IGF signaling directs ventricular cardiomyocyte proliferation during embryonic heart development

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SUMMARY

Secreted factors from the epicardium are believed to be important in directing heart ventricular cardiomyocyte proliferation and morphogenesis, although the specific factors involved have not been identified or characterized adequately. We found that IGF2 is the most prominent mitogen made by primary mouse embryonic epicardial cells and by a newly derived immortalized mouse embryonic epicardial cell line called MEC1. In vivo, Igf2 is expressed in the embryonic mouse epicardium during midgestation heart development. Using a whole embryo culture assay in the presence of inhibitors, we confirmed that IGF signaling is required to activate the ERK proliferation pathway in the developing heart, and that the epicardium is required for this response. Global disruption of the Igf2 gene, or conditional disruption of the two IGF receptor genes Igf1r and Insr together in the myocardium, each resulted in a significant decrease in ventricular wall proliferation and in ventricular wall hypoplasia. Ventricular cardiomyocyte proliferation in mutant embryos was restored to normal at E14.5, concurrent with the establishment of coronary circulation. Our results define IGF2 as a previously unexplored epicardial mitogen that is required for normal ventricular chamber development.

KEY WORDS: Compact zone, Ventricular chamber, Insulin-like growth factor, Cardiomyocyte proliferation, Mouse

INTRODUCTION

The heart at the time of its formation [around embryonic day (E)7.5 in the mouse] consists of an inner endothelium (endocardium) and an outer myocardium. A third cell type, the epicardium, forms over the myocardium during the E9.5-10.5 period. The epicardium originates from a progenitor structure, the proepicardium, that is adjacent to the septum transversum; vesicles delaminate from the proepicardium onto the surface of the myocardium to form a single-cell layered mesothelium on the outer surface of the heart (Manner, 1993; Sengbusch et al., 2002).

Morphologically, the myocardium is thin walled and uniform during the early E7.5-9.5 period. Starting at ~E10.5, immediately following the formation of the epicardium, the myocardium in the ventricular chamber expands significantly by cell proliferation and becomes partitioned into two distinct subdomains: trabecular myocardium facing the endocardium, and compact zone myocardium facing the epicardium. Compact zone myocardium is destined to form the ventricular wall, which provides the bulk of the force of ventricular contraction starting from midgestation (Sedmera and Thomas, 1996).

Several lines of evidence support the premise that cardiomyocyte proliferation during midgestation in the outer chamber wall, and thereby formation of the compact zone, is influenced by soluble factors secreted by the epicardium (for a review, see Sucov et al., 2009). For example, blocking the formation of the epicardium in chick embryos by physical obstruction resulted in a hypoplastic ventricular wall in the denuded region (Gittenberger-de Groot et al., 2000; Manner et al., 2005). Similarly, in explanted chick heart slices maintained in culture, removal of the epicardium resulted in a significant decrease in cardiomyocyte proliferation (Stuckmann et al., 2003).

The specific factors secreted from the epicardium and responsible for inducing mitogenic behavior in the myocardium have not been adequately clarified. Members of the fibroblast growth factor (FGF) family are involved in ventricular development, as mouse knockouts of ligand or receptor genes result in a hypoplastic phenotype (Lavine et al., 2005). Nonetheless, there are uncertainties related to their possible role as epicardial factors (see Discussion).

We showed previously that an immortalized adult rat atrial epicardial cell line called EMC secretes mitogenic activity into serum-free conditioned media (Chen et al., 2002). The primary mitogen expressed and secreted by these cells is PDGF-A (Kang et al., 2008). However, by conditionally mutating both PDGF receptor genes in the myocardium, we and others showed that PDGF signaling was not required for embryonic compact zone development or ventricular chamber morphogenesis (Kang et al., 2008; Mellgren et al., 2008). The expression of PDGF-A by EMC cells is appropriate for their derivation from adult atrial epicardium but is not characteristic of embryonic ventricular epicardium.

In this study, we observed that a newly derived embryonic epicardial cell line expresses high levels of the insulin-like growth factor IGF2. IGF1 and IGF2 are secreted ligands for the IGF1R and INSR receptors, which are members of the receptor tyrosine kinase family. These receptors activate proliferation via an intracellular phosphorylation cascade that generally includes ERK (mitogen-activated protein kinase; MAPK) and other components that

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regulate cell cycle progression. In cell culture, both IGFs stimulate the proliferation of primary fetal cardiomyocytes (Liu et al., 1996; Hertig et al., 1999), as do many other mitogens (Armstrong et al., 2000). In mouse embryos, treatment with exogenous IGF induces cardiomyocyte proliferation (Fraidenraich et al., 2004), and excess IGF2 caused by absence of the clearance receptor IGF2R leads to ventricular hyperplasia (Lau et al., 1994; Wang et al., 1994; Eggenschwiler et al., 1997). Although showing that embryonic cardiomyocytes have the capacity to respond to IGF, these observations do not define an actual in vivo role for IGF signaling. From mutational analysis, IGF1, IGF2 and both receptors are involved in general embryonic growth, although embryonic heart development has not been examined in these previous genetic investigations (DeChiara et al., 1990; Baker et al., 1993; Powell-Braxton et al., 1993). Postnatal *Igf1r* and *Insr* gene function in the myocardium has also been examined, although again with no insight or attention to embryonic heart development (Kim et al., 2000; Belke et al., 2002; Laustsen et al., 2007; Kim et al., 2008). Here, we demonstrate the crucial role of IGF2 as a mitogenic factor that influences cardiomyocyte proliferation and ventricular compact zone morphogenesis.

MATERIALS AND METHODS

Derivation of the MEC1 epicardial cell line
Ventricular tissue from several E13.5 wild-type hearts (ICR/CD1 strain background) was cut coarsely into pieces and plated collectively on gelatin-coated dishes in DMEM with 15% FBS. Following several days of outgrowth, the clumps of ventricular tissue were removed and the epicardial cells were allowed to continue to grow until reaching near confluence (Chen et al., 2002). The cells were then trypsinized and replated at low to moderate density; under these conditions, most cells differentiate into postmitotic fibroblastic-type cells that are easily recognized. Small colonies of cells with the morphology of early (pre-passaged) primary epicardial cells were identified by appearance and picked manually with a pipette, and re-plated individually in 48-well dishes. The MEC1 cell line was able to continue growth and maintained its morphology after repeated passages. Serum concentration was eventually reduced to 10% without adverse consequences. Primers used to validate gene expression in MEC1 cells or in E11.5 whole embryo RNA included: *Tcf21* (*epicardin*), F: TCGTTTCCGTTTCAGCGTCTCTCT, R: TAAAGGGCCACGTCAG-GTTGACT (515 bp); *Tbx18*, F: CACGAAATAGGCACCGAGAT, R: GGACAGATCATCTCCGCAAT (389 bp); *Wilms tumor 1 homolog* (*Wt1*), F: ACTCCTTCATCAAACAGGAGC, R: AGGGCGTCCTCAG-GAGCAG (455 bp); *Krt18*, F: TGCGAATTCTGTGGACAATGC, R: ACCAGTACTTGTCCAGTTCC (391 bp); *Myl2* (*Mlc2v*), F: AGCTC-CAACGTGTTCTCC, R: GATCCCTCAGTCCTTCTCTTCTCCG (462 bp); *Actc1* (cardiac actin), F: GACCTTCAATGTGCCTGCCA, R: GTG-GTGCCTCCAGATAGGAC (536 bp); *Myh7* (*Mhc*β), F: AGTTTGT-CAAGGCCAAGATCG, R: TTGACAGTCTTCCCAGCTCC (426 bp); *Pecam1*, F: CCTTCACCATCAACAGCATCC, R: GTGTCACCTT-GGGCTTGG (317 bp); *Tek* (*Tie2*), F: TAGAGCCAGAGACTACATAC, R: TAAGGGCCAGAGTTCCTCAG (305 bp).

