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HOXB7 Overexpression Promotes Differentiation of C3H10T1/2 Cells to Smooth Muscle Cells

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Abstract The presence of immature smooth muscle cells and ectopic tissues such as fully-formed bone in atherosclerotic lesions, may result from recapitulation of embryonic mechanisms in the artery wall. We hypothesized that expression of homeobox genes is triggered in atherogenesis and that these regulate proliferation and differentiation of multipotential progenitor cells along one or more specific lineages. We identified expression of the homeobox gene HOXB7 in clones of bovine aortic medial cells previously shown to be multipotent. HOXB7 was subsequently detected in human atherosclerotic plaques by RT-PCR and in situ hybridization. Expression was localized to areas adjacent to calcification and scattered in media and neointima, which may be reflective of a role in either osteoblastic or smooth muscle cell differentiation. To differentiate between these possibilities, we overexpressed HOXB7 in C3H10T1/2 cells, a multipotent cell line able to differentiate into vascular smooth muscle cells (SMC), as well as osteogenic and chondrogenic lineages. Results showed that overexpression of HOXB7 increased proliferation 3.5-fold, and induced an SMC-like cell morphology. In addition, expression of the early SMC markers calponin and SM22α increased 4-fold and 3-fold respectively by semi-quantitative RT-PCR. Expression of the intermediate SMC marker smooth muscle myosin heavy chain (SM-MHC) did not change. No increase in osteogenic or chondrogenic differentiation was detected, neither in the C3H10T1/2 cells nor in M2 cells, a bone marrow stromal cell line used to confirm this result. These findings suggest that HOXB7 plays a role in expansion of immature cell populations or dedifferentiation of mature cells. J. Cell. Biochem. 78:210–221, 2000.

Key words: atherosclerosis; smooth muscle cell; homeobox gene

Atherosclerotic lesions are characterized by disturbances in cell differentiation, including the appearance of smooth muscle cells (SMC) of the synthetic phenotype [Majesky et al., 1992; Villaschi et al., 1994; Schwartz, 1994] and other mesenchymal cells such as bone, cartilage, and marrow stromal cells [Bunting, 1906; Buerger and Oppenheimer, 1908; Haust and Moore, 1965; Haust and Geer, 1970]. It is known that adult connective tissues are populated by mesenchymal progenitor cells [Prockop, 1997], however, what factor(s) controls expansion of these cell populations and controls their lineage determination is not known. We hypothesized that embryonic tissue-identity developmental factors, such as homeobox genes, are triggered in atherogenesis and that these regulate proliferation and differentiation of multipotential progenitor cells in the artery wall along one or more specific lineages.

Homeobox genes, coding for homeodomain-containing factors, were first described in *Drosophila*, where expression is linked to the establishment of tissue identity [Dorn et al., 1994; Gehrig et al., 1994]. It has also been postulated that some of the homeodomain-containing factors activate tissue-specific genes [Bodner et al., 1988; Ingraham et al., 1988; Zhao et al., 1994; Lints et al., 1993; Komuro and Izumo, 1993]. Expression of homeobox genes has been demonstrated in vascular SMC and vascular tissues. Miano et al. [Miano et al., 1996] detected five nonparalogous homeobox genes in a fetal human smooth muscle cell library, HOXA5, HOXA11, HOXB1, HOXB7, and HOXC9, of which HOXB7 and HOXC9 showed preferential expression in fetal human SMC. No homeobox genes were detected in

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adult human smooth muscle cell lines or adult aorta. Cserjesi et al. [Cserjesi et al., 1992] also described a homeobox gene in the vasculature named Mhox, expressed in aortic SMC in developing mouse embryos. Patel et al. [Patel et al., 1992] found Hox1.11, and Gorski et al. [Gorski et al., 1993] identified Gax (growth-arrest-specific homeobox) in rat adult aortic tissue.

