8s embryos being less sensitive to increased Fgf signaling than the TB stage. For cell counts, see Supplemental Table 1. Asterisks indicate that there was a statistically significant difference in cell number from the heat-shocked control sibling hearts used for these comparisons (Supplemental Table 1). Images are frontal views. Red indicates ventricle. Green indicates atrium.

stages promoted an increase in cardiac cell number. To do so, homozygous *Tg(−5.1myl7:nDsRed)*^{f2} fish were crossed with heterozygous *Tg(hsp70:ca-fgfr1)*^{pd3} fish. The resulting embryos were heat-shocked at the TB and 8s stages and the hearts were examined at 48 hpf. We found that increasing Fgf signaling at the TB stage resulted in embryos with enlarged hearts that had more ventricular and atrial cells (Fig. 2A,C,E; Supplemental Table 1). Consistent with what has been previously reported (Marques et al., 2008), we found that increasing Fgf signaling at the 8s stage also resulted in embryos with larger hearts (Fig. 2B,D,E; Supplemental Table 1). However, embryos at the 8s stage

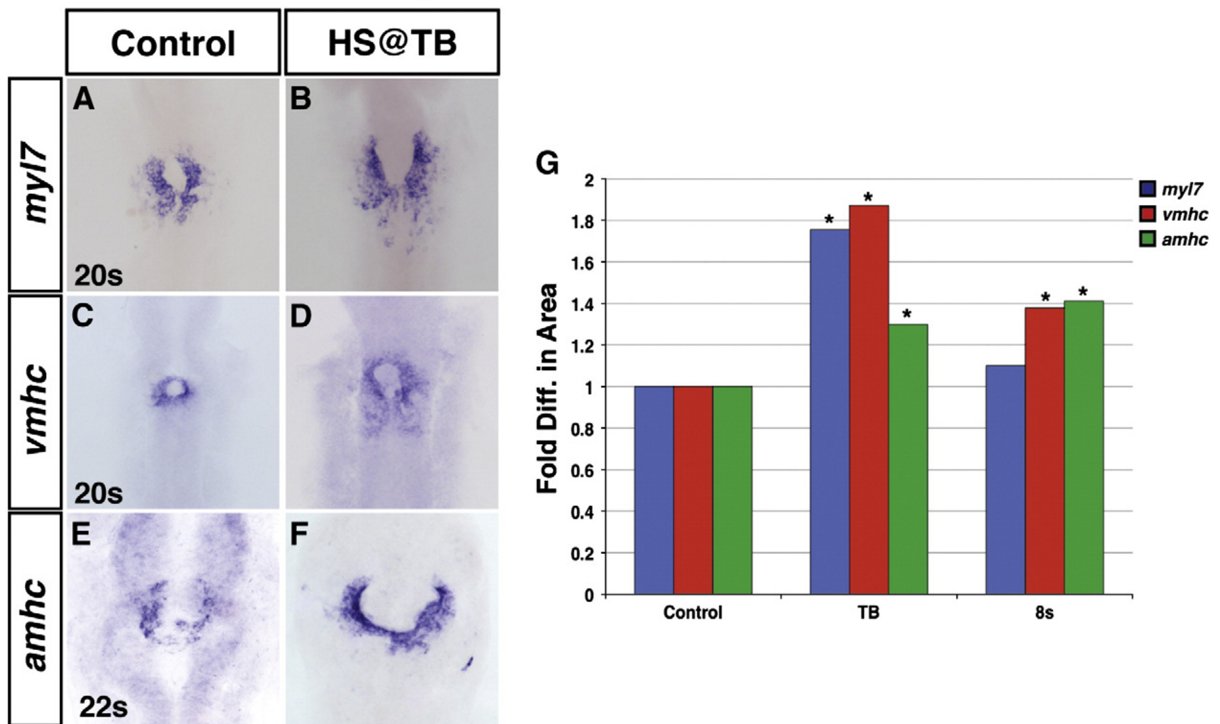

Fig. 3. Increased Fgf signaling expands early cardiac differentiation markers. (A–F) *myl7*, *vmhc* and *amhc* expression in heat-shocked control sibling embryos and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos. (A) *myl7*, (C) *vmhc* and (E) *amhc* expression in heat-shocked control sibling embryos. The amount of cells expressing (B) *myl7*, (D) *vmhc*, and (F) *amhc* is increased in *Tg(hsp70:ca-fgfr1)*^{pd3} embryos with increased Fgf signaling at TB stage. (G) Graph indicating the fold difference in total area of cells expressing *myl7*, *vmhc* and *amhc* from heat-shocked control sibling and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos at the TB and 8s stages. For area measurements, see Supplemental Table 2. Asterisks indicate that there was a statistically significant difference in the populations used for these fold comparisons (Supplemental Table 2). Images of ISH in A–F are dorsal views with anterior up.

were slightly less sensitive to increased Fgf signaling than at the TB stage (Fig. 2E; Supplemental Table 1), like what we have previously observed with sensitivity to loss of RA signaling (Waxman et al., 2008).

To complement the cardiac cell counting, we determined if the effect on cardiac cell number is evident in the earliest differentiation markers *myosin light polypeptide 7* (*myl7*; a pan-cardiac marker) at 20s, *ventricular myosin heavy chain* (*vmhc*; a marker of differentiated ventricular cells) at 20s, and *atrial myosin heavy chain* (*amhc*; a marker of differentiated atrial cells) at 22s. Although *myl7* will eventually mark all the cells of the myocardium, its initial expression is primarily in ventricular cells (Yelon et al., 1999). Similar to the cell counting at 48 hpf, we found that increasing Fgf signaling increased the amount of cells expressing these markers, as assayed by the total area of the expressed markers (Fig. 3A–G; Supplemental Table 2). Furthermore, using this assay, *myl7* and *vmhc* were less sensitive to increased Fgf signaling at 8s (Fig. 3G; Supplemental Table 2). However, *amhc* expression in response to increased Fgf signaling did not differ between the TB and 8s stages (Fig. 3G), which was reminiscent of the temporal sensitivity of atrial cells to loss of RA signaling (Waxman et al., 2008). Therefore, these results suggest that cardiac cell number is sensitive to increased Fgf signaling from the end of gastrulation through early to mid-somitogenesis, again paralleling the temporal sensitivity to loss of RA signaling.