Immunofluorescence
MEC1 cells were grown on glass coverslips coated with 1% gelatin and 0.01% poly-L-lysine until forming a monolayer, fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 30 minutes on ice or in methanol for 15 min at –20°C, and then washed with PBS. Permeabilization in 0.5% Triton X-100 was performed for WT1 detection for 5 minutes at room temperature, followed, for all antigens, by blocking for 1 hour at room temperature in PBS with 10% serum, 1% BSA and 0.1% Triton X-100, and incubation with specific antibodies for either 1 hour at room temperature or overnight at 4°C. Coverslips were washed with PBS and incubated with secondary antibodies for 1 hour at room temperature, then washed and mounted with mounting media containing DAPI (Invitrogen). Primary antibodies were: anti-ZO-1 (1:100, Invitrogen), anti-β-catenin (1:200, Santa

Cruz) and anti-Wilms Tumor-1 (1:50, Cell Marque). For F-actin staining, cells fixed with 4% PFA were incubated with Rhodamine Phalloidin (Molecular Probes) following the manufacturer's instructions. Images were obtained with Spot camera and imaging software (Diagnostic Instruments) attached to an Olympus BX41 microscope.

Microarray analysis
20 μg of total MEC1 cell RNA was converted into biotin-labeled cRNA using standard protocols recommended by Affymetrix. 15 μg of fragmented cRNA was applied to an Affymetrix mouse 430A microarray and hybridized for 12-16 hours at 45°C under standard conditions. Following hybridization, microarrays were washed using an Affymetrix Fluidics Station 400, stained with a streptavidin-phycoerythrin conjugate, and then read using a Hewlett Packard GeneArray Scanner.

Inhibition of *Igf2* by RNAi
Oligos for constructing *Igf2*-shRNAi were 5′-ACCGACGCCT-GCGCAGAGGCCTTTCAAGAGAAGGCCTCTGCGCAGGCGTCTTT-TTC-3′ and 5′-TCGAGAAAAAGACGCCTGCGCAGAGGCCTTC-TCTTGAAAGGCCTCTGCGCAGGCGT-3′. The core RNAi sequence (underlined nucleotides) was as reported previously (Sun et al., 2006). The oligos for scrambled RNAi were 5′-ACCGATATCCGGTACCGAA-GGTTTCAAGAGAACCTTCGGTACCGGATATCTTTTTC-3′ and 5′-TCGAGAAAAAGATATCCGGTACCGAAGGTTCTCTTGAAACCTT-CGGTACCGGATAT-3′. Oligos were annealed and then cloned behind a human U6 promoter in a vector described previously (Qin et al., 2003). The expression cassette containing the U6 promoter with the RNAi sequence was then excised and subcloned between the *Xho*I and *Xba*I sites in pAdTrack. Recombinant adenoviruses were generated and amplified in 293 cells as described (He et al., 1998).

Evaluation of mitogenic activity
Isolation of serum-free MEC1 cell-conditioned media was as described previously (Chen et al., 2002; Kang and Sucov, 2005). MEC1 cells were infected with RNAi adenovirus, then washed and switched to serum-free media in the same manner. Induction of mitogenic response in NIH3T3 cells as evaluated by thymidine incorporation was as described previously (Chen et al., 2002; Kang and Sucov, 2005). In general, NIH3T3 cells were grown to 50% confluence in the presence of serum, washed and switched to serum-free DMEM for 24 hours, and then exposed to serum-free conditioned media or recombinant factors in DMEM for 24 hours in the presence of tritiated thymidine. When used, responding cells were pretreated for 30 minutes with AG1024 (Calbiochem) in DMEM before replacement with media containing AG1024. Recombinant human IGF2 was from Imgenex (IMR-235).

Epicardium-cardiomyocyte co-culture
Primary epicardial cells were grown out from pieces of E13.5-14.5 ventricular tissue as described above, in gelatin- and FBS-coated 8-well chamber slides for several days until near confluence but were not passaged. Primary ventricular cardiomyocytes were isolated from E13.5-14.5 embryonic hearts by pancreatin-collagenase digestion and purified by Percoll gradient centrifugation as described previously (Chen et al., 2002; Kang and Sucov, 2005); purity was assessed by MF20 staining. Isolated cardiomyocytes were plated onto the epicardial cells, or into coated empty wells, in DMEM with 5% FBS and 10% horse serum for 48 hours. The media was replaced with serum-free DMEM and 20 hours later the media was replaced with fresh serum-free DMEM with 1 μM bromodeoxyuridine (BrdU) and AG1024 or solvent. After 20 hours, cells were fixed and immunostained for BrdU and Nkx2.5 as described above.

Quantification of IGF2
One-fourth volume of 50% trichloroacetic acid (v/w) with 1 mM deoxycholic acid was added to serum-free MEC1 cell-conditioned media and left on ice for 30 minutes. The mixture was then centrifuged at 22,000 g at 4°C for 30 minutes. The pellet was washed once with 1 ml acetone and air dried for 5 minutes before resuspension in SDS-PAGE loading buffer.

Mice

All mouse lines used in this study have been described previously: conditional *Igf1r/Insr* mice (Stachelscheid et al., 2008), *Igf2*$^{-/+}$ (DeChiara et al., 1990), *MLC2vCre* (Chen et al., 1998) and *Nkx2.5Cre* (Moses et al., 2001). The *Igf2* allele was maintained on a 129JS2 background; all other lines were on mixed and unspecified strain backgrounds.

In vitro embryo culture

Following a protocol described previously (Corson et al., 2003), wild-type embryos were isolated from their yolk sacs and cultured in a 5% CO_2 tissue culture incubator in 1 ml of pre-equilibrated RPMI with 1% BSA. AG1024 (Calbiochem) and PD173074 (Sigma) were dissolved in DMSO at appropriate concentrations and were added at a 1:1000 dilution into the culture media; control media contained a 1:1000 dilution of DMSO alone. Embryos were cultured on a rocking platform for 1 hour, then washed in cold PBS; ventricular and limb tissue was dissected, frozen in liquid nitrogen and stored at −70°C until further processing. Tissue samples were homogenized on ice in 5 mM HEPES, 1 mM EGTA (pH7.5) containing 1 mM phenylmethylsulfonyl fluoride and 0.5 mM orthovanadate to preserve the phosphorylated state. Protein concentrations were measured with a Biorad protein assay and 3 μg of protein were separated by electrophoresis in 10% SDS-polyacrylamide gel. For quantification, films were scanned and the intensity of the bands measured by ImageQuant software. The ratio of phosphoERK (pERK) to total ERK (tERK) was calculated for each sample; because of different film exposures and different affinities of antibodies, the ratio is expressed in arbitrary units (AU) and does not reflect the absolute percentage of pERK. The signal for DMSO-treated samples was defined as 100 for comparison between experiments.

