Extracardiac control of embryonic cardiomyocyte proliferation and ventricular wall expansion

Hua Shen1, Susana Cavallero1, Kristine D. Estrada1, Ionel Sandovici2,3, S. Ram Kumar4, Takako Makita5, Ching-Ling Lien5, Miguel Constancia2,3, and Henry M. Sucov1*

1Broad Center for Regenerative Medicine and Stem Cell Research, University of Southern California, 1425 San Pablo Street, BCC-511, Los Angeles, CA 90033, USA; 2MRC Metabolic Diseases Unit, Department of Obstetrics and Gynaecology and NIHR Cambridge Biomedical Research Centre, University of Cambridge Metabolic Research Laboratories, Cambridge, UK; 3Centre for Trophoblast Research, University of Cambridge, Cambridge, UK; 4Department of Surgery, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA; and 5Saban Research Institute, Children’s Hospital Los Angeles, Los Angeles, CA, USA

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1. Introduction

The midgestation period of mouse development, approximately between embryonic days E10 and 14, is a time of dramatic morphogenic change in many organ systems, including the heart. The mouse heart at E9.5 has already completed the specification and positioning of all four chambers, inflow and outflow tracts, and valves, but all domains are still quite rudimentary. By E14, the heart is essentially morphologically mature, with thickened ventricular walls, fully formed atrioventricular valves, septated outflow tract, and a functional coronary circulation. Further growth of the heart occurs after E14, but this is primarily enlargement with little change in morphogenic organization.

The ventricle wall, which is a primary focus of this study, accounts for the force of cardiac ejection. The ventricular wall at mouse E9.5 consists of a thin layer (1–2 cell diameters) of myocardium. By E14, the ventricle wall has expanded to 10–15 cell diameters and is often called the compact zone because of its tight organization. The importance of proper formation of the ventricular wall for midgestation embryonic

growth is evident in the number of mouse gene mutations which compromise this process and which result in midgestation embryo lethality.1

The epicardium, which is the outer mesothelial layer of the heart, migrates onto the surface of the myocardium during the E9.5–10.5 period.2,3 Thus, the formation of the epicardium is coincident with the onset of midgestation ventricular wall expansion. Many observations, including the underdeveloped compact zone that results from genetic2,4 or surgical5,6 elimination of the epicardium, have shown that the epicardium is in fact required for ventricular wall cardiomyocyte proliferation. We showed in co-culture assays that epicardial cells secrete a soluble activity that induces embryonic cardiomyocyte proliferation7 and recently identified the primary mitogenic factor made by mouse embryonic epicardial cells as IGF2.8 We further showed that mouse embryos globally lacking IGF2, and those conditionally lacking the IGF receptors IGF1R and IR in the heart, suffer from a midgestation deficiency in ventricular wall cardiomyocyte proliferation and a corresponding deficiency in compact zone morphogenesis. Using in vitro co-culture assays, we showed that blocking IGF signalling with a selective receptor antagonist

Corresponding author. Tel: +1 323 442 2563, Email: sucov@usc.edu
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prevented the induction of cardiomyocyte proliferation by epicardial cells. These results are consistent with IGF2 being an epicardial mitogen in vivo during midgestation heart development, although we did not have the genetic tools at that time with which to confirm this model.

Many signalling pathways potentially regulate or intersect with epicardial mitogenic activity. We have long been interested in retinoic acid (RA), which functions via a heterodimeric nuclear receptor transcription factor consisting of one RAR and one RXR. Three separate genes encode distinct RAR isoforms, and likewise, three genes encode RXR isoforms. Mouse mutants globally lacking RXRα show a dramatically underdeveloped compact zone and die around E14.5. An additional phenotype present in Rara−/− embryos is a transiently underdeveloped liver. We showed that the liver phenotype of Rara−/− embryos is the consequence of a delay in the expansion and differentiation of definitive erythroid progenitors that reside in the liver at this time. This process is controlled by the cytokine erythropoietin (EPO), and we showed that liver Epo expression was greatly reduced, albeit transiently, in Rara−/− embryos. We also showed that the Epo gene is a direct transcriptional target of RA signalling. The molecular explanation for the transient nature of the liver phenotype in Rara−/− embryos is a transition in Epo expression that is initiated by the onset of placental function.