We looked for homeobox gene expression in clones of bovine aortic medial cells, previously shown to be capable of differentiation along osteoblastic, chondroblastic, and marrow stromal cell lineages [Boström et al, 1993; Watson et al., 1994; unpublished observations]. HOXB7, one of the genes reported to be preferentially expressed in fetal cells [Miano et al., 1996], was identified in these clones using a degenerate homeobox probe in combination with RT-PCR. In atherosclerotic plaques, HOXB7 was identified in areas adjacent to calcification and scattered in the media and neointima. The pattern of expression may be reflective of a role in osteoblastic or in smooth muscle cell differentiation. To differentiate between these possibilities, we overexpressed bovine HOXB7 in C3H10T1/2 cells, a multipotent mouse cell line able to differentiate into vascular SMC [Hirschi et al., 1998], as well as osteogenic and chondrogenic lineages [Wang et al., 1993]. Overexpression of HOXB7 in C3H10T1/2 cells increased proliferation, and induced an SMC-like cell morphology associated with increased expression of the early SMC markers calponin and SM22α, but not the intermediate SMC marker smooth muscle myosin heavy chain (SM-MHC). No increase in markers for osteogenic or chondrogenic differentiation was observed. Similarly, no increase was observed in osteogenic or chondrogenic differentiation when HOXB7 was overexpressed in M2 cells, a bone marrow stromal cell line. These results suggest that HOXB7 plays a role in accumulation of immature cells either by expanding immature cell populations or dedifferentiation of mature cells.

MATERIALS AND METHODS

Cell Cultures

Subpopulations of bovine aortic SMC were cloned from primary cultures and were identified as previously described [Boström et al., 1993; Watson et al., 1994]. The cells were cultured in Dulbecco's Modified Eagle's medium (DME) (Irvine Scientific, Santa Ana, CA) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 U/ml), sodium pyruvate (1 mmol/L), and L-glutamine (2 mM). Clones were trypsinized before formation of nodules, plated in 60 mm Petri dishes (Costar, Pleasanton, CA) at a density of 16,000 cells/cm^2, and grown for up to three weeks. The cells were used at passage 10-18.

C3H10T1/2 mouse cells were obtained form the American Tissue Culture Collection (ATCC), and were cultured in α-Minimum Essential Medium with Earle's salt (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml), sodium pyruvate (1 mmol/L), and L-glutamine (2 mM). Transfections were performed using Superfect™ (Qiagen, Chatsworth, CA) as per manufacturer's instructions. Mixed mass, stable transfectants were selected by adding Zeocin™ (Invitrogen, Carlsbad, CA) at a concentration of 500 ng/ml to the culture medium. For differentiation experiments, the cells were plated at a density of either 6,000 cells per 60 mm-petridish or 10% confluency, and grown for 1–3 weeks.

Preparation of RNA and Poly(A) Enriched RNA, and Northern Blot Analysis

Total RNA was isolated from cultured cells using an RNA isolation kit (Stratagene, La Jolla, CA). Poly(A) enriched RNA was prepared using an Oligotex kit (Qiagen). Frozen human artery wall specimens and mouse aortas were homogenized in the initial denaturing solution and the RNA isolated according to manufacturer's instructions. Poly(A) enriched RNA (5 μg) was analyzed by Northern blots as previously described [Tintut el al., 1998]. The membranes were hybridized over night at 42°C with 5′-end-labeled oligonucleotide probe (HOX30 or HOXB7-antisense probe) in 10% formamide, 5XSSPE (5XSSPE = 0.9M NaCl, 0.05M NaH_2PO_4, 5mM EDTA, 47.5mM NaOH, pH 7.0), 5X Denhardt's solution (5X Denhardt's = 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% SDS, and 100 μg of salmon sperm DNA /ml. The filters were washed for 15 min at room temperature in 6XSSC (20XSSC = 3M NaCl, 0.3M Na citrate) and then for 15 min at 42°C in 6XSSC with

0.1% SDS, air-dried, and examined by autoradiography.

