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Proliferation via a Retinoic Acid-Inducible Trophic Factor

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Mouse embryos lacking the retinoic acid receptor RXRα properly undergo the early steps of heart development, but then fail to initiate a proliferative expansion of cardiomyocytes that normally results in the formation of the compact zone of the ventricular chamber wall. RXR$\alpha^{-/-}$ embryos have a hypoplastic ventricular chamber and die in midgestation from cardiac insufficiency. In this study, we have investigated the underlying mechanistic basis of this phenotype. We find that interference with retinoic acid receptor function in the epicardium of transgenic embryos recapitulates the hypoplastic phenotype of RXRα deficient embryos. We further show that wild type primary epicardial cells, and an established epicardial cell line (EMC cells), secrete trophic protein factors into conditioned media that stimulate thymidine incorporation in primary fetal cardiomyocytes, and thymidine incorporation, cell cycle progression, and induction of cyclin D1 and E activity in NIH3T3 cells. In contrast, primary epicardial cells derived from RXR$\alpha^{-/-}$ embryos and an EMC subline constitutively expressing a dominant negative receptor construct both fail to secrete activity into conditioned media. The production of trophic factors is induced by retinoic acid treatment and is inhibited by a retinoid receptor antagonist. Fetal atrial and ventricular myocytes both respond to epicardial-derived trophic signaling, although postnatal cardiomyocytes are nonresponsive. We therefore propose that the fetal epicardium, in response to retinoic acid and in a manner requiring the activity of RXRα, secretes trophic factors which drive fetal cardiomyocyte proliferation and promote ventricular chamber morphogenesis.

INTRODUCTION

Heart development, as for most aspects of organogenesis, requires the concerted interaction between cells of different lineage origin in order to promote the elaboration of complex structures. At least four independent primary cell types—myocardium, endocardium and endothelium, epicardium, and neural crest—contribute to the developing heart and are responsible for its morphogenesis. The myocardial and endocardial lineages are present and distinct even before the actual formation of the heart from splanchnic mesoderm (Cohen-Gould and Mikawa, 1996), which in mouse embryos, occurs at embryonic day 7.5 (E7.5). The neural crest cell lineage migrates into the heart around E9.5 and is restricted to populating the outflow region of the heart (Jiang *et al.*, 2000). The epicardial lineage originates from a region adjacent to the septum transversum, and migrates over the outer surface to ultimately (by E9.5–E10.5) surround the heart (Viragh and Challice, 1981). Subsequent interactions between these primary cell layers are responsible for the derivation of additional cardiac cell types [e.g., mesenchymal cells of the endocardial cushions are derived from the endocardium (Markwald *et al.*, 1977), and mesenchymal cells, which constitute the endothelium and smooth muscle of the coronary arteries, as well as the cardiac fibroblast lineage, are derived from the epicardium (Mikawa, 1999)].

The fetal ventricular chamber at E9.5 is a thin-walled and undivided structure; formation of a mature ventricular

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chamber requires two distinct proliferative and morphogenic processes, both of which initiate around E9.5–E10.5. Proliferation of the myocardium on the inner (endocardial) side of the ventricular chamber wall and the alignment of these cells into bundles forms the trabecular layer of the myocardium. Proliferation of the myocardium on the outer (epicardial) side of the chamber wall, and the alignment of these cells along the circumferential axis of the heart, forms the compact zone of the ventricular chamber wall. Enhanced compact zone proliferation and ingression of cardiomyocytes at the ventricular sulcus leads to formation of the interventricular septum, which divides the ventricular chamber into right and left sides. It has been suggested that the trabecular layer accounts for a greater percentage of the contractile force of the early heart (prior to approx. E12.5), although thereafter, the rapidly expanding compact layer accounts for an increasingly greater proportion of contractile strength (Sedmera and Thomas, 1996). This inference is borne out by mouse mutants which selectively affect either process individually, such that defects in trabecular formation result in embryo lethality around E10.5, whereas defects in compact zone formation result in lethality around E14.5–E16.5 (Sucov, 1998).

The signals which mediate these morphogenic processes are of obvious interest. Retinoic acid, the bioactive form of vitamin A, is known to be involved in compact zone formation, as rat embryos deficient in vitamin A (Wilson and Warkany, 1949) and mouse embryos lacking the retinoic acid receptor gene RXRα (Sucov *et al.*, 1994; Kastner *et al.*, 1994) both suffer from an inability to properly form the compact zone. This results in a thin-walled hypoplastic ventricular chamber with a poorly formed interventricular septum that leads to midgestation lethality. Direct counting of mitotic cells has demonstrated that this represents a proliferative defect, and there is no indication that elevated cell death contributes to this phenotype (Kastner *et al.*, 1997). Although this is ultimately a phenotype of the myocardium, a number of approaches have demonstrated that the myocardium does not utilize RXRα to respond to retinoic acid. Thus, tissue-specific knockout of the RXRα gene in the myocardium results in a normally formed heart (Chen *et al.*, 1998), individual cardiomyocytes lacking RXRα in chimeric embryos proliferate normally (Tran and Sucov, 1998), and transgenic expression of RXRα specifically in the myocardium does not rescue the defects of RXR$\alpha^{-/-}$ hearts (Subbarayan *et al.*, 2000). Consequently, the processes that are initiated by retinoic acid, and which result in cardiomyocyte proliferation and formation of the ventricular chamber compact zone, are inferred to be indirect.