In addition to their erythropoietic phenotype, Epo and Epo receptor (EpoR) global mutants die around E14 with an underdeveloped heart ventricular wall. The similar heart phenotypes seen in mouse mutants lacking EPO, EPOR, or RXRα, and the observation that the Epo gene is a direct downstream transcriptional target of RA signalling, suggested the possibility that compromised EPO signalling might be an explanation for the heart phenotype of Rara mutants. The EpoR gene is expressed in the epicardium and endocardium, but the Epo ligand gene is not expressed in the heart. We proposed that EPO from the midgestation liver might be an upstream regulator of epicardial IGF2 expression, although this model was based primarily on in vitro assays and was not evaluated by in vivo genetic manipulations.

Using a battery of genetic reagents, in this study, we demonstrate the epicardial function of IGF2 in supporting ventricular wall formation and directly demonstrate the importance of EPO signalling in the epicardium to support epicardial IGF2 expression. However, the role of EPO in supporting epicardial IGF2 expression is transient. We show that midgestation initiation of placental function supplements the requirement for EPO signalling in epicardial IGF2 expression, just as it also supplements control of liver Epo expression by RA signalling. Importantly, the effects of placental function on heart growth occur through a regulated programme of epicardial IGF2 gene expression and not simply by provision of growth substrates directly to the myocardium.

2. Methods

2.1 Mice

All mouse lines have previously been described: Nkx2.5Cre, Mesp1Cre, Tbx18Cre, Tcre, Myh6Cre, Tie2Cre, Rara403, conditional IGF2, conditional EpoR, and conditional R26R. In all experiments, mutant embryos were compared with littermate wild-type controls. Noon of the day of observation of a mating plug was defined as E0.5. Pregnant female mice were anaesthetized by isoflurane inhalation and euthanized by cervical dislocation prior to isolation of embryos or placental tissue. Animal procedures were reviewed and approved by the USC IACUC board (protocol 10080) and were followed in accordance with institutional guidelines.

2.2 Morphometric heart analysis

E14.5 embryos were fixed overnight in 4% PFA, permeabilized in PBS with 0.5% NP-40, dehydrated in a methanol series, incubated in methanol/hydrogen peroxide, rehydrated, and blocked in PBSST (PBS containing 5% BSA and 0.1% Triton X-100). The primary antibody was rat anti-mouse PECAm (BD, 1:100). Biotinylated goat anti-rat IgG (Santa Cruz, 1:500) was used followed by streptavidin-peroxidase complex (Vectastain ABC kit, Vector). Antibody and ABC reagent incubations were carried out in PBSST at 4°C overnight. Following overnight incubations, hearts were washed five times (1 h each at 4°C) with PBSST. Immunoreactivity was visualized with DAB substrate (Invitrogen).

2.3 Whole-mount PECAm immunohistochemistry

Digoxigenin (DIG)-labelled probes were made as described previously. Briefly, embryos or cultured thorax segments were cryopreserved in 30% sucrose, embedded in OCT, and then cryosectioned transversely at 10 μm. Control and mutant sections were placed on the same slides to ensure identical experimental conditions. Sections were fixed in 4% PFA in PBS, rehydrated, and treated with proteinase K and then triethanolamine in acetic anhydride. Hybridization was performed at 65°C for at least 16 h. Unhybridized probe was removed by RNaseA digestion. Signal was detected by POD-coupled anti-DIG primary antibody (Roche) and TSAplus Fluorescent Substrate Kit (PerkinElmer).

2.4 In situ hybridization

Wild-type embryos from an ICR background, or littermate TCre/Rara403 and control embryos, were dissected. The thorax section of each embryo was isolated by removal of the head and abdominal region (including the diaphragm) using dissecting forceps and transferred into DMEM medium containing 1% BSA with varying amounts of glucose in 12-well culture dishes. Tissue was incubated with shaking at 37°C for 1.5 h, then rinsed quickly with PBS and fixed with 4% PFA, and embedded in OCT for cryosectioning.

