Novel Embryonic Genes Are Preferentially Expressed by Autonomously Replicating Rat Embryonic and Neointimal Smooth Muscle Cells

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Abstract—We sought to identify and characterize the expression pattern of genes expressed by smooth muscle cells (SMCs) during periods of self-driven replication during vascular development and after vascular injury. Primary screening of a rat embryonic aortic SMC–specific cDNA library was accomplished with an autonomous embryonic SMC–enriched, nonautonomous adult SMC–subtracted cDNA probe. Positive clones were rescreened in parallel with embryonic SMC–specific and adult SMC–specific cDNA probes. We identified 14 clones that hybridized only with the embryonic cDNA (“emb” clones), 11 of which did not share significant homology with sequences in any of the databases. Five of these novel emb genes (emb7, emb8, emb20, emb37, and emb41) were selectively and only transiently reexpressed in vivo by neointimal SMCs during periods of rapid replication. The emb8:embryonic growth–associated protein (EGAP), which was studied the most extensively, was expressed at high levels by cultured, autonomously replicating embryonic and neointimal SMCs but was detected only at low levels even in mitogenically stimulated adult SMCs. Finally, the administration of antisense EGAP oligonucleotides markedly attenuated embryonic and neointimal SMC replication rates. We suggest that autonomous replication of SMCs may be essential for normal vascular morphogenesis and for the vascular response to injury and that these newly identified “embryonic” genes may be part of the molecular machinery that drives this unique growth phenotype. (Circ Res. 2000;87:608-615.)

Key Words: arteries ■ vasculature ■ restenosis ■ muscle, smooth ■ clones

During vascular development, aortic smooth muscle cells (SMCs) in vivo undergo a distinct phase of rapid proliferation, a time period during which the vessel wall acquires its complement of SMCs, followed by a period of extensive extracellular matrix production that contributes to the structural maturation of the vessel wall.1-5 We have documented in the rat that aortic SMCs replicate at a high rate (80%/d) throughout the embryonic period of life, demonstrate dramatic decreases in replication at the embryonic-to-fetal transition of intrauterine life (rates decrease to 40%/d), and gradually acquire a quiescent phenotype, reaching replication rates of <0.06%/d in the adult.1 Significant proliferation of SMCs in the adult animal is observed only during certain stages in the development of vascular fibroproliferative diseases such as atherosclerosis, in restenosis after angioplasty, and in transplant arteriopathies.6-9 For instance, Clowes et al10 have shown that after experimental arterial injury, neointimal SMCs demonstrate large increases in replication rates that reach levels similar to those observed during embryonic life. Furthermore, data from this laboratory and others suggest that after vascular injury, as during development, adult SMCs proceed through a period of rapid cell division followed by a period of extracellular matrix production.4,11-13 However, the factors that regulate SMC proliferation during vascular development and neointimal SMC replication after vascular injury to the adult blood vessel remain poorly defined.

Corresponding to the in vivo observations of high replication indices, embryonic aortic SMCs in culture exhibit a distinct growth phenotype characterized by their ability to replicate in an autonomous, mitogen-independent manner.1 The capacity for self-driven replication appears to be lost by fetal life, as demonstrated by the mitogen-dependent growth phenotype exhibited by fetal, neonatal, and adult rat aortic SMCs. The loss of autonomous growth potential suggests that important changes in gene expression and phenotype occur in developing SMCs between the embryonic and fetal periods of intrauterine life. In addition, we have demonstrated that adult SMCs that replicate in experimentally injured arteries transiently exhibit an autonomous, mitogen-independent growth
phenotype. This observation is consistent with the hypothesis that SMCs reiterate a pattern of gene expression during injury repair similar to that expressed by SMCs during early vascular development.