Western blotting

Proteins separated on SDS-PAGE gels were transferred to polyvinylidene difluoride membranes (Biorad). Membranes were blocked in PBS-Tween with 5% nonfat dry milk. Primary antibody for detection of IGF2 in MEC1 media was from R&D (AF792) and used at 0.2 μg/ml. Antibodies against PDGF-A (Santa Cruz sc-128; 1:400) and PDGF-B (Santa Cruz sc-7878; 1:400) were also used but did not detect target proteins in MEC1 cell-conditioned media. Antibodies for detection of ERK and pERK (Cell Signaling Technologies) in tissue lysates were used at 1:1000. Bound primary antibodies were visualized using secondary antibodies conjugated to horseradish peroxidase (1:2000, Santa Cruz Biotechnology) and chemiluminescent substrate (Supersignal West Pico, Thermo Scientific). For detection of ERK, pERK detection was performed first, then membranes were stripped by washing in 2% SDS, 0.1 M β-mercaptoethanol at 55°C for 15 minutes, then re-probed with antibody against tERK. Radiographic films were scanned and the intensity of the bands was measured with ImageQuant software.

Epicardium-ventricle tissue co-culture

Primary epicardial cells were grown in 24-well gelatin- and FBS-coated plates as described above and, at near confluence, media was switched from 15% FBS to serum-free DMEM. Control wells contained no epicardial cells. Following overnight incubation, the media was replaced with fresh serum-free DMEM containing AG1024 (2 μM) or DMSO as a control. Intact ventricles from E9.5 hearts were dissected and placed on 12 mm cell culture insert filters (Millipore), then cultured at the air-liquid interface. After 1 hour, the heart tissue was removed from the filters and processed for pERK western blotting as described above.

In situ hybridization

Digoxygenin (DIG)-labeled probes were made with an RNA Labeling Kit (Roche, 11175025910). Embryos were fixed overnight in 4% PFA, dehydrated and embedded in OCT for cryosectioning. Transverse sections (10 μm) were cut and kept at −80°C until use. Sections were rehydrated before treatment with HCl, proteinase K, and then triethanolamine/acetic anhydride. Hybridization was carried out at 60°C overnight. Unhybridized probes were digested with RNaseA. Signal was then detected using HRP-coupled anti-DIG primary antibody (Roche) and TSAplus Fluorescent Substrate Kit (PerkinElmer), or using AP-coupled anti-DIG primary antibody (Roche) and BM Purple substrate. Images were obtained using a Zeiss LSM510 confocal microscope.

Embryonic cell proliferation analysis

Pregnant females were treated with BrdU by intraperitoneal injection (100 mg/kg) 2 hours before sacrifice and isolation of embryos. Embryos were fixed and embedded in paraffin and sectioned. BrdU- and Nkx2.5-positive nuclei (antibodies from Abcam and Santa Cruz, respectively) were visualized using immunofluorescence; in some initial studies, BrdU-positive nuclei were alternatively visualized by immunohistochemistry with light Hematoxylin counterstaining. For quantification, photographs at the same magnification (40×) of the ventricular chamber lower lateral walls (compact zone), trabeculae and septum of at least three nonadjacent 10 μm transverse sections at a similar axial level (approximately midway between the inflow and outflow valves) from each embryo were taken; BrdU$^+$ and BrdU$^−$ nuclei were counted using ImageJ software, and the ratio of positive to total nuclei determined. Statistical significance was determined using a paired Student's *t*-test.

### Quantitative RT-PCR analysis

Ventricular tissue was dissected from embryos at E9.5 to E15.5. RNA was isolated from individual heart tissue with the ZR RNA MicroPrep Kit (Zymo Research) following the manufacturer's instructions and eluted in 6-10 μl of RNAse-free water. cDNA was synthesized from 200 ng of total RNA with MMLV Reverse Transcriptase (Invitrogen). Real-time PCR (RT-PCR) analysis was performed in iQ SYBR Green Supermix (Bio-Rad) using a CFX96 Real Time PCR Detection System (Bio-Rad). A standard curve was performed with serial dilutions of cDNA. Melting curves were constructed at the end of the run to ensure homogeneity of amplification products. Experiments were run in duplicate and expression levels were normalized to β-actin expression levels. Primer sequences used to amplify cDNA fragments were (5′-3′): *Igf2*: CCCTCAGCAAGTGCCTAAAG and TTAGGGTGCCTCGAGATGTT; *Igf1*: TGGATGCTCTTCAGTTCGTG and GTCTTGGGCATGTCAGTGTG; *Igf1r*: GTGGGGGCTCGTGT-TTCTC and GATCACCGTGCAGTTTTCCA; *Insr*: CGATATGGT-GATGAGGAGCT and TCGTCCGGCACGTACACAGA; β-*actin*: ATGGAGGGGAATACAGCCC and TTCTTTGCAGCTCCTTCGTT.

RESULTS

Derivation of a stable epicardial cell line (MEC1) from the mouse embryonic ventricle

Primary embryonic epicardial cells in culture secrete mitogenic activity (Chen et al., 2002), although these cultures tend to differentiate into postmitotic fibroblasts following their first passage and thus are not experimentally tractable. We sought to derive a stable epicardial cell line from the embryonic ventricle in order to identify mitogenic and secreted factors made by the embryonic epicardium. Starting from primary epicardial cell cultures from E13.5 wild-type ventricles, we manually subcloned individual colonies that retained the morphology of early primary epicardial cells. After several rounds of subcloning, we arrived at a stable cell line, which we named MEC1 (mouse epicardial cells). In addition to having an epicardial morphology (Fig. 1A), these cells expressed a number of epicardial-specific markers, including *epicardin* (*Tcf21*), *Tbx18* and *keratin 18*, but did not express markers of myocardial or endothelial cell lineages (Fig. 1B). MEC1 cells localized ZO-1 and β-catenin (TJP1 and CTNNB1 – Mouse Genome Informatics) in adherens junctions at the cell membrane and organized actin filaments in stress fibers and cortical filaments (Fig. 1C), as seen in prior studies of epicardial mesothelial cells (Dokic and Dettman, 2006; Austin et al., 2008). Furthermore, all MEC1 cells were immunopositive for nuclear WT1 (see Fig. S1 in the supplementary material), a marker that is expressed in epicardial epithelium and in epicardial cells that undergo epithelial-mesenchymal transformation, but which is downregulated as these cells differentiate (Perez-Pomares et al., 2002; Martinez-Estrada et
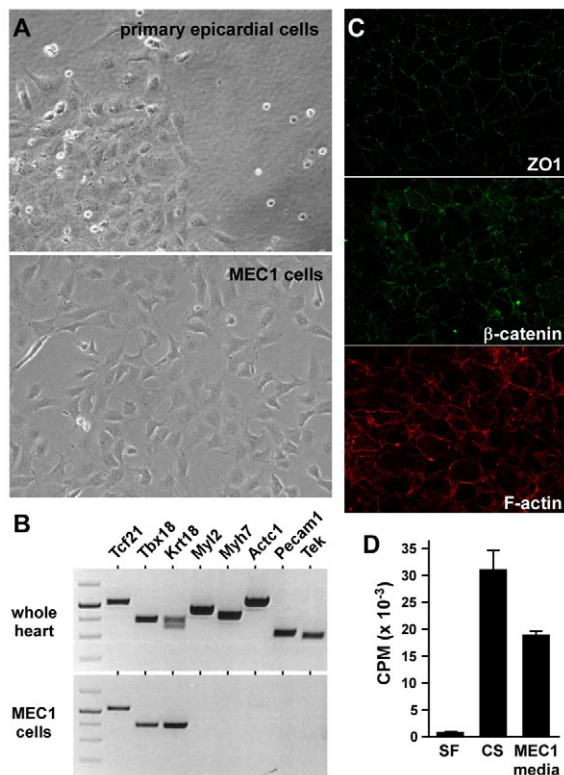
Fig. 1. Characterization of MEC1 cells. (**A**) Cell morphology. Shown are phase contrast photographs at the same magnification of primary epicardial cells (upper panel) growing out from an explanted piece of embryonic ventricle (not visible in this picture, which was taken at the edge of the outgrowth), and established MEC1 cells (lower panel). MEC1 cells have a typical cobblestone epicardial morphology. (**B**) Marker gene expression. cDNA was prepared from whole E11.5 embryonic heart (above) or from MEC1 cell RNA (below), and amplified for the indicated gene products. (**C**) MEC1 cells maintain an epithelial (mesothelial) organization. Immunofluorescence detection shows ZO-1 and β-catenin at the cell membrane in adherens junctions, and F-actin in stress fibers and cortical filaments. (**D**) Mitogenic activity. NIH3T3 cells were cultured in serum free media (SF), SF media with 10% calf serum (CS) as a positive control, or SF media conditioned by MEC1 cells (MEC1 media) in the presence of [^{3}H]thymidine. Error bars indicate s.d. CPM, counts per minute.