RT-PCR

Reverse transcription (RT) of 3 μg total RNA was carried out for 90 min at 37°C in 50 μl RT buffer (as supplied by Stratagene), supplemented with 0.5 mM of each dNTP, 50-80 units RNase Block (Stratagene), 50 U of Stratascript or M-MuLV reverse transcriptase (Stratagene), and 750 ng of oligo dT(10) (Boehringer-Mannheim, Indianapolis, IN) for priming. Polymerase chain reaction (PCR) using degenerate primers [Frohman et al., 1990] against the conserved regions of the homeobox region was carried out in a volume of 40 μl, using Pfu buffer (Stratagene), 25 U of Pfu polymerase (Stratagene), 100 ng of each primer, and 3.5 μl of template (from a 50 μl RT reaction). Thermal cycling was performed in GeneAmp® PCR System 2400 (Perkin-Elmer, Foster City, CA) as follows: (1) initial denaturation at 96°C for 1 min, (2) cycling for 45 cycles between 96°C for 1 min, 45°C for 2 min, and 72°C for 1 min, (3) final extension at 72°C for 5 min. PCR fragments were isolated by 8% polyacrylamide gel electrophoresis, ligated into pCRscript (Stratagene) that had been digested with EcoRV, and sequenced using the dideoxymethod. The sequence was compared against known sequences in GenBank using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc., Madison, WI).

PCR using primers to unique sequences in each cDNA was carried out in a volume of 10 μl, using Pfu buffer, 7 U of Pfu polymerase, 0.1 μl [P^{32}]-α-dCTP (Amersham), 25 ng of each primer, and 1 μl of template (from a 50 μl RT reaction). Thermal cycling was performed as follows: (1) initial denaturation at 96°C for 2 min, (2) cycling for cDNA-specific number of cycles (Table I) between 96°C for 1 min, 60°C for 2 min, and 72°C for 1.5 min, (3) final extension at 72°C for 5 min.

For semi-quantitative PCR, the number of cycles for each primer pair (see Table I) was chosen so that amplification remained well within the linear range, as assessed by densitometry (NIH Image, version 1.62, public domain program, Internet address http://rsb.info.nih.gov/nih-image/) of standard curves created for each primer pair by using different numbers of cycles. An equal volume from each PCR reaction was analyzed by 6% non-denaturing polyacrylamide gel electrophoresis and dried gels were examined by autoradiography. For non-quantitative PCR, the number of cycles was chosen to ensure detection of significant gene expression, usually 40–45 cycles. To verify that the HOXB7 primers recognized HOXB7 cDNA, PCR-fragments were prepared and sequenced as described above.

In Situ Hybridization

Human atherosclerotic artery specimens were obtained at autopsy and/or surgical endarterectomy with approval of the Institutional Review Board. Melanoma cells (positive control for HOXB7 expression), kindly provided by Dr. James Economou, UCLA, were grown in RPMI (Irvine Scientific) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 U/ml), sodium pyruvate (1 mmol/L), and L-glutamine (2 mM), trypsinized, and centrifuged into a dense pellet. The arterial specimens and the melanoma cell pellet were fixed in 4% paraformaldehyde and embedded in paraffin. Sections, 10 μm, were dewaxed in three changes of xylene, and two changes of industrial ethanol. They were hydrated by successive changes of 90%, 85%, and 70% ethanol followed by a quick rinse in double distilled water and then two changes in PBS. The hydrated sections were given the following pretreatments: sections were placed sequentially in 0.2 N HCl for 20 min, 0.3% Triton X-100 in PBS for 15 min, 50 μg/ml proteinase K in 0.1 M Tris-HCl, pH 8.0, 50 mM EDTA for 30 min at 37°C, and two changes of 2 mg/ml glycine in PBS. The slides were rinsed in PBS between each pretreatment and all procedures were carried out at room temperature (RT) unless otherwise stated. Sections were placed in prehybridization buffer (10% formamide, 5XSSPE (5XSSPE = 0.9M NaCl, 0.05M NaH_2PO_4, 5 mM EDTA, 47.5 mM NaOH, pH 7.0), 5X Denhardt's solution (5X Denhardt's = 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% SDS, 100 μg of salmon sperm DNA /ml, and 0.1 mg of poly(A)/ml for 30 min. Prehybridization buffer (100 μl) containing 0.3 ng/μl of HOXB7-antisense digoxigenin-labeled oligonucleotide probe was applied to the appropriate sections. The sections were covered with coverslips and hybridization was carried out in a sealed humid box at 42°C for 15–18 hours. Coverslips were removed in 6XSSC and sections washed. The post-