Because we found that the capacity for self-driven, autonomous replication contributes to high rates of SMC replication during vascular development and during early neointima formation after vascular injury, the purpose of the present study was to identify and characterize molecules capable of conferring autonomous growth capabilities to SMCs. Previous studies that cloned and identified genes expressed by vascular SMCs used animals from postnatal or later postinjury periods, stages at which changes in the vascular wall cells are characterized more by matrix synthesis than by extremely high rates of cell replication. Therefore, we sought to identify genes that are preferentially expressed during vascular development when SMCs express autonomous growth capabilities and to determine whether these same genes would be reexpressed in SMCs after injury to the adult blood vessel in the period characterized by high rates of intimal SMC proliferation. We used a subtractive hybridization approach with a cDNA library prepared from RNA isolated from cultured, autonomously replicating embryonic aortic SMCs and an embryonic SMC–enriched, adult nonautonomous SMC–subtracted cDNA probe. We report the cloning and expression patterns of 5 novel genes (referred to as “emb” genes) that are highly expressed in vivo and in vitro by SMCs during periods of vascular growth that are characterized by autonomous replication and suggest that these clones may be part of the molecular machinery that participates in autonomous replication of SMCs. The emb genes, therefore, may represent an entirely new class of genes reexpressed in the setting of vascular diseases characterized by excessive SMC replication.

Materials and Methods

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Using a subtractive hybridization cloning technique, we sought to identify novel genes expressed by SMCs during periods of self-driven growth observed during development and after vascular injury to the adult blood vessel. Primary screening of an embryonic SMC–specific library was accomplished with an embryonic SMC–enriched, adult SMC–subtracted cDNA probe as described in Materials and Methods (available in an online-only data supplement at http://www.circresaha.org). Approximately 9 × 10^3 clones, or 3% of the library, hybridized with the probe. Twenty clones (based on relative signal intensity) were picked for secondary screening. Of 76 positive secondary clones, 43 represented unique classes based on restriction digest patterns (data not shown). The 43 positive clones were pooled, replated, and lifted in duplicate as an embryo-enriched library. This embryonic SMC–enriched library was then screened in parallel with embryo-specific and adult-specific cDNAs with the use of hybridization probes prepared from autonomously replicating embryonic SMCs and from mitogenically stimulated adult SMCs. A total of 14 (33%) apparently independent clones of this group hybridized only with embryonic cDNA and hereafter are referred to as embryonic, or emb genes (emb1, emb2, emb3, emb7, emb8, emb12, emb15, emb20, emb22, emb29, emb36, emb37, emb39, and emb41).

These emb clones were sequenced at their 3’ and 5’ ends, and the sequences were compared against the GenBank database (data not shown). Of the 14 independent clones, 3 were found to exhibit significant homology to previously described genes, including the transcription factor pendulin (emb2), the S6 ribosomal protein (emb22), and rat cdc25A (emb39). The remaining 11 clones did not share significant homology in their 3’ and 5’ ends with any sequences in the databases and therefore represent potentially novel genes.

The 14 positive emb genes were simultaneously screened on tissue sections by in situ hybridizations for expression in embryonic aortas, in adult aortas, and in balloon-injured adult carotid arteries 9 days after injury. The relative amounts of each emb gene at the different time points were quantified as described in Materials and Methods (available in an online-only data supplement at http://www.circresaha.org). Five of the putative novel emb clones (emb7, emb8:embryonic growth–associated protein [EGAP], emb20, emb37, and emb41), as well as 2 previously identified clones (emb2:pendulin and emb22:S6 ribosomal protein), were highly expressed in the embryonic aorta, were essentially absent in the adult aorta, but were significantly reexpressed in the day 9 neointima (>4-fold over adult) (Figure 1).

