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Restricted expression of homeobox genes distinguishes fetal from adult human smooth muscle cells

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ABSTRACT **Smooth muscle cell plasticity is considered a prerequisite for atherosclerosis and restenosis following angioplasty and bypass surgery. Identification of transcription factors that specify one smooth muscle cell phenotype over another therefore may be of major importance in understanding the molecular basis of these vascular disorders. Homeobox genes exemplify one class of transcription factors that could govern smooth muscle cell phenotypic diversity. Accordingly, we screened adult and fetal human smooth muscle cell cDNA libraries with a degenerate oligonucleotide corresponding to a highly conserved region of the homeodomain with the idea that homeobox genes, if present, would display a smooth muscle cell phenotype-dependent pattern of expression. No homeobox genes were detected in the adult human smooth muscle cell library; however, five nonparalogous homeobox genes were uncovered from the fetal library (*HoxA5, HoxA11, HoxB1, HoxB7,* and *HoxC9*). Northern blotting of adult and fetal tissues revealed low and restricted expression of all five homeobox genes. No significant differences in transcripts of *HoxA5, HoxA11,* and *HoxB1* were detected between adult or fetal human smooth muscle cells in culture. HoxB7 and HoxC9, however, showed preferential mRNA expression in fetal human smooth muscle cells that appeared to correlate with the age of the donor. This phenotype-dependent expression of homeobox genes was also noted in rat pup versus adult smooth muscle cells. While similar differences in gene expression have been reported between subsets of smooth muscle cells from rat vessels of different-aged animals or clones of rat smooth muscle, our findings represent a demonstration of a transcription factor distinguishing two human smooth muscle cell phenotypes.**

Cultured arterial smooth muscle cells are able to display diverse phenotypes depending on whether the cells are cloned, isolated at early developmental stages, or isolated from an injured vessel. This apparent diversity *in vitro* may reflect the existence *in vivo* of smooth muscle variants with potential significance for the development of vascular diseases, especially atherosclerosis. Since the intima is the site where atherosclerosis develops, there is great interest in the possible role that intimal smooth muscle cells may play in the ontogeny of atherosclerotic lesions (1–3). For example, intimal smooth muscle cells overexpress a number of genes likely to be important in vascular occlusive disease, including those encoding tissue factor, thrombin receptor, osteopontin, and lipoprotein lipase (1, 4–20). Many of the genes that are overexpressed in the intima *in vivo* are maintained when intimal cells are placed into culture, and cultured intimal smooth muscle cells have a morphology, set of growth factor requirements, and pattern of gene expression that distinguish them from medial smooth muscle cells (7–9, 21, 22). Patterns of gene expression similar to those seen in neointimal smooth muscle cells are also observed in smooth muscle cells cultured from the 2-week-old rat pups (7–10, 22), while other distinct patterns of morphology and/or gene expression are seen in fetal arterial smooth muscle or smooth muscle from newborn animals (20, 23). Similarly, diverse morphologies and patterns of gene expression can be seen when smooth muscle cells are cloned from the arteries of rat pups (24) or adult rats (25).

The above correlations between *in vivo* and *in vitro* systems suggest that smooth muscle diversity could be an important consideration in vascular responses to injury. This is true whether diversity is due to the existence of true cell subtypes—that is, cells with stable mechanisms controlling differences in gene expression—or due to more transitory phenomena such as the release of growth factors and cytokines at sites of vascular injury (26, 27). In either case, smooth muscle cell phenotypic diversity is most likely governed by the action of transcription factors. Indeed, many such factors are rapidly induced in smooth muscle cells following balloon injury to the vessel wall and have been demonstrated in diseased human vessels (6, 9, 12, 15). The transcription factors modulated in smooth muscle cells after vascular injury include the immediate and delayed early genes which are considered important for cell cycle traversal as well as a homeobox gene product, Gax, that is normally present in quiescent smooth muscle cells but becomes transiently downregulated prior to DNA replication (6, 15, 21, 28). Gax, along with the upregulated components of the cell cycle, however, probably cannot explain the stable, cell cycle-independent differences in patterns of gene expression seen, for example, in smooth muscle cells cultured from arteries of animals of different ages or the diversity present in the normal intima or the intima of quiescent atherosclerotic lesions (4, 6–12, 14, 15, 18, 21, 24).

