Retinoic Acid and VEGF Delay Smooth Muscle Relative to Endothelial Differentiation to Coordinate Inner and Outer Coronary Vessel Wall Morphogenesis

Ana P. Azambuja, Victor Portillo-Sánchez, Mariliza V. Rodrigues, Samantha V. Omae, Deborah Schechtman, Bryan E. Strauss, Eugenia Costanzi-Strauss, José E. Krieger, José M. Perez-Pomares, José Xavier-Neto

Rationale: Major coronary vessels derive from the proepicardium, the cellular progenitor of the epicardium, coronary endothelium, and coronary smooth muscle cells (CoSMCs). CoSMCs are delayed in their differentiation relative to coronary endothelial cells (CoEs), such that CoSMCs mature only after CoEs have assembled into tubes. The mechanisms underlying this sequential CoE/CoSMC differentiation are unknown. Retinoic acid (RA) is crucial for vascular development and the main RA-synthesizing enzyme is progressively lost from epicardially derived cells as they differentiate into blood vessel types. In parallel, myocardial vascular endothelial growth factor (VEGF) expression also decreases along coronary vessel muscularization.

Objective: We hypothesized that RA and VEGF act coordinately as physiological brakes to CoSMC differentiation.

Methods and Results: In vitro assays (proepicardial cultures, cocultures, and RALDH2 [retinaldehyde dehydrogenase-2]/VEGF adenoviral overexpression) and in vivo inhibition of RA synthesis show that RA and VEGF act as repressors of CoSMC differentiation, whereas VEGF biases epicardially derived cell differentiation toward the endothelial phenotype.

Conclusion: Experiments support a model in which early high levels of RA and VEGF prevent CoSMC differentiation from epicardially derived cells before RA and VEGF levels decline as an extensive endothelial network is established. We suggest this physiological delay guarantees the formation of a complex, hierarchical, tree of coronary vessels. (Circ Res. 2010;107:204-216.)

Key Words: retinoic acid ■ heart development ■ coronary ■ smooth muscle ■ endothelium

Coronary morphogenesis has been receiving intense scrutiny because of its usefulness as a model to understand vascular development and as a source of cues for regenerative therapies. Coronary vessel morphogenesis is linked to the development of epicardium and proepicardium, a transient embryonic structure located between the sinus venosus and the liver primordium.1,2 Avian embryos have been instrumental to our knowledge on coronary development. Lineage-tracing studies indicate that the proepicardium contains precursors of the epicardium, coronary endothelial cells (CoEs) and coronary smooth muscle cells (CoSMCs).2,5

Proepicardial and primitive epicardial cells undergo epithelial-to-mesenchymal transformation (EMT),2,6 supplying mesenchymal cells to the outer surface of the heart. These epicardially derived cells occupy positions along the subepicardial space, where they will differentiate into coronary vessel cell types.7 Around Hamburger–Hamilton stage (HH)26, highly migratory, epicardially derived cells leave the subepicardium to invade the myocardium, thus disseminating part of CoE progenitors throughout the cardiac muscle.8,9

Coronary vessels are formed from primary subepicardial and intramyocardial endothelial plexuses. Subepicardial/intramyocardial coronary vessel morphogenesis display one of the most striking and least understood features of coronary development, ie, the pronounced delay between CoE/CoSMC differentiation. Already at stage HH24, precursors in subepicardium differentiate into CoEs and form endothelial tubes under the influence of myocardially secreted cytokines, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor.10,11 In contrast, full CoSMC differentiation is only apparent after HH31 to HH32, concomitant with connection of CoE network to the aorta, stabilization of directional blood flow, and the onset of shear stress on coronary endothelium.11 Curiously, CoSMC differentiation is
Remarkably efficient when proepicardial-cells are removed from embryos and cultured, suggesting that there are active embryonic/heart mechanisms preventing premature CoSMC differentiation before an endothelial vascular outline is formed.

The mechanisms underlying this characteristic physiological delay have not been addressed. Potential candidate inhibitors of precocious CoSMC differentiation are the signaling pathways operated by the Vitamin A/retinol-derived morphogen retinoic acid (RA) and by VEGF. In avian embryos proepicardium, epicardium and epicardially derived cells express RALDH2 (retinaldehyde dehydrogenase-2), the main RA aldehyde dehydrogenase catalyzing retinaldehyde conversion into RA during amniote cardiac development. Furthermore, epicardial derivatives activate RA reporters and downstream targets of RA signaling, indicating that early vasculogenic steps take place in an RA-rich environment. Along the migration into the heart, epicardially derived cells undergo progressive coronary differentiation and gradually lose RALDH2 expression, as documented by waning of RALDH2 immunoreactivity as epicardially derived cells migrate into myocardium, suggesting that these phenomena might be causally related. Moreover, it is well known that synthesis of VEGF by the developing myocardium peaks after epicardial formation and decreases after the onset of coronary circulation.

Therefore, we hypothesize that RA and VEGF signaling coordinate differentiation of CoE and CoSMC precursors, acting as brakes to slow smooth muscle differentiation until a network of endothelial tubes is established. To test this hypothesis, we developed in vitro and in ovo approaches to study the effects of RA and VEGF signaling pathways at the level of whole proepicardial populations and of single proepicardial cells. Here, we show that RA plays an important role in the physiological delay of CoSMC differentiation and, additionally, that VEGF inhibits CoSMC differentiation.

Methods
An expanded Methods section is available in Online Data Supplement at http://circres.ahajournals.org.