al., 2010). The MEC1 cell line has been passaged for many years without apparent loss of morphology or of marker gene expression and thus appears to be stably immortalized.

IGF2 is the major mitogen expressed in epicardial cells

One of the characteristic properties of epicardial cells is their secretion of mitogenic factors. We confirmed that the MEC1 cell line shares this feature (Fig. 1D). To identify the trophic agents produced by MEC1 cells, we first investigated, by a variety of means, the possible presence of a number of mitogens that have been previously implicated as epicardial factors, including PDGFs, EPO, FGFs, WNTs and SHH, but there was no indication that any of these candidates were present in MEC1 cell-conditioned media (data not shown). As an alternative approach, we used microarrays to determine the spectrum of genes expressed by these cells. This

profile reiterated that the above-mentioned candidates were not expressed at significant levels, and identified *Igf2* as the most abundantly expressed growth factor gene.

Next, we confirmed the presence of IGF2 in conditioned media from MEC1 cells. Western blot analysis with an anti-IGF2 antibody using total protein from serum-free MEC1 cell-conditioned media (Fig. 2A) visualized the 20 kDa and 11 kDa isoforms of IGF2 (Gowan et al., 1987; Zapf et al., 1992); this was verified by the almost-complete elimination of both bands when the MEC1 cells were treated first with an adenovirus expressing an anti-*Igf2* RNAi, but not when a control virus was used. By reference to known amounts of recombinant IGF2 (which is slightly smaller than the smaller of the two natural mouse proteins), we estimated the concentration of IGF2 (both isoforms together) in MEC1 cell-conditioned media under our typical conditions of preparation at ~100 ng/ml. This fairly high level of protein is consistent with the high level of RNA expression observed in the microarray survey. This is also consistent with the observation that IGFs are generally present at much higher levels in vivo than most other peptide hormones (Dupont and Holzenberger, 2003).

We took two complementary approaches to experimentally disrupting IGF2 expression in epicardial cells in culture, as a means of defining the relative contribution of IGF2 to the overall spectrum of mitogenic factors made by these cells. First, we used RNAi to knock down endogenous *Igf2* expression (Fig. 2A) and determined the extent to which mitogenic activity was reduced. In a mitogenic assay, the proliferative response to IGF2-deficient MEC1 cell-conditioned media was decreased by ~70%, although was not completely eliminated (Fig. 2B). Second, we used the inhibitor AG1024, a tyrosine mimic that selectively blocks IGF1R and INSR autophosphorylation and signaling but has no effect on many other receptor tyrosine kinases when used in the low micromolar range (Ohmichi et al., 1993; Parrizas et al., 1997). Using this compound, we achieved virtually complete inhibition of the mitogenic response induced by 100 ng/ml recombinant IGF2. However, when added to MEC1 cell-conditioned media, ~20% of the mitogenic activity remained (Fig. 2C). The significant reduction, but not complete elimination, of mitogenic activity by RNAi and by use of the AG1024 inhibitor suggests that IGF2 is the primary mitogen made by MEC1 cells, although additional mitogenic factors are expressed also.

To confirm the validity of these observations, we grew primary epicardial cells from mouse ventricular tissue explants, co-cultured with primary mouse embryonic ventricular cardiomyocytes, and measured cardiomyocyte proliferation (Fig. 2D). In the absence of epicardial cells, cardiomyocytes alone had a low proliferative index that was not affected by treatment with AG1024. Co-culture of cardiomyocytes with epicardial cells substantially increased the cardiomyocyte proliferative index, in a manner that was mostly (80%) eliminated in the presence of AG1024. In primary embryonic epicardial cells as in MEC1 epicardial cells, these results indicate that IGF is the most prominent secreted mitogen.