The emb clones were also screened by in situ hybridization for expression in adult testis, epidermis, brain, lung, skeletal muscle, kidney, intestines, and liver to assess the tissue specificity of expression and to determine whether emb genes are expressed in highly replicative adult tissues. The emb8:EGAP (Figure 2), which was widely distributed in the embryo, continued to be expressed at high levels in the adult in primary spermatocytes, in the basal epidermis, and in interstitial fibroblasts of skeletal muscle and in intestinal crypts. In contrast, the expression of emb37 (Figure 2) was more restricted in the embryo and, in the adult, was undetectable in primary spermatocytes, intestinal crypts, and bronchiolar epithelium, remaining detectable only in the basal epidermis and Purkinje cells of the brain. The characteristics and expression patterns of all 14 emb clones are outlined in Table 1 online (available in an online-only data supplement at http://www.circresaha.org). Although considerable variability obviously exists in the spatial pattern of expression of the mRNAs represented by these clones, all are expressed in SMCs specifically during periods of autonomous replication in the developing aorta, and 7 of the 14 (5 novel emb genes) are significantly reexpressed in the injured carotid artery during periods of autonomous growth. We chose to study these 5 novel clones in more detail, concentrating on emb8:EGAP. The emb clones that shared homology with sequences in the databases or were determined, through in situ hybridization, to be only moderately regulated between embryonic and adult developmental stages or to not be reexpressed after vascular injury were not analyzed further.

A complete developmental analysis of emb gene expression was performed on tissue sections obtained from whole...
embryos or fetuses (embryonic/fetal days 12 through 21) and from aortic tissues (postnatal days 1, 7, 16, 30, and 90). The typical pattern of expression in the developing aorta of the 5 clones is illustrated in Figure 3A, with emb8:EGAP given as an example. The emb8:EGAP mRNA was first detected in the embryonic aorta as early as embryonic day 12 (data not shown) and showed high levels of expression throughout embryonic life. There was a dramatic loss of signal intensity by fetal day 19, and expression of emb8:EGAP mRNA was essentially absent throughout postnatal development. We quantified the in situ signal for both emb8:EGAP and emb37 and plotted the results in conjunction with in vivo aortic SMC replication rates. As previously reported and illustrated in Figure 3B, aortic SMCs showed high rates of replication during embryonic life but exhibited dramatic decreases in replication by fetal life. The emb8:EGAP and emb37 mRNAs were expressed by \( \sim 70\% \) to \( 80\% \) aortic SMCs during the embryonic period, demonstrated marked decreases by embryonic day 18 (\( \sim 25\% \) positive SMCs), and were essentially undetectable by fetal day 19 (\( \leq 10\% \) positive SMCs). Because the embryo-to-fetus transition (embryonic day 17 to embryonic day 19) marks a period during which cultured SMCs lose the capacity to replicate autonomously, the loss of emb8:EGAP mRNA by fetal day 19 suggests that emb8:EGAP (and other emb clones) may play a functional role in the regulation of autonomous replication.

Previous data from our laboratory demonstrated that pulmonary artery and airway SMC and airway epithelial cell replication rates during development parallel those observed for aortic SMCs, suggesting that an intrinsic, developmentally timed mechanism controls the replication of a variety of cell types. We examined the expression of emb8:EGAP mRNA in the developing rat lung, a tissue known to undergo extensive remodeling throughout intrauterine and early postnatal life. Intense expression of emb8:EGAP mRNA was observed in embryonic extraparenchymal pulmonary arteries and airways, there was a marked decrease in signal intensity in all lung structures by fetal life, and virtually no emb8:EGAP signal was detected in pulmonary arteries or airways during early postnatal life (Figure 4). The decrease in signal intensity was typical for most tissues that express emb8:EGAP mRNA (data not shown), suggesting that along with its potential for regulating vascular SMC autonomous growth, emb8:EGAP may, in general, play an integral role in driving early developmental growth programs.