A reasonable model is that another tissue first responds to retinoid signaling through RXRα, and then in a second step, influences myocardial proliferation. Candidate tissues in the heart for the site of RXRα action include the endocardium, the epicardium, and the neural crest, as well as derivatives of these primary cell types. In principle, a tissue or process external to the heart might also be responsible for the RXR$\alpha^{-/-}$ cardiac phenotype. RXRα is ubiquitously expressed during early and midgestation (Dolle *et al.*, 1994; Mangelsdorf *et al.*, 1992), providing no insight into its site of action.

In this study, we have further investigated the underlying basis of the RXR$\alpha^{-/-}$ phenotype. We show that the epicardium, in response to retinoic acid and in a manner which requires RXRα, secretes trophic factors which stimulate cardiomyocyte proliferation and compact zone morphogenesis.

MATERIALS AND METHODS

Conditional and Chimeric Mouse Embryos

Embryos bearing the Wnt1-cre transgene (Jiang *et al.*, 2000) and homozygous for the conditional RXRα allele (Chen *et al.*, 1998) were isolated at E14.5 or later for histological assessment of cardiac morphogenesis. To create chimeric embryos, a previously described RXR$\alpha^{-/-}$ ES cell line (Tran and Sucov, 1998) was introduced by aggregation into recipient blastocysts derived by crosses of Flk1$^{-/+}$ parents. Embryos were isolated at E14.5, and the genotype of the recipient blastocyst was identified retrospectively by genotype determination of the visceral endoderm (Hogan *et al.*, 1994).

Generation and Analysis of Transgenic Mice

The dominant negative human RARα clone used in these studies (Saitou *et al.*, 1995) carries a point mutation (G303E) that is homologous to a point mutation in the thyroid hormone receptor that causes generalized thyroid hormone resistance. The human keratin 18 clone (Thorey *et al.*, 1993) used for transgenic studies (GenBank Accession No. AF179904) contains 2.5 kb of 5′ sequence, the entire transcribed region of the gene, and 3.5 kb of sequence 3′ to the polyadenylation site. The endogenous ATG site of the K18 clone was altered by mutagenesis into a *Eag*I site, and the RAR303E sequence, containing a consensus translation initiation sequence, was cloned into that site. Transgenic embryos were generated by pronuclear injection of excised K18-RAR303E DNA and were isolated at E14.5 for analysis. Genotyping was performed by PCR amplification using DNA extracted from yolk sac tissue.

Epicardial Cell Culture and Conditioned Media

To derive primary epicardial cells, fetal and neonatal hearts were dissected, cut coarsely into four to eight pieces, and plated onto gelatin-coated dishes in DMEM/15%FBS. Epicardial cells migrate away and form a monolayer surrounding the remaining cardiac tissue, which after 4 days, was removed using forceps. The epicardial cells were further cultured in DMEM/10%FBS, and trypsinized for further passaging. Primary cells were expanded by passaging at least four times without alteration in epithelial morphology. Cell purity was assessed at single cell level by using antibodies against pan-cytokeratin (Sigma, cat #2562), sarcomeric myosin (MF20), and PECAM (M-20, Santa Cruz Biotechnology). For RT-PCR evaluation, RNA was extracted from coarsely cut whole heart tissue or from passaged primary epicardial cells. Following random-primed reverse transcription, PCR amplification of the following segments of GenBank entries was done by using standard conditions: 139-626 of NM_009022.1 (RALDH2), 456-847 of M11686 (K18), 215-551 of

AF047418 (epicardin), 460-996 of NM_009608 (cardiac actin), 84-545 of XM_132324 (MLC2v), 275-701 of AY056464 (MHCβ), 1132-1489 of L06039 (PECAM1), and 2188-2490 of X71426.1 (Tie2).

The RAR303E construct described above was cloned into the plasmid pcDNA3 (Invitrogen), where its expression is driven by the CMV promoter. This construct, and a control lacking the insert, were stably transfected into EMC cells (Eid *et al.*, 1992) by calcium phosphate followed by selection in 250 μg/ml G418 for 2 weeks. Stable colonies were individually picked and expanded. The dominant negative subline EMCdn was chosen for use on the basis of expression of the full-length RAR303E sequence.

To prepare conditioned media, cells were grown in the presence of serum to near confluency, and then switched to DMEM alone for 1 day. The media was then replaced with fresh DMEM, retinoids [all-trans retinoic acid at 10^{-6} M; the retinoid receptor antagonist AGN193109 (Agarwal *et al.*, 1996) at 10^{-7} M (kindly provided by R. Chandraratna, Allergan Pharmaceuticals)] or solvent were added, and the cells were cultured 2 more days before the conditioned media was collected. Heat treatment of conditioned media was at 65°C for 30 min.

