Abstract—Recent work has demonstrated the importance of the epicardium in the development of the heart. During embryogenesis, these epithelial cells provide the progenitors for the epicardium, coronary smooth muscle, endothelium, and cardiac fibroblasts. The epicardium sends important signals to the developing myocardium. Still, analysis of these epithelial cells has lagged behind that of other cardiac cell types largely because of the lack of a defined experimental cell system in which epicardial cell differentiation can be studied. The present report examines the developmental potential of a cell line derived from rat epicardial mesothelial cells. These analyses demonstrate that the cell line retains many characteristics of the intact epithelium, including the ability to form a polarized epithelium and express many epicardial genes. Our data show for the first time that these cells retain the ability to produce mesenchyme in response to specific growth factors and, importantly, to generate smooth muscle cells. Thus, this study provides evidence that these cells can serve as an important model system for the analysis of the cellular and molecular mechanisms that govern epicardial development and function. (Circ Res. 2003;92:525-531.)

Key Words: epicardium ■ vasculogenesis ■ Bves protein ■ coronary development

The heart is composed of several different cell types, including cardiac myocytes, endocardial endothelial cells, fibroblasts, vascular smooth muscle, vascular endothelial cells, and epicardial epithelium. These cell types have varying functions in the normal adult heart and have distinct responses to disease and injury. In addition, these cells have distinct embryonic origins.

Myocardial and endocardial cells arise from the lateral splanchnic mesoderm1-4 and are the only resident cells in the heart until the looping phase of cardiac development (stage 15 in the chick and 9.5 postcoital days in the mouse).5-7 Numerous studies reviewed by Fishman and Chien8 have examined the differentiation potential of these two cell types. At the looping stage of heart development, the proepicardial organ (PEO), an epithelial structure derived from the septum transversum, migrates to and over the surface of the myocardium to form the epicardium/visceral pericardium and pericardium.6,9,10 Later, a subpopulation of epicardial cells undergoes an epithelial/mesenchymal transition (EMT) and migrates throughout the substance of the trabeculating myocardium.10,11 These mesenchymal cells give rise to cardiac fibroblasts, vascular endothelium, and smooth muscle of the coronary system, and the remaining epithelium becomes the epicardium.10 The generation of vasculogenic cells from a mesothelium is a unique mechanism of blood vessel formation in vertebrate development.

In the adult heart, the epicardium is a simple squamous epithelium and shares characteristics with other mesothelia, such as pleural epithelium and peritoneum. As stated above, the blood vessels and fibroblasts of the connective tissue space are derived from the epicardium. A major function of adult epicardial/pericardial epithelium is to provide a smooth surface on which the heart slides during contraction. In addition, this epithelium serves as a seal in the production and sequestration of pericardial fluid in the pericardial space.

Recent studies have focused attention on the importance of epicardial-myocardial interactions during development and on the essential role the epicardium plays in heart development. For example, the EMT of epicardium is regulated by a FOG2-dependent pathway from the myocardium that likely involves growth factor signaling from the myocardium to the epicardium.12,13 Although genetic and experimental analyses of heart development have identified factors that are essential in coronary vasculogenesis, the molecular basis of these developmental processes is unknown.

Little is understood about differentiation of the epicardium. This deficiency is particularly acute as data showing the essential interplay between epicardium and myocardium for proper heart development emerge. In addition, the potential for the epicardium to “reinitiate or recapitulate” its developmental program after injury and disease is an unexplored area of research. This gap in our current understanding of cardiac
biology is due in large part to the lack of a defined in vitro experimental cell model to analyze epicardial cell differentiation. In an effort to establish an in vitro system for the analysis of epicardial differentiation, we examined a cell line derived from rat epicardial mesothelioma by Eid and colleagues.\(^14,15\) (epicardial/mesothelial cells [EMCs]). Those studies detailed the derivation of EMCs from the epicardium and described the influence of EMCs on the contractility and differentiation of cultured cardiac myocytes. The epicardially derived EMC line potentially represents a unique possibility to examine epicardial differentiation and the mechanism to generate vasculogenic cells from this mesothelium. The present study focuses on elucidating the developmental potential of EMCs and establishing an in vitro system to study coronary vasculogenesis. Our data show that EMCs retain many characteristics of adult and embryonic epicardia, including the formation of a polarized epithelium and expression of many mesothelial/epicardial genes. Importantly, we demonstrate for the first time that these cells respond to specific growth factors to produce mesenchyme and, subsequently, smooth muscle cells. Thus, these results suggest that EMCs retain much of the differentiation potential of embryonic epicardium and demonstrate the utility of these cells as a model system for the analysis of epicardial development and coronary vessel differentiation.