Our previous data demonstrated that adult SMCs isolated from experimentally injured arteries during periods of peak in
vivo replication exhibit an autonomous growth phenotype similar to that expressed by embryonic SMCs. We next analyzed a complete series of balloon-injured rat carotid arteries to determine the temporal pattern of expression of emb8:EGAP mRNA after vascular injury (Figure 5). The emb8:EGAP mRNA was undetectable in uninjured carotid arteries and was first detected at high levels in the arterial media 4 days after injury, and expression remained high throughout the developing neointima at 7 and 9 days after injury. By 14 days after injury, emb8:EGAP mRNA remained detectable only on the growing, luminal edge of the neointima. By 3 weeks after injury, emb8:EGAP mRNA expression was undetectable in the arterial media and neointima. A similar pattern of expression was observed for emb37 (not shown). Therefore, reexpression of emb8:EGAP (and emb37) mRNA after vascular injury correlated temporally with large in vivo increases in neointimal SMC replication and with the reexpression of an autonomous growth phenotype by neointimal SMCs.

These data clearly demonstrate that emb8:EGAP mRNA is temporally expressed in vivo during periods of significant SMC replication. Northern blot analysis was used to examine the expression of emb8:EGAP mRNA in cultured embryonic, adult, and neointimal SMCs. RNA was isolated from highly replicative, autonomous embryonic and neointimal SMCs maintained in serum-free conditions and from highly replicative, serum-stimulated adult SMCs. As shown in Figure 6, emb8:EGAP mRNA was readily detectable in serum-deprived, but growing, embryonic and neointimal SMC cultures; was detected only at low levels in serum-stimulated, replicating adult SMCs; and was essentially undetectable in serum-deprived adult SMC cultures (not shown), consistent with the in vivo in situ hybridization data and consistent with its potential role in the regulation of autonomous replication of SMCs.

Northern blot analysis revealed a single emb8:EGAP transcript of \( \approx 2.6 \text{ kb} \), and this clone has been sequenced in its entirety (Figure 1 online; available in an online-only data supplement at http://www.circresaha.org). Translation analysis of the cDNA showed that the emb8:EGAP gene product contains an open reading frame encoding a 725-amino acid protein. The translated protein contains a consensus translation initiation signal sequence, a potential nuclear transloca-
tion signal, and putative tyrosine and serine phosphorylation sites but lacks a signal sequence for sorting through the secretory pathway. DNA and protein sequence analyses showed no significant homologies to known sequences in the GenBank database, suggesting that emb8:EGAP is a novel protein. However, a blast search of all deposited expressed sequence tags (updated monthly) showed significant homology to human clone RP11-379P1 located on chromosome 9. Homologies extended from bases 901 through 2278 and identities ranged from 83% to 100% over this region. Similarly, there was significant homology to Mus musculus clone MNCb-6493 extending from base 985 through 1833 and exhibiting 93% identity in this region. There also were significant homologies with Drosophila and Caenorhabditis elegans expressed sequence tags of unknown function (71% and 63%, respectively). Although the biological function of emb8:EGAP and its homologs is unknown, these data suggest that emb8:EGAP is a highly conserved gene.

To determine whether inhibition of the expression of emb8:EGAP could affect autonomous growth potential, we administered antisense emb8:EGAP oligodeoxynucleotides (ODNs) to embryonic and neointimal SMC cultures. Using FITC-labeled oligonucleotides, we found that the efficiency of uptake of ODNs by embryonic, neointimal, and adult SMCs was essentially equal and that ODNs were localized to the cytoplasm (Figure 7C). As shown in Figure 7A, under serum-free conditions, untreated and sense ODN-treated SMC cultures continued to demonstrate high rates of bromodeoxyuridine (BrdU) incorporation (replication index >70%). In contrast, a single administration of antisense emb8:EGAP ODNs (5 μmol/L) resulted in a significant decrease in BrdU-positive embryonic and neointimal SMCs (replication index 59±0.5% and 30.5±3.5%, respectively), suggesting that emb8:EGAP may play a functional role in the regulation of SMC autonomous growth. We detected no difference in results in parallel experiments with an interferon-γ-neutralizing antibody, suggesting that antisense ODN treatment did not result in nonspecific inhibition of SMC growth through double-strand DNA induction of interferon-γ (not shown). In contrast, emb8:EGAP sense and antisense
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Discussion