Increased Fgf signaling expands cardiac progenitor markers posteriorly within the LPM

We next examined a series of cardiac progenitor markers to determine if increased Fgf signaling at the TB stage affects these markers similarly to loss of RA signaling. Previously, we and others have found that RA signaling-deficient embryos exhibit a posterior expansion of the cardiac progenitor markers *nkx2.5* and *hey2* (Feng et al., 2010; Keegan et al., 2005; Waxman et al., 2008). Surprisingly, increasing Fgf signaling at the TB stage dramatically increased the expression of both *nkx2.5* and *hey2* in the anterior LPM (Fig. 4A–F). *nkx2.5* expression in particular was strongly and precociously induced throughout the anterior LPM (Fig. 4B,D). The expansion of cardiac progenitor markers throughout the anterior LPM made it difficult to determine if the cardiac progenitor markers were also being expanded posteriorly as well. To determine if there was a posterior expansion of cardiac progenitor markers, we determined the overlap of *nkx2.5* and *gata4*, which marks the entire anterior LPM, relative to the anterior most tip of the notochord, which was marked by *ntla*. Indeed, we found that increasing Fgf signaling at the TB stage caused an increase in overlap in the posterior extent of expression of both *nkx2.5* and *gata4* at the 8s stage (Fig. 5A–E), suggesting there is expansion into the posterior LPM as well as the anterior. Therefore, these results indicate a posterior expansion of cardiac progenitors, further supporting a correlation between the effects of increased Fgf signaling and loss of RA signaling in patterning the cardiac progenitor field.

We also examined *hand2* expression, which at the 8s stage is expressed in cardiac progenitors of the anterior LPM in addition to cells in the posterior LPM (Fig. 6A; Schoenebeck et al., 2007; Yelon et al., 2000). In embryos with increased Fgf signaling at the TB stage, there was no separation between these fields as there was strong ectopic *hand2* expression in this middle region of the LPM (Fig. 6B), which normally lacks expression (Fig. 6A). We also find a similar phenotype for *hand2* expression in RA signaling-deficient embryos (Supplemental Fig. 4). Therefore, one interpretation of these results is that increased Fgf signaling at the TB stage results in a posterior expansion of cardiac progenitors, consistent with what is observed with *nkx2.5* and *gata4*. However, we cannot rule out that the ectopic expression is also due to an anterior expansion of the posterior *hand2* expression domain, although we think it is unlikely based on the results with *nkx2.5* and *gata4*.

We next examined *tbx5a*, which is initially expressed in a broad region of the anterior LPM and contains both cardiac and forelimb progenitors (Ahn et al., 2002; Garrity et al., 2002; Grandel et al., 2002; Grandel and Brand, 2010; Waxman et al., 2008). Previously, we found that in RA signaling-deficient embryos the initial expression of *tbx5a* in the LPM was normal. However, shortly after this initial expression, when the two progenitor populations start to separate into their respective cardiac and forelimb fields, there is little or no posterior forelimb field (Waxman et al., 2008). Similar to loss of RA signaling (Waxman et al., 2008), increasing Fgf signaling at the TB stage resulted in no discernible difference in the initial length of *tbx5a*

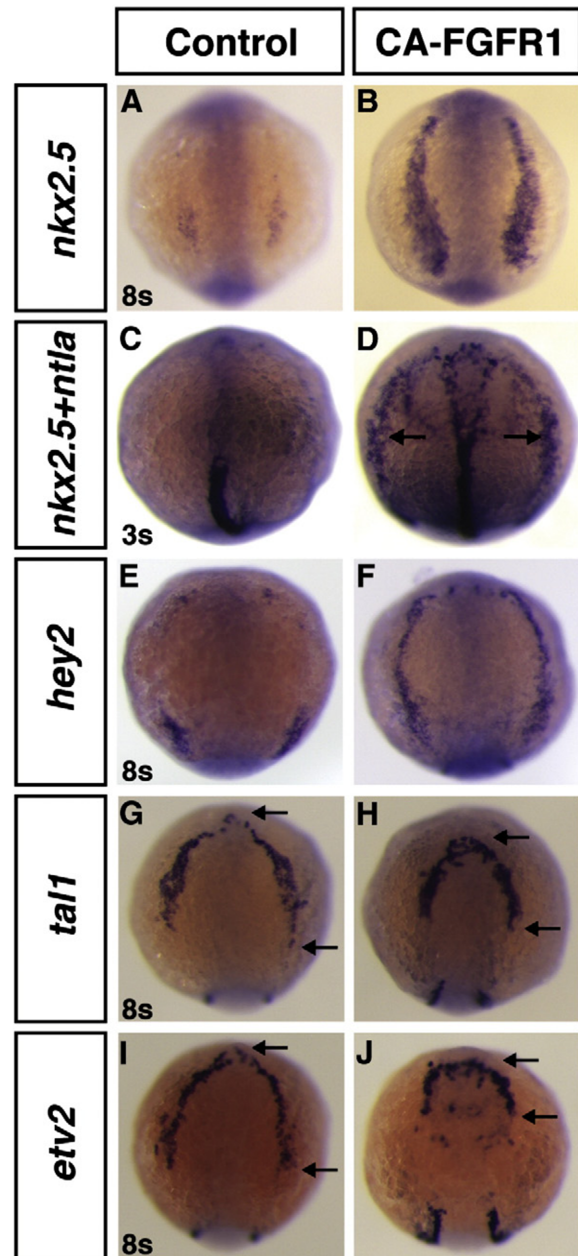

Fig. 4. Increased Fgf signaling induces cardiac progenitor marker expression throughout the anterior LPM. Expression of *nkx2.5* (A,B), *nkx2.5* and *ntla* (C,D), *hey2* (E,F), *tal1* (G,H) and *etv2* (I,J) in control and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos heat-shocked at the TB stage. Increased Fgf signaling controls an inverse relationship between the cardiac and hematovascular progenitor markers in the anterior LPM. Increased Fgf at the TB stage signaling dramatically induces the expression of *nkx2.5* and *hey2* (B,F) in the anterior LPM compared to non-transgenic control siblings (A,E) at 8s. (D) *nkx2.5* (arrows) is induced ectopically and precociously at the 3s when Fgf signaling is increased. Increased Fgf signaling at TB reduced the expression of the hematovascular markers *tal1* and *etv2* in the anterior LPM (arrows in H,J) compared to non-transgenic control siblings (arrow in G,I) at 8s. All views are dorsal with anterior up.