Proepicardia were dissected from quail embryos. To promote cell attachment, proepicardia were grown on DMEM +15% FBS during 30 hours. After, the medium was replaced by DMEM +0.5% FBS for 48 hours. Then, explants were supplemented with RA (1 μmol/L) or VEGF (75 ng/mL), in the presence (5% FBS) or absence of FBS, during 4 days. Control proepicardial explants were treated with DMSO.

Transductions of proepicardial explants were performed by replacing the medium, after cell adhesion, by DMEM +0.5% FBS containing 7×10⁵ viral particles per well. Treatments started after 24 hours. Treatments included retinol (5 μmol/L), retinaldehyde (5 μmol/L), DEAB (4-diethylaminobenzaldehyde, 5 μmol/L), or retinol/DEAB (5 μmol/L each) in DMEM or DMEM +5% FBS.

Proepicardial cultures and quail-to-chick chimeras (constructed after modification from⁸) were submitted to immunohistochemistry.

Results
Cultured Proepicardial Cells Express Markers of Coronary Cell Lineages
To investigate CoSMC differentiation in vitro, proepicardial explants were cultured in the presence of FBS. Cultured proepicardia spread, forming an epithelial monolayer. Around the third day of culture, free migratory cells appeared at the periphery of the culture (Online Figure 1).

Figure 1A and 1B shows that explants express the proepicardial markers cytokeratin and WT1. WT1 tends to disappear from proepicardium cells expressing significant levels of CoE (QH1) and CoSMC (smooth muscle α actin [SMαA]) differentiation markers, although small traces of WT1 expression can still be detected in some differentiating cells (Figure 1C and 1D). QH1 labeling shows that CoE progenitors concentrate in the culture center (Figure 1E), whereas SMαA labeling indicates that CoSMCs are abundant at the culture border (Figure 1F). The SMαA-positive ring of proepicardial cultures includes cells displaying typical EMT/migratory phenotypes (Online Figure 1), whereas the monolayer core is poor in those cells. Two cellular patterns of SMαA expression are evident. The first is characterized by dense SMαA expression, prominent in actin stress-fibers, which is typical of differentiated CoSMCs and of cells that have undergone EMT. The “dense” pattern is most often observed at the margin of the culture. The second pattern is characterized by SMαA expression in subcortical actin bundles at the periphery of the cell (Figure 1G). The “peripheral” pattern is most frequently observed close to culture center, where CoSMC differentiation is weaker. These 2 patterns will be henceforth referred to as “dense” and “peripheral.” Because CoSMC differentiation is associated with EMT in cultured proepicardium, we looked for focal adhesion kinase (FAK) expression as a further marker for dense and peripheral types. We observed that peripheral SMαA expression associates with a diffuse FAK expression, whereas dense SMαA correlates to FAK expression at focal adhesion sites (Figure 1H through 1M). The FAK expression patterns in cultured proepicardial cell suggest that the peripheral phenotype represents an intermediate state between epithelial and mesenchymal (dense) phenotypes.

Proliferation, CoSMC Differentiation, and Apoptosis in Cultured Proepicardial Cells
To establish the topology of differentiation and proliferation in cultured proepicardia we determined 5-bromodeoxyuridine

### Non-standard Abbreviations and Acronyms

- **CoE**: coronary endothelial cell
- **CoSMC**: coronary smooth muscle cell
- **DEAB**: 4-diethylaminobenzaldehyde
- **eGFP**: enhanced green fluorescent protein
- **EMT**: epithelial-to-mesenchymal transformation
- **FAK**: focal adhesion kinase
- **HH**: Hamburger–Hamilton stage
- **RA**: all-trans retinoic acid
- **RALDH2**: retinaldehyde dehydrogenase-2
- **SMαA**: smooth muscle α actin
- **SMγA**: smooth muscle γ actin
- **VEGF**: vascular endothelial growth factor

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(BrdUrd) immunoreactivity after a 12-hour exposure to BrdUrd. Figure 1F shows that the outer ring of CoSMC differentiation is matched by an opposite pattern of proliferation, which is higher at the center of the cell. This suggests that proepicardial cultures grow from inside-out, with cells dividing at the core and moving toward the periphery. To test this hypothesis, we labeled proepicardial explants with CMPTX, a vital dye diluted with cell division, but not shared...
among neighboring cells. At 30 hours, all cells are stained. However, die distribution gradually shifts to periphery, consistent with an inside-out growth (Figure 1N). To evaluate whether cultures are significantly affected by apoptosis we submitted proepicardium cultures to the annexin assay. Annexin-positive cells are not found in the presence of FBS (not shown) and are rare and limited to the culture core in its absence (see below), suggesting that apoptosis does not play significant roles in our cultures.

RA Inhibits Expression of Smooth Muscle Markers in Cultured Proepicardium

To test whether RA signaling is involved in the physiological delay of CoSMC differentiation, we treated proepicardial cultures with RA. Exogenous RA abrogated SMαA expression, except in rare cells at the periphery of the culture (Figure 2A and 2B). To establish whether RA effects on the density and distribution of SMαA-positive cells are contingent on the absence of FBS, a strong smooth muscle inducer (compare Figure 2A through 2E), we treated proepicardial cultures with RA in the presence of 5% FBS. As shown in Figure 1F, RA treatment produced a marked decrease in the number of SMαA-positive cells. RA effects were intense at the center of the cell, but comparatively weaker at the margins. Such marginal cells could represent a subpopulation of older progenitors already committed to the CoSMC fate. In sum, FBS did not prevent RA from reducing the relative number of SMαA-positive cells, indicating that the RA effects on SMαA expression are also observed in conditions

Figure 2. RA inhibits the expression of different CoSMC markers. Representative immunoreactivity to SMαA of proepicardium cultured without FBS (n=5) (A), with RA (n=4) (B), with 5% FBS (n=5) (E) and with 5% FBS plus RA (n=5) (F). RA treatment dramatically reduced SMαA-positive cells. C, D, G, and H, High-power fields of A, B, E, and F. The RA effect is also evident on SMγA immunoreactivity (I and J), K, Quantitative RT-PCR of 2 independent experiments using pools of 4 proepicardia each. RA treatment reduces expression of different smooth muscle markers.
that favor CoSMC differentiation. Similar effects were found in mouse proepicardial cultures (Online Figure II).