TABLE I. Oligonucleotide Probe and Primer Sequences for Northern Blotting, In Situ Hybridization and RT-PCR

Target gene	Primer sequence	Orientation	Position (reference)	Cycle number*
Probe for Northern				
HOX30	5′-GATCTTGATCTGTCTTTCTGTGAGGCAGAG-3′		648-619 (Simeone et al., 1987)	
Probes for in situ hybridization:				
HOXB7-antisense	5′-GGACTCGGACTTGGCGGCCGAGAGTAACTTCCGGATCTA-3′		479-441 (Simeone et al., 1987)	
HOXB7-sense	5′-GGACTCGGACTTGGCGGCCGAGAGTAACTTCCGGATCTA-3′		441-479	
Primers for RT-PCR				
HB7 (HOXB7)	5′-CCGACACTAAAACGTCCCTGCCTA-3′	Sense	57-80 (Simeone et al., 1987)	
	5′-TCCCTATTCGATTTGAGTTTCCTGA-3′	Antisense	849-825	
HOXB7**	5′-CCAGCCTCAAGTTCGGTTTTCGCT-3′	Sense	142-165 (Simeone et al., 1987)	35
	5′-TAGATCCGGAAGTTACTCTCGGCC-3′	Antisense	479-456	
GAPDH**	5′-ATCCCATCACCATCTTCCAGGAG-3′	Sense	275-297 (Tokunaga et al., 1987)	22
	5′-CCTGCTTCACCACCTTCTTGATG-3′	Antisense	853-831	
Calponin	5′-TGAGTCAACTCAGAACTGGCACCA-3′	Sense	5079-5102 (Miano and Olson, 1996)	28
	5′-ATCATAGAGGTGACGCCGTGTACC-3′	Antisense	8544-8521	
SM22α	5′-AGGTGTGGCTGAAGAATGGTGTGA-3′	Sense	234-257 (Li et al., 1996)	25
	5′-TGGGATCTCCACGGTAGTTTCCAT-3′	Antisense	524-501	
SM-MHC	5′-AGTTCTCTAAGGTGGAGGACATGG-3′	Sense	341-362 (Miano et al., 1994)	25
	5′-TCCAGACTCACCTGTGCACAGAAT-3′	Antisense	636-613	
Collagen IX	5′-TTTGAACTCCAGTGGATGCTGA-3′	Sense	841-862 (Abe et al., 1994)	38
	5′-ATCTATGCCCGGAACTCCAGGA-3′	Antisense	1011-990	
Osteocalcin	5′-CTCTGTCTCTCTGACCTCACAG-3′	Sense	91-112 (Celeste et al., 1986)	32
	5′-GGAGCTGCTGTGACATCCATAC-3′	Antisense	450-429	

*Cycle numbers refer to the number of cycles used for RT-PCR for the respective primer pair.
**The primer sequences for HOXB7 and GAPDH are identical in human and mouse. For the remaining primers, the mouse sequences are used.