**Materials and Methods**

**Cell Culture Conditions**

EMCs were obtained from Dr Eid (University of Ottawa, Canada)\(^4\) and grown in DMEM containing 4.5 mg/mL glucose, 4 mmol/L l-glutamine with 10% FBS, and 10 μg/mL penicillin/streptomycin. Incubation conditions were 37°C and 5% CO\(_2\). Cells were passaged by rinsing once with PBS, followed by trypsinization with 0.05% trypsin–EDTA for 2 minutes. We have found that EMCs can be seeded at very low or clonal densities in complete media. EMCs do not require special attention under normal growth conditions. They are also able to grow under low-calcium or serum-free conditions, forming a monolayer as observed in normal conditions. At present, we have only obtained low but successful transfection of EMCs using standard phosphate, lipofectamine, or electroporation methods. We have maintained these cells for a period of >3 years.

**Analysis of RNA Expression**

Tissues from rats used for RNA isolation were frozen at −70°C immediately after excision. Total RNA was prepared from cells and tissues by use of a homogenizer and Trizol reagent (GIBCO-BRL) per the manufacturer’s instructions, and RNA samples were stored at −70°C until the time of use.

Reverse transcription (RT)–polymerase chain reaction (PCR) analysis was used to analyze the expression of specific mRNAs in EMCs. Primer sequences are shown in the Table. The thermocycler profile used was as follows: room temperature: 48°C, 45 minutes; 94°C, 5 minutes; PCR: 95°C, 30 seconds; 53.8°C, 1 minute; 68°C, 1 minute 20 seconds; 40 cycles. Annealing temperatures varied with different primer sets (see Table).

**Immunofluorescence Microscopy**

Immunofluorescence procedures have been described previously.\(^11,16\) Primary antibodies used to detect epithelial markers and epicardial markers in these studies were as follows: B846 (anti-Bves, 1:100),\(^16\) monoclonal anti–E-cadherin (1:200, Transduction Laboratory), monoclonal anti–β-catenin (1:200, Sigma), polyclonal anti–ZO-1 (1:200, Zymed), monoclonal anti-connexin43 (1:200, Sigma), polyclonal anti–WT-1 (c–19, 1:200, Santa Cruz Biotechnology), and Alexa Fluor 488 Phalloidin (1:200, Molecular Probes). Primary antibodies used to detect smooth muscle marker were as follows: monoclonal anti–smooth muscle α-actin (1:200, Sigma), monoclonal anti-desmin (1:200, Sigma), monoclonal anti-mysin (smooth muscle) clone hSM-v (1:200, Sigma), and anti–platelet and endothelial cell adhesion molecule (anti-PECAM, gift from Dr Albelda, University of Pennsylvania, Philadelphia) for an endothelial marker. Secondary antibodies to rabbit or murine IgG were either Cy2- or Cy3-conjugated (Jackson Immunoresearch) and used at the manufacturer’s suggested dilutions. DAPI was used for nucleus detection. Fluorescence microscopes (Olympus BX60) were used, and pictures were taken with an attached camera system (Olympus PM-30).