Cardiomyocyte Proliferation Assay

Ventricular tissue was isolated from either E13.5 mouse or E7 chick embryos, or at other stages as indicated in the text. Following serial enzymatic digestion with collagenase (0.5 mg/ml for mouse; 0.44 mg/ml for chick) and pancreatin (1 mg/ml for mouse; 0.44 mg/ml for chick), the cell suspension was diluted with ADS buffer (1 × ADS buffer = 116 mM NaCl, 20 mM Hepes, pH 7.3, 1 mM NaH_2PO_4, 5.36 mM KCl, 0.83 mM $MgSO_4$, 0.1% dextrose), and layered on top of a Percoll step gradient. The bottom layer of this gradient contained a 4:1 ratio of Percoll stock [90% Percoll (Amersham-Pharmacia) + 10% 10× ADS buffer]: 1 × ADS buffer; the top layer contained a 9:11 ratio for E12.5–E15.5 mouse cells, a 19:21 ratio for E16.5–E18.5 mouse cells, a 1:1 ratio for cells from newborn to 3-day-old mouse pups, and a 17:23 ratio for chick heart cells. The step gradients were spun at 3000 rpm for 30 min, and cardiomyocytes were collected at the interface above the bottom layer. Using this method, we were able to routinely obtain cardiomyocytes with >98% purity (assessed by MF20 immunostaining). After purification, cardiomyocytes were plated on gelatin-coated 48-well dishes in plating media (4:1 DMEM/M199 + 5% FBS + 10% horse serum). After 24 h, the media was replaced with serum-free DMEM, and the cells were cultured for another 24 h. Thereafter, the media was replaced with fresh DMEM or with serum-free conditioned media, and 0.5 μCi [^{3}H]thymidine was added per well. When used, genistein was added to cardiomyocyte cultures at a concentration of 30 μM. After 48 h of incorporation, cells were washed three times with ice-cold PBS, fixed with ice-cold 10% TCA, and lysed in 1 N NaOH. Cell lysates were pH neutralized with 1 N acetic acid and transferred into scintillation vials to count radioactivity. A background subtraction (generally 50–100 cpm) was calculated for each experiment, measured by adding [^{3}H]-thymidine to cells cultured in DMEM alone 5 min before extraction. All data points represent at least duplicate samples when mouse cardiomyocytes were used, and at least quadruplicate samples when chick cardiomyocytes or NIH3T3 cells were used.

NIH3T3 Cell Culture

NIH3T3 cells were grown in DMEM/10% FBS, then switched to DMEM alone for 24 hr before replacement of media with fresh DMEM or with epicardial cell-conditioned media. Thymidine incorporation was exactly as above. For assessment of cell cycle by flow cytometry, cells were isolated by trypsinization, washed, and fixed overnight in 70% ethanol. Fixed cells were washed, and incubated with 100 μg/ml RNase and 20 μg/ml propidium iodide for 30 min at room temperature in the dark. For each sample, 10^4 cells were analyzed on an Epics XL/MCL flow cytometer (Beckman Coulter).

Cyclin Immunodetection Assays

Cells were washed three times with PBS and solubilized by rocking at 4°C for 30 min in lysis buffer (20 mM Tris, pH 8.0, 1% NP40, 137 mM NaCl, 10 mM EDTA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1 mM PMSF, 2 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_4$, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Following microcentrifugation for 20 min at 15,000*g*, the supernatant was assayed for protein content. For Western blotting, cell lysates were resolved by 10% SDS-PAGE (Coomassie-stained gels of parallel aliquots confirmed equal protein loading), followed by electrophoretic transfer onto Immun-Blot PVDF membranes (Bio-Rad). The membranes were then probed with anti-cyclin D1 (1:2000) or anti-cyclin E (1:2000) antibodies (Santa Cruz Biotechnology). After secondary incubation with an HRP-conjugated antibody, the signal was detected by using enhanced chemiluminescence (ECL) reagents.

RESULTS

As described above, the hypoplastic ventricular chamber phenotype seen in RXR$\alpha^{-/-}$ embryos does not represent a role of RXRα in the myocardium. To explore the potential involvement of RXRα in the endocardial and endothelial cell lineages, we employed chimera technology. We have previously demonstrated that ES cells lacking RXRα, when introduced into wild type recipient blastocysts, can contribute to all cell types of the developing heart in the resulting chimeric embryos, including the endothelium (Tran and Sucov, 1998). In contrast, other studies have shown that absence of the Flk1 gene prevents formation of the endothelial and endocardial lineages and causes embryo lethality at E9.5. This is a cell-autonomous phenotype, since only Flk1-positive cells can form endothelium in chimeric embryos (Shalaby *et al.*, 1997). We therefore introduced RXR$\alpha^{-/-}$ ES cells into blastocysts derived from crosses of Flk1$^{-/+}$ parents. We examined all chimeric embryos at E14.5 and recovered two embryos in which the RXR$\alpha^{-/-}$ ES cells had been introduced into Flk1$^{-/-}$ blastocysts (as determined by retrospective genotyping). In such chimeric embryos, the endothelial and endocardial cells must be derived from the RXR$\alpha^{-/-}$ ES cells. Both embryos were viable and normal upon isolation, and histology did not reveal any obvious deficiency in ventricular chamber morphogenesis (data not shown). To address the possible function of RXRα in the neural crest lineage, we crossed a conditional ("floxed") allele of RXRα (Chen *et al.*, 1998) against the Wnt1-cre transgene, which we have previously demonstrated drives highly efficient recombination in the neural