Because the vascular smooth muscle cell lineage represents such a diverse and plastic population of cells, we decided to screen for transcription factors that could potentially specify certain smooth muscle cell phenotypes. One such class of transcription factors is encoded by the homeobox gene family. Homeobox genes were first described in *Drosophila*, where their spatiotemporal pattern of expression during development has been linked to the establishment of a correct body plan (29, 30). In addition to directing proper body organization, the products of some homeobox genes have been shown or postulated to specify cell types through the activation of cell-specific genes (31–38). Cserjesi *et al.* (39) cloned a novel homeobox gene, MHox, by expression cloning using an oligonucleotide corresponding to the skeletal muscle creatine kinase enhancer. The expression of MHox in aortic smooth muscle cells prompted us to ask whether other, potentially smooth muscle cell-specific, homeobox genes exist in smooth muscle cells and, if so, whether their expression changes with the phenotype of smooth muscle cells. Here we describe the cloning of five homeobox genes heretofore not described in

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human smooth muscle cells. Expression of two of the homeobox genes is restricted to human fetal smooth muscle cells; no expression is observed in several human adult smooth muscle cell lines or adult aorta. The potential involvement of homeobox genes in specifying smooth muscle cell phenotypes is discussed in terms of how such factors may contribute to the development of smooth muscle cell diversity and, thus, vascular occlusive disease.

MATERIALS AND METHODS

Smooth Muscle Cell Culture. Human smooth muscle cells were derived by enzymatic dispersion (40) from thoracic aorta specimens obtained from heart transplant donors and aborted conceptus tissue obtained from the Central Laboratory for Human Embryology, University of Washington. Cells were grown in Waymouth's complete medium (GIBCO) supplemented with disodium pyruvate, nonessential amino acids and glutamate, 10% fetal bovine serum (HyClone), penicillin, and streptomycin. Smooth muscle cells from both fetal and adult donors were characterized by a spindle-shaped morphology and positive immunostaining for smooth muscle α-actin (SM-1; Sigma) and α-vimentin (Dako). The lack of reactivity with an antibody to von Willebrand factor showed that endothelial cell contamination was absent. A complete description of these cells has been published (40). Cells were used between passages 3 and 7. Rat smooth muscle cells were isolated and cultured as described (41) and were used between passages 12 and 20.

Library Construction. Oligo(dT)-primed cDNAs were prepared from fetal and adult aortic smooth muscle cells as described (8), with minor modifications. With the use of oligo(dT)-*Xho* I primers and *Eco*RI adapters, cDNAs were force-cloned into the *Eco*RI and *Xho* I sites of the λ Uni-Zap XR vector (Stratagene). cDNA libraries were packaged by using Gigapack II Gold packaging extract (Stratagene). With color selection by α-complementation, the fetal and adult smooth muscle cell libraries were found to contain 400,000 and 550,000 recombinants, respectively. Both libraries were utilized for these studies after one round of amplification.

Library Screening. Approximately 500,000 plaques from each library were screened as described, with minor modifications (42), by hybridization with a 768-fold degenerate oligonucleotide corresponding to the recognition helix of the homeodomain. The sequence of the degenerate oligonucleotide was 5′-AA(A/G)AT(A/C/T)TGGTT(C/T)CA(A/G)AA(C/T)(C/A)G(A/C/G/T)(C/A)G-3′. This degenerate oligonucleotide was expected to anneal to any one of 38 homeobox genes within the four clusters (43). The degenerate oligonucleotide ($\approx$100 ng) was end-labeled with polynucleotide kinase (Boehringer Mannheim) in the presence of 100 μCi (3700 kBq) of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). Duplicate filters were prehybridized in 5× standard saline citrate (SSC)/5× Denhardt's solution/0.5% SDS with salmon sperm DNA at 200 μg/ml for 24 hr at 39°C. Then end-labeled degenerate oligonucleotide was added at 1–2 × 10^6 cpm/ml to the prehybridization mixture and incubation was continued for another 48 hr at 39°C. Filters were washed at room temperature for 30 min each in 3× SSC/1% SDS and 1.5× SSC/0.5% SDS. Following autoradiography, duplicate signals were cored and rescreened until plaque pure.