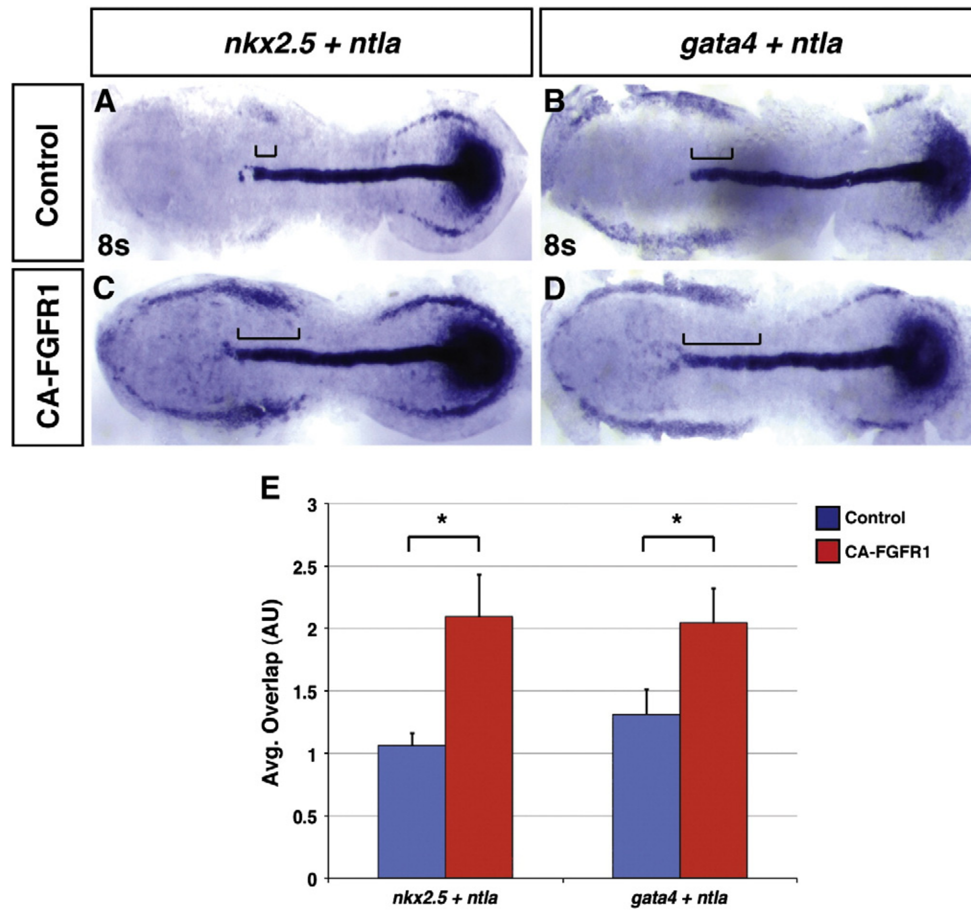

Fig. 5. Increased Fgf signaling causes a posterior expansion of cardiac progenitor markers. Heat-shocked control sibling embryos and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos with increased Fgf signaling at the TB stages probed for *nkx2.5* and *ntla* (A,C) and *gata4* and *ntla* (B,D) at 8s. (A,B) There is minimal overlap between the posterior limit of *nkx2.5* and *gata4* and the anterior limit of *ntla* expression. (C,D) Increased Fgf signaling extends the posterior limit of *nkx2.5* and *gata4* expression and the overlap with the anterior limit of *ntla* expression. Bars in A–D indicate the amount of overlap between the posterior limits of *nkx2.5* and *gata4* and anterior limit of *ntla*. All images are dorsal views with anterior left. (E) Measurements of the length of overlap in heat-shocked control sibling and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos in arbitrary units. Asterisks indicate statistically significant difference using Student's *t*-test.

expression in the LPM at the 8s stage (Fig. 7A, B). However, the length of *tbx5a* expression in the LPM was shorter by the 12s stage and embryos had little or no posterior *tbx5a* expression by the 14s stage (Fig. 7C–F). Altogether, these results suggest that increased Fgf signaling results in a posterior expansion of cardiac progenitor markers and loss of forelimb progenitors, similar to what has been observed in RA signaling-deficient embryos (Waxman et al., 2008).

Increased Fgf signaling results in the loss of anterior vascular marker genes

Although the expansion of cardiac progenitors into the anterior is not a trend consistently found in RA signaling-deficient embryos, we wanted to better understand the potential of Fgf signaling to affect the cardiac progenitor populations in the anterior LPM. The expansion of cardiac progenitor markers into the anterior suggested that this could be occurring at the expense of the more anterior myeloid and vascular populations marked by *etv2* and *tal1* (Schoenebeck et al., 2007; Sumanas et al., 2008; Sumanas and Lin, 2006). Indeed, increased Fgf signaling resulted in reduced expression, though not complete loss, of *etv2* and *tal1* in the anterior LPM (Fig. 4G–J). However, because neither *etv2* nor *tal1* was eliminated while the cardiac progenitor markers were ectopically expressed throughout the anterior LPM, this suggests that there must be some overlap in the cardiac and hematovascular progenitor populations when Fgf signaling is increased. Therefore, these results imply that most of the anterior LPM is sensitive to Fgf signaling and has the potential to express cardiac progenitor markers in part at the expense of hematovascular progenitor markers.

RA signaling-deficient embryos have expanded expression of Fgf ligands and responsive genes

The phenotypic correlation between increased Fgf signaling and loss of RA signaling in cardiac and forelimb progenitor formation suggested that increased Fgf signaling shortly after gastrulation may be a consequence of loss of RA signaling. To determine if Fgf signaling is increased in RA signaling-deficient embryos, we examined *fgf8a* and *fgf3* expression and the Fgf signaling responsive genes *dusp6*, *etv5b*, *pea3* and *spry4* at the 3s stage in embryos that were treated with DEAB, an inhibitor of the major RA producing enzyme Aldh1a2 (Russo et al., 1988). All of these genes, except for *fgf3*, are expressed in the LPM by 3s (Reifers et al., 2000; Znosko et al., 2010). In RA signaling-deficient embryos, both the Fgf ligands and responsive genes were expanded posteriorly relative to control sibling embryos (Fig. 8A–X), reminiscent of what has been reported in mice (Ryckebusch et al., 2008; Sirbu et al., 2008). A posterior expansion of the Fgf ligands is consistent with the role of RA signaling in posteriorizing the hindbrain (Hernandez et al., 2004; Maves and Kimmel, 2005). We also found that loss of RA signaling resulted in expansion of *fgf8a* and Fgf signaling responsive genes in the cardiac LPM at 8s (Supplemental Fig. 5). Although Fgf8a and Fgf