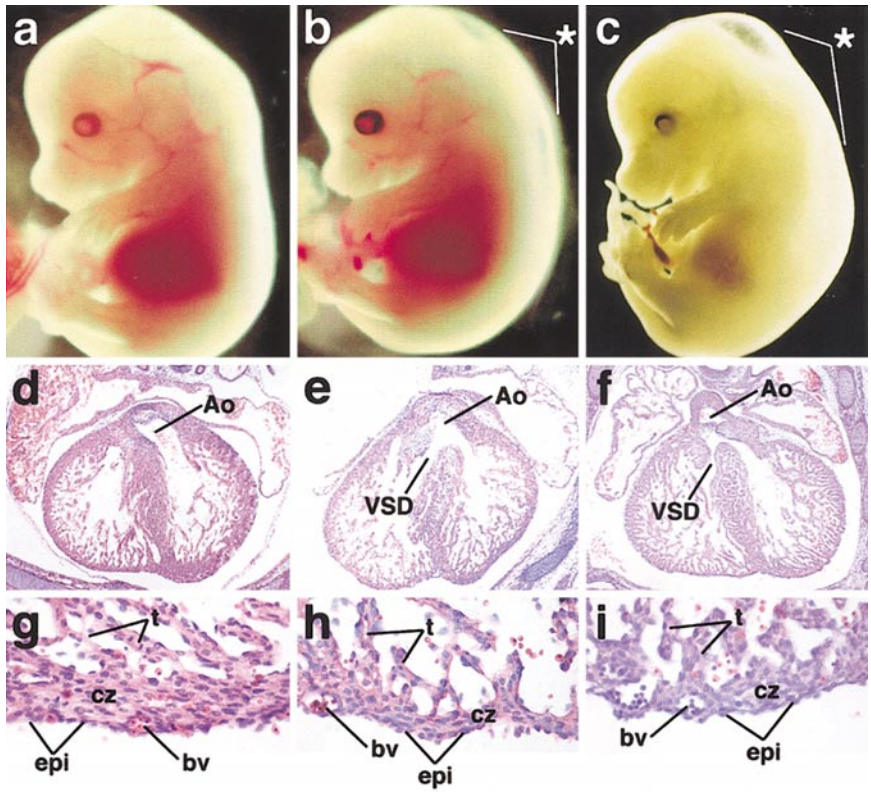

FIG. 1. Transgenic expression of K18-RAR303E recapitulates the RXR$\alpha^{-/-}$ cardiac phenotype. Shown are images of embryos and sections of a nontransgenic embryo (a, d, g), a K18-RAR303E transgenic (b, e, h), and a conventional RXRα-deficient embryo (c, f, i). The nontransgenic and transgenic embryos are littermates; all embryos were isolated at E14.5. (a–c) Whole-mount views of isolated embryos. Note the peripheral edema (indicated by an asterisk) in the transgenic (b) and knockout (c) embryos, which is suggestive of cardiac insufficiency; note also the presence of an eye defect in knockout embryos (Kastner *et al.,* 1994), which is not replicated by K18-RAR303E transgenic embryos. (d–f) Sections through the heart, taken at a level close to the aortic (Ao) valve. Note in both transgenic and knockout embryos the hypoplastic ventricular chamber wall, poorly formed ventricular septum, and interventricular septal defect (VSD). (g–i) High magnification views of the right ventricular chamber wall (same magnification in each panel). Note the thinner compact zone (cz) of the transgenic and mutant embryos, and the normal presence of forming coronary artery blood vessels (bv). Epi, epicardium; t, trabecular myocardium.

crest lineage beginning from the time that these cells are derived from the dorsal neural tube (Jiang *et al.,* 2000). In all of several [Wnt1-cre, RXR$\alpha^{f1/f1}$] embryos isolated at E14.5 or later, ventricular chamber morphogenesis was normal, and these embryos were viable at least to full term (data not shown; see also Jiang *et al.,* 2002). Thus, when the entire neural crest cell or endothelial cell lineage of an embryo is constituted by cells deficient in RXRα, normal ventricular morphogenesis still ensues.

We then turned our attention to the epicardial cell lineage. The epicardium (although not epicardial-derived mesenchymal cells) is positive for expression of EndoB (the mouse form of human keratin 18) *in vivo,* and a human keratin 18 (K18) promoter/enhancer construct has previously been shown to be expressed in the epicardium of transgenic mouse embryos, as well as in other K18-positive tissues outside of the heart (Thorey *et al.,* 1993). We used this promoter to drive expression of a dominant negative RAR construct (RAR303E), which has been previously used to induce phenotypes in skin and skeletal tissue when expressed by appropriate promoters in transgenic embryos (Saitou *et al.,* 1995; Yamaguchi *et al.,* 1998). Fertilized eggs were injected with the K18-RAR303E construct, implanted into recipient females, and the embryos then isolated at E14.5. Of 21 recovered embryos, 6 were visibly edematous upon isolation (Fig. 1), and only these 6 embryos were positive for the transgene construct upon genotyping. Peripheral edema is a typical sign of fetal cardiac insufficiency, and is seen in RXR$\alpha^{-/-}$ embryos at the same stage (Fig. 1c). Histology of these transgenic embryos revealed a ventricular chamber hypoplastic phenotype (thin compact zone, and poorly formed ventricular septum) that strikingly replicated what is seen in RXR$\alpha^{-/-}$ embryos (Fig. 1), whereas all nontransgenic littermate embryos exhibited normal ventricular morphology. In transgenic embryos, as in conventional RXR$\alpha^{-/-}$ embryos, the epicardial-derived