Sequencing. Plaque-pure clones were auto-excised from the λ vector by using R408 helper phage as described (Stratagene). Restriction digests were carried out with *Eco*RI and *Xho* I and the resulting fragments were Southern blotted. Clones that hybridized to the degenerate oligonucleotide were sequenced (Sequenase 2.0; United States Biochemical) with an automated sequencer (Applied Biosystems) using both the sense and antisense degenerate oligonucleotide as primers. Authentic homeobox genes were then used as probes to back-hybridize remaining positives for the identification of sister clones. All sequence analysis was performed with the FASTA program in the Genetics Computer Group software package (Biomathematics, University of Texas M. D. Anderson Cancer Center).

Northern Blotting. For isolation of total cellular RNA, cells were grown in T-75 flasks (Costar) and harvested 1–3 days after reaching confluence. Preconfluent cultures were isolated after cells had covered 70–80% of the flask surface. Frozen human or mouse tissues (0.1–1.0 g) were ground to a fine powder with a mortar and pestle cooled by liquid nitrogen, and RNA was extracted by the method of Chomczynski and Sacchi as modified by Majesky *et al.* (21). Northern blot analysis was performed as described (44). Radiolabeling of cDNA and oligonucleotide probes and conditions used for hybridization have been described (21, 44).

Reverse Transcription–PCR. One microgram of total RNA from cultured human fetal and adult smooth muscle cells was incubated for 15 min at 37°C with 40 units of RNasin (Promega) and 10 units of DNase I (Pharmacia) in 50 mM Tris·HCl, pH 8.3/8 mM $MgCl_2$/10 mM dithiothreitol and then heat-inactivated for 15 min at 80°C. DNase-treated total RNA was reverse-transcribed by incubation for 1 hr at 37°C with 40 units of Moloney murine leukemia virus reverse transcriptase (Pharmacia), 80 units of RNasin (Promega), and 10 units of DNase I (Pharmacia) in 50 mM Tris·HCl, pH 8.3/8 mM $MgCl_2$/10 mM dithiothreitol/1 mM dNTPs/2.5 pM random hexamers (Pharmacia). Each reaction mixture was heat-inactivated at 95°C for 5 min. One hundred nanograms of cDNA was amplified in 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/1.6 μM 5′ primer (5′-GCAGAACCTCTCCGGGGTGTG-3′)/1.6 μM 3′ primer (5′-CCTCTGCTTCAGCCCTGTCTTGG-3′)/200 μM dNTPs with 2 units of *Taq* DNA polymerase (Stratagene). PCR mixtures were heated at 94°C for 5 min and then amplified for 45 cycles of 94°C for 1 min and 72°C for 1 min, followed by an additional extension period at 72°C for 10 min.