To check whether the effects of RA affect smooth muscle markers other than SMαA, we performed immunohistochemistry to smooth muscle γ actin (SMyA) and used quantitative RT-PCR to examine expression of markers CRP2, GATA-6, SRF, and SM22A, which are sequentially activated during early smooth muscle differentiation. In Figure 2I through 2K, we show that SMyA immunoreactivity is reduced by RA and that the retinoid also inhibits expression of the other four smooth muscle markers. These results indicate that RA inhibits the expression of multiple markers of smooth muscle differentiation.

RA Inhibits Smooth Muscle Differentiation

To establish whether the decrease in smooth muscle marker expression after RA treatment is attributable to the effect of RA on CoSMC differentiation, proliferation, apoptosis, or EMT, we performed proliferation, apoptosis, and collagen gel transformation assays. RA treatment in the presence of 5% FBS changed neither the global rate of proliferation nor the specific rate of CoSMC proliferation (Figure 3C and 3D). Although RA treatment did not change global proliferation, analysis of proepicardial cultures indicate that it changed the topological distribution of BrdUrd-immunoreactivity, converting proliferation to a more homogeneous pattern (Figure 3A and 3B). RA did not alter the distribution of apoptotic...
cells (Figure 3E through 3G) nor changed EMT rates in proepicardial cultures (Online Figure III). Thus, the reduction in SMαA-positive cells by RA is not attributable to a decreased proliferation, increased apoptosis, or EMT inhibition, but to a direct effect on CoSMC differentiation.

A Nonreplicating Adenoviral Vector Provides RALDH2 Overexpression in Proepicardial Cells
Prompted by our results on RA inhibition of CoSMC differentiation, we investigated whether autocrine RA signaling would, likewise, inhibit CoSMC differentiation. Accordingly, we developed a nonreplicative adenoviral mouse (m)RALDH2 overexpression system in which high levels of mRALDH2 expression are driven by a CMV promoter in a mRALDH2-IRES-eGFP vector (Online Figure IV). Overall, transduced cells accounted for 10.5% of the culture. Using mouse-specific RALDH2 RT-PCR we provide evidence that transduced cells produce mRALDH2 at a level comparable to that attained by endogenous quail (q)RALDH2 expression in the whole proepicardium, suggesting that the average level of exogenous mRALDH2 expression by each transduced cell exceeds by 1 order of magnitude the endogenous level of qRALDH2 expression. In sum, the AdRalh2eGFP vector is an effective tool of mRALDH2 overexpression.

Activation of Autocrine RA Signaling by AdRALDH2eGFP Inhibits CoSMC Differentiation
Overexpression of mRALDH2 inhibited CoSMC differentiation in the presence of the RA precursor retinol (Figure 4A and 4B). mRALDH2 overexpression in retinol-supplemented proepicardium cultures reduced the relative number of enhanced green fluorescent protein (eGFP)/SMαA-positive cells approximately by half (Figure 4D). A slightly less intense effect of mRALDH2 overexpression plus retinol supplementation was observed with 5% FBS (Figure 4H through 4I and 4K). Similar results were obtained with retinaldehyde (Figure 4F and 4G).

Next, we tested whether the effects of mRALDH2 overexpression combined with retinol supplementation reflect a specific activation of autocrine RA signaling or a nonspecific consequence of adenosinergic infection and/or retinol treatment. As indicated in Figure 4N through 4P, transduction with a control AdLacZ vector did not alter the relative number of LacZ/SMαA-positive cells with or without retinol. This indicates that mRALDH2 overexpression effects are not attributable to unspecific effects of adenosinergic infection/retinol supplementation. These results also suggest that endogenous qRALDH2 expression in cultured proepicardia is not enough to drive significant levels of RA signaling.

To establish whether the retinol-induced CoSMC inhibition displayed by cells overexpressing mRALDH2 is dependent on increased aldehyde dehydrogenase activity, we performed experiments in the presence of DEAB, an aldehyde dehydrogenase inhibitor. As indicated in Figure 4D and 4K, DEAB abrogated the retinol-induced decrease in CoSMC differentiation with or without FBS. In contrast, treatment with DEAB in the absence of retinol did not change the relative number of eGFP/SMαA-positive cells, in line with the view that endogenous qRALDH2 expression is not sufficient to activate RA signaling in cultured proepicardia. This indicates that the effect of mRALDH2 overexpression combined with RA precursor supplementation on smooth muscle differentiation is mediated by an aldehyde dehydrogenase activity, consistent with an increased autocrine production of RA via RALDH2.

Activation of Autocrine RA Signaling Reduces the Number of Dense CoSMCs
To establish whether autocrine RA signaling reduces CoSMC differentiation acting on peripheral or dense CoSMC phenotypes, we assessed the effect of mRALDH2 overexpression/retinol supplementation on these cells. In FBS-free cultures (Figure 4E), the relative number of dense cells was significantly reduced by retinol treatment, whereas the relative number of peripheral CoSMCs was not significantly changed. Similar results were found with FBS (Figure 4L). Thus, activation of autocrine RA signaling in proepicardial cultures is primarily associated with a decrease in the number of dense CoSMCs.