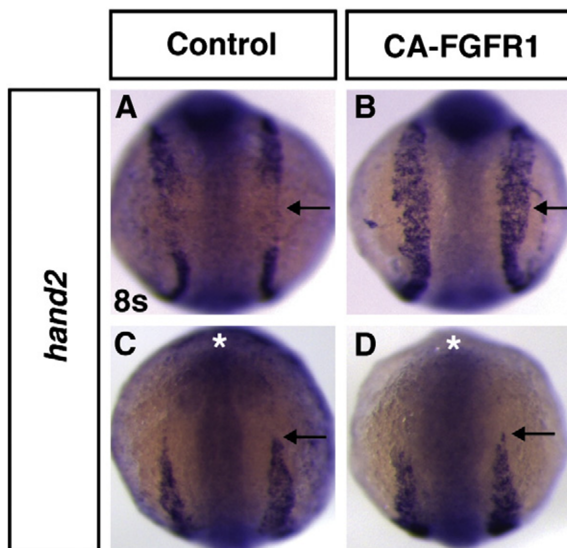

Fig. 6. Increasing Fgf signaling results in ectopic expansion of *hand2* expression. (A–D) Expression of *hand2* in heat-shocked control sibling and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos at 8s. (A) *hand2* is expressed in anterior (cardiac) and posterior domains of the LPM, but absent in between these domains (arrow). (B) In a *Tg(hsp70:ca-fgfr1)*^{pd3} embryo with increased Fgf signaling at the TB stage, expression of *hand2* is expanded into this intermediate region of the LPM (arrow). (C,D) The anterior limit of *hand2* expression (arrows) is the same in heat-shocked control sibling and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos with increased Fgf signaling at the TB stage. Asterisks in C and D indicate the anterior of the head. (A,B) Dorsal views of the trunk region. (C,D) Dorsal views of the anterior. In all images anterior is up.

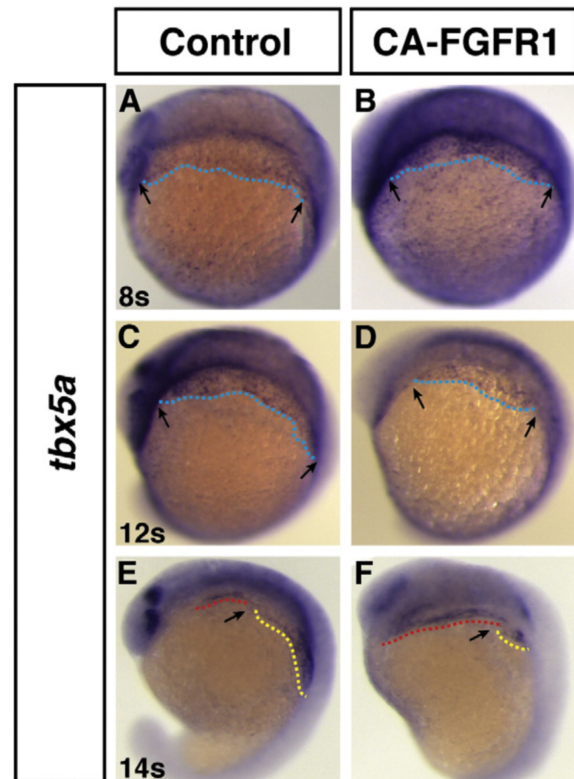

Fig. 7. Increased Fgf signaling reduces posterior forelimb expression of *tbx5a*. (A–F) Expression of *tbx5a* in heat-shocked control sibling and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos. (A,B) The expression of *tbx5a* in heat-shocked control sibling and *Tg(hsp70:ca-fgfr1)*^{pd3} embryos at 8s is indistinguishable. Compare length between arrows in A,B. (C,D) By the 12s stage, in *Tg (hsp70:ca-fgfr1)*^{pd3} embryos with increased Fgf signaling, the length of *tbx5a* expression is shorter (arrows). (E) By the 14s stage, the tbx5a expressing cardiac cells have begun to migrate medially, while the forelimb progenitors remain lateral and occupy a large portion of the LPM. (F) In *Tg(hsp70:ca-fgfr1)*^{pd3} embryos at the 14s stage, the *tbx5a* expressing cardiac progenitors are expanded relative to controls, while the *tbx5a* expressing forelimb progenitors are extremely reduced. The lateral edge of *tbx5a* expression in the LPM is outlined with a dashed blue line in A–D. The lateral edges of the cardiac and forelimb *tbx5a* expression domains are outlined with red and yellow dashed lines respectively in E and F. Arrows in E and F indicate the separation point between the two *tbx5a* expressing domains. All views are dorso-lateral with anterior to the left.

responsive genes were expanded in the LPM, we did not find an effect on the Fgf receptors Fgfr2 or Fgfrl1b, which are both expressed in the LPM (Hall et al., 2006; Nechiporuk et al., 2007; Trueb et al., 2005), in RA signaling-deficient embryos (data not shown; Supplemental Fig. 6). Thus, loss of RA signaling results in a posterior expansion of Fgf ligands and downstream Fgf signaling components shortly after gastrulation, suggesting loss of RA signaling is resulting in an increase in Fgf signaling in this region of the LPM.

Thus far, our results indicated there was a correlation between heart and forelimb phenotypes of increased Fgf and loss of RA signaling. Moreover, loss of RA signaling results in increased Fgf signaling in temporally and regionally appropriate places. Consistent with a role for Fgf8a signaling in promoting cardiac specification, the cardiac phenotype of *nls*/*ace* double mutants resembles *ace* mutants (Keegan et al., 2005). Therefore, we hypothesized that a more moderate reduction in Fgf signaling in RA signaling-deficient embryos may be able to simultaneously rescue both heart and forelimb phenotypes. To test this hypothesis, RA signaling-deficient embryos were injected with a suboptimal dose of *fgf8a* MOs (i.e. a dose that did not overtly induce a *fgf8a* loss-of-function phenotype or had a hypomorphic *fgf8a* loss-of-function phenotype; Supplemental Fig. 1) and were treated with DEAB from 40% epiboly. We chose *fgf8a* because it is the only Fgf ligand so far suggested to be required to promote normal cardiac cell number in zebrafish (Marques et al., 2008; Reifers et al., 2000). We found that RA signaling-deficient embryos with reduced Fgf8a signaling had normal or partial forelimb formation (Fig. 9A, C,D), while RA signaling-deficient embryos alone never had forelimbs (Fig. 9B,D). We were also able to rescue forelimb development when DEAB treatment was initiated at TB (data not shown). At the same time, the expression of *myl7*, an indicator of differentiated cardiac cells, in RA signaling-deficient embryos injected with the *fgf8a* MOs was reduced relative to RA signaling-deficient embryos (Fig. 10A–E; Supplemental Table 3). Therefore, these results suggest that increased Fgf signaling is likely acting downstream of RA signaling and that RA signaling must temper the amount of Fgf8a signaling after gastrulation to allow for proper heart and forelimb development.

