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Retinoic acid stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial *Igf2*

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SUMMARY

Epicardial signaling and *Rxra* are required for expansion of the ventricular myocardial compact zone. Here, we examine *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ mouse embryos to investigate the role of retinoic acid (RA) signaling in this developmental process. The heart phenotypes of *Raldh2* and *Rxra* mutants are very similar and are characterized by a prominent defect in ventricular compact zone growth. Although RA activity is completely lost in *Raldh2*$^{-/-}$ epicardium and the adjacent myocardium, RA activity is not lost in *Rxra*$^{-/-}$ hearts, suggesting that RA signaling in the epicardium/myocardium is not required for myocardial compact zone formation. We explored the possibility that RA-mediated target gene transcription in non-cardiac tissues is required for this process. We found that hepatic expression of erythropoietin (EPO), a secreted factor implicated in myocardial expansion, is dependent on both *Raldh2* and *Rxra*. Chromatin immunoprecipitation studies support *Epo* as a direct target of RA signaling in embryonic liver. Treatment of an epicardial cell line with EPO, but not RA, upregulates *Igf2*. Furthermore, both *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ hearts exhibit downregulation of *Igf2* mRNA in the epicardium. EPO treatment of cultured *Raldh2*$^{-/-}$ hearts restores epicardial *Igf2* expression and rescues ventricular cardiomyocyte proliferation. We propose a new model for the mechanism of RA-mediated myocardial expansion in which RA directly induces hepatic *Epo* resulting in activation of epicardial *Igf2* that stimulates compact zone growth. This RA-EPO-IGF2 signaling axis coordinates liver hematopoiesis with heart development.

KEY WORDS: Retinoic acid, Erythropoietin, Heart development, Epicardium, Myocardial compact zone, Liver, *Raldh2*, *Epo*, *Igf2*, Mouse

INTRODUCTION

Retinoic acid (RA) synthesis is catalyzed by three retinaldehyde dehydrogenases (RALDHs) encoded by *Raldh1*, *Raldh2* and *Raldh3* (*Aldh1a1*, *Aldh1a2* and *Aldh1a3*, respectively – Mouse Genome Informatics), with RALDH2 being the main enzyme providing RA for early organogenesis (Duester, 2008). As expression of these enzymes is restricted in a temporal and spatial manner, RA synthesis is a major point of control for the RA signaling pathway. RA binds to nuclear retinoic acid receptors (RARα, RARβ and RARγ) which form heterodimers with retinoid X receptors (RXRα, RXRβ and RXRγ) to regulate the transcription of target genes (Kastner et al., 1995; Mangelsdorf and Evans, 1995; Niederreither and Dolle, 2008). Binding of a retinoid ligand to one of the RXRs appears to be unnecessary for RA signaling (Mic et al., 2003; Calleja et al., 2006), and it remains unclear whether physiological ligands exist for RXR (Mascrez et al., 2009). Expression of the six RAR and RXR genes is very broad, resulting in redundant functions, illustrated by the fact that among all six mutants only *Rxra*$^{-/-}$ mice exhibit an embryonic lethal phenotype (Mark et al., 2009).

RA signaling is a key factor in heart development. This was first demonstrated by depriving rat and avian embryos of dietary vitamin A, resulting in a vitamin A deficiency syndrome characterized by defects in many organs, including the heart, which exhibits abnormal early development and a thinning of the ventricular myocardium (Wilson et al., 1953; Dersch and Zile, 1993; Zile, 2001). Genetic loss-of-function studies have demonstrated that *Rxra*$^{-/-}$ embryos exhibit ventricular myocardial thinning (Kastner et al., 1994; Sucov et al., 1994), whereas *Raldh2*$^{-/-}$ embryos exhibit an early heart defect (Niederreither et al., 2001). Recent studies using *Raldh2*$^{-/-}$ embryos demonstrated that RA limits the size of the second heart field population along the anteroposterior axis by negatively regulating cardiac *Fgf8* (required for expression of *Isl1*), thus defining the posterior boundary of the heart field (Ryckebusch et al., 2008; Sirbu et al., 2008). Also, loss of *raldh2* (aldh1a2 – Zebrafish Information Network) function in zebrafish increases the heart progenitor population (Keegan et al., 2005).

The mechanism through which RA regulates expansion of the ventricular myocardium is unknown. After the four-chambered heart has formed, the naked myocardium is covered from embryonic day (E)9.5-10.5 by the epicardium, an epithelial cell layer derived from the proepicardial organ that originates from the septum transversum mesenchyme (Komiyama et al., 1987; Manner et al., 2001). Some epicardial cells undergo an epithelial-to-mesenchymal transition in response to signals from the ventricular myocardium (Snarr et al., 2008). These epicardial-derived cells create the subepicardial mesenchyme and differentiate further into smooth muscle cells of the coronary vessels and cardiac fibroblasts (Lie-Venema et al., 2007). Signals from the endocardium and the epicardium ensure normal maturation of the myocardium in subsequent developmental stages, with the endocardium primarily governing the development of the trabecular myocardium, and the epicardium being an important source of mitogens for compact zone myocardium (Smith and Bader, 2007; Lavine and Ornitz, 2008). This instructive role of the epicardium is emphasized by the

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fact that mouse mutants displaying impaired epicardial development have an underdeveloped compact zone myocardium (Kwee et al., 1995; Yang et al., 1995). Data from chicken embryos, in which the formation of the epicardium has been disrupted mechanically, show that ventricular regions devoid of the epicardium do not undergo myocardial expansion (Manner, 1993; Gittenberger-de Groot et al., 2000). Mouse *Rxra*$^{-/-}$ embryos fail to expand the ventricular myocardial compact zone (Kastner et al., 1994; Sucov et al., 1994), but *Rxra* function in cardiomyocytes is not required (Chen et al., 1998; Subbarayan et al., 2000). Conditional *Rxra*$^{-/-}$ embryos, obtained using a *Gata5-Cre* expressed in epicardium and other derivatives of the septum transversum, exhibit thinning of the myocardial compact zone, suggesting that RXRα might function in the epicardium to induce secretion of a myocardial proliferative factor (Merki et al., 2005). However, RA has never been linked directly to epicardial function, and the factor secreted by the epicardium that stimulates myocardial expansion has remained elusive.

Several growth factors have been suggested to function in communication between the epicardium and myocardium. Conditional deletion of *Rxra* and β-catenin in epicardial and septum transversum mesenchyme derivatives using *Gata5-Cre* suggested a WNT connection, but direct evidence for the cooperation of RA and WNT signaling pathways during myocardial expansion has not been reported (Merki et al., 2005; Zamora et al., 2007). Another study showed that *Fgf9* is required for myocardial development and that cardiac *Fgf9* expression can be induced by pharmacological RA doses in heart organ cultures; however, as *Fgf9* is expressed primarily in the endocardium and not expressed in the epicardium after E10.5, it seems unlikely that FGF9 signaling alone can induce compact zone proliferation (Lavine et al., 2005). Another protein implicated in ventricular compact zone growth is erythropoietin (EPO) as demonstrated by similar myocardial defects in null mutant mice for *Epo* (expressed in the liver but not heart) and *Epor* (encoding the EPO receptor; expressed in epicardium and endocardium, as well as liver) (Wu et al., 1999). Interestingly, *Epo* expression in embryonic liver is greatly reduced in *Rxra*$^{-/-}$ embryos (Makita et al., 2001).