Inhibition of RA Signaling Induces Premature SMαA Expression In Vivo
To establish whether RA controls CoSMC development in vivo, we tested whether systemic inhibition of RA signaling would induce premature SMαA activation. DEAB-treated quail embryos display multiple SMαA-positive cells in the atrioventricular junction (Figure 5C) that are not present in controls. This premature SMαA activation associates with reduction of immunoreactivity to cytokeratin, a marker of noncommitted proepicardial/epicardial and epicardially derived cells.

To test whether localized changes in RA signaling would also be consistent with an in vivo role for RA in CoSMC differentiation we combined DEAB-soaked bead grafting with quail-to-chick proepicardial chimeras, so that both the fate of bona fide proepicardial cells and of resident cardiac cells could be simultaneously studied. Figure 5H through 5J shows that quail (donor) proepicardial cells, as well as host subepicardial cells, express SMαA and SMαI under DEAB released from the carrier, whereas control beads did not (Figure 5E through 5G). Moreover, host chicken hearts display increases in SMαA immunoreactivity at distance from the DEAB bead, suggesting inhibition of endogenous RA signaling. These results indicate that in vivo inhibition of RA synthesis affects transplanted proepicardial cells and resident host subepicardial CoSMC precursors.

Coculture of Proepicardia With Cardiomyocytes Inhibits CoSMC Differentiation
We tested whether additional signaling mechanisms are also implicated in physiologically delayed CoSMC differentiation. Because the myocardium has been suggested to control coronary progenitor fate, we evaluated whether coculture of proepicardial explants onto myocardial feeders would reduce CoSMC differentiation. In Figure 6A, double cytokeratin (red) and SMαA (green) staining indicate that proepicardia cultured onto myocardial feeders (SMαA-positive) hardly ever coexpress both markers (yellow). This contrasts with
Figure 4. Autocrine RA signaling inhibits CoSMC differentiation. Immunoreactivity to SMoA/eGFP in AdRaldh2eGFP-transduced proepicardium cultured without RA precursor supplementation (A and H), supplemented with retinol (ROL) (B and I), retinaldehyde (RAL) (F and G), or with retinol plus DEAB (C and J). D and K, Autocrine RA signaling decreases the relative number of
results in fibroblast feeders, in which a distinct yellow external ring marks proepicardial cells SMαA/cytokeratin-positive, reminiscent of the marginal ring of SMαA expression displayed by proepicardial cultures under FBS supplementation (Figure 6D). Figure 6C and 6F shows that proepicardium cultured on myocardial feeders display more QH1-positive cells than proepicardium cultured on fibroblast feeders and that endothelial cells are more homogeneously distributed in the former than in the latter. This strongly suggests that cardiomyocyte feeders possess specific mechanisms that inhibit the CoSMC differentiation and support the endothelial one.

Because the myocardium is a source of VEGF,19 VEGF signaling could account for the decreased immunoreactivity to SMαA in our cocultures by shifting the differentiation of proepicardial cells toward the endothelial phenotype. To check whether VEGF was implicated in the differential effect of cardiomyocytes and fibroblasts on CoSMC differentiation, we measured VEGF expression in these monolayers. Using quantitative RT-PCR, we show VEGF expression by cardiomyocytes and fibroblasts on CoSMC differentiation, only 25% of transduced cells expressed SMαA, whereas AdLacZ vector did not. Although autocrine activation of VEGF signaling reduced CoSMC differentiation, we transduced proepicardium cultures with AdVegfeGFP (Figure 7E). We determined that autocrine VEGF significantly reduced CoSMC differentiation with (Figure 7F and 7G) or without FBS (not shown), whereas AdLacZ vector did not. Although autocrine VEGF signaling reduces CoSMC differentiation, only 25% of transduced cells expressed QH1 (Figure 7H and 7I), suggesting that autocrine VEGF does not trigger efficient endothelial differentiation.

VEGF Inhibits Smooth Muscle Differentiation by Paracrine and Autocrine Modes
To test the role of VEGF in proepicardial differentiation, we treated our cultures with exogenous VEGF. VEGF decreases the number of cells expressing SMαA, while increasing the number of QH1-expressing cells displaying the typical cobblestone endothelial phenotype (Figure 7A through 7D). To establish whether autocrine VEGF signaling reduces CoSMC differentiation, we transduced proepicardial cells with AdVegfeGFP (Figure 7E). We determined that autocrine VEGF significantly reduced CoSMC differentiation with (Figure 7F and 7G) or without FBS (not shown), whereas AdLacZ vector did not. Although autocrine activation of VEGF signaling reduced CoSMC differentiation, only 25% of transduced cells expressed QH1 (Figure 7H and 7I), suggesting that autocrine VEGF does not trigger efficient endothelial differentiation.
To evaluate the effects of VEGF signaling in a more physiological context, in which local extracellular concentration gradients are formed by cytokine secretion, diffusion, and degradation, we scored the number of SMaA-positive or QH1-positive cells in the immediate vicinity of cells transduced with the AdVegfaGFP (Figure 7J). We determined that fewer SMaA-positive cells and more QH1-positive cells are found in a radius of 130 µm from cells transduced with AdVegfaGFP than in cells transduced with control AdLacZ/AdGFP, consonant with a paracrine effect of VEGF (Figure 7K and 7L).