2.5 Thorax culture

Mouse MEC1 cells were grown in DMEM containing high glucose with 10% FBS. After reaching 80% confluence, cells were seeded into 6-well culture dishes and were cultured to near confluence. Cells were then cultured in high (25 mM glucose; Gibco) or no glucose (Gibco 10% FBS. After reaching 80% confluence, cells were seeded into 6-well culture dishes and were cultured to near confluence. Cells were then cultured in high (25 mM glucose; Gibco 11 965) or no glucose (Gibco 11 966) media, or a mixture of the two to yield intermediate glucose concentrations, with 1% BSA for 24 h. For hypoxic cultures, media were first sparged with 94% N2;5% CO2;1% O2 gas, and plated cells were incubated in the same atmosphere in a sealed container in the incubator.

2.6 Cell culture

Mouse MEC1 cells were grown in DMEM containing high glucose with 10% FBS. After reaching 80% confluence, cells were seeded into 6-well culture dishes and were cultured to near confluence. Cells were then cultured in high (25 mM glucose; Gibco 11 965) or no glucose (Gibco 11 966) media, or a mixture of the two to yield intermediate glucose concentrations, with 1% BSA for 24 h. For hypoxic cultures, media were first sparged with 94% N2;5% CO2;1% O2 gas, and plated cells were incubated in the same atmosphere in a sealed container in the incubator.

2.7 Polymerase chain reaction

RNA from MEC1 cells was isolated using RNA Mini Kit (Ambion). Equal amounts of RNA were used to synthesize cDNA using M-MLV reverse transcriptase (Invitrogen). The following primer sequences were used to
amplify Igf2 cDNA fragments: 5′-GGCCTCGCTCTTGCTGCA TC-3′ and 5′-GGATCCAGATCAAGGTGCGAG-3′. 18S
classic II (Ambion) and primers for beta-actin were used for the internal
control. Q-PCR was performed in iQ SYBR Green Supermix (Bio-Rad)
using LightCycler 480 (Roche). The primers used for Igf2 Q-PCR were
as follows: 5′-CCCTCAGCAAGTGCCTAAAG-3′ and 5′-TTAGGG TGCCCTGAGATGTT-3′. Quantification was normalized to GAPDH
expression.

2.8 X-gal staining
Embryos or placentas were fixed with 2% PFA, cryopreserved, and
embossed in OCT. Ten micrometre cryosections were stained with X-gal
at 37°C overnight. For whole-mount staining, the samples were stained
immediately after fixation.

2.9 Placenta IF
E13.5 placentas were fixed with 4% paraformaldehyde in PBS at 4°C
overnight. After cryoprotection in 10% and 30% sucrose, the tissue
was embedded and frozen in OCT. Eight micrometre sections were
used for IF. Slides were briefly fixed with 4% PFA, washed with PBS,
and permeabilized in 0.1% Triton X-100 at room temperature for
30 min. After blocking with 1× casein solution (Vector Labs SP-5020)
at room temperature for 1 h, sections were incubated in rabbit
anti-GFP (Invitrogen A6455; 1:500) and rat anti-mouse CD31 (BD Phar-
mingen 550274; 1:100) in PBS containing 1% BSA and 10% donkey
serum at 4°C overnight. Secondary antibodies donkey anti-rabbit (Invi-
rogen Alexa Fluor 488) and donkey anti-rat (Invitrogen Alexa Fluor
594) were used to detect GFP and CD31 with 45 min incubation at
room temperature. Slides were mounted with mounting media contain-
ing DAPI.

2.10 Glucose measurement
Embryos were dissected in PBS on ice, then quickly frozen in liquid nitro-
gen, and kept at −80°C until use. Embryos of the appropriate genotypes
were freeze-thawed three times to promote lysis, and then homo-
geinizd in 450 μl of glucose assay buffer (BioVision). Eight microlitres of
lysat per sample was used for glucose measurement, using a glucose
colormetric assay kit (BioVision K606) and following the manufacturer’s
recommended protocol. A standard curve was made using a reference
solution provided in the kit. Total embryo protein was determined with the
Bio-Rad reagent. Calculation of whole embryo glucose concentra-
tions used the glucose/embryo measurements and embryo volumes
of 25 μl at E10.5, 110 μl at E12.5, and 265 μl at E14.5 as measured
directly and consistent with previously reported measurements. 26,27
These total embryo volumes do not take into account excluded volume,
so free glucose concentrations are likely to be somewhat higher than
calculated.