In this study, we focused on how RA signaling influences the epicardial/myocardial signaling needed for myocardial compact zone development. Although *Raldh2*$^{-/-}$ embryos normally die at E9.5, we show that *Raldh2*$^{-/-}$ embryos rescued with a small dose of RA prior to E9.5 survive to later stages, similar to *Rxra*$^{-/-}$ embryos, and exhibit a severe heart defect characterized by a thin compact zone myocardium. Surprisingly, although *Raldh2*$^{-/-}$ embryos lose cardiac RA activity, *Rxra*$^{-/-}$ embryos retain normal cardiac RA activity, suggesting that an RA signaling event outside the heart is responsible for the ventricular phenotype. We demonstrate that the RA target gene *Epo* is downregulated in hepatic tissues of both *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ embryos, and that EPO function is necessary for epicardial induction of insulin-like growth factor-2 (IGF2). We propose a new mechanism for compact zone maturation in which EPO, induced by hepatic RA signaling, travels to the epicardium and induces IGF2, which is secreted by the epicardium to stimulate myocardial compact zone development.

MATERIALS AND METHODS

Transgenic mice

Generation of *Raldh2* null embryos and *Raldh2* null embryos carrying the *RARE-lacZ* reporter transgene were described previously (Mic et al., 2002). Germline *Rxra* null embryos were obtained from mice carrying a floxed *Rxra* gene (Chen et al., 1998) crossed with mice that express *Cre* recombinase under the control of the protamine promoter, which enables homologous recombination in the male germline (O'Gorman et al., 1997). Conditional *Rxra* null mutants were obtained by crossing *Rxra* floxed mice with a line expressing *Cre* recombinase under the control of the chicken *Gata5* promoter (Merki et al., 2005). Genotyping of embryos was performed by PCR analysis of yolk sac DNA. All mouse studies were performed according to regulatory standards adopted by the Animal Research Committee at the Sanford-Burnham Medical Research Institute.

Dietary rescue of *Raldh2* null mutants

The protocol for rescue of *Raldh2*$^{-/-}$ lethality by dietary supply of RA to a pregnant dam was described previously (Zhao et al., 2010). Briefly, a 50 mg/ml stock solution of all-trans-RA (Sigma) in 100% ethanol was mixed with corn oil to obtain a 5 mg/ml RA solution, which was mixed thoroughly with ground mouse chow to yield 0.1 mg RA/g mouse chow. RA-supplemented food was provided from E6.75 to E9.25 and was changed every 12 hours to ensure activity. At E9.25 mice were returned to normal food until collection of embryos at E10.5-E13.5. This low dose of RA provides a physiological amount of RA (Mic et al., 2003) and is cleared within 12-24 hours of the end of treatment at E9.25 (Mic et al., 2002), thus allowing analysis of embryos from E10.5 onwards that are RA-deficient.

Whole-mount and section in situ hybridization, immunohistochemistry and histology

In situ hybridization of whole-mount hearts or paraffin-embedded heart sections (5 μm) was performed as described (Wilkinson and Nieto, 1993). Staining of wild-type and mutant samples was performed for an equal length of time and experiments were repeated at least three times. For whole-mount hearts, sectioning was performed with a vibratome after staining; tissues were embedded in gelatin-bovine serum albumin (BSA) solution (0.5% gelatin, 30% BSA, 20% sucrose in PBS, polymerized by addition of 50% glutaraldehyde) and sectioned at 20 μm. The riboprobes used for this study included *Raldh2* (Mic et al., 2002), *Tbx18* (Kraus et al., 2001) and *Igf2* (generated here by RT-PCR). Immunohistochemistry on paraffin sections was performed as previously described using an affinity-purified anti-RALDH2 antibody at 1:200 (Haselbeck et al., 1999), anti-MF20 (Developmental Studies Hybridoma Bank; 1:20) and anti-Ki67 (Sigma Chemical; 1:100). For histological analysis, paraffin sections were stained with Hematoxylin and Eosin. *RARE-lacZ* transgenic mice and β-galactosidase staining have been described previously (Rossant et al., 1991); hearts were dissected from embryos, stained for the same length of time and vibratome sectioned as described above.

Semi-quantitative RT-PCR

RNA was isolated from frozen specimens using Trizol (Invitrogen) according to the manufacturer's guidelines. Equal amounts of RNA were used to synthesize cDNA using Superscript III reverse transcriptase (Invitrogen) and standard protocols, and 1 μl cDNA was used for subsequent PCR reactions. The following primer sequences were used to amplify cDNA fragments: GAPDH: Gapdh_f: CAGCTTCGGCACAT-ATTTCA, Gapdh_r: GACCACCTGGTCCTCTGTGT; EPO: EPO_f: CCACCCTGCTGCTTTTACTC, EPO_r: CCTCTCCCGTGTACAG-CTTC; FGF-9: Fgf_9_f: TGGACACCGGAAGGAGATAC, FGF_9_r: TTGAAGTATGGCAGCTGTGC. The following PCR program was used for amplification: 95°C for 2 minutes; 95°C for 30 seconds; 60°C for 30 seconds; 72°C for 45 seconds; 72°C for 5 minutes; 30 cycles in total for all genes analyzed.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to the manufacturer's ChIP protocol (Active Motif, Carlsbad, CA, USA). Five livers from E13.5 wild-type mouse embryos were dissected and cross-linked with 1% formaldehyde at room temperature for 15 minutes. Isolated nuclei (in 650 μl) were sonicated on ice for 20 pulses of 10 seconds each at 40% power output using a microtip probe from a Misonix Digital Sonicator 4000 (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) to shear the DNA to an average size of 500 bp followed by centrifugation

at 13,000 ***g*** for 10 minutes. At this point, a small portion of supernatant was stored as the input control. For each ChIP reaction, immunoprecipitation was performed using 150 µl of sheared chromatin mixed with 3 µg of either anti-RAR-α (sc-551, Santa Cruz Biotechnology), anti-RAR-β (Affinity Bioreagents), anti-RAR-γ (sc-550, Santa Cruz Biotechnology) or control IgG, along with 25 µl pre-blocked protein G-coated magnetic beads (Active Motifs, Carlsbad, CA, USA) for 4 hours at 4°C. Beads were washed and eluted DNA-protein complexes were reverse cross-linked and purified. The immunoprecipitated DNA was analyzed by PCR, and was compared with input DNA that was diluted 100-fold. For each antibody, ChIP analysis was performed in at least two independent experiments. PCR products were verified with 3% agarose gel electrophoresis. RARE specific and non-specific primer sequences for mouse erythropoietin gene used in this study were as follows: Epo-RARE-Fwd: CAGCTGAAATCACCA-ACCAGACTC; Epo-RARE-Rev: CTCTGTACTGGCTCCTGTTTCCT; Epo-NS-Fwd: GCATGTGCCATCGTACTTAGCTG; Epo-NS-Rev: CTGGTCTGCCTTCAGTAGCATCA.