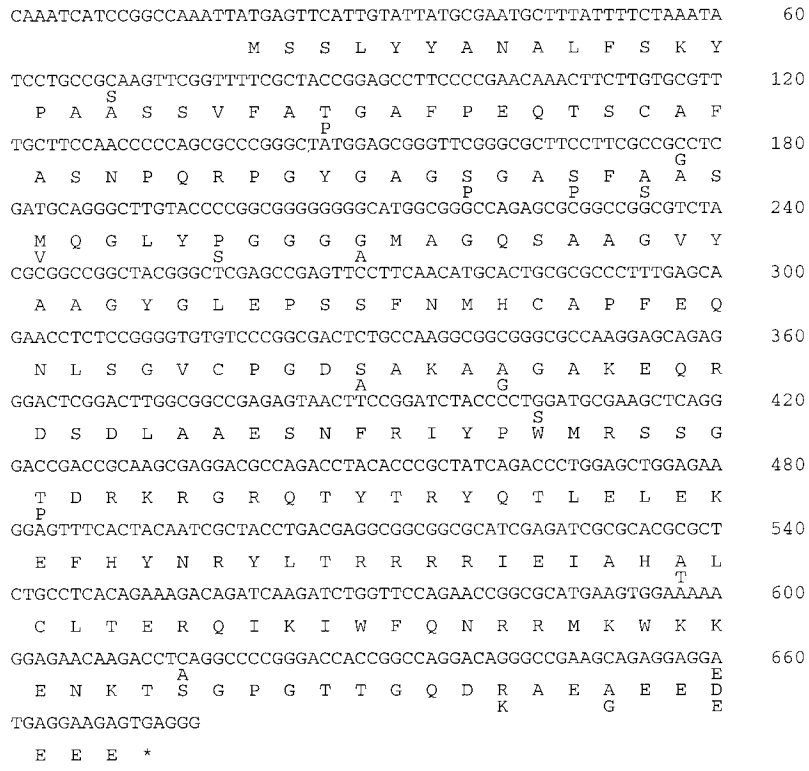

Fig. 1. Nucleotide sequence of the bovine HOXB7 cDNA and deduced amino acid sequence of the encoded protein. The sequence has been submitted to GenBank under Accession number AF200721. The bovine cDNA sequence has 96% and 93% sequence homology with the human and mouse cDNA sequence respectively. Amino acid residues that differ in the human (Simeone et al., 1987) and the mouse (Meijlink et al., 1988) protein sequence are indicated *above* and *below* the bovine protein sequence respectively.

hybridization washes were: two changes of 6XSSC, 10 min each at RT, then two changes of 6XSSC with 0.1% SDS, 20 min each at 58°C, followed by one change of 6XSSC at RT. The controls used were (i) hybridization with HOXB7-sense digoxigenin-labeled oligonucleotide probe; (ii) pretreatment with 100 mg/ml RNase A (Sigma) in 2XSSC, 10mM $MgCl_2$ for two hours at 37°C prior to hybridization with the antisense probe; (iii) omission of labeled probe.

Labeling of oligonucleotide probes was performed as per manufacturer's instructions using an oligonucleotide tailing kit (Boehringer-Mannheim) containing terminal transferase and digoxigenin-labeled dUTP. For detection of bound digoxigenin-labeled oligonucleotide probe, sections were washed once in buffer 1 (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl), blocked for 30 min in buffer 1 containing 0.5% blocking agent (Boehringer-Mannheim) and 15% fetal bovine serum. Alkaline phosphatase-conjugated sheep anti-digoxigenin (Boehringer-Mannheim) was applied at a dilution of 1:500 for two hours at room temperature. Unbound conjugate was removed by washing in three changes of buffer 1 (15 min each) followed by two washes in 0.1 M Tris-HCl pH 9.5 with 0.1 M NaCl and 0.05 M $MgCl_2x7H_2O$. Binding was visualized using nitroblue tetrazolium dye solution (Boehringer-Mannheim) as substrate.

Construction of Expression Vector for Bovine HOXB7

The coding region of HOXB7 cDNA was amplified by RT-PCR using primers (HB7-5′ and HB7-3′) recognizing sequences upstream and downstream of the coding region respectively. The PCR-fragment (792 base pairs) was then subcloned into a unique EcoRV site in pcDNA3.1(+)Zeo (Invitrogen) under the transcriptional control of the CMV-promoter. The HOXB7 sequence was confirmed by nucleotide sequencing (Fig. 1). pcDNA3.1(+)Zeo containing a CAT construct (Invitrogen) was used as transfection control.

Probes and Primers

The degenerate homeobox primers used for RT-PCR are the same as in [Frohman et al., 1990). For other probes and primers, see Table I.

Immunohistochemistry

Immunohistochemistry was performed on serial sections of the same specimens used for

in situ hybridization. The sections were dewaxed and immunohistochemistry was performed as previously described [Boström et al., 1993]. Primary mouse antihuman antibodies (Dako, Carpinteria, CA) were used at the following dilutions: anti-α-smooth muscle actin 1:2,000, anti-B lymphocyte antigen 36kD (BLA.36) 1:500, and anti-T cell CD43 1:500.