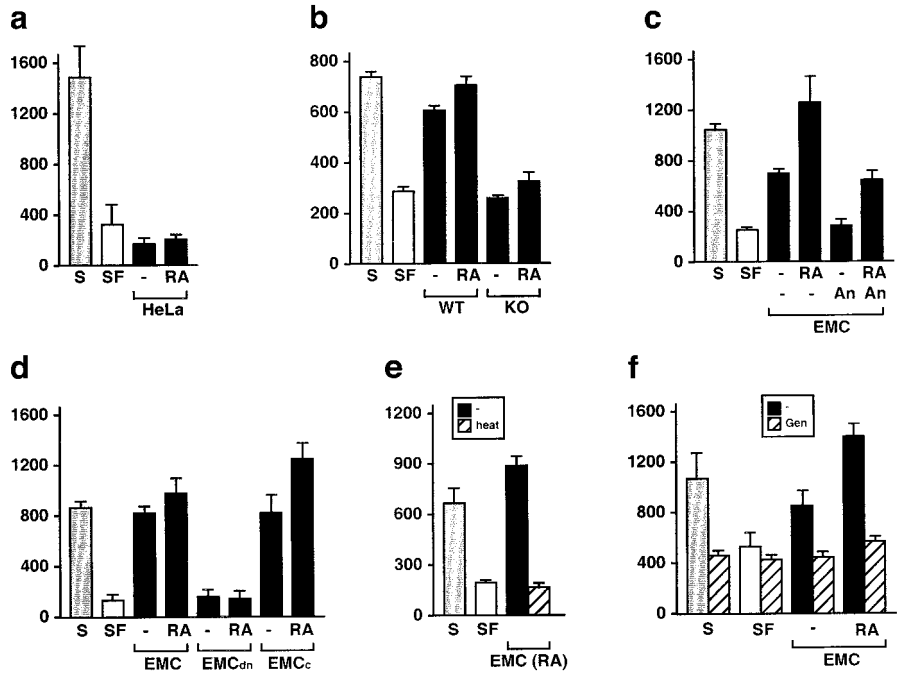

FIG. 2. Retinoic acid- and retinoid receptor-dependent induction of cardiomyocyte trophic activity in epicardial cells. All panels represent results of tritiated thymidine incorporation in cultured chick cardiomyocytes exposed to serum-free DMEM media (SF), DMEM plus 10% FBS (S), or to serum-free conditioned media as indicated. All data points were done in quadruplicate, and the ordinate axis for all panels represents CPM incorporated. (a) Establishment of baseline conditions for the assay. HeLa cells, whether untreated (−) or retinoic acid treated (RA; at 10^{-6} M), produce no activity in conditioned media that is trophic for cardiomyocytes. (b) Positive activity from wildtype (WT) but not RXR$\alpha^{-/-}$ (KO) primary epicardial cells. Wild type cells express a relatively high basal level of activity in the absence of treatment, whereas RXRα-deficient epicardial cells express little or no activity even following RA treatment. (c) Positive activity from EMC cells is RA-inducible and is suppressed by exposure to the retinoid receptor antagonist AGN193109 (An; 10^{-} M). (d) Normal expression of activity in parental epicardial cells (EMC) and in a control subline (EMCc), and suppression of basal and inducible activity in EMC cells constitutively expressing a dominant negative retinoic acid receptor construct (EMCdn). (e) The activity present in conditioned media from EMC cells treated with RA is sensitive to heat inactivation (heat). (f) Genistein (Gen; 30 μM) inhibits the proliferative response of cardiomyocytes exposed to conditioned media from EMC cells.

mesenchyme still forms and nascent coronary arteries are apparent in the hypoplastic ventricular chamber wall. This indicates that epicardial expression of the dominant negative receptor does not interfere with formation of epicardial-derived cells, and because epicardial-derived cells do not express cytokeratins (including K18; Thorey *et al.*, 1993), this suggests that defects in the epicardial-derived mesenchymal population are unlikely to account for the phenotype of RXR$\alpha^{-/-}$ hearts. We therefore conclude that interference in retinoid receptor function within the domain of expression of the K18 transgenic promoter is sufficient to induce the ventricular hypoplastic phenotype, and because the epicardium is one such site, we suggest that the site of action of retinoid receptors during heart development is in the epicardium.

To further explore this model, we established an *in vitro* assay to explore the proliferative behavior of fetal cardiomyocytes. We isolated wild type primary fetal cardiomyocytes to >98% purity from either E12.5–E13.5 mouse embryos or E7 chick embryos, plated these cells overnight in the presence of serum to promote attachment, further cultured in serum-free media for 24 h, and then cultured for an additional 48 h in the presence of [^{3}H]thymidine. Cardiomyocytes cultured without any additional factors in the media have a modest basal level of thymidine incorporation, whereas treatment of these cells with serum induces a substantial increase in incorporation of the label (Fig. 2). Treatment of cardiomyocytes with retinoic acid did not have any proliferative effect (data not shown). We then exposed cardiomyocyte cultures to serum-free conditioned media prepared from a variety of sources. As a negative control, conditioned media from HeLa cells, whether untreated or treated with retinoic acid, had no activity in this assay (Fig. 2a).