**Growth Factor Treatment**

EMCs were seeded at low density in chamber slides (Nalge Nunc International Corp) in standard culture medium. Cells were incubated in growth factor–supplemented media (epidermal growth factor [EGF], platelet-derived growth factor [PDGF], transforming growth factor–β1, and basic fibroblast growth factor [bFGF], all from ICN) without serum for 24 hours, at which time the medium was replaced with fresh medium containing the same conditioned growth factors and incubated for another 24 hours. Control experiments determined the range of concentrations over which these growth factors are effective; 10 to 40 ng/mL was used for all factors in these concentrations.

**Primers Used for RT-PCR in Gene Expression**

<table>
<thead>
<tr>
<th>Primers used to detect epicardial marker expression</th>
<th>Sequence, 5′→3′</th>
<th>Annealing Temperature, °C</th>
<th>Expected Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbves F11</td>
<td>CATCTTGCGGAGGAGCTATAC</td>
<td>58.3</td>
<td>620</td>
</tr>
<tr>
<td>rbves R11</td>
<td>TGATTGTGACGGCTGCTGTAAC</td>
<td>54.2</td>
<td>403</td>
</tr>
<tr>
<td>Wilms tumor-1 F1</td>
<td>ATCCCCCTGTTGCCAAAAAT</td>
<td>52</td>
<td>461</td>
</tr>
<tr>
<td>Wilms tumor-1 R1A</td>
<td>CTGAAAGGCTGCTGCTGTAAC</td>
<td>54</td>
<td>230</td>
</tr>
<tr>
<td>Tbx-18 F1</td>
<td>GGTAGTTTACCACTAGCTCT</td>
<td>54</td>
<td>240</td>
</tr>
<tr>
<td>Tbx-18 R1A</td>
<td>AGGTGAAATCCGCTAGTGAT</td>
<td>54</td>
<td>240</td>
</tr>
<tr>
<td>Myogenin F1</td>
<td>CAGGAAGGCGGATCGTCCGCTAC</td>
<td>52</td>
<td>461</td>
</tr>
<tr>
<td>Myogenin R1</td>
<td>CAGGAATGGATGGCGATGGCTACCA</td>
<td>54</td>
<td>230</td>
</tr>
<tr>
<td>rGAPDH F1</td>
<td>TTAACAGGCGACACGTGAAAG</td>
<td>54</td>
<td>240</td>
</tr>
<tr>
<td>rGAPDH R1</td>
<td>TGATTGTGACGGCTGCTGTAAC</td>
<td>54</td>
<td>240</td>
</tr>
</tbody>
</table>

The sequence of each oligo used and annealing temperature are listed. The predicted sizes of the products for each primer set are provided.
studies. Cells were scored for mesenchymal transition by morphological changes, rearrangement of the actin cytoskeleton using phalloidin, and translocation of E-cadherin from the cell surface. After 24 or 48 hours of incubation in growth factor-containing medium, the cells were fixed with 70% methanol, permeabilized with 0.25% Triton X-100, and subjected to immunofluorescence analysis as described above.

**Smooth Muscle Differentiation**

EMCs were cultured under normal growth conditions, and the expression of smooth muscle markers was examined by RT-PCR, as described above, and by immunofluorescence analysis using the antibodies listed above. Primer sequences and annealing temperatures are listed in the Table. To detect smooth muscle–positive cells on growth factor stimulation, cells were cultured in low-calcium medium to maximize the frequency of EMT, and EGF, PDGF, and PDGF-AA (all from ICN) were added at 10 ng/mL. Cultures were incubated for 48 hours and examined for smooth muscle gene expression by immunofluorescence.