H^3-Thymidine Incorporation

Cells were seeded in 24-well plates at a density of 100,000 cells per well, and allowed to attach for 4-6 hours. H^3-Thymidine was added at 1 μCi/ml for two hours, and H^3-thymidine incorporation was determined as previously described [Tintut et al., 1998]. The data were normalized to total cell number per well at the beginning of incubation with H^3-thymidine, and calculated as the mean ± S.D. of three wells.

RESULTS

Homeobox gene expression was detected in clones of bovine vascular SMC by Northern blot analysis of poly(A) enriched RNA using a probe recognizing the conserved homeobox sequence (probe HOX30, see Methods). The probe recognized at least two transcripts in the size range 1.0 to 1.9 kb in CVC (data not shown). To identify specific homeobox genes, RT-PCR was performed using degenerate primers constructed against the most conserved regions of the homeobox [Frohman et al., 1990]. This yielded a product of the expected size, 115 bp, which was subcloned into the pCRscript vector and sequenced. Eight clones were sequenced, all of which contained a sequence homologous to that of HOXB7 (previously called homeotic protein c1 or Hox2.3 [Simeone et al., 1987; Scott, 1992; Meijlink et al., 1987]. HOXB7 expression was confirmed by RT-PCR using specific HOXB7 primers upstream of the homeobox, and by sequencing of the resulting 337 bp PCR-product. Specific primers for the human HOXB7 sequence upstream and downstream of the coding region were also used to obtain the full-length coding sequence for bovine HOXB7 (Fig. 1), which is 96% and 93% homologous to the human and the mouse sequence respectively. HOXB6 has the sequence most similar to HOXB7 in the homeobox region. However, RT-PCR using primers to unique sequences in HOXB6 did not detect any HOXB6 expression.

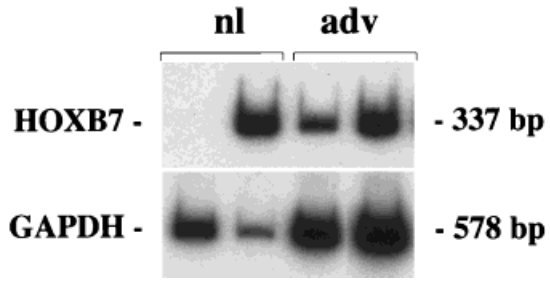

Fig. 2. HOXB7 expression in human artery wall. Total RNA and cDNA was prepared from human artery specimen, either grossly normal (nl) or with advanced atherosclerosis (adv). Expression of HOXB7 by PCR was identified consistently in the atherosclerotic specimen; grossly normal arteries were positive in four of six specimens. GAPDH is shown for comparison.

In vivo expression of HOXB7 was studied in human artery wall specimens by RT-PCR and in situ hybridization. HOXB7 expression was observed consistently (six of six) by RT-PCR in advanced, fibrous, and calcified atherosclerotic plaques. Grossly normal arteries were positive in four of six specimens. Figure 2 shows two specimens from each category, with GAPDH shown for comparison. In situ hybridization was perfomed to localize HOXB7 expression in atherosclerotic lesions. A total of nine specimens at various stages of atherosclerosis were processed. Hybridization with antisense oligonucleotide demonstrated scattered expression of HOXB7 in the media and, in addition, distinct expression in discrete groups of contiguous cells adjacent to calcium deposits within advanced plaques (Fig. 3A). No signal was detected in control specimens processed with sense oligonucleotide, or after RNase digestion. Paraffin-embedded melanoma cell pellets were sectioned and used as positive controls for HOXB7 in situ hybridization (Fig. 3B). Carè et al. [Carè et al., 1996] reported constitutive expression of HOXB7 in melanoma cells, and RT-PCR using melanoma cell cDNA and specific HOXB7 primers resulted in the expected 337 bp band.