3. Results

3.1 Epicardial IGF2 controls ventricular wall expansion
We previously reported that global deficiency of Igf2 resulted in dimin-
ished ventricular cardiomyocyte proliferation and a poorly developed
ventricular wall. 8 Igf2 is expressed in epicardium and endocardium
(Supplementary material online, Figure S1), may be expressed at a very
low level in the myocardium, 28 and is expressed in a number of other
embryonic and extraembryonic tissues outside of the heart. 29 To genet-
ically define the tissue source of IGF2 in heart development, we com-
ponent a conditional Igf2 allele with a number of Cre drivers. Nkx2.5Cre
drives highly efficient recombination in all three primary mesodermal
cell types of the heart (epicardium, myocardium, and endocardium),
but it is active in only a very small number of non-cardiac tissues. 16
We replicated the global Igf2 null heart phenotype in Nkx2.5Cre/Igf2
d conditional mutants (Figure 1), implying that Igf2 function in heart de-
velopment is intrinsic to the heart. The same phenotype was observed using
Tbx18Cre, which is active in the epicardium, 16 but not when using
Tie2Cre, which is active in endocardium and endothelium, 21 nor with
Myh6Cre, which is active only in the myocardium 20 (Figure 1). Our
results and measurements confirm the epicardium as the primary
source of IGFR2 that supports ventricular wall formation. If the endocar-
dium or myocardium have any additional contributions to this process,
these must be very small incremental roles.

The coronary vasculature initially forms in the subepicardial space, 30
and it is theoretically possible that epicardial IGFR2 might support
heart development through promotion of coronary vasculogenesis.
However, whole-mount PECAM1 staining at E14.5 demonstrated a
normal distribution and degree of maturation of coronary vasculature
in Nkx2.5Cre/Igf2 and Tbx18Cre/Igf2 mutants (Supplementary material
online, Figure S2), that these vessels are functional is implied by the
viability of Igf2- and IGFR2 receptor-deficient mutant embryos in the
late gestation period. 8,31

3.2 Extracardiac RA signalling regulates
epicardial Igf2 expression
Embryos compromised in IGF or RA signalling have a similar ventricular
wall phenotype, suggesting a possible relation between these pathways.
Epicardial Igf2 expression is substantially reduced in Rara global mutant
embryos (Supplementary material online, Figure S1A) and in embryos
globally lacking expression of the RA synthetic enzyme RALDH2; 15
the elimination of this domain of expression in Rara or Raldh2 mutant
embryos is consistent with epicardial IGFR2 serving as a downstream
effector of RA signalling in heart development. Igf2 is expressed normally
in the endocardium of Rara−/- mutants (Supplementary material online,
Figure S1A), which implies a different mode of regulation of this gene in
the endocardium vs. epicardium.

We used several Cre lines combined with a conditional dominant
negative RA receptor allele (designated Rara403) 22 to address the
site(s) of RA signalling relevant to epicardial Igf2 expression and heart
development. Mesp1Cre 17 is highly active in all mesoderm of the heart
and throughout mesoderm in the anterior region of the embryo, although
it is inefficient in mesoderm of the liver and of the caudal half
of the embryo; Brachyury (T)-Cre 19 has mostly a reciprocal pattern of
recombination efficiency (including efficient liver mesoderm activity)
and is inefficient in the heart (Supplementary material online, Figure
S3). We observed normal ventricular chamber morphology in
Rara403 embryos combined with Mesp1 Cre or with Nkx2.5Cre (Supple-
mentary material online, Figure S4), but all TCre/Rara403 embryos at
E14.5 displayed peripheral oedema (a sign of impaired heart function)
and an underdeveloped ventricular wall (Figure 2A and B). Furthermore,
epicardial but not endocardial Igf2 expression was abolished in TCre/
Rara403 embryos at all examined midgestation time points (Figure 2C),
although the epicardium was intact (Supplementary material online,
Figure S5). The combination of Mesp1 Cre with Rara403 results in highly
penetrant outflow tract and arch artery phenotypes, 35,33 both of

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which involve RA signalling processes earlier in development than the formation of the epicardium, implying that the absence of a ventricular phenotype cannot be trivially explained on the basis of timing or recombination efficiency. Thus, the sites of RA signalling relevant to ventricular wall formation are external to the heart and to the Mesp1Cre domain, and lie within the TCre domain.