### Results

**EMCs Form a Polarized Epithelium and Express Epicardial Cell Markers**

The epicardium is a polarized simple squamous epithelium, with the apical surface directed toward the pericardial cavity; thus, we first determined whether EMCs retain the ability to form and maintain a simple epithelium. EMCs were passaged, plated at low density, and allowed to grow. At confluence, EMCs remain in a single layer and do not form multilayered structures. EMCs were tested for their ability to traffic E-cadherin and Bves to the lateral cell surface as seen in vivo. EMCs grown in standard medium with serum do not undergo significant levels of EMT. Immunocytochemical analysis of dispersed single EMCs shows that E-cadherin is widely dispersed within the cytoplasm (Figures 1A and 1D). However, when EMCs make contact with one another, E-cadherin is localized to the membrane at points of contact (Figures 1A, 1B, 1D, and 1E). E-cadherin and β-catenin, which are components of the adherens junction, are localized around the circumference of cells in confluent EMCs (Figures 1C, 1F, and 1J), indicating that a polarized epithelium has formed. Bves, which is also a marker of epithelial sheet formation, localizes to cell-cell contact sites (G, arrows) and is not seen in contact-free areas (H, arrowhead). Additional polarized epithelial markers are detected. β-Catenin, a component of the adherent junction, is localized in the same pattern as E-cadherin (J), ZO-1, a tight junction marker, is also detected at the cell periphery in epithelial sheets (K).

WT-1 protein, which is detected in the mesothelia, including the epicardium, during embryogenesis, is detected in the EMC nuclei. Thus, this expression profile suggests that EMCs retain a phenotype characteristic of the epicardium.

**Specific Growth Factors Induce Mesenchyme Formation From EMCs**

Epicardium plays an essential role in the maturation of the myocardium and generation of the coronary vasculature. Mesenchyme production from the epicardium is one of the critical events in the generation of coronary vessels. Analysis of its regulation has drawn interest recently; thus, we sought to determine whether EMCs could be induced to undergo EMT using selected growth factors under serum-free conditions. Cells were allowed to form epithelial sheets before growth factor addition and were assayed for mesenchyme production after an additional 24 hours. As used in prior studies, cell morphology (the transition from epithelial to elongated fibroblast-like shape), rearrangement of the actin cytoskeleton with production of stress fibers, and disruption...
of cadherin localization at the cell membrane were assayed as indicators of EMT. Under serum-free conditions without growth factor addition, EMCs form epithelial sheets within 24 hours and continue to grow as epithelial sheets until the termination of the experiment at 48 hours. If cells are allowed to grow, cultures become confluent monolayers. E-cadherin and Bves were detected at cell-cell borders, demonstrating that the epithelial sheet is complete in the absence of serum (Figures 3Aa and 3Ba). In addition, EMT was not observed from these sheets at any time in the absence of added growth factors.

Morabito et al. recently demonstrated that bFGF induced EMT from the epicardium in vivo and in cultured PEO explants; thus, we began our studies with this growth factor. At 10 ng/mL, only minimal disruption of the epithelial sheets was observed at 24 or 48 hours. E-cadherin localization was seen at the cell membrane, and cells maintained epithelium-like morphology similar to that seen in control cells without growth factor in serum-free conditions (authors’ unpublished data, 2002). In contrast, when cultures were incubated with 20 or 40 ng/mL bFGF for 24 hours, numerous fibroblast-like cells became detached from the edge of the epithelial sheet, where the most prominent region of EMT is observed (Figure 3Ac). Epithelial sheet formation in treated cultures proceeds more slowly because of the prominence of EMT and freely migratory mesenchyme. Cells in the middle of epithelial sheets established before growth factor treatment produced stress fibers (as visualized with phalloidin) and showed disruption of cadherin at the cell membrane (Figures 3Bc and 3Bf). Interestingly, after 48 hours of incubation, stress fiber formation was reduced (data not shown), and immunocytochemistry demonstrated the presence of E-cadherin at the membrane of most EMCs regardless of growth factor concentration. This suggests a time dependence of the response.

EGF has also been shown to be a potent inducer of EMT in many cell types. When cells were incubated with 20 or 40 ng/mL EGF, very large numbers of cells were converted to an...
EMCs Can Differentiate Into Smooth Muscle Cells

One of the most important developmental properties of embryonic epicardium is the ability to produce progenitor cells that later differentiate into components of the coronary vasculature.7,10,29 To determine whether EMCs retain this essential developmental potential, expressions of markers of smooth muscle and endothelial lineage were assessed by RT-PCR and immunocytochemistry.