To identify HOXB7-expressing cells in the artery wall, immunohistochemical staining for α-smooth muscle cell actin, B lymphocyte antigen 36kD (BLA.36), and T cell CD43 was performed on sections serial to those used for in situ hybridization. There was decreased staining for α-smooth muscle cell actin in areas with HOXB7 expression compared to surrounding media, and no staining for B lymphocyte anti-

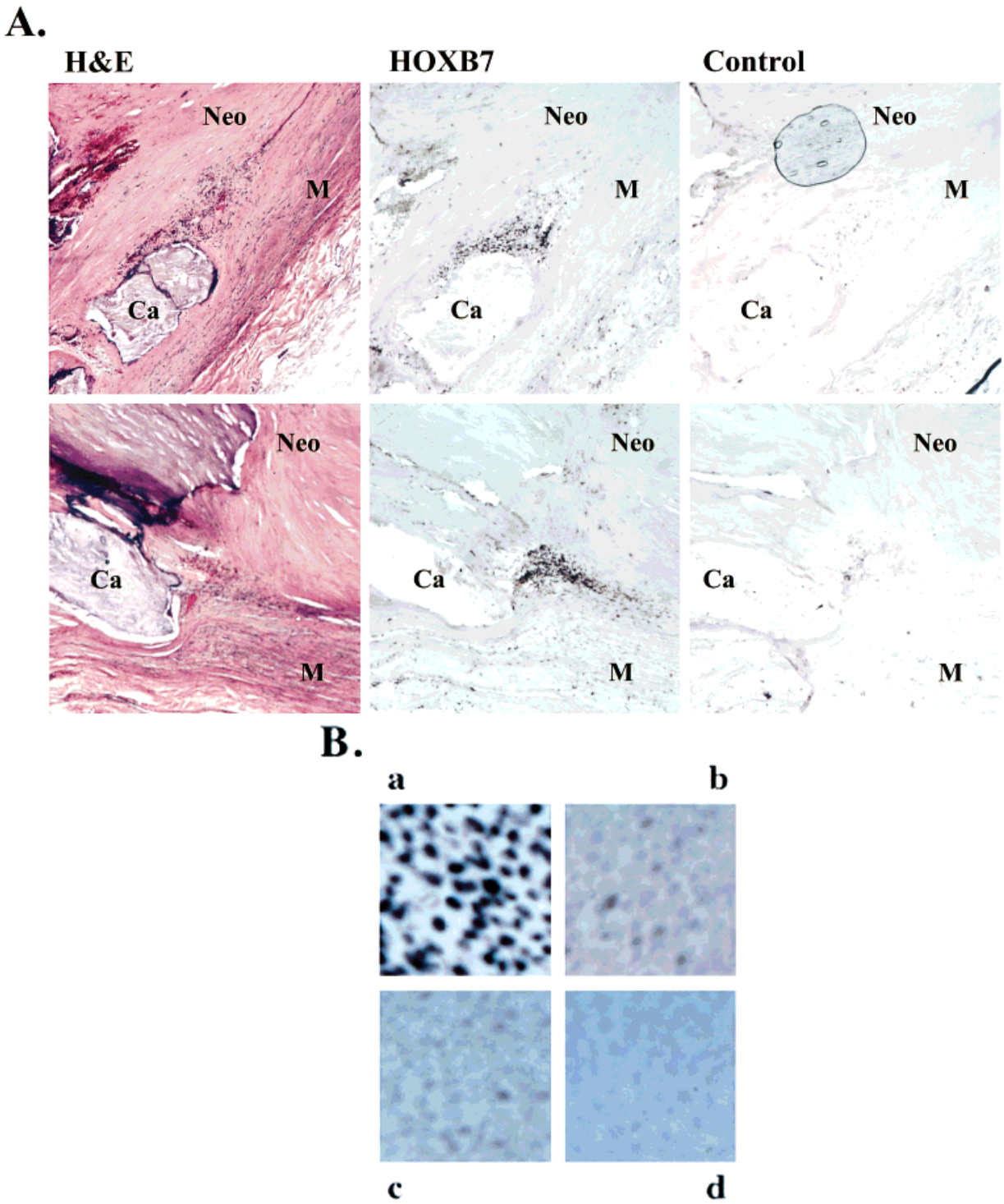

Fig. 3. **A**: In situ hybridization for HOXB7 in advanced human atherosclerotic plaques. Adjacent paraffin-embedded sections were stained with hematoxylin and eosin (left panels), and subjected to in situ hybridization with HOXB7-antisense (center panels) and HOXB7-sense (control) probes (right panels). HOXB7 expression was detected in a population of cells between the media (M) and the neointima (Neo) adjacent to calcium deposits (Ca). Hybridization with no probe, or RNase-digestion before hybridization with HOXB7-antisense yielded results similar to that of the HOXB7-sense probe. Original magnification 31.25×. **B**: Positive control in situ hybridization for HOXB7 in sectioned melanoma cell pellet. Paraffin-embedded pelleted melanoma cells known to express HOXB7 (Carè et al., 1996) were hybridized with the HOXB7-sense and antisense probes, and detected by digoxigenin staining. Panels show hybridization with (a) antisense probe before RNase digestion, (b) antisense probe after RNase digestion, (c) HOXB7-sense probe, and (d) no probe. Original magnification 62.5×.

gen 36kD (BLA.36) or T cell CD43 (data not shown) suggesting presence of modified smooth muscle cells or cells of other non-identified lineages.

The distinct expression of HOXB7 adjacent to calcium deposits may be consistent with HOXB7 having a role in mesenchymal differentiation, either SMC or osteoblastic differentiation. To assess these possibilities, we overexpressed bovine HOXB7 in C3H10T1/2 cells. C3H10T1/2 cells are multipotent mouse mesenchymal cells which are able to act as vascular precursor cells, and become incorporated into the media of newly formed vessels in induced angiogenesis in mice [Hirschi et al., 1998]. Once incorporated, they express SMC specific markers including calponin, SM22α, and SM-MHC. The C3H10T1/2 cells also have the capacity to differentiate along adipogenic, osteogenic and chondrogenic lineages when stimulated with selected factors, such as bone morphogenetic proteins [Wang et al., 1993].