We next isolated primary epicardial cells from individual midgestation mouse embryos isolated from crosses of RXR$\alpha^{-/+}$ parents. Such cells can be expanded for at least several passages in culture and retain their epithelial mor-

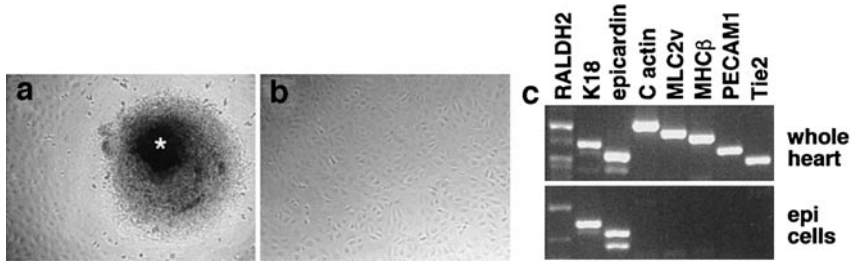

FIG. 3. Derivation of primary epicardial cells. Cells from a wild type heart are shown, although cells from RXR$\alpha^{-/-}$ hearts were identical in all examined respects. (a) Initial outgrowth of epicardial cells after two days in culture. The large mass of cardiac tissue (indicated by the asterisk) stays coherent in culture, whereas epicardial cells are seen migrating away from this mass. (b) Epithelial morphology of primary epicardial cells. A morphologically uniform culture of epithelial cells is seen after removal of the cardiac tissue mass and after three passages of the remaining epicardial cells. (c) Expression of appropriate molecular markers in primary epicardial cells. RNA was extracted from whole heart tissue or from primary epicardial cells, and analyzed by RT-PCR for expression of epicardial markers (retinaldehyde dehydrogenase 2, keratin 18, epicardin), cardiac muscle markers (cardiac actin, myosin light chain 2v, myosin heavy chain β), and endocardial/endothelial markers (platelet endothelial cell adhesion molecule 1, Tie2). All markers are expressed in the mixed population of the whole heart, whereas primary epicardial cells express only epicardial markers.

phology (Figs. 3a and 3b). These cells express several epicardial markers and do not express markers of cardiac muscle or endocardium (Fig. 3c, and data not shown). Unlike our observations with HeLa cells, when conditioned media was prepared from wild type primary epicardial cells, a relatively high basal level of trophic activity on cardiomyocytes was observed (Fig. 2b). This level of activity was increased when the epicardial cells were treated with retinoic acid; because the incorporation of tritiated thymidine by cardiomyocytes is saturable, the increased activity induced by RA is not apparent in the experiment shown in Fig. 2b, although serial dilution indicated a two- to threefold increase in activity caused by RA treatment of the epicardial cells (data not shown). The high basal level probably reflects endogenous production of retinoic acid, since these cells express the RA synthetic enzyme RALDH2 (Fig. 3c), as do epicardial cells *in vivo* (Moss *et al.*, 1998), and because the basal activity could be suppressed by treatment with a retinoid receptor antagonist (data not shown; see below). Importantly, epicardial cells derived from RXR$\alpha^{-/-}$ embryos produced little or no trophic activity into conditioned media, either basally or when treated with retinoic acid. Thus, the essential feature of the hypoproliferative ventricular chamber in RXR$\alpha^{-/-}$ embryos is replicated by RXR$\alpha^{-/-}$ epicardial cells in this *in vitro* assay of cardiomyocyte proliferation.

A stable rat epicardial cell line, called EMC, has previously been derived (Eid *et al.*, 1992, 1994). These cells express RALDH2 and other markers of the fetal epicardium (data not shown), similar to the primary fetal epicardial cells described above. We next determined whether EMC cells also secreted activity into conditioned media that would be trophic for primary fetal cardiomyocytes. Indeed, in a manner comparable to the activity of primary mouse epicardial cells, EMC cells produced a relatively high basal level of trophic activity, which could be further induced by treatment with retinoic acid, and which was suppressed by treatment with a retinoid receptor antagonist (Fig. 2c). We then derived a stable subline of these cells, in which the RAR303E dominant negative receptor described above was stably and constitutively expressed. This subline, designated EMCdn, and a control subline (EMCc) expressing an irrelevant product, were tested for the production of trophic activity in the cardiomyocyte proliferation assay. Indeed, we found that forced expression of the dominant negative RA receptor blocked the basal production of cardiomyocyte trophic activity, and prevented the induction of activity in response to RA treatment (Fig. 2d). The EMC and EMCdn epicardial cell lines therefore behave in a manner comparable to wild type and RXR$\alpha^{-/-}$ primary epicardial cells, respectively.

As part of an exploration of the biochemical properties of the trophic factor(s) made by epicardial cells, we found that the activity is abolished by heat treatment (Fig. 2e) and is ammonium sulfate precipitatable (data not shown), both suggestive of a protein or peptide. We also found that treatment of cardiomyocytes with genistein, a tyrosine kinase inhibitor, abolished their response to epicardial cell-produced trophic stimulation, suggestive of the involvement of a receptor tyrosine kinase or a cytoplasmic tyrosine kinase in transducing this activity (Fig. 2f).

Most known cytokines and growth factors have activity on multiple cells types. We found that conditioned media from RA-treated EMC cells was also trophic for 3T3 cells, assayed both by thymidine incorporation as well as by flow cytometry (Figs. 4a and 4b). The response of 3T3 cells to the epicardial cell-derived trophic activity involved the upregulation of cyclin D1 and E expression (Fig. 4c), although for lack of sufficient material we cannot yet say whether the same or similar responses occur in primary fetal cardiomyocytes. Nonetheless, these additional assays suggest that the induction of thymidine incorporation in treated cells is a true proliferative response to trophic factors present in epicardial cell conditioned media.