Culture of epicardial cells

Mouse epicardial cells (MEC1) were grown in DMEM supplemented with 10% fetal bovine serum until 80% confluence was reached, then were switched to serum-free DMEM for 24 hours. The media was then replaced with fresh DMEM including all-trans-RA (1 µM; Sigma) or recombinant mouse EPO (10 ng/ml; R&D Systems) for 24 hours. RNA was isolated using Trizol (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from equal amounts of total RNA with the iScriptTM cDNA Synthesis Kit (Bio-Rad). Real-time PCR analysis was performed using iQ SYBR Green Supermix (Bio-Rad) with an Opticon2 thermocycler (MJ Research). A standard curve was performed with serial dilutions of cDNA. Experiments were run in duplicate and expression levels were analyzed using Opticon Monitor Analysis Software and normalized against β-actin expression levels. The following primer sequences were used to amplify cDNA fragments: Igf2_f: CCCTCAGCAAGTGCCTAAAG; Igf2_r: TTAGGGTGCCTCGAGATGTT; β-actin_f: ATGGAGGGGAA-TACAGCCC; β-actin_r: TTCTTTGCAGCTCCTTCGTT.

Heart organ culture

Hearts were dissected at E11.5, cultured overnight at 37°C in 5% CO_2 for 18 hours in 6-well plate transwell insets (Millipore) in serum-free medium consisting of DMEM F12/Glutamax (Gibco) supplemented with 2 µg/ml heparin and, where indicated, with 8 U/ml of human recombinant erythropoietin (Calbiochem). Hearts were fixed overnight in 4% paraformaldehyde in PBS, paraffin sectioned at 5 µm and subjected to in situ hybridization or immunohistochemistry (see above). Quantification of proliferating cardiomyocytes was performed by counting total cardiomyocytes ($MF20^+$ cells) and cardiomyocytes undergoing mitosis ($Ki67^+$ cells) in 40× images of three sections for each genotype treated with or without EPO from at least two different hearts; counts were performed by two researchers independently using ImageJ software. Percentages of proliferating cardiomyocytes for each genotype were plotted and statistical analysis using *t*-test was carried out using GraphPad Prism software.

RESULTS

Cardiac phenotypes of rescued *Raldh2*$^{-/-}$ mutants and *Rxra*$^{-/-}$ mutants

A distinct growth phase of the compact zone myocardium begins at ~E10.5, after the formation of the epicardium is completed, and by E11.5 the compact myocardium becomes morphologically recognizable (Smith and Bader, 2007). At E13.5, wild-type hearts exhibited a well-developed ventricular compact zone myocardium (Fig. 1A,D), whereas *Rxra*$^{-/-}$ hearts displayed a thin compact zone myocardium (Fig. 1B,E) as reported previously (Kastner et al., 1994; Sucov et al., 1994). As *Rxra*$^{-/-}$ myocardial defects might be due to defective RA signaling, we examined *Raldh2*$^{-/-}$ embryos, which exhibit loss of RA synthesis. *Raldh2*$^{-/-}$ embryos display early embryonic lethality at ~E9.5, but low-dose RA treatment

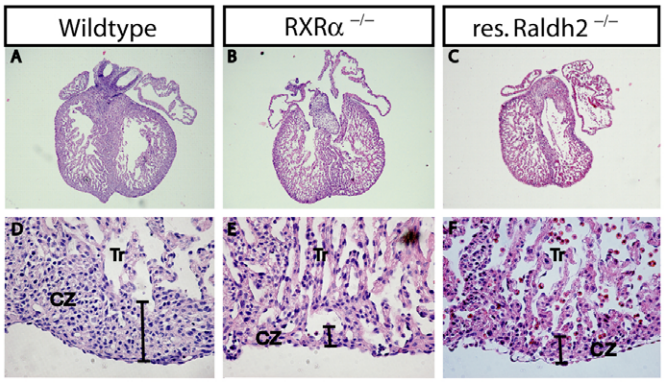

Fig. 1. Comparison of heart phenotype in rescued *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ mouse embryos. (**A-F**) Sections of Hematoxylin and Eosin-stained E13.5 hearts from wild-type (A,D), germline *Rxra*$^{-/-}$ (B,E) and rescued *Raldh2*$^{-/-}$ (C,F) embryos. Representative heart sections are shown at 40× magnification in A-C and detailed photographs of the compact zone at 400× magnification in D-F. Both mutants exhibit a severe reduction in the size of the compact zone myocardium (CZ) whereas the trabecular myocardium (Tr) is only mildly affected; the black bar indicates the thickness of the compact zone. Similar results were observed for all mutants analyzed (*n*=3 for each genotype).

from E6.75 to E9.25 delays lethality, allowing analysis of E13.5 *Raldh2*$^{-/-}$ embryos that lack RA activity in tissues where RALDH2 is the source of RA (Zhao et al., 2010). Rescued *Raldh2*$^{-/-}$ embryos exhibited a very similar heart morphology at E13.5 compared to *Rxra*$^{-/-}$ embryos, characterized by a thin underdeveloped ventricular compact zone (Fig. 1C,F). This striking similarity in phenotypes suggests that a common RA signaling event stimulating compact zone growth has been affected in both mutants.

Cardiac RA signaling activity is lost in *Raldh2* mutants but not in *Rxra* mutants

In order to determine whether the underdeveloped ventricular myocardial compact zone observed in *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ embryos is associated with a loss of epicardial RA signaling, we examined cardiac RA signaling using embryos carrying the *RARE-lacZ* RA-reporter transgene (Rossant et al., 1991). In E10.5 wild-type hearts, RA signaling was evident in the atria and the outflow tract but was either absent or detected at very low levels in the ventricle (Fig. 2A); vibratome sections revealed little or no detection of RA activity in the ventricular epicardium at E10.5 (Fig. 2D). *Rxra*$^{-/-}$ hearts exhibited no difference in cardiac *RARE-lacZ* expression at E10.5 compared with wild type (Fig. 2B,E). By contrast, *RARE-lacZ* expression in E10.5 rescued *Raldh2*$^{-/-}$ hearts was nearly eliminated (Fig. 2C,F). In E12.5 wild-type hearts, RA signaling activity was detected in clusters of cells throughout the ventricular epicardium, whereas in the atria lower levels of RA activity were observed compared with E10.5 hearts (Fig. 2G,J). Surprisingly, E12.5 *Rxra*$^{-/-}$ hearts exhibited no significant reduction in RA signaling activity in the ventricular epicardium (Fig. 2H,K) compared with E12.5 rescued *Raldh2*$^{-/-}$ hearts, which completely lacked RA activity in the ventricular epicardium (Fig. 2I,L). These findings suggest that the compact zone defect in *Rxra*$^{-/-}$ hearts is not due to a loss of epicardial RA signaling.