RESULTS

Cloning of Five Homeobox Genes from Human Fetal Smooth Muscle Cells and mRNA Expression in Adult and Fetal Tissues. No homeobox genes were identified from the adult human smooth muscle cell library. Primary screening of the fetal human smooth muscle cell library, however, revealed 121 duplicate signals. Of these, 52 remained positive through successive rounds of screening. Upon sequence analysis with the degenerate oligonucleotide probe the clones were classified as follows: *HoxA5* (4 clones); *HoxA11* (1 clone); *HoxB1* (1 clone); *HoxB7* (24 clones); *HoxC9* (1 clone); *Alu* repeats (17 clones); cDNAs with partial homology to the degenerate oligonucleotide (4 clones). Northern analysis of adult mouse tissues generally showed a low level of expression for all the *Hox* genes examined (Fig. 1). Of notable importance was the absence of *Hox* transcripts in adult aorta, a finding that supports our negative *Hox* screen of the adult smooth muscle cell library. Interestingly, *HoxA11* transcripts, which have previously been reported only in embryonic chicken limbs (45), were detected in adult uterus, as were *HoxB1* messages (Fig. 1). Although the bulk of uterine tissue is smooth muscle, it is possible that these *Hox* genes are expressed by endometrial cells. *In situ* hybridization will be necessary to pinpoint the expression of *HoxA11* and *HoxB1* in adult uterus.

In contrast to adult tissues, we found that levels of two *Hox* genes (*HoxB7* and *HoxC9*) were abundantly expressed in certain fetal tissues (Fig. 2). Thus, *HoxB7* mRNA was present in human fetal lung at day 62 through day 76. *HoxB7* transcripts were also found in kidney at day 67 through day 76 and in thigh at day 83. *HoxC9* was found in kidney at day 67 through day 72 and in thigh at day 83 (Fig. 2). Both *HoxB7* and *HoxC9* probes hybridized to fetal brain/spinal cord (data not shown). Like adult lung (Fig. 1), fetal lung tissue showed a prominent *HoxA5* signal (data not shown).

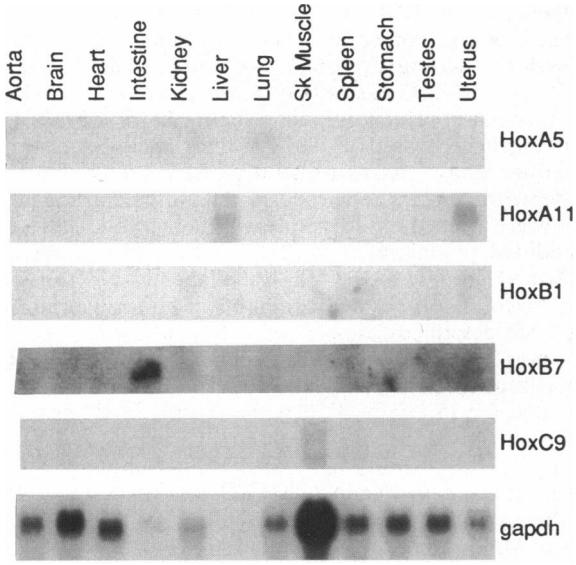

FIG. 1. Representative panel of adult mouse tissue Northern blots showing steady-state mRNA expression of five homeobox genes cloned from a human fetal smooth muscle cell library. Total RNA (20 μg per lane) was prepared as described under *Materials and Methods* and hybridized to the indicated *Hox* cDNA clones. The apparent *HoxB7* signal under intestine is a blot artifact. Exposure times were 3 days for *HoxA5, HoxA11*, and *HoxB1*; 4 days for *HoxB7*; and 36 hr for *HoxC9*. A glyceraldehyde-3-phosphate dehydrogenase (gapdh) probe was used to control for variations in sample integrity and loading.

Homeobox Gene Expression in Cultured Smooth Muscle Cells. To date, no transcription factors have been demonstrated to distinguish human fetal versus adult smooth muscle cells. To determine whether the cloned *Hox* genes displayed a phenotype-dependent pattern of expression, total RNA (from the same human fetal and adult smooth muscle cell lines used to construct the cDNA libraries) was hybridized to the five cloned *Hox* genes. No *HoxB1* transcripts were observed in either cell line. *HoxA5* and *HoxA11* mRNAs were expressed in both fetal and adult smooth muscle cell lines (data not shown). However, expression of *HoxB7* (and to a lesser degree, *HoxC9*) showed a striking restricted pattern of expression in fetal smooth muscle cells (Fig. 3*A*). This finding is consistent with the number of *HoxB7* clones obtained from the fetal smooth muscle cell cDNA library (24 of 31 *Hox* clones). The expression of *HoxB7* and *HoxC9* mRNAs was independent of fetal smooth muscle cell growth, as both preconfluent and confluent cultures displayed transcripts of both *Hox* genes (Fig. 3*B*).