In the present study, we sought to identify genes preferentially expressed by embryonic and neointimal SMCs with autonomous growth potential, with the ultimate goal of elucidating the molecular mechanisms responsible for conferring this unique and important growth property to SMCs. We hypothesized that this growth phenotype may involve the biologically timed expression of specific growth-associated genes controlled by a mechanisms inherent to the SMCs themselves. Using a subtractive hybridization technique that was aimed at specifically identifying only those genes expressed by cells with autonomous growth potential, we identified 14 clones in embryonic SMCs that were not expressed by highly proliferative adult SMCs. Eleven embryonic clones are presumably novel genes, because no significant sequence homologies to known genes were found in database screenings.

The expression pattern of each of the 14 genes was not exclusive to vascular structures, because 10 embryonic genes were ubiquitously expressed in all embryonic tissues evaluated. Importantly, however, the vast majority of embryonic genes were not expressed in any of a variety of adult tissues and specifically were never expressed by any cells in adult uninjured blood vessels. When detected in adult tissues, these genes were expressed only by rapidly replicating cells in tissues that undergo continuous renewal (eg, intestinal crypts, spermatocytes, and basal epidermis), a process that occurs via mechanisms as yet largely unknown. We found that 7 embryonic genes were specifically, but only transiently, upregulated in SMCs in response to balloon catheter–induced injury. The expression patterns of these 7 embryonic genes correlated to extremely high rates of in vivo replication during development and after injury and to autonomous growth capabilities of SMCs in vitro. However, the existence of 7 genes in embryonic cells that are not reexpressed after vascular injury suggests that the reexpression of embryo-specific genes is not all inclusive and that certain unique properties of embryonic SMCs are not observed or reexpressed after balloon catheter injury. Finally, antisense constructs to emb8:EGAP specifically reduced the autonomous proliferative potential of embryonic and neointimal SMCs. Thus, our data suggest that unique gene products may be necessary to confer autonomous growth potential to SMCs. Elucidation of the function of these genes could provide important insight into the mechanisms that control SMC growth during critical periods of development and in response to injury.

Both emb2:pendulin and emb22:S6 ribosomal protein, reexpressed by autonomously growing cells in the setting of vascular injury, have been shown in other cell systems to play integral roles in processes related to cell growth. For example, *Drosophila* strains defective in ribosomal protein synthesis exhibit a delayed developmental program manifested by slower rates of cell growth and division. The mammalian S6 ribosomal protein, rapidly phosphorylated when cells are stimulated to grow or divide, is essential for normal development. Likewise, pendulin, a member of a superfamily of proteins that contain Armadillo repeats, is required for the nuclear import of DNA-binding proteins involved in cellular proliferation. Furthermore, emb39:cdc25A was highly expressed in embryonic, autonomously replicating SMCs but not in adult SMCs or in injured arteries. Cdc25 phosphatases act during G1/S or G2/M as critical links between various developmental signals and cell cycle control. Although emb39:cdc25 was not significantly reexpressed in injured arteries, our observation that emb39:cdc25A is highly expressed in embryonic, autonomously replicating SMCs and not in adult SMCs is strongly suggestive of a functional role for this gene product in self-driven replication and again supports the idea that not all embryonic processes are reiterated in adult SMCs that respond to injury. Furthermore, that fact that the protein products of these known embryonic genes are regulators of cell growth is supportive evidence that our cloning method yielded genes that are functionally involved in the regulation of certain aspects of self-driven replication rather than merely being markers of an embryonic SMC.