As epicardial RA signaling activity was not observed in rescued *Raldh2*$^{-/-}$ hearts, we determined whether the epicardium develops in these mutants by analyzing the expression of *Tbx18*, which is a

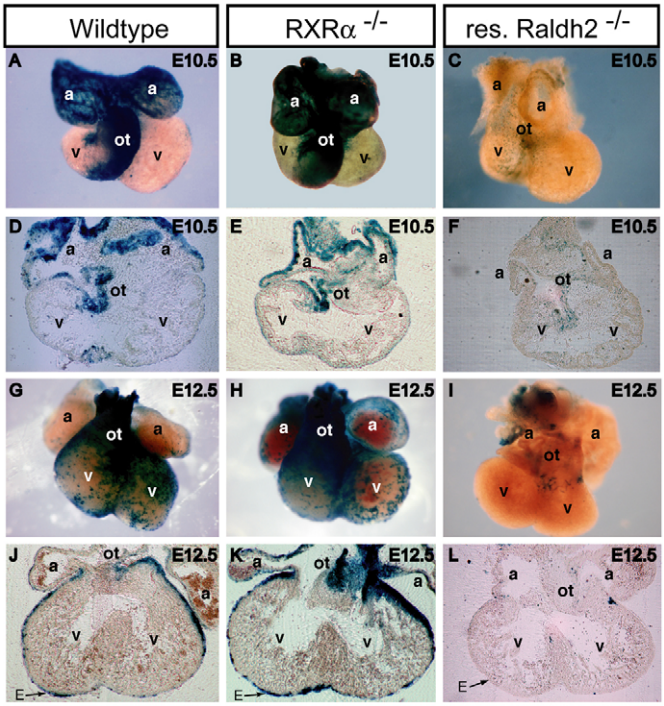

Fig. 2. Detection of cardiac RA signaling in rescued *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ mouse embryos. (**A-L**) *RARE-lacZ* staining of wild-type (A,D,G,J), germline *Rxra*$^{-/-}$ (B,E,H,K) and rescued *Raldh2*$^{-/-}$ (C,F,I,L) whole hearts and sections at E10.5 (A-F) and E12.5 (G-L). Similar results were observed for all mutants analyzed (n=3 for each genotype and stage). RA signaling is not altered in germline *Rxra*$^{-/-}$ hearts at E10.5 and E12.5 relative to wild-type hearts, whereas loss of *Raldh2* leads to severe impairment of RA signaling in cardiac tissues at both stages, including loss of epicardial RA signaling at E12.5 (compare J,K,L). a, atrium; E, epicardium; ot, outflow tract; v, ventricle.

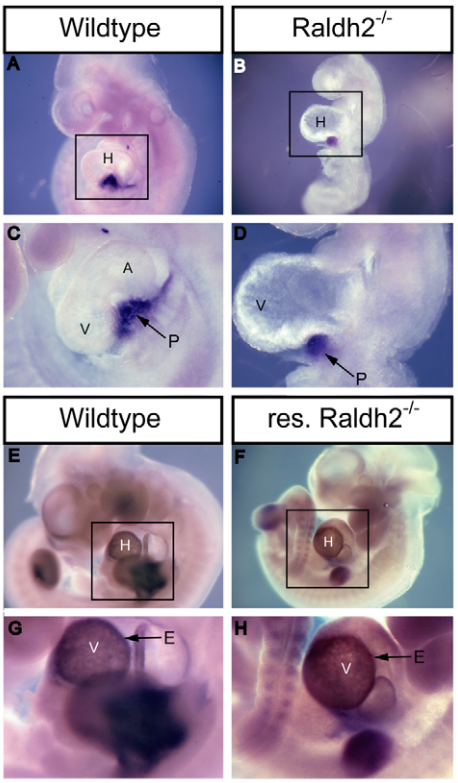

Fig. 3. Epicardium develops in rescued *Raldh2*$^{-/-}$ mouse embryos. (**A-D**) At E9.5, detection of *Tbx18* mRNA indicates that the proepicardial organ is present in both wild-type (A,C) and unrescued *Raldh2*$^{-/-}$ (B,D) embryos. C and D are higher magnifications of the boxed areas in A and B, respectively. (**E-H**) E10.5 hearts of both wild-type and rescued *Raldh2*$^{-/-}$ mutants exhibit *Tbx18* mRNA expression demonstrating that the epicardium has developed in the mutant. G and H are higher magnifications of the boxed areas in E and F, respectively. Similar *Tbx18* detection was observed for all mutants analyzed (n=3 for both stages). A, atrium; E, epicardium; H, heart; p, proepicardial organ; V, ventricle.

marker for the proepicardial organ and the epicardium (Bussen et al., 2004). At E9.5, unrescued *Raldh2*$^{-/-}$ embryos exhibited a proepicardial organ marked by *Tbx18* expression, demonstrating that formation of this tissue can occur in the absence of *Raldh2* function (Fig. 3A-D). Analysis of E10.5 *Raldh2*$^{-/-}$ embryos (rescued to avoid lethality) revealed that *Tbx18* mRNA was expressed in the epicardium at a level similar to that observed in wild type (Fig. 3E,F). Thus, the loss of RA activity observed in rescued *Raldh2*$^{-/-}$ hearts is not due to a failure to generate the epicardium.

The late appearance of RA activity in the ventricular epicardium (E12.5) is inconsistent with a role for epicardial RA in compact zone formation, which initiates at ~E10.5 (Smith and Bader, 2007). Previous studies have demonstrated that detection of RALDH2 protein in the ventricular epicardium is not observed until E12.5 (Moss et al., 1998), consistent with our observations of RA activity in this tissue (Fig. 2A,G); we observed scattered *RARE-lacZ* expression in the wild-type ventricular epicardium at E11.5 (data not shown). We further examined whether epicardial *Raldh2* expression appears late after epicardium formation. We found that *Raldh2* mRNA was not detectable in cardiac tissues at E9.5, but was observed in the adjacent splanchnic mesoderm (see Fig. S1A-B in the supplementary material). At E10.5, after epicardial formation has completed, *Raldh2* mRNA was still not observed in the epicardium (see Fig. S1C-E in the supplementary material). Also, immunohistochemical staining did not detect RALDH2 protein in the heart at E10.5 (see Fig. S1F,G in the supplementary material). At E12.5, RALDH2 protein was detected in the epicardium, pericardium, diaphragm and liver mesothelium (see Fig. S1H-K in the supplementary material). Our findings thus demonstrate that epicardial RA synthesis by RALDH2 initiates between E11.5 and E12.5. A cell-autonomous role for RA in the ventricular epicardium at E10.5 has been proposed to regulate the expression of a secreted factor that in turn induces cardiomyocyte proliferation and compact zone development (Lavine et al., 2005). However, our analysis of *Raldh2* mRNA and protein expression as well as detection of RA signaling using *RARE-lacZ* does not support this hypothesis. Moreover, we now demonstrate that RA signaling observed in the ventricular epicardium at E12.5 does not require *Rxra*. Normal RA activity in *Rxra*$^{-/-}$ hearts might be due to the redundant nuclear receptor RXRβ, which is also expressed in heart (Wendling et al., 1999), but loss of *Rxra* function alone impairs compact zone growth (Kastner et al., 1994; Sucov et al., 1994). Taken together, these findings implicate an RA signaling event outside the heart, requiring both *Raldh2* and *Rxra*, that controls ventricular compact zone expansion.