**Figure 6.** Cardiomyocytes inhibit CoSMC differentiation of cocultured proepicardium. A, D, G, J, and C through L are representative immunostainings for cytokeratin/SMaA and QH1, respectively. CoSMC differentiation is rare in proepicardium cultured onto cardiomyocytes (n=6) (A) and exuberant in proepicardium cultured onto neonatal fibroblast feeders (n=6) (D). Myocardial cocultures support increased endothelial differentiation from proepicardium cells (n=6) (C) as compared to fibroblast feeders (n=5) (F). VEGF complementation of fibroblast feeders with AdVegfaGFP (n=5) (G through I) but not with a control AdLacZ (n=4) (J through L), resemble cardiomyocyte cocultures. B, E, H, and K, High-power fields of dotted areas in A, D, G, and J. Quantitative RT-PCR of cardiomyocytes and fibroblasts monolayers presenting murine (M) and human (N) VEGF expression relative to fibroblast shows strong VEGF expression in cardiomyocyte and VEGF-complemented fibroblast monolayers which is confirmed by immunoreactivity (ELISA) of conditioned media (O). NA indicates nonapplicable.

**Discussion**

The differentiation and organization of blood vessel layers (endothelium/smooth muscle) is a sequential, pivotal event in early vascular development, especially during coronary vasculogenesis. CoSMCs highly expressing SMaA are only detected among the myocardial layers at HH32, whereas CoE tubes form from HH24 onwards.2,6,7 How multipotent coronary progenitor cells are able to differentiate following this strict chronological sequence remains unclear. We herein show that RA and VEGF signaling restrain CoSMC differentiation before a primary CoE plexus is formed by
vasculogenesis. Therefore, delayed CoSMC differentiation allows the development of a well-defined hierarchy of coronary vessels in an optimal, flexible, provasculogenic environment before vessel structure is stabilized with recruitment and addition of extracellular matrix-producing CoSMCs.

**RA Signaling Is Well Poised to Delay CoSMC Differentiation Until a CoE Network is Formed**

The delay in CoSMC differentiation in respect to CoEs is especially puzzling, considering that coronary progenitors easily initiate their differentiation into CoSMCs in vitro and that some cells expressing markers of smooth muscle differentiation are already present in the late proepicardium. Moreover, cells in proepicardium, epicardium, subepicardium, and myocardium express growth factors and receptors such as SRF, TGFβR, and PDGFR that could induce smooth muscle differentiation well before physiological CoSMC differentiation actually starts. Thus, it is likely that premature CoSMC differentiation is continuously repressed by signaling mechanisms that must operate from very early on, during proepicardium formation, up until an endothelial outline of coronary circulation is organized. Few molecules with strong signaling properties fit this role better than RA. Retinoids are synthesized in the heart and are known modulators of cell differentiation, whereas dysregulation of retinoid signaling in early embryo produces vascular dysmorphogenesis. In avians, RA signaling is initiated in coronary precursors at stages HH16 to HH17, when proepicardial cells activate RALDH2 expression. In avians and mammals, RA is produced by proepicardial-derived cells during formation of the epicardium, which is a continuous source of RA for the subepicardium and myocardium. Furthermore, epicardial cells undergoing EMT gradually lose expression of RALDH2 and WT1 as they migrate into the myocardium. Because this sequence of events is inversely...
related to coronary marker acquisition, RA is an excellent candidate to keep epicardial progenitors in an undifferentiated state, controlling the balance between proliferation and differentiation in coronary precursors.

RA Inhibits CoSMC Differentiation

By using viral/cellular/systemic methods to activate RA signaling, we showed that RA delays CoSMC differentiation through autocrine, paracrine, and systemic modes without disturbing cell proliferation, apoptosis, or EMT. Our data offer evidence that epicardially derived cells expressing RALDH2 produce their own RA and activate RA signaling in an autocrine fashion, being biased against CoSMC differentiation. By analyzing cell fates in the vicinity of cells transduced with the AdRaldh2eGFP, we suggest that the RA diffuses and inhibits CoSMC differentiation in their neighbors. This paracrine mode is supported by in vivo experiments in which SMαA and SMαA expression was precociously activated following systemic or local inhibition of RA synthesis.

The inhibitory effects of RA signaling on CoSMC differentiation offer a plausible explanation for the ease with which cultured proepicardial cells initiate their differentiation into CoSMCs. As we have shown, proepicardial cultures display an inefficient RA signaling. In fact, we showed that endogenous expression of qRALDH2 by cultured proepicardia containing ∼4000 cells is roughly equivalent to the level of mRALDH2 expression achieved through transduction of ∼10 times less cells. This level of qRALDH2 expression is apparent below the threshold for effective RA production, as indicated by the failure of retinol supplementation, or of DEAB, to change the numbers of CoSMCs in cultured proepicardia.

The combined effects of autocrine and paracrine RA on CoSMC differentiation can be modeled according to two schemes, depending on whether one believes that smooth muscle cells display a continuous range of differentiated phenotypes, or that each smooth muscle phenotype derives directly from a common CoSMC progenitor. Given the fact that we have identified a number of intermediate phenotypes between a promesenchymal, peripheral CoSMC and a more differentiated dense CoSMC, we currently favor the idea that RA inhibition of CoSMC differentiation reflects a global arresting effect of retinoids on CoSMC differentiation.