Fgf signaling autonomously promotes cardiac specification

We next wanted to determine which cells require Fgf signaling to promote cardiac specification and inhibit forelimb specification. Because Fgf8a and Fgf signaling responsive genes overlap with cardiac progenitors (Reifers et al., 2000; Znosko et al., 2010), but appear to be excluded from the forelimb field, this would suggest a hypothesis that Fgf signaling may promote cardiac progenitor specification cell autonomously, but affect forelimb progenitors non-autonomously. Furthermore, this model would be the converse of what we have previously found with RA signaling, which promotes forelimb formation autonomously, but restricts cardiac cell specification non-autonomously (Waxman et al., 2008). To determine which cells within the LPM require Fgf signaling, we performed cell transplantation experiments using embryos injected with *ifgfr* mRNA. The iFgfr can be induced to dimerize and signal only when the dimerization agent AP20187 is added (Supplemental Fig. 2; ARIAD Pharmaceuticals; Londin et al., 2007; Pownall et al., 2003). In hosts embryos treated with AP20187 at the TB stage for 2 h, we found that cells contributed to the atria and ventricles approximately twice as often as for hosts receiving their untreated control sibling cells (Table 1; Supplemental Fig. 3). In contrast, the frequency with which control untreated and AP20187 treated donor

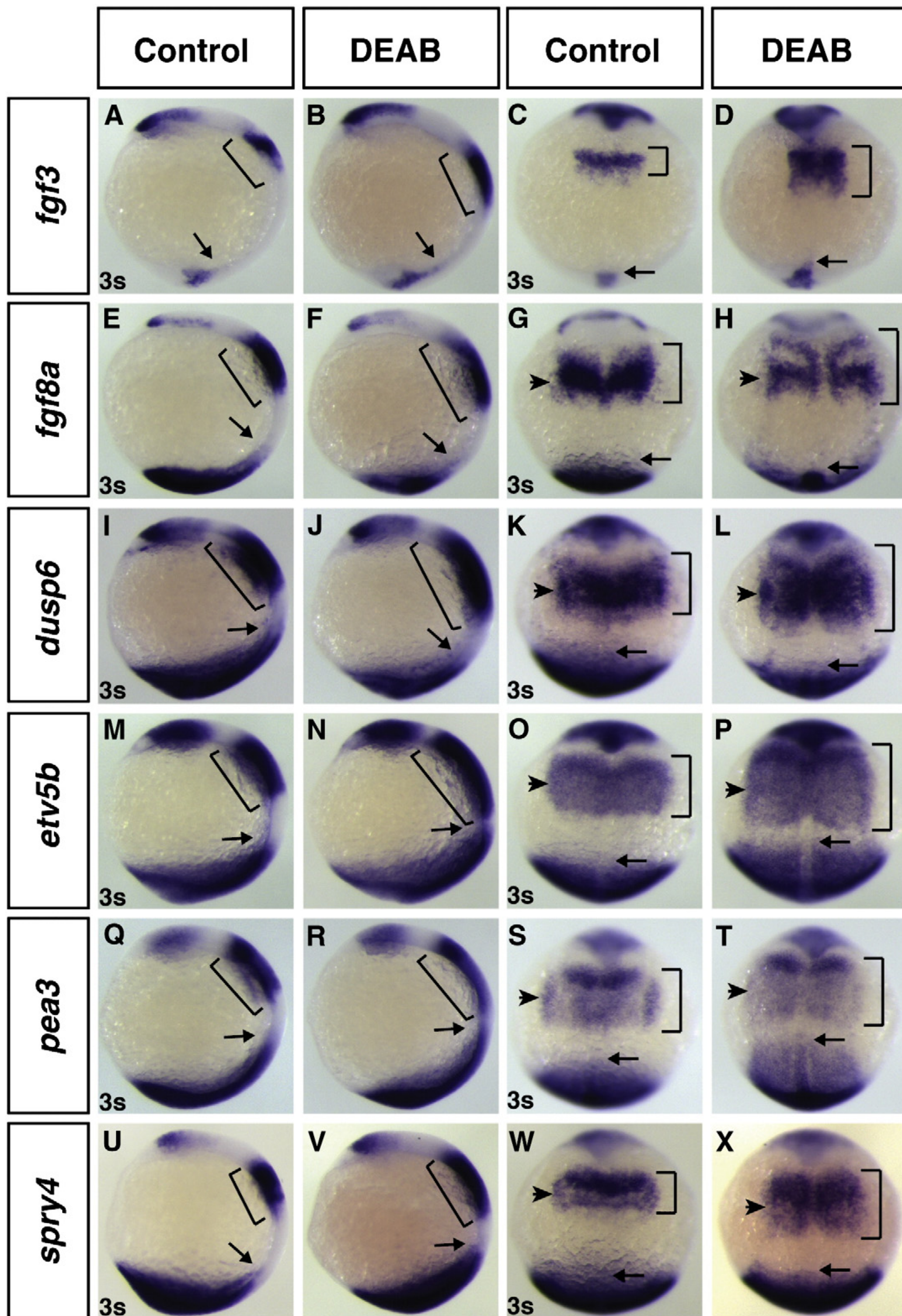

Fig. 8. RA signaling-deficient embryos have a posterior expansion of Fgf ligands and responsive genes. Control sibling and DEAB treated embryos at 3s probed for the Fgf ligands *fgf3* (A–D) and *fgf8a* (E–H) and the Fgf responsive genes *dusp6* (I–L), *etv5b* (M–P), *pea3* (Q–T) and *spry4* (U–X). DEAB treated embryos display a posterior expansion of the Fgf ligands and Fgf responsive genes (B, D, F, H, J, L, N, P, R, T, V, X) compared to control siblings (A, C, E, G, I, K, M, O, Q, S, U, W). Bars indicate the overall length of expression within the anterior of the embryo. Arrow indicates the anterior limit of the posterior expression domains. Arrowheads in C, H, K, L, O, P, S, T, W, and X indicate LPM expression. A, B, E, F, I, J, M, N, Q, R, U, and V are lateral views with anterior up and dorsal right. C, D, G, H, K, L, O, P, S, T, W, and X are dorsal views with anterior up.

cells contributed to the forelimb progenitors in host embryos was the same (Table 1; Supplemental Fig. 3). Together, these results support a model where Fgf signaling acts autonomously to promote cardiac progenitor specification, but non-autonomously to restrict forelimb progenitor specification.