Analysis of *Epo* expression in hepatic and ventricular tissues

Epicardial development and liver organogenesis are intricately linked as it has been shown that endodermally derived hepatoblasts migrate into the septum transversum mesenchyme to form a liver bud surrounded by coelomic mesothelium (Zorn and Wells, 2009), and the septum transversum mesenchyme also gives rise to the proepicardial organ and epicardium (Komiyama et al., 1987). RALDH2 is detected in the liver mesothelium at E10.5-E13.5 (Ijpenberg et al., 2007). We also detected RALDH2 immunoreactivity in the liver mesothelium at E12.5 (see Fig. S1J,K in the supplementary material). It has been demonstrated previously that RXRα and RA synthesis is necessary for normal liver development. *Rxra*$^{-/-}$ embryos (Makita et al., 2001) and rescued *Raldh2*$^{-/-}$ embryos (Wang et al., 2006) both exhibit variable reductions in liver size. Also, previous studies on *Wt1*$^{-/-}$ and *Rxra*$^{-/-}$ mouse embryos and analysis of chick embryos treated with RA synthesis inhibitors have demonstrated that RA/RXRα signaling functions downstream of *Wt1* in liver mesothelium, and that loss of RA signaling disrupts development of the liver mesothelium leading to reduced progenitors for hepatic stellate cells (Ijpenberg et al., 2007), which are known to secrete EPO (Maxwell et al., 1994). Additionally, there is evidence that expression of EPO in hepatic tissues is regulated by RA, as shown in *Rxra*$^{-/-}$ embryos that exhibit a large reduction in hepatic *Epo* expression (Makita et al., 2001). Furthermore, *Epo*$^{-/-}$ and *Epor*$^{-/-}$ embryos exhibit growth arrest of the ventricular compact zone myocardium, consistent with the observed expression of *Epor* in the epicardium and endocardium (Wu et al., 1999). As our findings above suggest that an RA signaling event outside the heart is responsible for induction of an epicardial mitogen, we further examined the expression of *Epo* in liver.

We analyzed *Epo* mRNA levels in hepatic and cardiac tissues of *Rxra*$^{-/-}$ and rescued *Raldh2*$^{-/-}$ embryos. We also examined a conditional *Rxra*$^{-/-}$ model using a *Gata5-Cre* that is expressed in the epicardium as well as other derivatives of the septum transversum mesenchyme, including the liver coelomic mesothelium (Merki et al., 2005). The morphological heart phenotype of *Gata5-Cre:Rxra*$^{-/-}$ conditional mutants recapitulates that observed in germline *Rxra*$^{-/-}$ and rescued *Raldh2*$^{-/-}$ embryos (Merki et al., 2005). A semi-quantitative RT-PCR approach was used to analyze expression of *Epo* in ventricular and hepatic tissues from wild-type and mutant embryos at E11.5 and E12.5. We found that *Epo* mRNA was significantly downregulated in hepatic tissues at E11.5 and E12.5 from all three mutants (Fig. 4). *Epo* mRNA was undetectable in wild-type and mutant ventricular tissue. Thus, hepatic *Epo* expression is dependent upon RA signaling and could be responsible for the heart phenotype in all three mutants. Our observation that hepatic *Epo* expression is severely downregulated in the *Gata5-Cre:Rxra*$^{-/-}$ conditional mutant strongly suggests that *Gata5-Cre* activity in the liver mesothelium is responsible for the heart phenotype, rather than *Gata5-Cre* activity in the epicardium as proposed originally (Merki et al., 2005).

Earlier studies implicated FGF9 as a potential mitogen induced in the epicardium upon treatment with 10 μM RA (Lavine et al., 2005). We did not detect any change in *Fgf9* expression in ventricles or livers of the three mutants compared with wild type, demonstrating that heart *Fgf9* expression is independent of RA signaling (Fig. 4). We suggest that high levels of RA might ectopically induce *Fgf9* but that endogenous levels of RA are unnecessary for its expression. In addition, previous studies on mouse and chick embryos with impaired FGF receptor function suggest that FGF signaling might not play a major role in ventricular compact zone expansion (Mima et al., 1995; Corson et al., 2003).

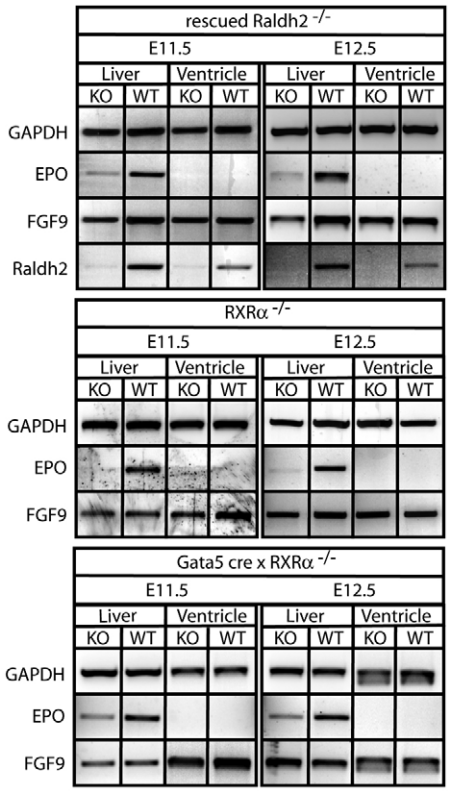

Fig. 4. Analysis of *Epo* and *Fgf9* expression in hepatic and ventricular mouse tissues. Semi-quantitative RT-PCR shows that *Epo* mRNA is reduced in the liver of rescued *Raldh2*$^{-/-}$ embryos at E11.5 and E12.5 relative to wild type, whereas *Epo* expression is not detectable in ventricular tissues; as a control, rescued *Raldh2*$^{-/-}$ embryos exhibit a loss of *Raldh2* mRNA. *Epo* mRNA is also reduced in livers from germline *Rxra*$^{-/-}$ embryos and *Gata5-Cre* x *Rxra*$^{-/-}$ conditional mutant embryos. Expression of *Fgf9* mRNA is not altered in any of the analyzed tissues and mutants relative to wild type. GAPDH, glyceraldehyde 3-phosphate dehydrogenase mRNA control.

Epo is a direct target of RA signaling in embryonic liver

To determine whether *Epo* is a direct transcriptional target of hepatic RA signaling in vivo, we performed chromatin immunoprecipitation (ChIP) on E13.5 mouse liver samples. Previous studies have shown that *Epo* harbors a conserved DR2 RARE element in its 3′ enhancer that is required for RA-regulated transcription in transfected cell lines (Makita et al., 2005). Therefore, we carried out ChIP studies to ascertain whether RAR proteins are recruited to this RA response element in embryonic liver. ChIP results demonstrated that all three RA receptors (RARα, RARβ and RARγ) exhibit robust binding to the *Epo* RARE, whereas no association was observed in a region upstream of *Epo* and no signal was observed with IgG control (Fig. 5A,B). These findings provide strong evidence that *Epo* expression in embryonic liver is directly induced by RA signaling.