The Physiological Delay of CoSMC Differentiation Also Involves VEGF Signaling

We show that autocrine and paracrine RA act preferentially on CoSMC precursors to retard their differentiation, whereas leaving the timing of CoE differentiation unaffected (not shown). Using cocultures with myocardium, or fibroblasts, we provided evidence that the myocardium (but not fibroblasts) produces factors at levels able to inhibit CoSMCs and promote endothelial differentiation. We have shown that fibroblast cocultures complemented with virally delivered VEGF display decreased CoSMC differentiation and increased CoE differentiation, suggesting that VEGF plays a complex role in regulation of CoSMCs and CoE fates. This was confirmed in vitro by showing that VEGF controls CoSMC differentiation through autocrine, paracrine, and systemic modes.

The simplest way through which VEGF could decrease CoSMC differentiation is indirect, by shifting the fate of epicardially derived cells toward the endothelial phenotype. Our results on the activation of autocrine VEGF signaling by viral transduction clearly support this possibility. This indirect and autocrine, mode, however, does not appear to be the most physiological, or important one, because only 25.9% of VEGF-expressing cells activates endothelial marker expression, whereas CoSMC differentiation is reduced from 96.2% to 49.7%. This suggests that VEGF also works by directly inhibiting CoSMC differentiation. Thus, it is possible that VEGF produced by transduced cells diffuses and directly represses CoSMC differentiation in a paracrine fashion. We confirmed this by showing that although paracrine VEGF reduces CoSMC
differention by 35.4%, it only marginally increases endothelial differentiation by 12.3%.

A Model for Coordinated CoE and CoSMC Differentiation

Coronary progenitors that reach subepicardial and intramyocardial domains are either under autocrine RA signaling because of their RALDH2 expression or within range of paracrine RA produced by neighboring cells. Thus, in our model (Figure 8), CoSMC differentiation is first checked by epicardial/subepicardial RA and later on by the direct action of myocardially secreted VEGF, which also orients uncommitted epicardially derived cells toward the CoE phenotype. Importantly, our results provide clues about the poorly understood mechanisms that trigger differentiation of CoSMCs, recruit them for medial layer formation, and synchronize these processes with the establishment of directional blood flow and shear stress in the newly formed coronary endothelial network. In our model, CoSMC differentiation is derepressed by the well-documented downregulation of RALDH2 expression in cardiac development and by reduced VEGF production secondary to efficient myocardial perfusion. It remains to be established whether RA and VEGF signaling inhibit CoSMCs additively, or synergistically, or whether they are in the same, or in parallel pathways. Moreover, although changes in HIF-1α activity secondary to the reduction of cardiac hypoxia induced by coronary perfusion offer a likely molecular explanation accounting for progressively reduced VEGF expression, the molecular pathways leading to progressive decreases in RALDH2 expression remain unknown. In summary, we provide evidence that RA is a crucial signal involved in the spatiotemporal control of myocardial blood flow and shear stress in the newly formed coronary endothelial network. In our model, CoSMC differentiation is derepressed by the well-documented downregulation of RALDH2 expression in cardiac development and by reduced VEGF production secondary to efficient myocardial perfusion. It remains to be established whether RA and VEGF signaling inhibit CoSMCs additively, or synergistically, or whether they are in the same, or in parallel pathways. Moreover, although changes in HIF-1α activity secondary to the reduction of cardiac hypoxia induced by coronary perfusion offer a likely molecular explanation accounting for progressively reduced VEGF expression, the molecular pathways leading to progressive decreases in RALDH2 expression remain unknown. In summary, we provide evidence that RA is a crucial signal involved in the spatiotemporal control of CoSMC differentiation, acting coordinately with VEGF to regulate the formation of multilayered coronary blood vessels, an idea supported by recent demonstration of reduced VEGF receptor 2 and PECAM-1 expression in RALDH2 knockout embryos. Finally, our results provide important information on the cellular and molecular regulation of blood vessel morphogenesis, raising prospects for the discovery of additional regulatory networks during normal and abnormal coronary development.

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Disclosures

None.

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References


Novelty and Significance

What Is Known?

- The proepicardium is a transient embryonic structure that gives rise to epicardium and to coronary precursors.
- Epicardially derived coronary precursors ingress into the myocardium, where they promptly undergo endothelial differentiation under the influence of cytokines such as fibroblast growth factor and vascular endothelial growth factor (VEGF) secreted by the heart.
- Smooth muscle differentiation of cells derived from the epicardium is delayed in relation to endothelial differentiation, occurring only after a primitive endothelial outline of the coronary circulation is formed.
- Before the endothelial network is established, epicardially derived cells are exposed to VEGF produced by cardiomyocytes and to intense RA signals triggered by expression of RALDH2, the major cardiac retinoic acid (RA)-synthesis enzyme.
- After the onset of coronary circulation, the strength of both RA and VEGF signals is markedly reduced.

What New Information Does This Article Contribute?

- The pronounced physiological delay between coronary endothelial and smooth muscle differentiation from their multipotent epicardial progenitors is controlled by both RA and VEGF signals.
- Activation of autocrine and paracrine RA signaling inhibits coronary smooth muscle differentiation.
- Activation of VEGF signaling, chiefly through a paracrine pathway, inhibits smooth muscle differentiation and further biases the differentiation of epicardial coronary progenitors to an endothelial fate.