Figure 1  Epicardial IGF2 promotes ventricular wall morphogenesis. Shown are H&E stained sections of control and conditional Igf2 mutant E14.5 hearts, with the boxed regions shown at higher magnification. Scale bars = 100 μm. Brackets and cz, compact zone. Panels below show quantification of right and left ventricular wall thickness in E14.5 Igf2 conditional mutant embryos (mean ± SEM). *P < 0.0002; ns, not significant (P > 0.05) using an unpaired two-tailed t-test. The numbers of conditional mutant embryos examined were as follows: Nkx2.5Cre/Igf2 (10 from 5 litters); Tbx18Cre/Igf2 (8 from 4 litters); Tie2Cre/Igf2 (11 from 3 litters); Myh6Cre/Igf2 (6 from 2 litters).

Figure 2  Phenotype following abolition of RA signalling in the TCre domain. (A) Whole mount views of control and TCre/Rara403 littermate E14.5 embryos; note oedema (arrows) in the conditional mutant. (B) Morphology of the heart at E14.5; note hypoplasia of the ventricular wall in the conditional mutant. (C) Igf2 expression by fISH; note absence of epicardial (but persistence of endocardial) Igf2 expression in the conditional mutant at all midgestation time points. (D) Restoration of epicardial Igf2 expression in E12.5 TCre/Rara403 mutants in thorax culture in the presence of 5 mM glucose. n ≥ 3 for both genotypes for histology and n ≥ 3 for all in situ hybridization analyses.
3.3 EPO signalling transiently regulates epicardial Igf2 expression

Epo and EpoR global nulls are compromised in ventricular wall development. To test whether this phenotype is related to the epicardium and to epicardial Igf2 expression, we combined a conditional EpoR allele with Nkx2.5Cre to eliminate EPO function in all mesoderm of the heart and with Tbx18Cre to eliminate EPO function in the epicardium. Neither manipulation had any impact on the presence or morphology of the epicardium, but in both cases, epicardial Igf2 expression at E10.5–11.5 was reduced to near undetectable levels (Figure 3; Supplementary material online, Figure S6). Endocardial Igf2 expression was not affected in either case. Subject to the assumption that EPO function in the epicardium mediates EPO signals, these observations directly demonstrate the involvement of EPO signalling in promoting epicardial Igf2 expression at these time points. Epo is not expressed in the heart, and a prominent site of expression during the E10.0–11.5 period is the liver. In principle, EPO protein from the liver (or elsewhere in the abdominal cavity) could come directly into contact with the epicardium by transport through large openings in the incompletely formed diaphragm (the pericardioperitoneal canals) that are present through E11.5 and which close around E12.5 (see also Discussion).

Although epicardial Igf2 expression was compromised in Nkx2.5Cre/EpoR and Tbx18Cre/EpoR mutants at E10.5–11.5, we noted restoration of expression in both mutant backgrounds at E12.5 and continuing through E14.5 (Figure 3; Supplementary material online, Figure S6). Consistent with this observation, both Nkx2.5Cre/EpoR and Tbx18Cre/EpoR mutants had normal heart morphology by E14.5 (Supplementary material online, Figure S7). Thus, while epicardial Igf2 expression from E10 to 14 is required for normal ventricular morphogenesis, EPO signalling only transiently controls epicardial Igf2 expression and is not required for the ultimate formation of the ventricular wall. The developmental time during which EPO regulates epicardial Igf2 expression (from the time of epicardium formation at E10 up to E11.5) is also the time during which Epo is expressed at high levels in the fetal liver in response to RA, and when the pericardioperitoneal canals are open.

3.4 A transition to placental control of epicardial Igf2 expression and heart morphogenesis

The delayed onset but ultimately normal level of epicardial Igf2 expression in Nkx2.5Cre/EpoR and Tbx18Cre/EpoR mutants implies that a different mode of regulation of epicardial Igf2 expression becomes established starting between E11.5 and 12.5. Importantly, though, this new mode of regulation must still be dependent on RA signalling, because epicardial Igf2 expression through E14.5 and ventricular morphology at E14.5 are both compromised in Rara mutant backgrounds (see also Supplementary material online, Figure S1A) and Tcre/Rara043 (Figure 2) mutants. The transition in control of epicardial Igf2 expression at E11.5–12.5 is coincident with the onset of placental function. Placental organization, specifically of the labyrinthine layer that is the major site of exchange between maternal and fetal blood, was previously noted to be compromised in Rara -/- mutants. We observed a substantially diminished labyrinthine layer in Tcre/Rara043 mutants (Figure 4A; Supplementary material online, Figure S8), similar to that previously reported for Rara global mutants. Tcre is active extensively in endothelial cells of the labyrinthine layer of the placenta, and in no other cell type present in appreciable quantity (Figure 4B), implying that the endothelial cell lineage is a likely target of RA signalling in the placenta.