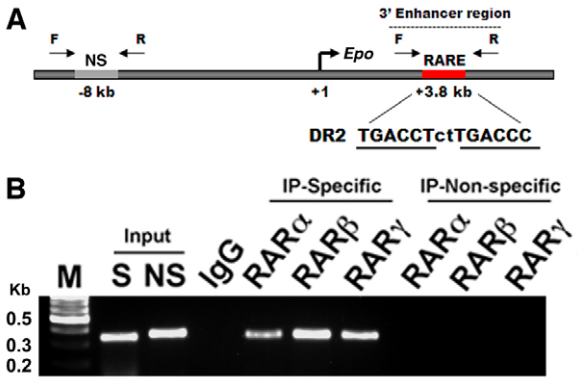

Fig. 5. Recruitment of RA receptors to the *Epo* 3′ enhancer in mouse embryonic liver. (**A**) Schematic representation of the 3′ enhancer region of mouse *Epo* showing the location of a DR2 RARE (direct repeat with 2 bp spacer) and PCR primers used for chromatin immunoprecipitation (ChIP) analysis. (**B**) E13.5 liver ChIP results demonstrating robust binding of all three RARs detected with primers flanking the *Epo* RARE; no signal was obtained with IgG control or non-specific primers located several kb upstream from the RARE. M, DNA ladder; S, RARE specific primers; NS, specific primers.

EPO signaling induces expression of *Igf2* in mouse epicardial cells

Next, we determined whether secreted EPO acts in the epicardium to stimulate production of a downstream mitogen that could facilitate compact zone growth. To date, which mitogen(s) are produced by the epicardium to induce compact zone formation remains elusive. We have recently derived a mouse embryonic ventricular epicardial cell line called MEC1 and tested expression of various mitogens (P.L. and H.M.S., unpublished). We found that expression of *Igf2* is upregulated in MEC1 cells upon EPO treatment. Our data shows that 10 ng/ml EPO in serum-free culture medium increased IGF2 mRNA levels by approximately 3.5-fold in MEC1 cells, whereas treatment of MEC1 cells with 1 μM RA did not increase IGF2 mRNA above control levels (Fig. 6A). We conclude from these findings that EPO, but not RA, can induce *Igf2* in epicardial cells in vitro.

Loss of *Raldh2* or *Rxra* function results in loss of *Igf2* expression in the epicardium

We investigated whether *Igf2* expression in epicardium is affected by loss of RA signaling in vivo. Heart sections from wild-type, rescued *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ E12.5 embryos were examined by in situ hybridization to visualize *Igf2* mRNA. We observed that E12.5 rescued *Raldh2*$^{-/-}$ mutants and that *Rxra*$^{-/-}$ mutants exhibited a loss of *Igf2* mRNA in the epicardium (Fig. 6B). These findings demonstrate a dependency of epicardial *Igf2* expression on RA signaling. However, RA does not act directly in the epicardium as our findings above indicate that *Rxra*$^{-/-}$ embryos retain epicardial RA signaling, and RA treatment of MEC1 epicardial cells does not induce *Igf2*. Together, these in vivo and in vitro data point to a possible RA-EPO-IGF2 signaling axis from liver to heart.

EPO treatment rescues epicardial *Igf2* expression and cardiomyocyte proliferation

In order to establish the existence of an RA-EPO-IGF2 signaling axis, we analyzed EPO-treated heart organ cultures for epicardial *Igf2* induction and ventricular cardiomyocyte proliferation. In order to determine whether EPO can stimulate *Igf2* expression in embryonic epicardium, we cultured E11.5 wild-type and *Raldh2*$^{-/-}$

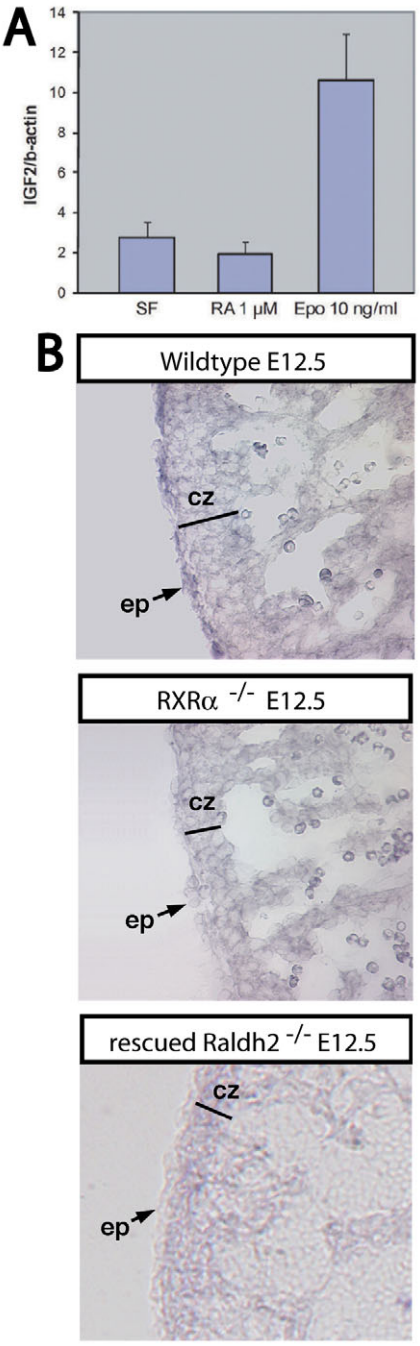

Fig. 6. RA controls cardiac IGF2 expression indirectly through EPO. (**A**) Treatment of epicardial MEC-1 cell line with EPO (10 ng/ml) significantly induced *Igf2* mRNA compared with cells grown under serum-free conditions (SF), whereas RA treatment (1 μM) had no effect; expression based on RT-PCR analysis was normalized to β-actin mRNA. Data are mean ± s.d. (**B**) *Igf2* mRNA was detected by in situ hybridization in E12.5 mouse hearts. Rescued *Raldh2*$^{-/-}$ and *Rxra*$^{-/-}$ hearts exhibit a reduction in epicardial *Igf2* mRNA detection compared with wild type. The bar represents the thickness of the compact zone. Similar results were observed for all mutants analyzed (n=3 for each genotype). cz, compact zone myocardium; ep, epicardium.

hearts with or without the addition of EPO to the culture medium. After 18 hours in culture, hearts were sectioned and analyzed for expression of *Igf2* by in situ hybridization. Both control and EPO-

treated wild-type hearts exhibited *Igf2* mRNA in the epicardium (Fig. 7A,B). Control *Raldh2*$^{-/-}$ hearts lacked *Igf2* mRNA in the epicardium but EPO treatment of *Raldh2*$^{-/-}$ hearts was able to rescue the appearance of *Igf2* mRNA in the epicardium (Fig. 7C,D). These findings provide further evidence that EPO can stimulate induction of epicardial *Igf2*.