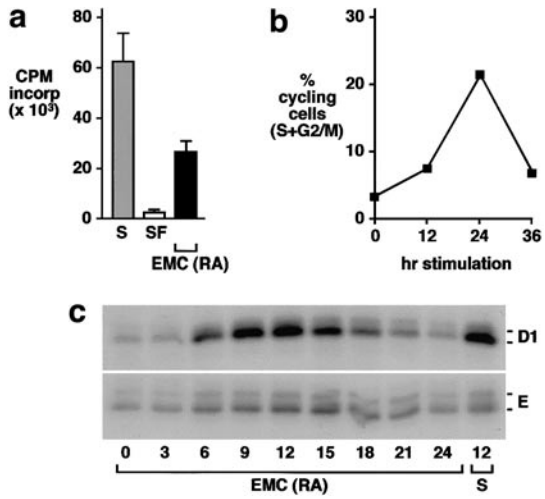

FIG. 4. Response of NIH3T3 cells to trophic stimulation by conditioned media from RA-treated epicardial cells. (a) Thymidine incorporation assay. Serum-free and serum controls are as in Fig. 2. (b) Flow cytometry assay. Cells were cultured in serum-free media supplemented with conditioned media from RA-treated EMC cells for the indicated times, then propidium iodide-stained and passed through a cell sorter. Cells were categorized as being in the resting (G_0 or G_1) or cycling (S, G_2, or M) phase of the cell cycle, based on DNA content. (c) Expression of cyclin D1 and cyclin E is induced by exposure to epicardial cell-conditioned media. Shown are Western blots of equal levels of loaded protein (36 μg) taken from cells exposed to conditioned media [EMC(RA)] or 10% serum (S) for the indicated times (in h).

Finally, we addressed how the production of and response to trophic activity changes during development. We found that conditioned media prepared from wild type mouse epicardial cells derived from late gestation embryos was comparably active to that produced by epicardial cells derived from midgestation embryos (Fig. 5a). However, epicardial cells obtained from early postnatal animals produced substantially less of the activity (although there was still detectable activity made from these cells). Midgestation fetal atrial cardiomyocytes were comparably responsive to stimulation as ventricular cardiomyocytes from embryos of the same age (Fig. 5b). However, the ability of cardiomyocytes to respond to stimulation decreases progressively as development proceeds, being highest in cells isolated at the earliest developmental stages and falling off as development proceeds. In the postnatal period, cardiomyocytes isolated from newborn pups were only marginally responsive to trophic stimulation, and cells isolated from 3-day-old pups were refractory to proliferation.

DISCUSSION

Based on the results described in this study, we propose that the fetal epicardium secretes one or more protein factors that stimulate fetal cardiomyocyte proliferation. The production of this factor is induced by retinoic acid, which is probably produced in an autocrine or paracrine manner by epicardial expression of RALDH2, and this induction requires the involvement of RXRα. We propose that the pathology seen in the hearts of RXR$\alpha^{-/-}$ embryos reflects a failure in the production of this activity and the resulting lack of cardiomyocyte proliferation.

A prediction of this model is that ablation of the epicardium should result in a hypoplastic ventricular chamber wall. This appears to be the case in VCAM-1 (Gurtner *et al.*, 1995; Kwee *et al.*, 1995) and possibly also α4 integrin (Yang *et al.*, 1995) mutant mice, where a physical interaction between these two molecules is normally responsible for adherence (and persistence) of the epicardium to the myocardium. Similarly, in chick embryos, formation of the epicardium can be partially prevented by the insertion of a piece of egg shell membrane in front of the proepicardial organ (the precursor of the epicardium). This results in patches of absent epicardium, with the myocardium underneath becoming hypoplastic (Gittenberger-de Groot *et al.*, 2000).

The existence of an epicardial proliferative signal accounts for two aspects of fetal ventricular development that

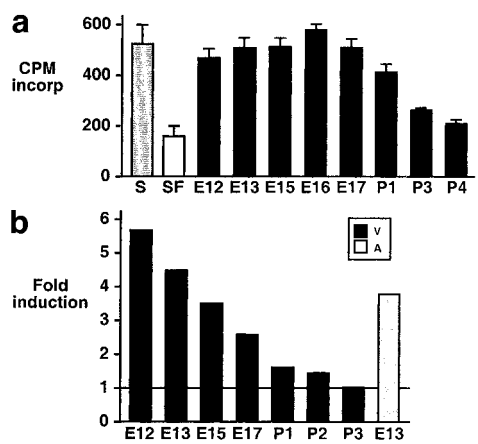

FIG. 5. Changes in production of and response to trophic activity during development. (a) Production of trophic activity in primary wild type mouse epicardial cells isolated from embryos or pups at various stages as indicated. Conditioned media was prepared from RA-treated cultured primary epicardial cells and collected and stored; all samples were assayed on chick cardiomyocytes in the same experiment. Solvent-treated cells showed lower but proportional levels of activity (not shown). Data is expressed as CPM of tritiated thymidine incorporated. (b) Response of primary mouse cardiomyocytes isolated from embryos or pups at the indicated stages. All cardiomyocytes were treated with the same batch of conditioned media from EMC cells treated with RA, although because all assays were done at different times, the data is expressed in terms of fold induction caused by conditioned media relative to the basal level of thymidine incorporation in cardiomyocytes exposed only to serum-free DMEM. Cardiomyocytes were prepared from ventricular (V) or atrial (A) tissue as indicated.

have previously been obscure. First, it has been noted that cardiomyocyte proliferation is greater on the epicardial side of the chamber wall than on the endocardial side, which is suggestive of a proliferative signal originating from the epicardium (Rumyantsev, 1977; Tokuyasu, 1990). Second, the utilization of the epicardium as the source of a proliferative signal provides a simple developmental mechanism for coordinating the timing of ventricular chamber growth and for formation of the compact layer of the ventricular chamber wall, which in mouse embryos begins around E10.5, immediately following formation of the epicardial layer.