Igf2 is expressed in vivo in the epicardium during compact zone expansion

We examined the expression of the genes encoding the two IGF ligands and the two IGF receptors during mouse heart development. By in situ hybridization, *Igf2* was clearly expressed in the epicardium from the time of its formation at E10.5 (see Fig. S2 in the supplementary material) and throughout the E11.5-14.5 period (Fig. 3). The prominent expression of *Igf2* in MEC1 cells, therefore, is typical of midgestation epicardium from which this
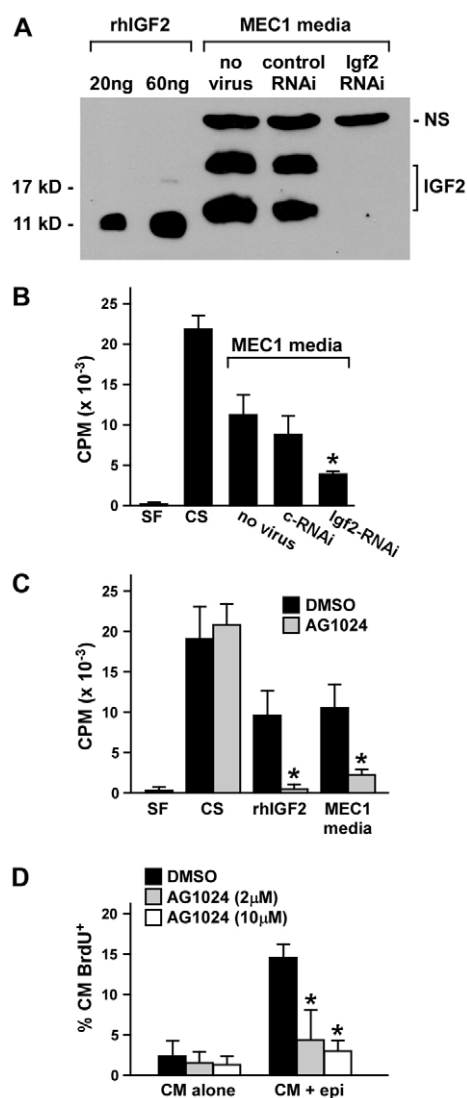
Fig. 2. MEC1 and primary epicardial cells secrete IGF2.
(**A**) Western blot. Serum-free media from MEC1 cells was TCA
precipitated and evaluated by western blotting. MEC1 cells were
treated with no adenovirus, with a control virus expressing an
irrelevant sequence (control RNAi), or with virus expressing an RNAi
specific for *Igf2*. Two specific bands for IGF2 (20 kDa and 11 kDa)
were detected, plus a 35 kDa nonspecific (NS) band. Recombinant
human IGF2 (rhIGF2) was used as a reference for size and quantity.
Sizes at left are the migration of standards. (**B**) Inhibition of *Igf2*
expression reduces mitogenic activity. NIH3T3 cells were grown in
serum-free media (SF), treated with 10% calf serum (CS) as a positive
control, or treated with serum-free media isolated from MEC1 cells
grown in the absence or presence of the indicated viruses.
Proliferation was measured by [^{3}H]thymidine incorporation.
(**C**) Inhibition of IGF signaling reduces mitogenic activity. NIH3T3 cells
were grown in the presence of solvent (DMSO) or the IGF receptor
inhibitor AG1024 (0.2 μM) in serum-free media (SF), in the presence
of 10% calf serum (CS) as a positive control, in the presence of 100
ng/ml recombinant human IGF2, or in serum free media from MEC1
cells. (**D**) Epicardium-cardiomyocyte co-culture. Embryonic ventricular
cardiomyocytes were cultured alone (CM alone) or co-cultured with
primary embryonic epicardial cells (CM + epi), and proliferation of
Nkx2.5$^+$ cardiomyocytes measured by BrdU incorporation in the
absence or presence of AG1024. For all graphs, asterisks indicate
statistically significant differences (*P*<0.05) relative to control
treatments and error bars indicate s.d. CPM, counts per minute.
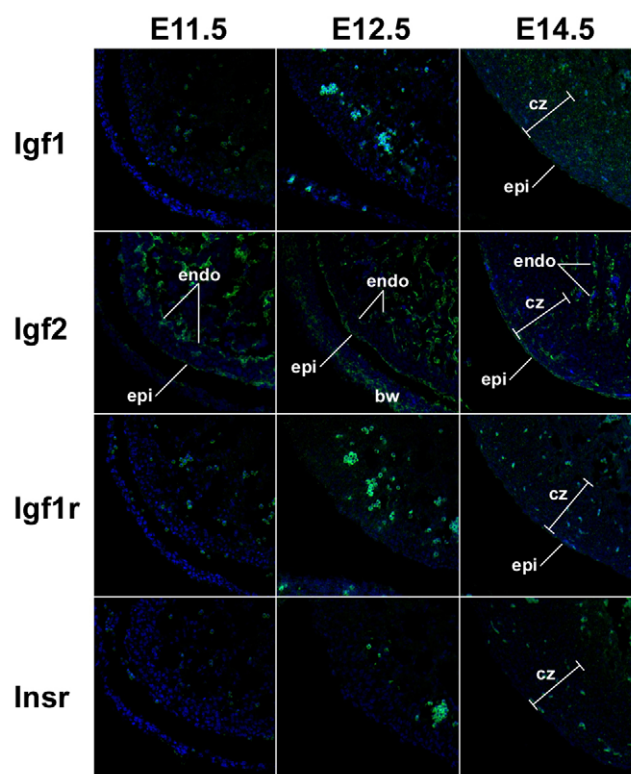
Fig. 3. Expression of IGF components in the developing heart
detected by fluorescence in situ hybridization. Fluorescent
riboprobes are shown in green, DAPI counterstaining is in blue.
Nonspecific red blood cell epifluorescence is seen with varying intensity
in all panels. bw, body wall; cz, compact zone; endo, endocardium; epi,
epicardium.

cell line was derived. *Igf2* was also expressed in the endocardium
at all examined stages of heart development (E9.5-14.5; Fig. 3; see
Fig. S2 in the supplementary material). With lengthy color
development, a low level of staining in the myocardium was
apparent (data not shown). By quantitative RNA analysis of
ventricular tissue over the E9.5-15.5 period, we found that the
normalized level of *Igf2* expression in the ventricle did not change
appreciably during this period (see Fig. S3 in the supplementary
material). Elsewhere in the embryo, the staining we observed was
consistent with published reports (Stylianopoulou et al., 1988;
Bondy et al., 1990; Murrell et al., 2001).

Igf1 expression was weak and marginally detectable in the heart
at E11.5 and E12.5, although expression was more evident by
E14.5 throughout the ventricular tissue, and clearly including the
epicardium (Fig. 3). Expression of *Igf1* in midgestation epicardium
was also noted in previous papers (Bondy et al., 1990; Fraidenraich
et al., 2004). Quantification of *Igf1* mRNA levels in ventricular
tissue indicated that *Igf1* message abundance increased slightly
(threefold) between E9.5 and E15.5 (see Fig. S3 in the
supplementary material), perhaps accounting for its easier detection
by in situ hybridization at later stages. Throughout the E11.5-14.5
period, *Igf1r* was expressed broadly, and perhaps ubiquitously, in
the heart, including the epicardium, and quantification revealed
very little change in overall abundance during the E9.5-15.5
interval. The *Insr* gene was expressed in ventricular tissue, and the
relative amount of *Insr* transcript increased threefold between E9.5
and E15.5 (see Fig. S3 in the supplementary material), although
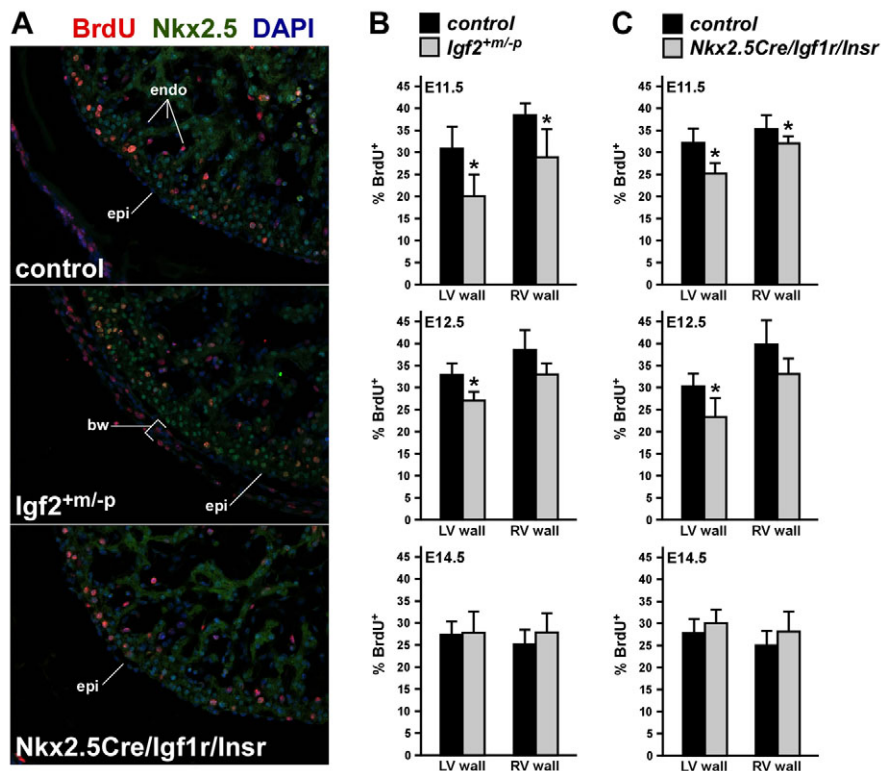
Fig. 4. Proliferative consequences of *Igf2* and IGF receptor deficiency. (**A**) Proliferation in the right ventricular wall in control, *Igf2*-null, and conditional IGF receptor-null embryos at E11.5, visualized by merged three-color fluorescence. Nkx2.5-positive cardiomyocytes appear green, BrdU-positive (proliferating) cardiomyocytes appear orange-yellow, proliferating epicardial or endocardial cells are red and nuclei are blue (DAPI). Individual single color panels are shown in Fig. S5 in the supplementary material. (**B,C**) Quantification of compact zone cardiomyocyte proliferation in control versus mutant embryos. *n*=4-5 embryos for each genotype at E11.5 and E12.5, and *n*=3 at E14.5. Asterisks indicate statistically significant differences (*P*<0.05) relative to control hearts. Error bars indicate s.d. LV, left ventricle; RV, right ventricle.

expression was not detectable by in situ hybridization even at E14.5. In these analyses, a control hybridization with a probe for the myocardium-specific *Mlc2v* (*Myl2* – Mouse Genome Informatics) gene showed the expected myocardial staining and no signal in the epicardium or endocardium (see Fig. S4 in the supplementary material).