Our initial studies into the vasculogenic potential of EMCs in standard growth medium with 10% serum used RT-PCR analysis to detect smooth muscle gene products. As shown in Figure 4, α-smooth muscle actin, calponin, and α-smooth muscle tropomyosin transcripts were clearly apparent in these analyses (Figure 4A). These results suggest either that all EMCs express smooth muscle genes or that a specific population of cells converted to a smooth muscle phenotype. To distinguish between these two possibilities, antibodies directed against smooth muscle markers were to stain EMC cultures. As seen in Figures 4B through 4D, smooth muscle myosin, desmin, and α-smooth muscle actin were present at high levels in isolated patches of cells in the epithelial sheet and not at low levels in the majority of cultured cells (Figures 4B through 4D). The frequency of these cells in culture was <5% of the total number of cells and was maintained at that rate after repeated passage. Smooth muscle cell differentiation was rare or not observed under serum-free conditions over the time course of study (48 hours). These data suggest that factors within normal growth medium with serum could induce a smooth muscle phenotype from EMCs.

To determine whether specific growth factors could promote smooth muscle cell differentiation from this epicardial cell line, we cultured EMCs in serum-free conditions with the addition of selected factors. With the addition of EGF, PDGF-AA, or PDGF-AB, smooth muscle cells were readily detected 48 hours after treatment. Patches of cells were observed within the epithelial sheet, much like that seen in standard serum-containing medium (data not shown). The frequency of these cells was again ~5% of the culture. If calcium was depleted from the growth factor–containing medium to promote EMT, smooth muscle–positive cells were readily detected and had clearly altered cell morphology and apparently detached from the epithelial sheet (Figures 4E through 4G). Taken together, these data clearly demonstrate that EMCs retain the potential to produce smooth muscle cells. Interestingly, PECAM-positive endothelial cells were not detected in serum or growth factor–treated cultures on a consistent basis within any experimental group. At present, it is not clear whether these data suggest that EMCs have either a limited potential or no potential to form endothelial cells or that the proper growth factor(s)/condition(s) for this process has not been identified.

Discussion

Recent data have demonstrated that the epicardium plays an essential role in the development of the coronary vascular system and is an important regulator of myocardial differentiation. Although insight into the role of the epicardium in heart development has been gained from in vivo studies, an in vitro system is clearly needed to advance the field. Our present data demonstrate that EMCs retain much of the developmental potential seen in embryonic epicardium, including cellular and molecular mechanisms, to produce...
smooth muscle cells. Furthermore, these findings suggest that EMCs may serve as a model for the in vitro analysis of epicardial cell growth and differentiation. This cell line will provide a powerful tool to investigate the cellular and molecular mechanisms regulating epicardial differentiation during coronary and heart development.

Expression of Epicardial Gene Products in EMCs
The epicardium is a mesothelium. Mesothelia line or cover body spaces such as the peritoneal, pleural, and pericardial cavities and provide a smooth surface on which organs move. In addition, this specialized simple squamous epithelial structure provides a barrier in the compartmentalization of body spaces. In general, mesothelia arise “late” during the generation of organs.20–22 migrating over an already formed primordium. Recent data have shown that a diverse array of gene products is expressed in these distinct, but functionally related, epithelia.20,22,24,33 The present study reveals that EMCs express specific genes that are enriched in mesothelia, such as the epicardium. In addition, EMCs retain the ability to form a polarized simple epithelium and produce smooth muscle progenitors; both are characteristic of the epicardial phenotype. Other recent studies suggesting the interaction of the epicardia and myocardia also point to the potential utility of EMCs.12,13 These cells may prove invaluable in the analysis of tropic factors emanating from epicardium that play a role(s) in the generation and maintenance of myocardium, and they may be a reliable source of “pure” epicardium for the production of gene arrays.