The expression patterns of at least 7 genes in our study were similar to those of another gene, EVEC, that was recently described by Kowal et al. The authors cloned a novel calcium-binding, EGF-repeat–like protein from PAC-1 cells, a highly differentiated SMC line, but suggested that its structure and expression pattern are consistent with a role for EVEC in the regulation of vascular growth during develop-
ment and in lesions in injured vessels. In our study, the expression pattern and signal intensity of the most extensively studied emb gene, emb8:EGAP, were also highly suggestive of a functional role for EGAP in SMC growth during development and in response to injury. However, the structure of the emb8:EGAP-translated product did not lend insight into the specific functions of this protein. We therefore used an antisense approach to determine whether emb8:EGAP was participating in the autonomous growth process. Inhibition of emb8:EGAP in embryonic and neointimal SMCs decreased the autonomous growth capacity of these cells. However, the loss of function of only 1 emb gene in embryonic and neointimal SMCs did not result in the same degree of growth arrest as observed in adult SMCs under mitogen-free conditions, suggesting that ≥2 emb gene products may coordinately interact to determine an autonomous growth phenotype. These results are not surprising given that the development of a mature vascular system or the formation of a neointima after injury likely involves the integration of multiple intracellular signals. Inasmuch, future studies have been designed to analyze the interactions of multiple emb genes on the autonomous growth potential of SMCs.

Interestingly, the degree of growth inhibition by emb8:EGAP ODN was consistently greater in neointimal SMCs than in embryonic SMCs, suggesting that although the autonomous growth characteristics of embryonic and neointimal SMCs are similar, the factors that ultimately regulate the growth phenotypes of these 2 cell types might differ slightly. Based on our fluorescent-labeled ODN experiments, it is unlikely that these results are due to more efficient uptake of antisense ODNs by the neointimal SMC populations used in this study. It is currently unclear whether all SMCs in the vascular media are capable of expressing emb genes and the autonomous growth phenotype or whether this is the unique capacity of only certain SMC subpopulations. Several investigations not only point out the heterogeneous nature of SMCs in the adult vessel wall but also have suggested that only certain unique cell populations contribute to the intimal thickening process.25–27 The finding that SMCs may originate from sources other than traditional mesoderm-derived cells is highly suggestive of the possible existence of several distinct subtypes of SMCs.28,29 Nevertheless, the transient reexpression of an autonomous growth phenotype after injury11 in conjunction with the reexpression of several embryo-specific genes suggests that at least some SMC populations exhibit plasticity in relation to their growth properties.30 Our data

Figure 7. Emb8:EGAP-specific antisense oligonucleotides attenuate autonomous replication of embryonic and neointimal SMCs. E17 and Neo7 (A) and Ad SMCs (B) were plated at 5 × 10^4 cells/1.9 cm^2 in 10% CS, allowed to attach and spread overnight, maintained in SFM for 72 hours, and then labeled for an additional 24 hours with BrdU in SFM or in 10% CS. Single-stranded sense or antisense ODNs were added to cultures when the media were changed at the beginning of the growth arrest period. Replicating SMCs were identified with BrdU immunocytochemistry. The data were determined in triplicate and are presented as mean ± SEM. C, SMC cultures were treated as described with FITC-labeled ODN (green); cells were fixed, stained with DAPI to identify total cell nuclei, and analyzed with fluorescent microscopy.
therefore suggest that although the self-driven growth of neointimal SMCs is similar to that embryonic SMCs, it is unlikely that an entire developmental growth program is reexpressed after vascular injury.

The treatment of vascular diseases characterized by excessive SMC proliferation has in large part been unsuccessful and continues to be an important clinical issue. However, strategies designed to inhibit the growth of SMCs and decrease the formation of the neointima after experimental injury have been only variably successful, supporting the idea that neointimal SMCs use unique growth pathways that have not yet been fully defined. This could explain the failure to identify an exogenous factor responsible for neointimal SMC replication despite intense efforts to find one. The findings of the present study point to the possibility that a combination of novel genes may be used to drive SMC proliferation during critical early periods of embryonic growth and neointima formation. Studies are ongoing in our laboratory to further characterize the structure and function of these embryonic gene products, and future studies aimed at mutational deletion of these genes could confirm their roles in proliferation, thereby providing potential alternative treatment strategies for fibroproliferative vascular diseases.