A proliferative deficiency in heart development in *Igf2* deficient embryos

To address directly the role of IGF2 as a potential mitogen in embryonic heart development, we undertook a proliferation analysis in control and *Igf2*-null embryos. Because the *Igf2* gene is imprinted and only the paternal allele is expressed (DeChiara et al., 1991), our mating strategy was to cross heterozygous males with wild-type females, such that heterozygous embryos ($Igf2^{+m/-p}$) were functional nulls and were compared with littermate wild-type controls. Pregnant females were injected with BrdU and the embryos isolated 2 hours later for analysis.

Our results revealed a substantial and statistically significant decrease in ventricular wall cardiomyocyte proliferation in $Igf2^{+m/-p}$ embryos at E11.5 and to a somewhat lesser extent at E12.5 (Fig. 4A,B; see also Fig. S5 in the supplementary material). For the left and right trabecular myocardium and in the ventricular septum, proliferative differences between mutant and control were minimal and were not statistically significant (see Fig. S6 in the supplementary material). The overall morphology of the heart (shape, thickness of compact zone, size of ventricular septum, amount of trabeculae, etc.) in control and *Igf2* mutant embryos at E11.5 was indistinguishable (Fig. 4; data not shown). Note, however, that this developmental time point is only one day after formation of the epicardium and when compact zone expansion is just initiated, and the myocardial wall is still quite thin at this stage. By E12.5, the compact zone in *Igf2* mutants was noticeably underdeveloped generally, although not uniformly, throughout the

ventricular myocardium (see Fig. S7 in the supplementary material). Hypoplasia was also apparent in E14.5 embryos (Fig. 5). There was only a minimal level of apoptosis in $Igf2^{+m/-p}$ hearts, equivalent to control embryos (data not shown; see also below).

A developmental heart phenotype in IGF receptor mutants

The partial expansion of the compact zone in *Igf2* deficient embryos by E14.5 (Fig. 5) might be because of compensatory action of IGF1. Although both ligand-encoding genes have been mutated individually, the combined knockout of *Igf1* and *Igf2* together has not been reported. IGFs signal through IGF1R and INSR, two closely related members of the receptor tyrosine kinase family. Thus, mutation of both receptors together should eliminate all IGF signaling.

To address directly the involvement of IGF receptors in ventricular development, we conditionally mutated the *Igf1r* and *Insr* genes together in the heart. For this purpose, we used *Nkx2.5Cre* and *MLC2vCre*, which are active starting from ~E8.0 and ~E8.5, respectively (Chen et al., 1998; Moses et al., 2001). *MLC2vCre* activity is restricted to ventricular myocardium and is known to be incompletely inefficient in driving gene recombination (see Fig. S8 in the supplementary material). *Nkx2.5Cre* is active in ventricular and atrial myocardium and also in the endocardium and epicardium, and achieves very high efficiency recombination (see Fig. S8 in the supplementary material); several previous studies have confirmed that *Nkx2.5Cre* and certain other *Cre* drivers cause more severe heart phenotypes than does *MLC2vCre* when crossed to the same target genes (Shai et al., 2002; Hakim et al., 2007; Peng et al., 2008; Ieda et al., 2009).

Analysis of *MLC2vCre/Igf1r/Insr* conditional mutants at E14.5 revealed obvious, albeit variable, thinning of the ventricular wall, similar to *Igf2* nulls (Fig. 5). By contrast, a more severe and more widely distributed phenotype was observed in *Nkx2.5Cre*
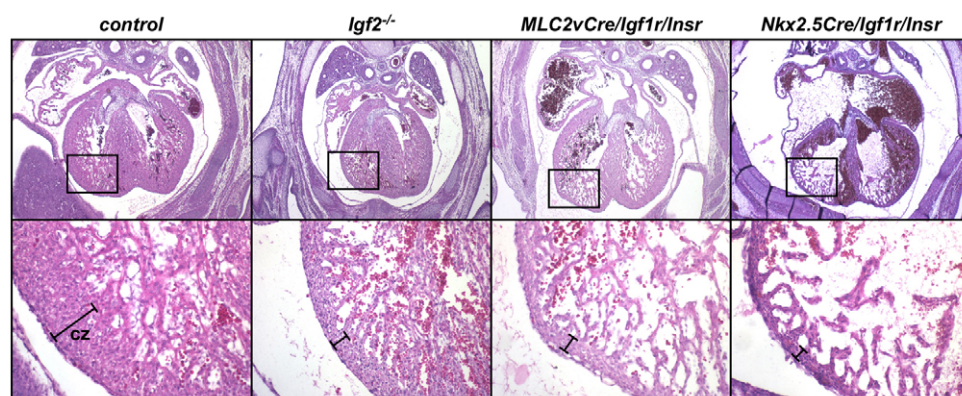
Fig. 5. Morphology of embryonic hearts at E14.5. Hematoxylin and Eosin stained sections of hearts of the indicated genotypes; the boxed regions in the low magnification views above are shown at higher magnification below. Corresponding photographs are at the same magnification. The compact zone (cz) is indicated by brackets.

conditional receptor mutants (Fig. 5). This phenotype was also more pronounced than in *Igf2* nulls, which suggests the involvement of IGF1 (or another ligand that can activate through these receptors) in supporting proliferation in the period prior to E14.5. Approximately half of E14.5 *Nkx2.5Cre/Igf1r/Insr* conditional mutant embryos had edema (see Fig. S9 in the supplementary material), which is often an indication of impaired cardiac function; this was only rarely seen in *Igf2*-null embryos (data not shown). Analysis of E11.5 and E12.5 *Nkx2.5Cre/Igf1r/Insr* conditional null embryos revealed a significant decrease in compact zone proliferation (Fig. 4A,C), with no difference in apoptosis between control and *Nkx2.5Cre* conditional receptor-null hearts (see Fig. S10 in the supplementary material). The depth of the compact zone was correspondingly diminished at E12.5 (see Fig. S7 in the supplementary material).

At E14.5, compact zone cardiomyocyte proliferation on a per cell basis was normal in global $Igf2^{+m/-p}$ nulls and in *Nkx2.5Cre/Igf1r/Insr* conditional nulls. In both cases, at E14.5 the compact zone was clearly diminished in cell number, presumably because of the decreased proliferative rate over the preceding few days. This implies that the dependence on IGF signaling to support compact zone cardiomyocyte proliferation is restricted to the E10.5-14.5 period. Between E13.5 and E14.5, coronary circulation is established (Viragh and Challice, 1981) and we suggest that proliferative factors other than IGF are brought to the myocardium via the coronary vasculature at this time to influence cardiomyocyte proliferation (see Discussion).