We next isolated four independent human fetal and adult smooth muscle cell lines to ascertain whether the restricted expression of *HoxB7* and *HoxC9* mRNAs for fetal smooth muscle cells was a general finding. *HoxB7* mRNA was detected in three of the four fetal smooth muscle cell lines (Fig. 3*A*). Of the fetal smooth muscle cell lines expressing *HoxB7*, two exhibited transcripts for *HoxC9* (Fig. 3*A*). The variable expression of *HoxB7* and *HoxC9* in fetal smooth muscle cell lines could relate to the age of the donor. It will be necessary to further characterize the phenotypic differences in these and other fetal smooth muscle cell lines, since they may represent a continuum of phenotypes related to their stage of differentiation. In contrast, none of the four individual human adult smooth muscle cell lines showed any evidence for *HoxB7* and *HoxC9* transcripts. Thus, at least two cell-restricted transcription factors show preferential expression in human fetal smooth muscle cell lines.

Gene expression was further assessed in rat aortic smooth muscle cells (Fig. 3*C*). Both *HoxB7* and *HoxC9* mRNAs showed signal expression by Northern blotting in cultured rat pup, but not adult, aortic smooth muscle cells (Fig. 3*C*). Again, this restricted expression pattern did not appear to be related to the growth state of the cells examined. Thus, the expression pattern of *Hox* genes in fetal versus adult smooth muscle cells does not appear to be species-specific.

Homeobox Gene Expression in Fetal Aortic Tissue. Despite the presence of *HoxB7* and *HoxC9* mRNAs in cultured fetal smooth muscle cell lines, Northern analysis of fetal aortic tissue failed to reveal these messages even with prolonged autoradiographic exposure (data not shown). Since this might be due to the lack of sensitivity, we used reverse transcription–PCR to analyze the expression of *HoxB7* and *HoxC9* mRNAs in human fetal aorta. No PCR product was detected in any aortic tissue when *HoxC9* strand-specific primers were used. Strand-specific primers to *HoxB7*, however, yielded a 330-bp amplified product in fetal aortic tissue from days 59, 67, 78, and 124 (Fig. 4). Curiously, no *HoxB7* amplified band was detected in fetal aortic tissue from days 53, 87, and 99 (Fig. 4). Thus, as with our culture data, there does appear to be some variability in expression of *HoxB7* with the age of the donor. This could represent a true developmental difference in *HoxB7* expression but might also reflect interindividual and/or regional differences in *HoxB7* mRNA synthesis. Nevertheless, the presence of *HoxB7* transcripts in several fetal aortic samples indicates that its *in vitro* expression is not a culture artifact. Of most interest, no *HoxB7* signal was found in three separate human adult aortic tissue samples (Fig. 4). Thus, consistent with the cell culture data, *HoxB7* expression is restricted to the fetal smooth muscle cell phenotype *in vivo*.

DISCUSSION

Understanding the molecular basis of smooth muscle cell diversity requires the identification of transcription factors that direct the expression (or repression) of genes defining unique smooth muscle cell phenotypes. Uncovering such factors could have important implications for the potential management of vascular disease, the predisposition to which is thought to involve variable changes in smooth muscle cell phenotype (46). In the present study, we have cloned five homeobox genes (*HoxA5, HoxA11, HoxB1, HoxB7*, and *HoxC9*) from a human fetal smooth muscle cell library by using a degenerate oligonucleotide corresponding to a region of the homeobox that is highly conserved among the more than 340 homeobox genes described to date (43). Two of the cloned genes (*HoxB7* and *HoxC9*) display restricted mRNA expression in human fetal