Some aspects of coronary development, such as the pronounced temporal gap that separates endothelial from smooth muscle differentiation, are puzzling. This gap is critical to ensure the capacity for extensive remodeling before a complex, hierarchical vessel network is formed by the recruitment of extracellular matrix–producing smooth muscle cells. We show that this physiological delay is controlled by RA and VEGF signals. Using adeno-viral vectors for RALDH2 and VEGF, and in vivo inhibition of RA synthesis, we show that RA acts as a brake to slow smooth muscle differentiation from coronary precursors. We show that besides inducing endothelial differentiation, VEGF acts directly on coronary precursors to decrease the expression of smooth muscle markers. Our results support a model in which epicardial/subepicardial RA production by RALDH2 and myocardial VEGF expression create an environment that prevents early smooth muscle differentiation, thus allowing the formation of an extensive endothelial network. Our results suggest that normal/abnormal coronary development involves novel cross-regulatory networks and suggest new approaches to direct differentiation of precursors toward specific coronary fates. We suggest that revascularization/regenerative therapies can be improved by taking into account the multistep nature of coronary development that coordinates inner and outer coronary vessel wall morphogenesis.
Retinoic Acid and VEGF Delay Smooth Muscle Relative to Endothelial Differentiation to Coordinate Inner and Outer Coronary Vessel Wall Morphogenesis

Ana P. Azambuja, Víctor Portillo-Sánchez, Mariliza V. Rodrigues, Samantha V. Omae, Deborah Schechtman, Bryan E. Strauss, Eugenia Costanzi-Strauss, José E. Krieger, José M. Pérez-Pomares and José Xavier-Neto

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Detailed Methods

Proliferation and cell death analysis

In proliferation assays PE-cultures were supplemented with 10μM BrdU during the last 12 hours of each treatment (5%FBS+DMSO and 5%FBS+RA). After that, cultures were washed, fixed in 4% paraformaldehyde and submitted to immunohistochemistry.

For the apoptosis analysis we utilized the annexin assay¹ using ApoAlert® Annexin V Kit (Clontech, Cat#630109) according to the manufacturer’s manual, followed by signal amplification by anti Polyclonal rabbit anti fluorescein Alexa 488 conjugate.

Immunohistochemistry

Cultured PE explants were fixed in 4% paraformaldehyde for ten minutes at room temperature, permeabilized with 1% Nonidet P40 and blocked with 1% BSA in PBS. Explants were incubated for one hour with primary antibodies in 1% BSA. After four washes in PBS, explants were incubated for ninety minutes with secondary antibodies. Explants were counterstained with Hoechst 33258 for thirty minutes. Cover slides mounted with glycerol 50% in PBS.

Paraplast sections (8µm) were rehydrated, washed, soaked in 0.1M glycine/PBS, washed two times in Triton 0.5%, blocked in 1% BSA for 30 minutes, and incubated overnight with primary antibodies. Slides were washed four times in PBS, blocked in 1% BSA for 15 minutes and incubated for 90 minutes at room temperature with secondary antibodies in PBS.

Primary antibodies used were: monoclonal mouse IgG2a anti Smooth Muscle a-Actin (1:500, Sigma), monoclonal mouse IgG1 anti QH1 (1:50, DSHB), monoclonal mouse IgG1 anti BrdU (1:100, Sigma), polyclonal rabbit IgG anti Cytokeratin (1:100, Dako), polyclonal rabbit IgG anti eGFP (1:1000, AbCam), monoclonal mouse IgG anti eGFP (1:500, Sigma), monoclonal mouse IgG anti βGal (1:100, DSHB), Gamma-Actin (1:100, MP Biomedicals). Secondary antibodies were anti mouse IgG Cy3 conjugate (1:700, Sigma), anti rabbit IgG Cy3 labeled (1:700, Sigma), alexa fluor 488 anti mouse IgG (1:700, Molecular Probes), alexa fluor 488 goat anti mouse IgG1 (1:700, Molecular Probes), alexa fluor 594 goat anti mouse IgG2a (1:700, Molecular Probes).

Adenoviral construction

Replication-incompetent human adenoviral type 5 ΔE1/ΔE3 (AdRaldh2eGFP, AdVegfeGFP, AdLacZ) was constructed using the Adeno-X™ Expression System 1 (Clontech, Cat#631513). AdVegfeGFP and AdLacZ were kindly gifted by Dr. Krieger, J.E.². For AdRaldh2 construction, the murine RALDH2 cDNA was subcloned into the hybrid vector pShires (modified from pShuttle) between a CMV promoter and the internal ribosome entry site (IRES) followed by the eGFP sequence. All the subsequent steps followed manufacturer’s specifications. Adenoviral constructs were then transfected into the packaging HEK 293 cells and adenoviral particles were harvested from the culture supernatants. The cDNA for eGFP (AdeGFP) was isolated from pCLeGFP³ and inserted in the ViraPower Adenoviral Expression System following the manufacturer’s protocol (Cat # V493-20, Invitrogen, Carlsbad, CA). Virus production was performed as described above.

RT-PCR

RT-PCR assays were carried out on RNA prepared from cultures derived from single PEs. PE explants were washed twice with PBS. For RNA isolation the TRizol® Reagent (Invitrogen) was employed according to manufacturer’s manual. cDNA was synthesized using oligo d(T) primers and reverse transcriptase. RT-PCR amplification was carried out using the following primers: mRaldh2 5’ – CTGGTTCAGTGCCGAGA – 3’ and 5’ – TGGGATGATCTGCTCCACA – 3’, qRaldh2 5’ – GTGGGGGTAAAGGCTA – 3’ and 5’ – CTCTACCATTGCCAGACA – 3’.
DEAB treatment

HH17 quail embryos were treated in ovo with 200µL of 0.1mmol/L DEAB in PBS (stock solution 10mmol/L DEAB in DMSO). Control embryos were treated with equal volumes of DMSO. Embryos were reincubated and collected after 48 hours. Embryos were then washed in PBS; fixed overnight in 4% paraformaldehyde in PBS, dehydrated, embedded in paraplast, sectioned (8µm) and submitted to immunohistochemistry.