Epicardial IGF activates ERK signaling in heart development

Our results clearly confirm a requirement for IGF signaling in compact zone cardiomyocyte proliferation. Activation of mitogenic pathways generally results in the phosphorylation of ERK as well as many additional signaling intermediates, and activation of the ERK signal transduction pathway is essential in fetal cardiomyocytes in cell culture (and in most other cell types) for a proliferative response to mitogenic stimulation (Kang and Sucov, 2005). A previous analysis by another group evaluated the distribution of pERK in E10.5 mouse embryos subjected to short-term in vitro culture in the presence of FGF receptor inhibitors, and concluded that FGF signaling was responsible for almost all pERK in the embryo (Corson et al., 2003). Interestingly, one of the few exceptions where pERK staining was not abrogated by the FGFR inhibitor was the heart. This suggested that other ligands and receptors activate pathways that lead to phosphorylation of ERK in the developing heart, although no other receptor-specific inhibitors were utilized in that study. We repeated this same experimental

approach, examining levels of pERK in ventricular and limb tissue of E10.5-11.5 embryos in whole embryo culture by western blotting (Fig. 6). We confirmed that the FGFR inhibitor PD173074 had only limited efficacy in reducing pERK levels in the heart, although the drug was fully able to suppress pERK levels in the limb (and elsewhere; data not shown). By contrast, treatment with the IGFR inhibitor AG1024 resulted in a substantial decrease in pERK levels in the heart, with no apparent effect in the limb. AG1024 treatment reduced, but did not completely eliminate, pERK levels, implying the presence of additional factors in the developing heart that cause the phosphorylation of ERK. The combination of PD173074 and AG1024 together did not eliminate
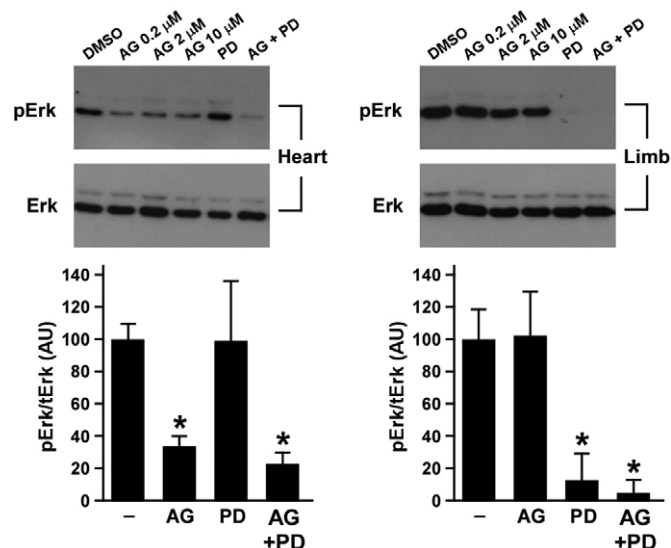
Fig. 6. Inhibition of phosphorylation of ERK in short-term in vitro cultured embryos. E11.5 wild-type embryos were cultured in the presence of solvent (DMSO), the IGFR inhibitor AG1024 (at three different concentrations), the FGFR inhibitor PD173074 (500 nM), or a combination of AG1024 (10 μM) plus PD173074 (500 nM). Protein extracts from the heart (left) or forelimb (right) were analyzed by western blotting for phosphorylated ERK (pErk; above) or total ERK (tErk; below). At the bottom, results from several experiments were combined graphically. The ratio is expressed in arbitrary units (AU) and does not reflect the absolute percentage of pERK; the signal for DMSO-treated (–, negative control) samples was defined as 100 for comparison between experiments. Asterisks indicate statistically significant differences ($P<0.05$) for heart samples relative to DMSO and to PD173074 treated; for limb samples relative to DMSO and to AG1024 treated. Error bars indicate s.d.

pERK levels, suggesting that FGF signals do not account for the residual activity. It was not possible to undertake this experimental approach with older embryos, which did not survive in culture (presumably because of their larger size). At least for the E10.5-11.5 period, these observations indicate that activation of the ERK pathway in the developing heart is primarily driven by IGF signaling.

We repeated the whole embryo culture assay using E9.5 embryos. IGF2 is expressed in the endocardium at E9.5 (see Fig. S2 in the supplementary material), but the heart at E9.5 consists only of myocardium and endocardium, with no epicardium yet formed on the outer surface. In E9.5 embryos, before the epicardium has formed, AG1024 treatment had no effect on pERK levels (see Fig. S11A in the supplementary material). This is in contrast to the consequence of similar treatment of E10.5-11.5 embryos, in which AG1024 caused a pronounced decrease in pERK levels (Fig. 6), and indicates that mitogenic activity in the heart at E9.5 is not dependent on IGF signaling.

Finally, we used E9.5 ventricular tissue in an explant assay, cultured alone or co-cultured with primary epicardial cells, and measured pERK levels. As in the intact embryo (see Fig. S11A in the supplementary material), E9.5 ventricular tissue explanted into culture had a basal level of pERK that was not impacted by treatment with AG1024 (see Fig. S11B in the supplementary material). By contrast, in the presence of epicardial cells, there was a significant increase in pERK in the ventricular explant that was blocked by AG1024 treatment. The combination of E9.5 ventricular tissue with epicardial cells essentially recreates the organization and interaction of the three cell types seen in the heart from E10.5 onward, and furthermore demonstrates that the E9.5 tissue has the competence to respond to IGF. We conclude that the amount or type of IGF2 that is made by the endocardium (at least at E9.5) does not account for a significant level of mitogenic signaling, and that epicardial IGF induces mitogenic signaling and cardiomyocyte proliferation in the ventricle.

DISCUSSION

In mouse heart development starting around E10.5, the ventricular myocardium undergoes a significant expansion and becomes partitioned into trabecular and compact zones. It is generally thought that trabecular morphogenesis occurs in response to factors secreted from the endocardium, and that compact zone morphogenesis occurs in response to factors secreted from the epicardium (for a review, see Sucov et al., 2009). Both transformations require cardiomyocyte proliferation, coupled to the establishment of a trabecular or compact tissue organization, respectively. We have suggested (Kang and Sucov, 2005) that endocardial and epicardial signaling pathways converge in cardiomyocytes to activate a common set of intracellular mediators of proliferation, including ERK and many other mitogenic components that are also involved in cell proliferation in most cell types. By contrast, the endocardial and epicardial signals must also diverge to achieve the different morphological characteristics of the trabecular and compact myocardium. Although this model is conceptually appealing, the definition of the specific factors that promote proliferation and/or morphological differentiation has been uncertain.

One of the factors considered to be an epicardial mitogen is FGF9, possibly also in association with FGF16. Mutation of either gene caused a thin myocardium phenotype and, similarly, conditional mutation of *Fgfr1* and *Fgfr2* (genes encoding FGF receptors) together in the myocardium resulted in a thin-walled ventricle (Lavine et al., 2005; Lu et al., 2008). FGF2 is prominently expressed in the embryonic heart, although mutation of *Fgf2* alone has no impact on heart development, and does not further enhance the heart phenotype when combined with *Fgf9* deficiency (Lavine et al., 2005). At E10.5, *Fgf9* and *Fgf16* are both expressed in the epicardium and endocardium, but at E12.5 both genes are expressed only in the endocardium. Furthermore, as noted above, inhibition of FGF signaling at E10.5 and E11.5 did not reduce pERK levels in the heart, and FGFs do not account for much, if any, of the mitogenic activity in MEC1 and primary epicardial cells. Thus, the role of FGFs as candidate epicardial factors is uncertain. FGF signaling in the myocardium also regulates coronary vasculogenesis via the expression of several angiogenic factors (Lavine et al., 2006), and this might contribute to, or even explain, the ventricular wall phenotype seen in these mutant backgrounds.