Epicardial Differentiation and EMT In Vitro
The importance of EMT in epicardial and coronary vessel differentiation has been discussed.10,12,13,29,34–37 Our present data clearly demonstrate that EMCs respond to specific growth factors with the acquisition of a fibroblast-like phenotype, rearrangement of the actin cytoskeleton, and disruption of cadherins at the cell membrane, all indicators of EMT.17,26,28,38 The response of EMCs to bFGF mirrors that of embryonic epicardium in vivo.13 In addition, although EGF has not yet been shown to induce EMT from embryonic epicardium, the response of cells to EGF is particularly interesting inasmuch as this growth factor signals through the receptor tyrosine kinase pathway and has been shown to induce EMT by disruption of the intercellular adhesion16,39 that we observed in the present study. It will be critical to unravel how these signaling pathways are synchronized in cells. Studies on this topic may lead to a more thorough understanding of how some of these cells remain in the epicardium while a subpopulation undergoes EMT, eventually becoming coronary vascular components.

EMCs Can Differentiate Into Smooth Muscle
Production of vasculogenic cells via EMT from the epicardium is a unique situation in vertebrate development. Many studies indicate that endothelial cells induce smooth muscle differentiation from “local mesenchyme.”14 Our current data conclusively show that EMCs (epithelial cells) can differentiate into smooth muscle cells that express several proteins characteristic of PEO-derived smooth muscle.37 Although EMC-derived smooth muscle cells may go through a mesenchymal phase, the clonal nature of this cell line predicts that the original progenitors of these cells were epithelial in nature. This unique mode of smooth muscle development seen from the epicardium is retained in EMCs. These smooth muscle cells constitute <5% of the cells after growth in serum or growth factor treatment, but it is clear that EMCs have retained their ability to produce smooth muscle cells at this frequency after repeated passage. This finding suggests that the production of smooth muscle from this cell line is a heritable trait. In addition, EMCs are capable of producing smooth muscle cells in the absence of cardiac myocytes and differentiated endothelial cells, albeit within a serum-dependent or growth factor-dependent manner. This suggests that epicardial cells are capable of response to specific factors to produce smooth muscle in the absence of complex intercellular contacts or interaction with large numbers of endothelial cells. Whether EMCs can give rise to endothelial cells is an issue to be resolved. Our study to determine whether EMCs differentiate into endothelial cells was not conclusive. At present, we have not determined whether these cells are incapable of producing endothelium or that the correct signaling molecule has not been identified. Further analysis of EMC differentiation may lead to insights into the unique form of vasculogenesis carried out by the embryonic epicardium.

It is interesting to note that EMCs produce smooth muscle cells at a fairly consistent frequency even after repeated passages. During the generation of coronary arteries, the epicardium produces smooth muscle cells at some constant rate, whereas other epicardial cells retain their epithelial state. The cellular and molecular mechanisms underlying the selection of smooth muscle precursors from the epicardium are not currently understood but may involve intercellular interactions between cells in the epicardium. One might speculate that Notch-Delta41–43 or other paracrine signaling systems (such as transforming growth factor-β)44 that have been implicated in other epithelial cell lineage decisions might be involved in the epicardial differentiation pathway. It would be of interest to determine whether such a system is involved in regulating the production of mesenchyme and/or smooth muscle from the epicardium. The production of smooth muscle from EMCs at a predictable frequency as demonstrated in the present study suggests that some or all regulatory mechanisms present in the epicardium are retained by this cell line. The present system may prove to be a valuable model to unravel these developmental processes.

Possible Stem Cell Populations in Epicardium
In the present study, we have shown that EMCs, a cell type isolated from the pericardium/epicardium, retains the ability to produce smooth muscle cells. The possibility that the epicardium may contain/give rise to a vasculogenic stem cell population is raised. From the point of view of coronary disease, this is an intriguing hypothesis. To generate vessels in the damaged heart, it is important to have a cell population that is capable of producing all of the vascular cell types. Our data support the hypothesis that a homogeneous population of cells can differentiate into a smooth muscle cell type and potentially other cell types while maintaining the epicardial cell phenotype.
Acknowledgments

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References


Epicardial/Mesothelial Cell Line Retains Vasculogenic Potential of Embryonic Epicardium

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