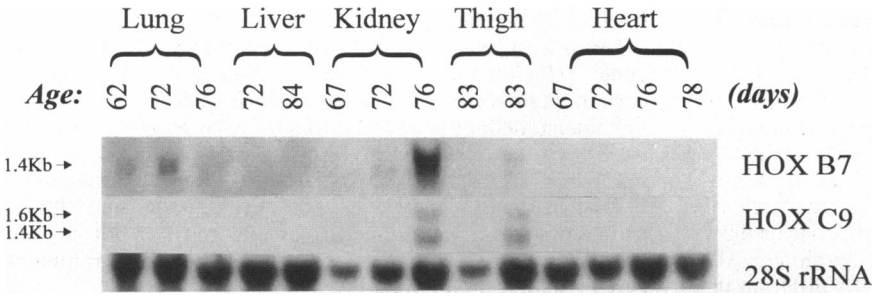

FIG. 2. Survey of expression of *HoxB7* and *HoxC9* in various human fetal tissue as a function of age. *HoxC9* expression is more restricted than *HoxB7* expression, but neither message is detected in the heart. This is intriguing because the developmental distinction between heart and smooth muscle should be an important fork in the lineage of the two muscle cells making up the cardiovascular wall.

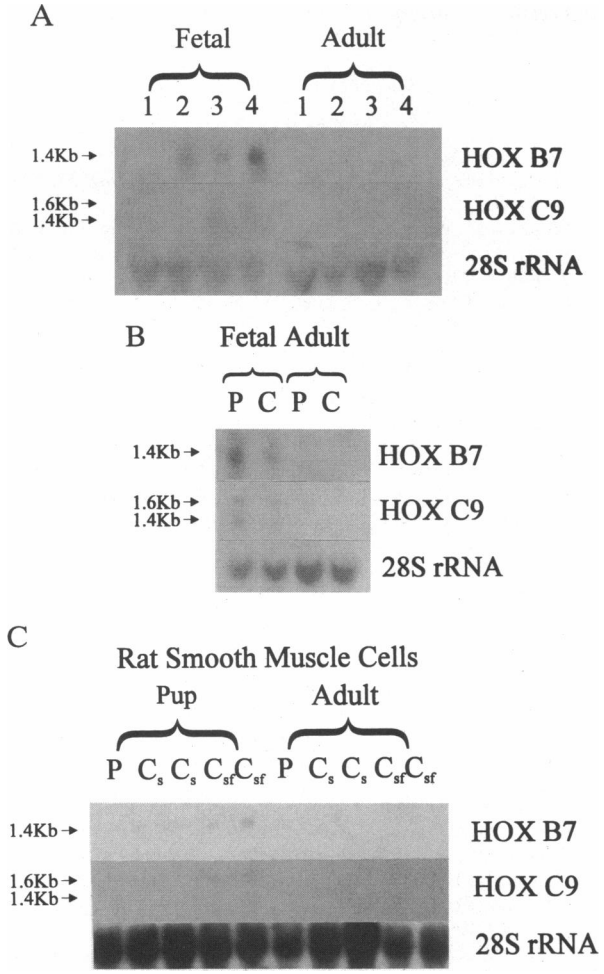

FIG. 3. (*A*) Northern analysis of *HoxB7* and *HoxC9* transcripts in cultured human fetal and adult smooth muscle cell lines. Northern analysis of four human fetal and adult cultured smooth muscle cell lines (passage no. 3–5) grown to confluence detected *HoxB7* mRNA in human fetal smooth muscle cell lines, but not in adult smooth muscle cell lines. *HoxC9* mRNA was detectable as a faint signal only in two of the four fetal lines. Autoradiographic signal was detected between 12 and 21 days. All cells are from lesion-free regions of aorta. Fetal line 1, HfSMC 8 T5 (age 75 days); fetal line 2, HfSMC 7 T5 (age 57 days); fetal line 3, HfSMC 16 T3 (age 115 days); fetal line 4, HfSMC 23 T3 (age 85 days). Adult line 2 is from a 16-year-old; all other adult lines are from middle-aged or older subjects. (*B*) Northern analysis of expression of two fetal-specific homeotic genes in RNA from preconfluent (P) and postconfluent (C) cells. Signals for *HoxB7* and *HoxC9* were present at the same levels in pre- and postconfluent human fetal cells. This suggests that the difference in expression between fetal and adult cells is not due to growth state. Neither *HoxB7* nor *HoxC9* was found in adult cells regardless of their growth state. (*C*) Northern analysis of *HoxB7* and *HoxC9* transcripts in rat cultured smooth muscle cells. Weak signals were detected in RNA from rat pup, but not adult, cultured smooth muscle cells. Signals are very weak and it is difficult to determine a difference due to growth state. RNA was isolated from passaged pup and adult smooth muscle cells. Autoradiographic exposure was 21 days. P, preconfluent cells (80% confluent); C_s, confluent cells with 10% calf serum; C_{sf}, confluent cells incubated 5 days in serum-free medium.