Next, we examined the proliferation rate of cardiomyocytes in E11.5 wild-type and *Raldh2*$^{-/-}$ heart organ cultures. The proliferation rate of ventricular cardiomyocytes in untreated *Raldh2*$^{-/-}$ mutant hearts was significantly lower than that observed in wild-type hearts, but we detected a significant increase in the proliferation rate of *Raldh2*$^{-/-}$ ventricular cardiomyocytes following EPO treatment (Fig. 8). The cardiomyocyte proliferation rates for wild-type hearts (EPO-treated and untreated) and EPO-treated *Raldh2*$^{-/-}$ hearts were not significantly different (Fig. 8). Taken together with the findings above, we conclude that EPO treatment can rescue the observed loss of *Igf2* expression and reduced cardiomyocyte proliferation in RA-deficient hearts, further strengthening our proposed model of ventricular compact zone growth.

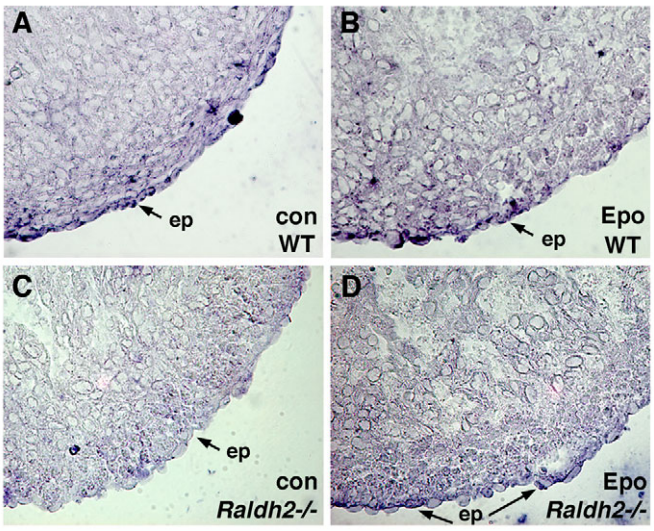

Fig. 7. Rescue of epicardial *Igf2* expression. (**A-D**) Wild-type and *Raldh2*$^{-/-}$ mouse hearts were cultured in the absence or presence of EPO, then sections were analyzed for *Igf2* expression by in situ hybridization. Wild-type hearts (A,B) exhibited *Igf2* mRNA with (Epo) or without (Con) added EPO. A control (con) *Raldh2*$^{-/-}$ heart (C) displays no epicardial *Igf2* mRNA, but EPO treatment rescues epicardial *Igf2* expression in an *Raldh2*$^{-/-}$ heart (D; *n*=3 for each genotype). ep, epicardium.

DISCUSSION

RA-EPO-IGF2 signaling axis from liver to heart is required for myocardial expansion

The epicardium is thought to secrete one or more diffusible factors that stimulate proliferation of ventricular cardiomyocytes but the identity of the secreted factor(s) remains elusive and upstream regulators of epicardial mitogen production are unclear. The findings presented in this study suggest a novel mechanism for ventricular compact zone expansion through a RA-EPO-IGF2 signaling axis extending from liver to heart (Fig. 9). Thus, we suggest that RA signaling affects epicardial function in a tissue-nonautonomous fashion by inducing *Epo* expression in hepatic tissues, leading to secretion of the glycoprotein hormone EPO and binding to the EPO receptor in the epicardium. EPO produced in the liver might preferentially reach the epicardium by diffusion from the nearby liver mesothelium through the pericardioperitoneal canals that persist until E13.5-E14.5 (Kaufman, 1992), or EPO could reach the heart by secretion into the bloodstream. We propose that EPO signaling in the epicardium then induces production of IGF2, which acts as a promoter of cardiomyocyte proliferation in the ventricular compact zone. This model is supported by genetic studies demonstrating ventricular myocardial thinning in mice carrying null mutations of *Epo* or *Epor* (Wu et al., 1999). In addition, we demonstrate that EPO treatment of *Raldh2*$^{-/-}$ hearts can rescue epicardial *Igf2* expression and ventricular cardiomyocyte proliferation. Signaling between heart and liver has also been observed earlier in development in studies demonstrating that FGF signaling from the heart can induce endoderm to undergo liver formation (Jung et al., 1999).

RA signaling in the epicardium is not required to stimulate myocardial expansion

Previous studies suggested that an RA signaling event in the epicardium is necessary for ventricular myocyte proliferation (for a review, see Lavine and Ornitz, 2008). This mechanism was supported by two previous genetic experiments that resulted in ventricular myocardial thinning, one employing a dominant-negative RARα construct expressed in the epicardium under the control of the human keratin-18 promoter (Chen et al., 2002), and another using *Gata5-Cre* for conditional deletion of *Rxra* in the epicardium (Merki et al., 2005). These mouse models used promoter elements driving *Cre* expression in the epicardium but not other cardiac tissues, however, both promoters are also expressed in other septum transversum mesenchyme (STM) derivatives including the mesothelium around the liver and the adjacent liver tissue potentially derived from ingression of the mesothelium (Thorey et al., 1993; Merki et al., 2005). Therefore, both the dominant-negative RARα and *Gata5-Cre:Rxra*$^{-/-}$ conditional mutant could have acted in STM-derived liver progenitors to reduce hepatic *Epo* expression; here, we demonstrate that the latter genetic model does exhibit a loss of hepatic *Epo* expression.

As *Rxra*$^{-/-}$ hearts exhibit myocardial compact zone thinning but maintain epicardial RA signaling [potentially through a redundant function of RXRβ (Wendling et al., 1999)], the studies reported here suggest that RA synthesis detected in the epicardium does not function to stimulate compact zone expansion. This conclusion is further strengthened by data demonstrating that epicardial RA activity initiates marginally at E11.5 and robustly only at E12.5 after compact zone expansion (which begins at E10.5) is well underway. By contrast, *Raldh2* expression and RA activity are detected by E10.5 in the liver mesothelium (Wang et al., 2006; Ijpenberg et al., 2007), which our findings suggest is the RA target tissue required for myocardial compact zone expansion. A recent study suggested that differentiation of cardiac progenitors contributing to compact zone growth is regulated by epicardial RA (Lin et al., 2010) but our studies suggest that this is due to RA signaling outside the heart as we demonstrate normal epicardial RA signaling in *Rxra*$^{-/-}$ hearts. Although we demonstrate that epicardial RA signaling is unnecessary for compact zone growth, epicardial RA might function in coronary vessel development as recently suggested (Azambuja et al., 2010; Lin et al., 2010), or in a regenerative response to heart injury as suggested by studies in zebrafish (Lepilina et al., 2006). However, as the epicardium and liver mesothelium are both derived from the STM, which expresses