RA synthesis block on proepicardial quail-chick chimeras

Donor quail embryos were incubated at 37ºC until stages HH16-17, excised, and washed in sterile EBSS (Gibco). Proepicardia were carefully dissected using tungsten needles, small iridectomy forceps and scissors. AG 1-X2 resin beads (Bio-Rad) were extensively washed in EBSS+0.1% DMSO, then in EBSS and finally embedded for 45 minutes in a DEAB (1µM from a 1mM stock in DMSO) solution or in EBSS+0.1% DMSO (control embryos).

Proepicardia and resin beads were then transplanted onto chick embryo hosts incubated for 60h (HH16-17). Chimeras were fixed in Dent’s fix (methanol, DMSO 4:1), dehydrated in a graded series of ethanol, cleared in butanol, carefully oriented, and embedded in Histosec (Merck). Finally, 5µm serial sections were mounted on microscope slides (Menzel-Gläser).

Immunohistochemical characterization of the chimeric tissues was performed using the QCPN antibody (quail pan-nuclear marker) to identify the donor cells. QCPN signal was amplified with a tyramide (TSA) kit following the indications of the supplier (Perkin-Elmer). Counterstaining with antibodies against smooth muscle markers was performed using a Cy5-conjugated secondary antibodies (Jackson).

Co-cultures

Two feeder monolayers were utilized: mitotically-inactivated mouse embryonic fibroblasts or neonatal rat cardiomyocytes. Mouse fibroblast and rat cardiomyocytes were isolated according to established protocols. Fibroblasts were treated with Mitomycin C as in. Feeder cells were placed on cover slits on 24-well plates. After 24 hours, cultures were washed twice with PBS. PE explants were placed and cultured on feeder for 72 hours in FBS-free DMEM.

VEGF complementation of fibroblasts layers was performed by transducing the monolayer cultures with AdVegfeGFP. Control cultures were transduced with AdLacZ. After 24 hours medium was replaced and transduced cells were kept in the absence of serum for more 24 hours. PE explants were placed without medium replacement.

Quantitative RT-PCR

VEGF expression on feeder cells and smooth muscle differentiation markers were verified by quantitative RT-PCR as in. Primers employed were: Mouse/Rat (TaqMan® Gene Expression Assays Applied Biosystems): Vegf 5’ – GAGCAGAAAGCCCATGAAGTG – 3’ and 5’ – GGCAATAGCTGCCTGGTA – 3’; reporter sequence – ACGTCCATGAACTTCA; Cyclophilin 5’ – GGCAATAGCTGCCTGGTA – 3’; reporter sequence – CACCCCTGACATGAA.

**Image analysis**

PE-cultures and embryo sections were photographed on a Nikon fluorescence microscope (Eclipse E600) with a Roper Scientific Photometrics digital camera using Image-ProPlus software or with confocal microscope Leica SP-5.

**Quantitative analysis and statistics**

Global rates of proliferation were scored as the number of proliferative cells over the total number of cells (BrdU-positive nuclei over total nuclei) in one radial section of at least three independent PE-cultures. Specific rates of CoSMC proliferation represent the number of SMαA-positive cells that are proliferative over the total number of cells (SMαA/BrdU doubly-positive over total nuclei) in one radial section of at least three independent PE-cultures.

The autocrine effects of AdRaldh2eGFP and of AdVegfeGFP were scored as the number of eGFP/SMαA-positive or eGFP/QH1-positive, doubly-positive cells over the total number of transduced cells counted in 21 fields (40x objective) per treatment in at least three independent experiments.

The paracrine effects of AdRaldh2eGFP and AdVegfeGFP represent the number of SMαA-positive or QH1-positive over total number of cells within a radius of 130 µm of a transduced cell.

All values shown represent means ± standard error. Where appropriate, statistical analysis was performed with two-tailed Student’s t-test, or with One-Way Analysis of Variance with the Tukey-Krahe Multiple Comparisons Test as a post-hoc test, p< 0.05 was considered statistically significant.
Supplemental Figures and Legends

Online Figure I. Cultured PE explants form monolayer and display migratory cells. (A) 48 hours PE-culture. (B-D) Third day PE-culture. Arrows, early EMT. Arrowhead, free migratory cell.
Online Figure II. RA inhibits CoSMC differentiation in mouse-derived proepicardial explants. PE cultured without FBS (A), supplemented with RA (B), in the presence of 5%FBS (C) and supplemented with 5%FBS and RA (D).
Online Figure III. RA did not affect EMT in PE-cultures. PE-cultures surface on collagen gels in absence (A) or presence of RA (C). (B,D) 25µm-deep sections of cultures showed in (A,C) depicting migratory cells invading collagen gel.
Online Figure IV. Adenoviral RALDH2 expression system (AdRaldh2eGFP). (A) AdRaldh2eGFP. (B) AdRaldh2eGFP-transduced proepicardium. RT-PCR for mouse RALDH2 (C) and quail RALDH2 (D) show that the AdRaldh2eGFP vector is an effective tool for mRALDH2 expression in proepicardium cells. (E) Control AdLacZ vector. (F) βgal immunoreactivity of a proepicardium transduced with control AdLacZ. HH18 quail are positive controls for qRALDH2 and negative controls for mRALDH2 RT-PCR. 9.5dpc mice are positive controls for mRALDH2 and negative controls for qRALDH2 RT-PCR.
Supplemental References