Because glucose transport is one of the primary functions of the placenta, we measured embryonic glucose levels to confirm the impairment of placental function in Tcre/Rara043 mutants (Figure 4C). Approximate whole embryo glucose concentrations calculated from these numbers (see Methods) for wild-type embryos at E10.5, E12.5, and E14.5 are <50 μM, 0.5 mM, and 1 mM, respectively. In Tcre/Rara043 mutants, we observed a 40% decrease in whole embryo glucose at E12.5, and more than two-fold decrease at E14.5, confirming deficient placental function.

We reasoned that glucose or other materials transported from the placenta to the midgestation embryo might regulate epicardial Igf2 expression and heart development after E11.5, and their deficiency might explain the heart phenotypes of Tcre/Rara043 mutants. We used in vitro embryonic thorax cultures to model this response (Figure 5A). Epicardial Igf2 expression in cultured wild-type thorax...
Figure 4 Placental phenotype in TCre/Rara403 mutants. (A) H&E sections of placentas reveal a smaller labyrinthine (L) layer in the TCre/Rara403 mutant. Scale bars = 500 μm. Higher magnification views of these sections are in Supplementary material online, Figure S8. n ≥ 3 for both genotypes at all time points. (B) Endothelial recombination in E13.5 placentas from embryos bearing paternally inherited TCre and the R26-YFP conditional reporter. Most cells are co-labelled with Pecam1; white arrowheads point to rare Pecam1−, GFP+ cells. Recombination occurred also in rare non-endothelial placental cells (blue arrowheads), the identity of which is unknown. S, spongiotrophoblast layer. Scale bars = 500 μm (upper) and 100 μm (lower). n = 2. (C) Measurement of midgestation whole embryo glucose levels, on a per embryo basis or normalized to recovered protein. *P = 0.024; **P = 0.008; ns, not significant, using an unpaired two-tailed t-test. The number of embryos used for each determination was, for control embryos, 11 at E10.5, 13 at E12.5, and 10 at E14.5; for TCre/Rara403 embryos, 5 at E10.5, 9 at E12.5, and 6 at E14.5.

Figure 5 Placental substrates control epicardial Igf2 expression. (A) Epicardial Igf2 expression in normoxic thorax culture is glucose responsive; detected by ISH. Scale bars = 50 μm. n ≥ 3 for all time points and conditions. (B and C) Igf2 expression in MEC1 epicardial cells is glucose responsive. Quantitative PCR analyses of glucose response of Igf2 expression in MEC1 cells are shown in B’ and C’; n = 3 for all samples. Asterisk in B’: P < 0.04 for all glucose-treated sample compared with no glucose; asterisk in C’: P < 0.02 for 5 mM glucose compared with no glucose and for 25 mM glucose compared with 5 mM glucose (unpaired two-tailed t-test). (D) Glucose-induced Igf2 expression in MEC1 epicardial cells requires normoxia. (E) Glucose induces pErk activation in ventricular tissue in thorax culture; detected by Western blotting. n = 2 for each condition.
segments was undetectable in no glucose conditions, induced in the presence of glucose in the physiological range of the midgestation embryo (see above), and further induced in high glucose conditions. In all cases, endocardial Igf2 expression was not impacted. Glucose restored epicardial Igf2 expression in TCre/Rara403 thorax cultures (Figure 2D), supporting the conclusion that abolished epicardial Igf2 expression and heart defects in this mutant in vivo (Figure 2B and C) are the indirect result of impaired placental function. In MEC1 mouse embryonic ventricular epicardial cells, Igf2 expression was minimal in the absence of glucose and increased with increasing glucose (Figure 3B and C), demonstrating that glucose directly induces epicardial Igf2 expression. Glucose did not induce Igf2 expression under hypoxic conditions (Figure 5D), implying that both placental substrates support epicardial Igf2 expression. Finally, in ventricular tissue isolated from thorax cultures, the proliferation intermediate phosphoErk was activated by increasing glucose levels (Figure 5E), consistent with placental glucose-induced activation of a proliferative response in the myocardium.