Previous studies have given rise to the suggestion that the RXR$\alpha^{-/-}$ cardiac phenotype may result indirectly from placental dysfunction (Kastner *et al.*, 1997; Sapin *et al.*, 1997). Our results are not incompatible with this model, and in fact, the hypoplastic ventricular phenotype may represent the combined effects of placental deficiency as well as local epicardial deficiency. We cannot say at this time which of these effects is more important in defining the overall phenotype.

A number of reciprocal interactions occur between adjacent layers of the developing heart, mediated by secreted factors. The myocardium secretes factors, including TGFβ3, that act on the endocardium to induce an epithelial–mesenchymal transformation that allows endocardial-derived cells to populate the atrioventricular and conotruncal cushions (Brown *et al.*, 1999; Runyan *et al.*, 1992). Similarly, the myocardium produces additional factors, including FGFs, that act on the epicardium to induce an epithelial–mesenchymal transformation that creates the vascular endothelium of the coronary arteries, the smooth muscle cells that surrounds these arteries, and the cardiac fibroblast lineages (Morabito *et al.*, 2001). In return, the endocardium secretes neuregulin, which acts at the inner surface of the myocardium to induce cardiomyocyte proliferation and trabecular ingrowth (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995). Based on our observations, we propose a fourth axis of signaling, in which factors secreted by the epicardium act on the outer surface of the myocardium to induce cardiomyocyte proliferation and compact zone formation.

The identity of the factor (or factors) produced by the epicardium in response to retinoic acid signaling is currently unknown. EMC cells are known to express endothelins (Eid *et al.*, 1994), but endothelin expression is not retinoic acid regulated in these cells, and blocking antibodies against endothelins do not suppress cardiomyocyte proliferation induced by epicardial cell conditioned media (unpublished observations). We have also explored the possibility that erythropoietin, which our prior work has shown to be a primary target gene of retinoic acid and RA receptor action (Makita *et al.*, 2001), may be involved in the cardiac defect of RXR$\alpha^{-/-}$ embryos. However, erythropoietin is expressed at only trace levels by epicardial cells, and transgenic expression of erythropoietin in the fetal heart from a β myosin heavy chain promoter does not rescue the cardiac defects of RXR$\alpha^{-/-}$ embryos (unpublished observations). We have also explored a number of additional candidate factors, including IGF1, midkine, PDGF, and several others, but none of these appear to be regulated by retinoic acid in epicardial cells. It is of course possible that retinoic acid induces the expression of a processing enzyme that acts on a constitutively expressed precursor to create an active factor, although we currently have no evidence that would support this model. We have initiated biochemical and molecular strategies to identify the factors responsible for the cardiomyocyte trophic response, to identify the receptors of these factors, and to define the target genes that are immediately retinoic acid regulated at the onset of this process. These investigations may also help understand why the atrial compartment does not undergo the proliferative expansion that occurs in the ventricular chamber *in vivo*, even though atrial cardiomyocytes seems equally able to respond to epicardial trophic stimulation *in vitro* (Fig. 4). Possibly, the production of trophic factors by the epicardium *in vivo* differs between the atrial and ventricular chambers.

It has long been known that fetal cardiomyocytes proliferate, whereas postnatal cardiomyocytes are mostly if not completely postmitotic. Our results with cardiomyocytes isolated from different developmental stages and exposed to epicardial cell conditioned media indicate that the myocardium itself changes over time in its ability to respond to trophic stimulation. In fact, our results are most easily explained by a stochastic loss of proliferative potential in the cardiomyocyte population as development progresses. This was previously understood based on the response of fetal and postnatal cardiomyocytes to serum stimulation, but our results represent a more biologically relevant context from which to draw this conclusion. The identification of the receptor of the epicardial trophic signal, which as described above may be a member of the receptor tyrosine kinase family, should enable the definition of the signaling pathways which are triggered by trophic stimulation in fetal cardiomyocytes, and the alterations in signal transduction that occur in postnatal cells that prevent a proliferative response.

A number of mouse gene mutations result in a ventricular hypoplastic phenotype which is comparable if not identical to that of RXRα deficiency (Rossant, 1996; Sucov, 1998). One of these, the N-myc gene product, acts cell autonomously in fetal cardiomyocytes to support compact zone formation, and we have previously shown that RXRα function is not epistatic to N-myc expression (i.e., myc expression does not rescue the RXR$\alpha^{-/-}$ phenotype) (Tran and Sucov, 1998). A long-term goal of these studies is to assemble a pathway of genetic control of cardiomyocyte proliferation and compact zone morphogenesis. Our demonstration of the epicardium as a critical source of trophic stimulation, and the in vitro culture assays we have employed in this study, should facilitate the experimental dissection of the roles of these other genes in these processes.

Ischemic heart disease is the leading cause of death in the Western world and represents myocardial cell death caused by coronary artery obstruction and the inability of adult myocardium to repair itself via proliferation. Recent investigations have led to the conception that there may exist cardiomyocyte stem cells, either within the heart or derived from extracardiac sources, which to a limited extent may initiate proliferation within the scar of ischemic damage (Anversa and Nadal-Ginard, 2002). The factors described in this study may be useful in stimulating such cells to further or more rapidly divide, in a manner which could be therapeutically meaningful.

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