Note Added in Proof
After submission and acceptance of this manuscript, another group (Yi XJ, Li XF, Yu FS. A novel epithelial wound-related gene is abundantly expressed in developing rat cornea and skin. Curr Eye Res. 2000;20:430–440) described the cloning and expression of a novel corneal wound healing–related protein from rat healing corneal tissue. These results will be included in the final version of the manuscript of another group and studies are in progress to characterize this gene.

Acknowledgments
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MATERIALS AND METHODS ONLINE

Animals, In Vivo Tissue Preparation and Cell Culture: Whole embryos or arterial tissues from Sprague-Dawley rats were collected and were processed as previously described (1,2). Testis, epidermis, brain, lung, skeletal muscle, kidney, intestinal, and liver tissues were collected from adult male rats and processed as described above. The procedures followed were in accordance with institutional guidelines. For cell culture, the aortic media from adult (Ad SMC) and from embryonic day 17 (e17 SMC) rats and the carotid arterial media and neointima from 7 day balloon catheter injured adult rats (Neo7 SMC) were aseptically dissected and SMC were obtained by explant technique as described previously (2). E17 SMC which were used as a source of RNA for cDNA library construction were passed at low density, allowed to reach 40%-50% confluency, and then cultured in DMEM with 0.1% CS for 72 hours. RNA used for Northern analysis was isolated from autonomously replicating e17 and Neo7 SMC (maintained under serum-deprived conditions), from growth-arrested Ad SMC, and from highly replicative Ad SMC (maintained in DMEM containing 10% CS). Experiments were performed with SMC in passage 9-14. Growth phenotypes were determined by BrdU immunocytochemistry as described by Cook et. al.(1). All tissue culture supplies were purchased from Gibco BRL (Gaithersburg, MD).

cDNA Library Construction: A custom-made cDNA library was constructed in the Lambda ZAP Express™ EcoRI/XhoI replacement vector (Stratagene). Poly(A⁺) RNA (10 µg) from e17 SMC was oligo d(T)-primed and cDNA was size selected (>400 bp) prior to ligation and packaging. A total of 1.3×10⁶ primary plaques were generated and amplified prior to screening. Maintenance, propagation and genetic manipulation of the
lambda library, resulting phagemids, associated bacterial host strains and f1 helper phage were according to the manufacturer's protocol (Stratagene, LaJolla, CA).

**e17-Specific Subtracted cDNA and Library Screening:** Adult cDNA was synthesized on magnetic beads (DynabeadsR Oligo(dT)25, Dynal Inc.) using the method of Rodriguez and Chader (3). Adult cDNA (200 ng on beads) was resuspended in 100 ml 5X SSC with 2 µg of tRNA included as carrier. e17 poly(A+)RNA (100 ng/100 ml 5X SSC) was added and allowed to denature at 94ºC for 2 min and to anneal at 55ºC for 6-12 hours. The supernatant containing subtracted, e17-specific mRNA was removed for rehybridization to the adult cDNA under the same conditions. Beads containing adult cDNA-e17 mRNA hybrids were regenerated for rehybridization by destroying the mRNA by alkaline hydrolysis and reequilibrating them in 5X SSC. The library was screened by plating out approximately 3x10⁵ plaques which were lifted onto nylon membranes (Duralon-UV™, Stratagene, LaJolla, CA) and probed with ³²P-dCTP-oligo(dT) primer labeled cDNA using twice-subtracted e17-specific mRNA as template.

**DNA Sequencing and Sequence Analysis:** Phagemid DNA was prepared by the alkaline lysis method. The 5’ and 3’ ends of each clone were sequenced using the SequenaseR kit (United States Biochemical Corp., Cleve., OH), ³⁵S-dATP, and either the T3- or T7-specific promoter primers found in the pBK phagemid (Stratagene, LaJolla, CA). For some clones, cDNA inserts were cloned into M13 for single stranded sequencing (4). Sequence data obtained was compared to those in the Genbank nucleic acid and protein databases by BLAST searching on the NCBI server.