Our studies support an overall model (Figure 6) in which epicardial Igf2 expression is controlled independently in two distinct phases, first by EPO from E10 to E11.5 and then by glucose and oxygen (and perhaps also additional materials) from the placenta. Both processes involve signals from a distant tissue acting on the epicardium, which then produces IGF2 that acts locally to induce cardiomyocyte proliferation and ventricular wall growth.

4. Discussion

Our prior results indicated the importance of IGF2 signalling in ventricular wall morphogenesis. Through tissue-specific Igf2 gene disruption, we confirm that the epicardium is the source of this mitogen.

The unexpected realization, made possible because of our genetic approach, is that ventricular growth during midgestation mouse development occurs in two independent phases (Figure 6). The E10–11.5 pre-placental phase occurs in the context of hypoxia and very low glucose, and employs EPO signalling via EPOR to regulate epicardial Igf2 expression. Epo is not expressed in the heart, and a prominent site of expression in the E10–11.5 period is the fetal liver, where it is expressed at high level under the transcriptional control of RA and hypoxia. EPO can reach the epicardium via coelomic fluid that moves easily through the pericardioperitoneal canals. With the onset of placental function around E11.5, Epo expression in the liver diminishes greatly, and glucose and oxygen (and perhaps additional factors) reach the heart through circulation and maintain epicardial Igf2 expression, presumably by diffusion from the heart lumen. Unlike large proteins that do not diffuse deeply into tissues and must be presented directly to responding cells, small molecules like glucose and oxygen diffuse over tens of cell layers. The specific intracellular mechanisms by which glucose and oxygen support Igf2 expression in the epicardium are unknown but are under ongoing investigation. According to this understanding, the severe heart phenotype seen in Epo and EpoR mutants is explained by the initial loss of liver-epicardium EPO signalling followed by persistent hypoxia associated with the absence of definitive erythropoiesis. The similar heart phenotype in Rraa−/− and TCre/Rara403 mutants is explained by the initial loss of liver-epicardium EPO signalling followed by persistent hypoxia and reduced glucose associated with impaired placental function.

A large number of gene mutations in mice result in compromised placental development. In severe cases, these cause early or midgestation embryo lethality, whereas less severe placental impairment is associated with general embryo growth deficiency in late gestation. The placental phenotype of TCre/Rara403 mutants seems to fall somewhere in the middle of this spectrum, and as we show in this study, this is associated with impaired ventricular wall formation. A partially underdeveloped ventricular wall may have been present in some other mutant lines with intermediate placental severity and simply not recognized. Alternatively, epicardial Igf2 expression and heart morphogenesis might require the combined activity of glucose and oxygen, plus an additional placental product that is specifically absent in TCre/Rara403 mutants, such that placental defects in other mutants might not be associated with the same impairment of heart development.

While epicardial IGF2 signalling to the myocardium occurs at close proximity across the subepicardial space, the factors that control epicardial Igf2 expression reach the heart from a distance and thus qualify as endocrine signals. Endocrine interactions between organs at a distance are the norm in adult physiology but have not previously been recognized in heart development and are rarely employed in the embryo; for this reason, almost all developmental studies focus on local signalling events between adjacent and nearby tissues. Even placental function is generally thought to involve the passive provision of growth substrates...
rather than regulating a morphogenic programme of gene expression, such as occurs in the epicardium to influence heart growth. Our observations demonstrate that the embryo does indeed employ endocrine signalling mechanisms to facilitate development and specifically in heart morphogenesis.

The onset of placental function around E11.5 explains the transition in control of epicardial Igf2 expression and heart growth. Several other developmental transitions occur at the same time, including the dramatic reduction in liver Epo expression, maturation of hepatoblasts to hepatocytes, and closure of the pericardio-peritoneal canals (completion of the diaphragm). Liver Epo expression is clearly also regulated by the onset of placental function, and we speculate that other developmental events at this same time may also be responsive to placental activity, perhaps also via glucose and oxygen acting as developmental endocrine signals. This might serve to co-ordinate the timing of distinct processes in multiple organs, including the heart, during midgestation mouse development.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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