Discussion

In the present study, we have examined the relationship of RA and Fgf signaling in heart and forelimb development. We found that increased Fgf signaling phenocopies multiple aspects of loss of RA

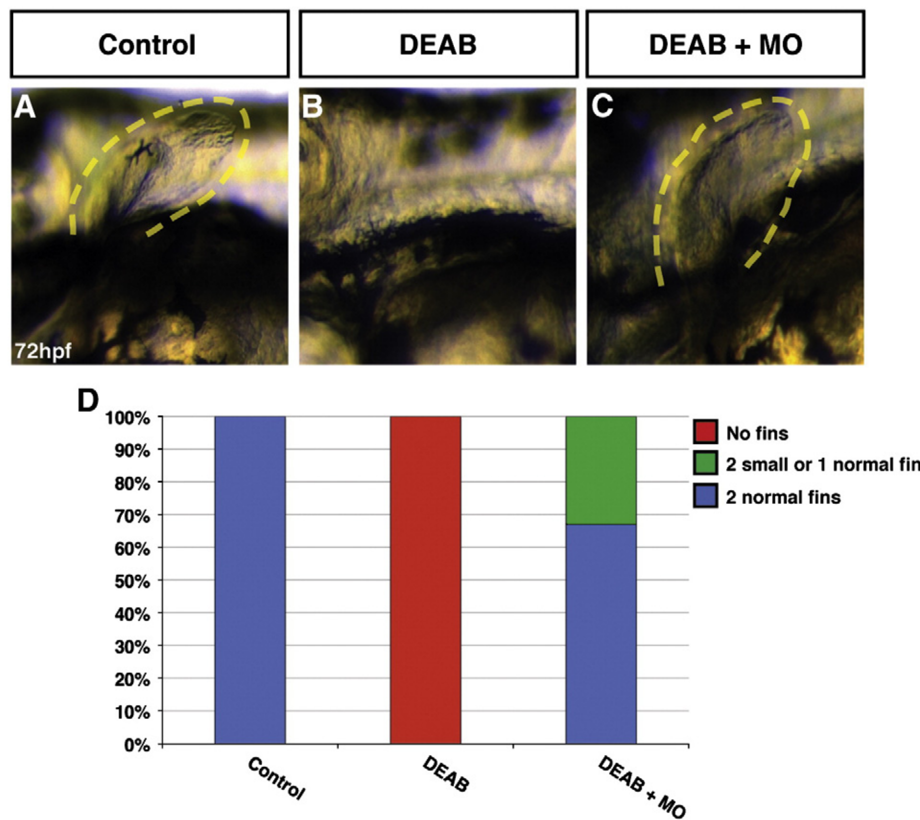

Fig. 9. Moderate reduction of Fgf8a can rescue forelimb development in RA signaling-deficient embryos. (A) Forelimb (pectoral fin) of control sibling embryo. (B) Representative embryo treated with DEAB lacks forelimbs. (C) Representative embryo treated with DEAB and injected with 2 ng *fgf8a* MO mixture that has forelimbs. Views are lateral at 72 hpf. (D) Graph indicating the percentage of control sibling (n = 27) embryos, DEAB treated embryos (n = 34), and DEAB treated embryos that were also injected with 2 ng *fgf8a* MO mixture (n = 21) with forelimbs from a representative experiment. 100% (n = 13) of embryos injected with 2 ng *fgf8a* MO mixture had forelimbs (not shown). Injection of a 6 ng *fgf8a* MO mixture into DEAB treated embryos also restored fins as 63% (10 had forelimbs; n = 16).

signaling in patterning cardiac and forelimb progenitors and development. Notably, increased Fgf signaling results in increased CM number and loss of forelimbs over a similar developmental period as loss of RA signaling. Furthermore, there is a posterior expansion of the cardiac progenitor markers *nkx2.5* and *gata4* into the region normally occupied by forelimb progenitors, similar to what we have recently observed in RA signaling-deficient embryos (Waxman et al., 2008). RA signaling-deficient embryos have a posterior expansion of *fgf8a* and Fgf signaling responsive genes. Importantly, we find that modest suppression of *fgf8a* signaling in RA signaling-deficient embryos can simultaneously rescue both the forelimb and heart phenotypes. Furthermore, we find that Fgf signaling acts cell autonomously to promote cardiac progenitor formation, but non-autonomously to inhibit forelimb progenitor formation, which is the converse of what is found for RA signaling (Waxman et al., 2008). Therefore, these results indicate that increased *fgf8a* signaling downstream of RA signaling is a contributor to both the increased heart size and loss of forelimb in RA signaling-deficient embryos.

Fgf signaling and cardiac cell induction

Our results indicate that increased Fgf signaling promotes an inverse relationship between the cardiac and forelimb progenitor fields, similar to RA signaling-deficient embryos (Waxman et al., 2008). In addition to a posterior expansion of the cardiac progenitor markers, we find that increased Fgf signaling can promote cardiac progenitors precociously in the anterior LPM, suggesting this expansion may be to some extent at the expense of hematopoietic and vascular markers. Thus, our results confirm other studies in zebrafish and other vertebrates that suggest that Fgf signaling strongly induces cardiac progenitors markers, particularly *nkx2.5* (Alsan and Schultheiss, 2002; Barron et al., 2000; Reifers et al., 2000; Ryckebusch et al., 2008). We note though that the previous study in zebrafish using Fgf soaked beads found that *nkx2.5* expression was always induced more posteriorly (Reifers et al., 2000). Although both studies support the same fundamental conclusion, that cardiac progenitor markers including *nkx2.5* in the anterior LPM are responsive to Fgf signaling, these slightly different results are likely due to the difference between global overexpression of the constitutively active Fgfr transgene at the TB stage and the more restricted effect of using Fgf soaked beads implanted at the 5–7s stages (Reifers et al., 2000). Moreover, together these studies suggest that the anterior LPM loses competence to respond to Fgf signaling by the 5–7s stages, while the adjacent more posterior LPM maintains a competence to respond to Fgf signaling. Therefore, our results extend previous observations in zebrafish through suggesting that nearly the entire anterior LPM is highly sensitive to Fgf signaling shortly after gastrulation and is capable of expressing cardiac progenitor markers.