smooth muscle cells, fetal aorta, and cultured rat pup aortic smooth muscle cells; no transcripts were observed in several human adult smooth muscle cell lines, human adult aorta, or adult rat aortic smooth muscle cells. The restricted expression of *HoxB7* and *HoxC9* in fetal smooth muscle cells suggests that these transcription factors play some role in specifying the fetal smooth muscle cell phenotype (see below).

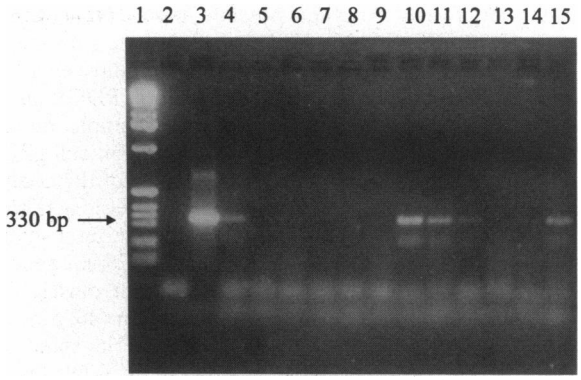

FIG. 4. Agarose gel of *HoxB7* amplified cDNA from human fetal and adult aortic tissue RNA. cDNA was detectable by PCR cDNA amplification in fetal aortic tissue RNA on days 59, 67, 78, and 124. No signal was detectable on days 53, 87, or 99 or in any of the three human adult aortic tissue RNAs. This is consistent with the Northern analysis of the cultured human fetal and adult smooth muscle cells. Lane 1, 1-kb ladder (BRL); lane 2, water control; lane 3, plasmid control *HoxB7*; lane 4, human fetal smooth muscle cell cDNA; lane 5, human adult smooth muscle cell cDNA; lanes 6–8, adult aortic tissue cDNA; lane 9, human fetal aortic tissue cDNA, day 53; lane 10, day 59; lane 11, day 67; lane 12, day 78; lane 13, day 87; lane 14, day 99; lane 15, day 124.

No homeobox genes were isolated from the human adult smooth muscle cell library. Two recent papers, however, reported the cloning of several homeobox genes from an adult rat aortic smooth muscle cell library (28, 47). The homeobox genes cloned from adult rat aortic smooth muscle cells include *HoxA2, HoxA4, HoxA5*, and rat homeobox *R1b* and *Gax*, which is the rat homologue of Mox-2 (48). It is not known whether these homeobox genes show smooth muscle cell phenotype-restricted expression, although one of them (*Gax*) appears to be transiently downregulated in cultured rat aortic smooth muscle cells following growth stimulation (28). Unlike *Gax*, none of the homeobox genes isolated from the fetal smooth muscle cell library were regulated by growth state when we compared mRNA expression in confluent versus growing cells. Thus, fetally restricted expression of homeobox genes may be intrinsic to the cells themselves rather than an inductive process due to extrinsic factors.