**In Situ Hybridizations and Northern Blot Analysis:** In situ hybridizations were performed as previously described (5). Slides were developed after 21 days and
counterstained with hematoxylin and eosin. For Figure 1, the relative amounts of emb mRNA expression was assessed by counting exposed silver grains per square micrometer using NIH IMAGE software and are reported as fold signal over background sense probe signal. Counts were performed on three animals per time point for each message. For Figure 3B, quantitation of emb8 and emb37 gene expression was performed by counting a minimum of 400 cells per section. Cells were considered positive for emb8 or emb37 expression if five or more exposed silver grains were present over their nucleus and cytoplasm. Total RNA for Northern analysis was isolated from e17, Neo7, and Ad SMC cultures using Trizol Reagent (Sigma Chemicals, St. Louis, MO). The quantity and purity of the RNA samples were determined by spectrophotometric analysis. Northern blots were prepared, hybridized with 32P-labeled riboprobes using the Strip-EZ RNA probe kit (Ambion, Austin, TX) and were exposed to a phosphoimager screen for 3 days. Densitometry readings of the emb8:EGAP signal were obtained and normalized to a GAPDH signal (analysis performed on a Macintosh computer using the public domain NIH Image program; developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Riboprobes:** The riboprobes used for Northern blot and in situ hybridizations were as follows: emb8:EGAP and emb37: 568 bp and 600 bp, respectively, rat cDNA fragments generated by PCR using emb8:EGAP- or emb37-specific oligonucleotide primers and embryonic rat aortic RNA as template; GAPDH: a 403 bp rat cDNA fragment generated by PCR using GAPDH-specific oligonucleotide primers and adult rat aortic RNA as template (6). PCR-generated cDNA fragments were subcloned into the pCRII vector (Invitrogen Corp., San Diego, CA) and were sequenced prior to use to verify their
sequence specificity and to determine the direction of cloning. \(^{32}\text{P}-\text{UTP}\)-labeled antisense riboprobes for Northern analysis and \(^{35}\text{S}-\text{UTP}\)-labeled sense and antisense riboprobes for in situ hybridizations were generated by in vitro transcription using the T7 and/or Sp6 promoters (pCRII vector). For initial in situ hybridization screening of all emb clones, riboprobes were prepared as described above using the cloned emb inserts in the pBK phagemid vector, the T7 and T3 promoters, and \(^{35}\text{S}-\text{UTP}\).

**Antisense Oligonucleotide Analysis:** Single-stranded 24 bp antisense and sense oligodeoxynucleotides (ODN) were designed against the 5’ region of the emb8:EGAP gene flanking the start codon and were synthesized and purified by Oligos Etc., Inc. (“Optimized oligomers”; Wilsonville, OR). E17, Neo7, and Ad SMC were plated in triplicate at 5 x 10^4 cells per 1.9 cm^2 in 10% CS and were allowed to attach and spread overnight. The media were changed and cells were maintained in SFM for 72 h, then were labeled for an additional 24 h with BrdU in SFM. ODN (5 or 10 μM final concentration) were added to cultures when the media were changed. Replicating SMC were determined by BrdU immunocytochemistry.
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FIGURES ONLINE

Online Figure 1: DNA sequence of emb8:EGAP cDNA. emb8:EGAP cDNA was sequenced as described in Materials and Methods. The predicted amino acid sequence (estimated 725 amino acids) for the open reading frame extending from nucleotide 92 to 2266 is shown below the DNA sequence using the one letter code. The AUG start codon is highlighted in **bold** and the consensus sequence for initiation of translation is *underlined* in bold. The polyadenylation signal and poly A+ tail are *underlined*. Potential tyrosine phosphorylation and myristoylation sites are underlined with dotted and **double** lines, respectively.
<table>
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