The anterior expression of cardiac progenitor markers also suggests that the CM surplus induced by increased Fgf signaling may derive in part from anterior LPM at the expense of the hemangioblast cells, in addition to the LPM posterior to the cardiac progenitors. However, it is difficult at this point to determine to what extent CMs would derive from this source because *hand2*, whose expression has been suggested to best reflect cardiac progenitor populations from fate mapping (Schoenebeck et al.,

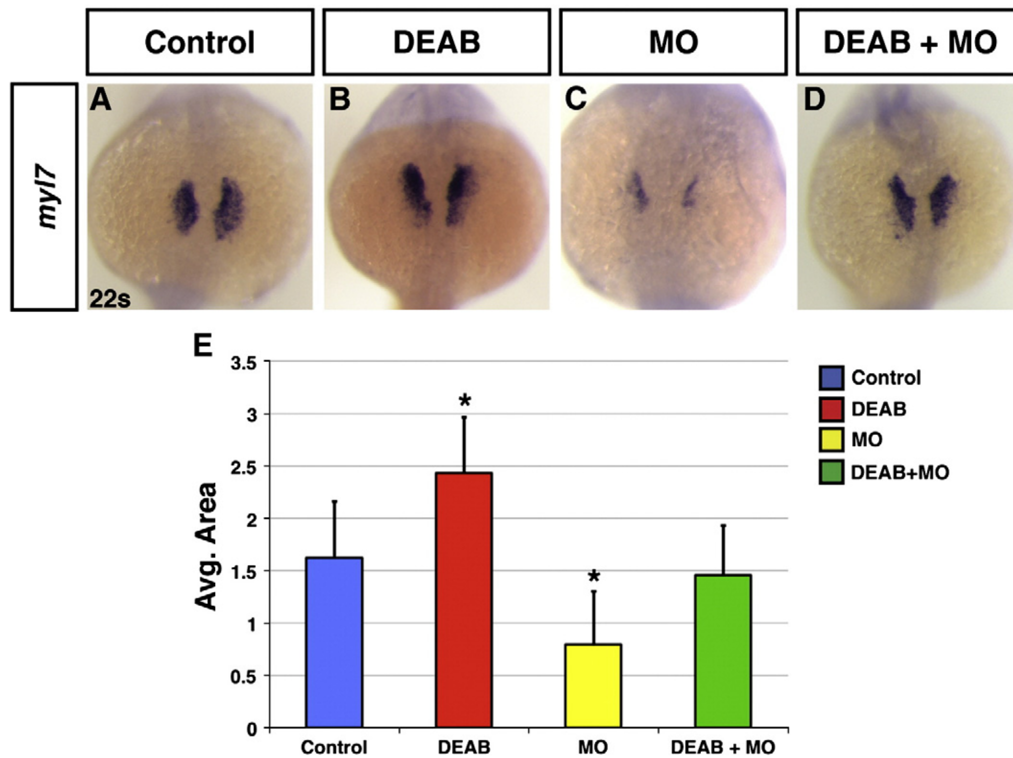

Fig. 10. Inhibition of Fgf8a function can rescue cardiac differentiation marker expression in RA signaling-deficient embryos. Expression of *myl7* at 22s in (A) control, (B) DEAB treated, (C) 2 ng *fgf8a* MO mixture injected, and (D) DEAB treated with 2 ng *fgf8a* MO mixture injected embryos. (B) Expression of *myl7* is increased in DEAB treated embryos. (C) Expression of *myl7* is reduced in embryos injected with the 2 ng *fgf8a* MO mixture. (D) Expression of *myl7* is rescued in embryos treated with DEAB and injected with 2 ng *fgf8a* MO mixture. (E) Area of total cells expressing *myl7* in control, DEAB treated, *fgf8a* MO mixture injected, and DEAB treated plus 2 ng *fgf8a* MO mixture injected embryos. See Supplemental Table 3. Asterisks indicate statistically significant difference from sibling control embryo measurements ($p<0.05$) using Student's *t*-test. Single experiments that examined heart and forelimb phenotypes in sibling embryos and experiments that only examined heart or forelimb phenotypes gave equivalent results (not shown). Images are dorsal views with anterior up.

2007), is not expanded into the anterior (Fig. 6C,D). The induction of CM progenitor markers by Fgf signaling in the anterior LPM is also interesting in light of recent studies of *gata* factors and Fgf responsive genes in zebrafish. Analysis of *gata4–6* in zebrafish suggests that initially the whole anterior LPM, which harbors both hemangioblast and cardiac progenitors, may derive from a precursor with similar potential (Peterkin et al., 2009; Warga et al., 2009). In addition, co-depletion of multiple Fgf responsive genes results in the loss of cardiac progenitors with a concomitant expansion of more anterior hemangioblast markers (Znosko et al., 2010). Together with these other studies, our results highlight the possibility that the distinction between multiple anterior and posterior fates within the LPM may be largely controlled through the presence or absence of Fgf signaling.

Despite the overt similarity of increased Fgf signaling in the post-gastrula embryo to some of the major characteristics of RA signaling-deficient embryos, we have also found important phenotypic differences with respect to patterning the LPM. For instance, the aforementioned expansion of cardiac progenitor markers into the anterior is not typically observed in RA signaling-deficient embryos (Waxman et al., 2008). Another difference is that not all RA responsive genes in the LPM are responsive to increased Fgf signaling. Increased Fgf signaling had no discernable effect on *cdc42ep* or *dhrs3a* (data not shown), which RA signaling regulates negatively and positively, respectively, in the LPM (Feng et al., 2010; Waxman et al., 2008). Moreover, the effects of increased Fgf signaling only seem to delay *hoxb5b* (data not shown), a RA signaling target gene that we have recently found to limit atrial cell number (Waxman et al., 2008). Therefore, these observations suggest that other factors downstream of RA signaling in addition to the moderation of Fgf signaling must be coordinated to ultimately allow for the proper development of the heart and forelimb fields.