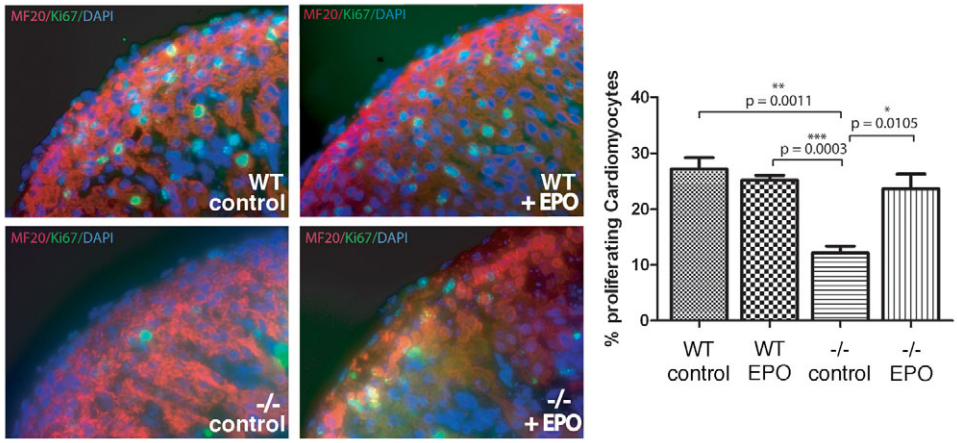

Fig. 8 Cardiomyocyte proliferation in heart organ culture is rescued by EPO treatment. Hearts dissected from E11.5 wild-type (WT) or *Raldh2*$^{-/-}$ mutant mice were cultured for 18 hours in the absence of EPO (control) or with EPO (+EPO), then examined immunohistochemically for cardiomyocytes (MF20; red), dividing cells (Ki67; green), and nuclei (DAPI; blue). Control or EPO-treated wild-type hearts exhibited significantly more proliferating cardiomyocytes (MF20+, Ki67+) compared with control *Raldh2*$^{-/-}$ hearts, but EPO treatment of *Raldh2*$^{-/-}$ hearts restored the cardiomyocyte proliferation rate to wild-type levels (n=2 for each genotype). Data are mean ± s.d.

Raldh2, one might speculate that expression of *Raldh2* and synthesis of RA in the epicardium could be an unnecessary by-product of their common lineage. Although we show that *Raldh2* expression in liver STM derivatives performs a necessary function, expression of *Raldh2* in epicardial STM derivatives might have no significant function in mouse, and could exist as a vestigial evolutionary remnant.

Septum transversum derived progenitor cells play important roles during organogenesis

Recent findings have made it increasingly clear that STM-derived progenitor cells are required for maturation and growth of both heart and liver. Independent studies have emphasized parallel developmental mechanisms between these two organs and have specifically focused on the STM-derived pluripotent progenitors. The liver bud, which develops after cardiogenic mesoderm formation, is required for proepicardial organ induction in naive chicken mesoderm (Ishii et al., 2007). Mesenchymal cells originating in the STM are able to differentiate into hepatic stellate cells and sinusoidal endothelial cells that migrate into the liver, providing vasculature and mitogens for liver expansion (Asahina et al., 2009). Also, RA synthesis controlled by *Raldh2* downstream of *Wt1* is required for the expansion of these hepatic stellate cell progenitors (Ijpenberg et al., 2007), which are known to express *Epo* (Maxwell et al., 1994). Furthermore, RA generated by *Raldh2* is necessary for remodeling of subcoelomic mesenchyme during formation of the cardiac sinus horns (Norden et al., 2010), thus demonstrating that RA signaling in STM derivatives is required for organogenesis. Liver hypoplasia observed in rescued *Raldh2*$^{-/-}$ embryos (Wang et al., 2006) might therefore be the result of disrupted STM-derived cell differentiation and expansion. During ventricular maturation, the STM-derived epicardium facilitates an analogous process. The epicardium provides essential mitogens for compact zone proliferation and epicardial-derived cells differentiate into smooth muscle cells and cardiac fibroblasts, indispensable cell lineages for cardiac architecture and coronary vessel formation (Lie-Venema et al., 2007). However, our findings suggest that RA signaling in STM cells contributing to the epicardium is dispensable for compact zone formation.

Hepatic RA signaling acts through EPO to stimulate epicardial IGF2 expression

Our studies provide evidence for an RA-EPO signaling pathway from liver to heart that stimulates expansion of the ventricular compact zone. We demonstrate that liver expression of *Epo* requires not only *Rxra* but also RA synthesis controlled by *Raldh2* in liver mesothelium. EPO signaling in the epicardium has been shown to stimulate myocardial expansion (Wu et al., 1999; Stuckmann et al., 2003), but target genes for EPO signaling in the epicardium have not been described prior to this study. Here, we show that treatment of MEC1 cells with EPO (but not RA) induces *Igf2* expression. Also, loss of *Raldh2* or *Rxra* function in vivo results in loss of hepatic *Epo* expression and epicardial *Igf2* expression. Furthermore, EPO treatment of RA-deficient hearts rescues both epicardial *Igf2* expression and ventricular cardiomyocyte proliferation. Together, these findings provide evidence that hepatic RA signaling induces *Epo*, which results in

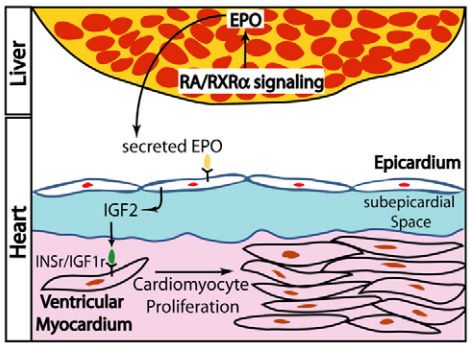

Fig. 9. RA-EPO-IGF2 signaling axis from liver to heart. We propose a model for control of myocardial compact zone formation that involves a sequence of three signaling pathways extending from liver to heart: (1) RA signaling generated by hepatic RALDH2 and RXRα induces hepatic *Epo*; (2) EPO secreted by the liver results in epicardial EPO signaling needed to induce *Igf2*; (3) IGF2 secreted by the epicardium results in myocardial IGF2 signaling needed to stimulate ventricular myocardial growth. INSr/IGF1r represents the receptors for IGF2.

epicardial EPO signaling that induces the *Igf2* needed for ventricular compact zone growth. Our conclusion is supported by studies demonstrating that loss of IGF2R, a receptor that negatively regulates IGF2 signaling, leads to cardiac hypertrophy characterized by a 3-fold larger heart with a markedly increased compact zone myocardium (Lau et al., 1994). Lastly, cardiomyocytes derived from human embryonic stem cells require IGF signals for proliferation (McDevitt et al., 2005). Taken together with our findings, we suggest that IGF2 functions as an important epicardial-derived mitogen needed for cardiomyocyte expansion downstream of EPO and RA signaling.

It is intriguing to speculate about the evolutionary implications of our model of myocardial growth. Cross-talk between the developing heart and liver at mid-gestational stages appears to satisfy the growing need of the embryo for sufficient oxygen and nutritional supply carried by the bloodstream. Embryonic hematopoiesis begins shifting from the yolk sac to the liver when ventricular compact zone expansion begins. Therefore, RA-EPO-IGF2 signaling provides a mechanism to coordinate embryonic liver hematopoiesis with cardiac growth, a prerequisite for embryonic survival. Moreover, coelomic mesothelial cells originating in the lateral plate mesoderm and giving rise to the STM and other types of mesenchyme, contribute cells and signaling cues not only to hepatic and cardiac tissues but also to the lung and gut (Wilm et al., 2005; Que et al., 2008). A broader understanding of signals governing coelomic organ development and multipotent precursor contributions will further deepen our insight into mechanisms vital for normal embryonic growth and could open avenues for new strategies to counteract congenital birth defects.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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