Our studies reported in this paper document the crucial role of IGF signaling in heart development. IGF2 is the most prominent mitogen made by epicardial cells, and compact zone formation and compact zone cardiomyocyte proliferation were clearly compromised in both *Igf2* ligand deficient and *Igf1r/Insr* conditional receptor deficient embryos. Similarly, using phosphorylation of ERK as a marker of mitogenic signaling, we showed for E10.5-11.5 embryos that IGF is a major mitogenic signal in vivo (Fig. 6). The crucial IGF in this latter assay is most likely to be IGF2, as *Igf1* is not expressed at high levels in the heart at this time (Fig. 3). Supporting this conclusion, the phenotypic consequences and the suppression of compact zone cardiomyocyte proliferation were comparable in *Igf2*-deficient and receptor-deficient embryos at E11.5-12.5. We presume that *Igf1* function contributes to ventricular development at stages past E12.5, because conditional mutation of the *Igf1r* and *Insr* receptor genes together caused a more severe phenotype in the heart at E14.5 than was seen in the absence of IGF2 alone, and *Igf1* expression in the heart increases at E13.5-14.5 (Fig. 3; see Fig. S3 in the supplementary material). Indeed, based on the consequences on general embryo growth rates, IGF2 is thought to be more important earlier in development, with IGF1 assuming a progressively more important role from midgestation onwards (Baker et al., 1993; Louvi et al., 1997). Insulin is another possible ligand, but is not thought to be important in embryonic development until immediately prior to birth (Louvi et al., 1997).

Our studies also suggest that additional mitogens are likely to be expressed in the embryonic epicardium, although with a less significant role in compact zone formation. MEC1 cells express other secreted signaling molecules (at the RNA level) in addition to *Igf2*, although we have not yet assessed their quantitative contribution or their developmental importance. These additional epicardial factors are presumably responsible for the residual mitogenic activity in epicardial cell-conditioned media when IGF signaling was blocked (Fig. 2). Similarly, these factors might be responsible for the residual pERK signal seen when IGF and FGF signaling were both blocked in embryos (Fig. 6), although endocardial factors could also explain this in vivo observation.

Multiple observations implicate IGF2 as a true epicardial mitogen: it is expressed in the epicardium during midgestation heart development, it is the most active mitogen made in MEC1 cells and in primary ventricular embryonic epicardial cells, and our direct observations (Fig. 2D; see Fig. S11 in the supplementary material) show that epicardial-derived IGF induces mitogenic signaling and cardiomyocyte proliferation. Nonetheless, in addition to its epicardial expression, *Igf2* is also expressed in the

endocardium throughout the E9.5-14.5 period, and this source of IGF might, in principle, also be involved in heart development. Our studies indicate that this is unlikely, at least to a significant extent. Thus, ventricular tissue isolated prior to the formation of the epicardium did not show any impact of inhibition of IGF signaling, even though *Igf2* is expressed in the endocardium and the myocardium is competent to respond to IGF at this stage. In *Igf2* nulls and in conditional receptor nulls, cardiomyocyte proliferation was selectively compromised in the compact zone but did not change in the trabecular myocardium (Fig. 4; see Fig. S6 in the supplementary material), which is suggestive of specific disruption of an epicardial-myocardial signaling axis. Furthermore, embryos lacking the epicardium show a diminished compact zone (Kwee et al., 1995; Gittenberger-de Groot et al., 2000; Manner et al., 2005), demonstrating the importance of the epicardium and the inability of the endocardium to support compact zone growth. From our studies, it is not obvious whether there is any role for endocardial *Igf2* expression, either alone or in synergy with the validated endocardial factor neuregulin as previously proposed (Hertig et al., 1999). There is no endocardial cell line, analogous to MEC1 epicardial cells, with which to undertake experiments comparable to those in this study. A definitive assignment of *Igf2* as functioning genetically in the epicardium and the resolution of the role of endocardial *Igf2* expression will require tissue-specific gene disruption, which is not yet possible to undertake.

In *Igf2*-null and conditional receptor-null embryos, the magnitude of the reduction in compact zone cardiomyocyte proliferation varied between the left and right chambers and with embryo age. The reduction was greatest (>30%) in the left ventricle at E11.5, but was not as severe in the right ventricle and at later stages. Even a modest reduction in proliferation can have a significant consequence. In one study (Pennisi et al., 2003) in which interference with epicardium formation in chick embryos led to a clearly evident ventricular wall phenotype, there was only an 11% reduction in proliferation rate as measured by BrdU incorporation; however, clonal analysis revealed the loss of one cycle of cell division, and those authors emphasized that one fewer round of cell division over a period of a few days would result in a 50% reduction in compact zone thickness. In experimental models with severe compact zone thinning, the extent to which compact zone proliferation is decreased does not exceed 50% (Kastner et al., 1997; Bushdid et al., 2003) and, in more typical cases, still with dramatic compact zone hypoplasia, reductions in the range of 30-45% were seen (Lavine et al., 2005; Zamora et al., 2007; Lavine et al., 2008; Nanka et al., 2008; Peng et al., 2008). IGF signaling deficiency has a phenotypic impact of this magnitude (Fig. 5), although it does not lead to the extreme hypoplasia seen in some models. The additional epicardial mitogenic activity mentioned above might explain why a larger impact on proliferation was not observed when IGF signaling was eliminated.

Compact zone cardiomyocyte proliferation is compromised in many mouse mutant backgrounds, including those that interfere with retinoic acid (RA) and erythropoietin (EPO) signaling (Kastner et al., 1994; Sucov et al., 1994; Wu et al., 1999). RA induces *Epo* expression in the fetal liver but not in the fetal heart (Makita et al., 2001; Brade et al., 2011). We recently showed that EPO induces *Igf2* expression in MEC1 cells and in the fetal epicardium, and proposed that EPO made in the liver in response to RA is the primary inducer of epicardial *Igf2* expression (Brade et al., 2011). Other genes that also impact compact zone formation might intersect with this pathway.

During the E10.5-14.5 period, the ventricular wall grows from approximately two cell diameters to approximately 15 cell diameters in thickness. Coronary vasculogenesis occurs during this same interval, and becomes coupled to systemic circulation at ~E14 (Viragh and Challice, 1981). We speculate that passive diffusion of factors, including IGFs, from the epicardium to the myocardium becomes limiting around this same time, and that the imposition of coronary circulation supports further cardiomyocyte cell division in a manner that does not depend on diffusible factors from the epicardium. Indeed, a recent analysis of β1-integrin mutant mice (Ieda et al., 2009) supports the concept of a different (non-epicardial) mechanism that controls ventricular cardiomyocyte proliferation starting at ~E14. Based on our observation that compact zone cardiomyocyte proliferation in conditional IGF receptor mutants is compromised at E11.5 and E12.5 but is restored to normal at E14.5, we infer that the relevant factors that support heart growth after E14 are not IGFs. This model would explain the observation that *Igf1r/Insr* global double mutant embryos show pronounced edema at E14.5 that was no longer apparent by E18.5 (Louvi et al., 1997). Although heart morphology was not specifically examined in that study, edema is often a sign of impaired cardiac function, and was seen frequently in E14.5 *Nkx2.5Cre/Igf1r/Insr* mutants (see Fig. S9 in the supplementary material). Our results demonstrate the important role for IGF in supporting cardiomyocyte proliferation during the crucial period of compact zone formation prior to E14 and the establishment of coronary circulation.

Acknowledgements

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.054338/-/DC1

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