Model of Fgf and RA signaling in defining the cardiac and forelimb progenitor fields

Incorporating the observations here with our recent study of RA signaling (Waxman et al., 2008), we propose a model where RA signaling acts on the more posterior forelimb fields to indirectly restrict Fgf8a, while Fgf8a acts directly on the more anterior cardiac progenitor field and indirectly restricts the forelimb field (Fig. 11). Together, these feedback interactions allow for appropriate specification of the cardiac and forelimb fields. This hypothesis is supported by the different locations of Fgf and RA signaling responsive genes, in addition to the effects on cardiac and forelimb progenitor markers, the epistasis analysis, and the cell transplantation experiments presented here.

In zebrafish, *fgf8a* is expressed in the anterior LPM by the 3s stage and significantly overlaps with *nkx2.5* at 8s, when *nkx2.5* expression is initiated, in the anterior LPM of zebrafish (Reifers et al., 2000). Consistent with the direct role for Fgf signaling in cardiac progenitor induction demonstrated in this study, it has recently been shown that Fgf signaling responsive genes, also overlapping with cardiac progenitors in the anterior LPM, are required for proper cardiac progenitor expression (Znosko et al., 2010). Here, we find that these Fgf responsive genes are expanded posteriorly in RA signaling-deficient embryos, presumably into the forelimb progenitor field. By comparison, direct RA responsive genes, such as *dhrs3a* and *hoxb5b*, are located posterior to cardiac

Table 1
Frequency of cardiac and forelimb cells found in cell transplant experiments.

	Atrial	Ventricular	Forelimb	n
Untreated	12% (4)	15% (5)	21% (7)	33
AP20187	*26% (18)	*36% (25)	17% (12)	69

Untreated and AP20187 indicate host embryos receiving donor cells injected with the *ifgfr* mRNA that were not treated with AP20187 and treated with AP20187, respectively. Number of embryos with cells contributing to the different tissues is indicated in the parentheses. Asterisks indicate statistically different frequency ($p<0.001$) of cells contributing to the different tissues between the control untreated and AP20187 treated hosts.

progenitor markers *nkx2.5* and *gata4* in anterior LPM (Waxman et al., 2008). Despite the expansion of cardiac progenitor markers with increased Fgf signaling, our cell transplantation analysis supports that there is not a direct fate transformation between cardiac and forelimb progenitors (Table 1), consistent with what we have previously found in RA signaling-deficient embryos (Waxman et al., 2008). Therefore, the locations of RA and Fgf signaling responsive genes lay in opposition to each other, largely marking the respective forelimb and cardiac progenitor fields.

Our epistasis analysis is consistent with and significantly extends the suggestions of a previous study, which found that treating RA signaling-deficient embryos with SU5402 (an Fgfr inhibitor; Mohammadi et al., 1997) could restore forelimb development (Zhao et al., 2009). However, this study did not examine the underlying mechanisms of Fgf and RA signaling interactions in zebrafish, leaving open the nature of this genetic interaction. Moreover, the basis of those experiments was to corroborate experiments in mice, which demonstrated an increase in Fgf signaling in RA signaling-deficient mice with respect to forelimb development, although no epistasis analysis was reported in mice. Therefore, our results dramatically expand our current understanding of the relationship of Fgf and RA signaling in patterning the forelimb and cardiac progenitor fields in vertebrates through demonstrating that modest reduction of *fgf8a* alone in RA signaling-deficient embryos can restore both cardiac and forelimb development.

Although our data suggest an antagonistic relationship between Fgf and RA signaling in the heart and forelimb fields, it is important to recognize that this relationship occurs largely in the context of loss of RA signaling. For instance, *ace/fgf8a* mutants have loss of cardiac progenitor marker expression but do not have forelimb defects or a discernible anterior expansion of the forelimb field into regions normally occupied by the cardiac progenitors (Marques et al., 2008; Reifers et al., 2000). Similarly, while increased RA signaling posteriorizes embryos and can reduce or eliminate the cardiac progenitor field, there is not a discernible increase in forelimb size (data not shown; Waxman and Yelon, 2009). Therefore, these results suggest that there is not a simple inverse relationship between the requirements and potential of RA and Fgf signaling in appropriately patterning these progenitor fields. With respect to *ace/fgf8a* mutants, it is conceivable that other additional factors are necessary to cause an anterior expansion of the forelimb progenitor field, which would be consistent with Fgf signaling not affecting all RA responsive genes. Alternatively, loss of Fgf signaling in this context may primarily affect more anterior hematovascular lineages (Znosko et al., 2010). Moreover, that aspects of forelimb and cardiac phenotypes are separable in certain developmental contexts is not completely unexpected. Our previous results suggest that there is not a fate transformation between these two cell types, which is corroborated by results presented here, and that RA signaling acts directly on the forelimb progenitors and indirectly on the cardiac progenitors (Waxman et al., 2008). Therefore, that forelimbs and cardiac phenotypes are separable in certain contexts only makes these complex developmental processes more interesting.

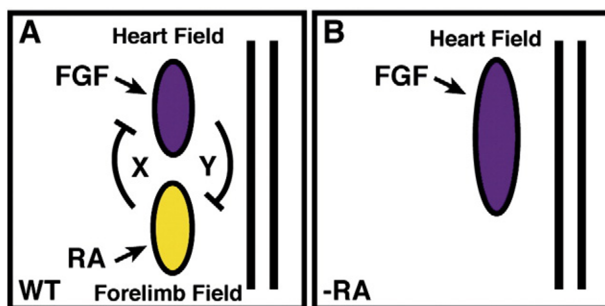

Fig. 11. Model of Fgf and RA signaling interactions that define the cardiac and forelimb progenitor fields. Schematics of anterior LPM on a single side of an embryo. (A) RA signaling promotes the expression of RA responsive genes in the forelimb field (yellow oval). Through a hypothesized factor X, RA signaling indirectly represses the extent of Fgf signaling and the cardiac progenitor field (purple oval). Fgf signaling is able to directly promote the specification of the cardiac progenitor field, while through a reciprocal interaction indirectly restrict the size of the forelimb field through a hypothesized factor Y. (B) In the absence of RA signaling, Fgf signaling and cardiac progenitors are expanded into the forelimb field.

Conclusions

Setting this study in the context of the current literature, this is the first study to use multiple genetic tools to explicitly examine the hypothesis that RA signaling restrains Fgf signaling to allow for the coordinated development of the heart and forelimb. Altogether, these data significantly extend our understanding of the relationship of RA and Fgf signaling in patterning the cardiac and forelimb progenitors in vertebrates, shedding new light on the mechanisms of developmental syndromes with congenital defects affecting both the forelimb and heart. Future studies will be aimed at further elucidating the complex interactions of these and other signaling pathways in the coordinated development of the heart and forelimb.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.07.022.

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