The restricted expression of *HoxB7* and *HoxC9* in human fetal smooth muscle cells suggests that their encoded proteins, in some manner, specify this unique, potentially pathologic smooth muscle cell phenotype. Several homeobox genes have been shown or proposed to specify cell identity by activating cell-specific genes. The prototypic example is the gene encoding Pit-1, a protein that defines two cell types of the anterior pituitary gland by directly transactivating either growth hormone (somatotrophs) or prolactin (lactotrophs) (49). Several other novel homeobox genes have been cloned and shown to be essentially cell-specific (31–38). By analogy with Pit-1, it is likely that these homeodomain proteins specify cell types by stimulating the expression of cell-specific genes.

If HoxB7 and HoxC9 do play some role in defining the fetal smooth muscle cell phenotype, at least two modes of action may be envisioned. First, as alluded to earlier, HoxB7 and HoxC9 could directly or indirectly stimulate the expression of other fetally restricted genes, such as those encoding the B chain of platelet-derived growth factor (PDGF-B), osteopontin, tropoelastin, or cytochrome P450IA (7–9, 44). Precedent for this concept is derived from the finding that tenascin, a fetally restricted gene which is induced following balloon injury to the vessel wall (50), is activated by a homeodomain protein, Evx-1 (51). Evx-1 mRNA is expressed by smooth muscle cells *in vivo* following balloon injury (J.M.M. and E.N.O., unpublished observation). Thus, it is possible that the dramatic increase in tenascin mRNA after vascular injury is

mediated by Evx-1. Another possible mode of action for HoxB7 and HoxC9 defining fetal smooth muscle cells may be related to the growth advantage these cells may have over their adult counterparts. Despite the presence of PDGF in the medium of rat pup smooth muscle cells, for example, neutralizing antibodies to PDGF were not able to inhibit cell growth even when the antibodies were given in excess of the concentration required to inhibit all detectable mitogenic activity in 3T3 cells (unpublished data). Moreover, human fetal smooth muscle cells are readily immortalized with the E6/E7 genes of human papilloma virus, whereas adult smooth muscle cells show only a prolongation of replicative lifespan (40, 52). It is possible, therefore, that HoxB7 and HoxC9 in some way facilitate the growth advantage exhibited by fetal smooth muscle cells and by smooth muscle cells from the rat pup (23, 41, 53), perhaps by directly interacting with DNA replication factors as has been proposed for other transcription factors. Possibly relevant to this discussion is the reported oncogenic potential of several homeobox genes (including *HoxB7*) in murine fibroblasts (54).

Finally, since the vascular tree has an obligate pattern colinear with the major segments of vertebrate anatomy, it may be worth considering the possibility that homeobox genes play a role in the complex patterning of the vascular tree. All five of the homeobox genes cloned in this report lie within the four *Hox* gene clusters (43). It is well established that the homeobox genes within these clusters are expressed in discrete regions along the anteroposterior axis of the developing embryo such that the 5′-most homeobox genes are expressed anteriorly and the 3′-most homeobox gene transcripts accumulate in more posterior regions of the embryo (30, 43). It is important to note, however, that secondary sites of expression are often observed during organogenesis. Paralogous homeobox genes are those clustered genes whose spatial and temporal patterns of expression are overlapping. The homeobox genes we cloned from fetal smooth muscle cells are not paralogs, suggesting that smooth muscle cells of the fetal aorta arise from distinct regions along the anteroposterior axis, perhaps in a discontinuous manner as has been postulated for coronary artery development (55). Such a process is compatible with the concept of smooth muscle cell diversity, which may be essential for correct establishment of the major segments of the vascular tree. Alternately, fetal aortic smooth muscle cell *Hox* gene expression could be involved in the elimination of subpopulations of cells that are no longer necessary for vascular wall homeostasis.

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