Overexpression of bovine HOXB7 in the C3H10T1/2 cells resulted in a 3.5-fold increase in HOXB7 expression assessed by semi-quantitative RT-PCR (Fig. 4A), and a 3.5-fold increase in proliferation as assessed with H^3-thymidine incorporation. There was a change in cell morphology to spindle shape reminiscent of SMC-morphology (Fig.. 4B). Areas with spontaneous contraction were also observed. To further identify changes in differentiation, we performed semi-quantitative RT-PCR for cell-lineage specific markers for smooth muscle, osteogenic, and chondrogenic lineages. For SMC, we used the early SMC markers calponin and SM22alpha, induced in mice on embryonic day 8.5 and 9.5 respectively [Miano and Olson, 1996; Li et al., 1996], and the intermediate SMC-marker SM-MHC induced on embryonic day 10.5 [Miano et al., 1994]. Osteocalcin [Celeste et al., 1986], and collagen IX [Abe et al., 1994; Kolettas et al., 1995] were used for detection of osteogenic and chondrogenic differentiation respectively. The results showed a 4-fold and 3-fold induction of calponin and SM22α respectively, corresponding to the induction of HOXB7, but no significant change in expression of the intermediate SMC marker SM-MHC (Fig. 5), or in the markers for osteogenic and chondrogenic differentiation (Fig.. 5).

Two other observations are consistent with the hypothesis that HOXB7 promotes expression of early SMC markers rather than osteoblastic markers. First, one of the stable control transfectants (CAT construct) spontaneously lost most of its baseline HOXB7 expression during the selection process, which led to barely detectable levels of SM22α (Fig. 6), and slightly increased osteocalcin expression (Fig. 6). Second, overexpression of HOXB7 in M2 cells, a bone marrow stromal cell line which spontaneously undergoes osteoblastic differentiation and calcification in vitro when supplied with ascorbic acid and β-glycerophosphate, did not enhance osteoblastic differentiation (data not shown).

The results show immature SMC markers in HOXB7 overexpressing cells suggesting that HOXB7 plays a role in expansion of immature cell populations of dedifferentiation of mature cells.

DISCUSSION

The presence of immature smooth muscle cells and ectopic tissues such as fully-formed bone in atherosclerotic lesions [Bunting, 1906; Buerger and Oppenheimer, 1908; Haust and Moore, 1965; Haust and Geer, 1970], may be a result of recapitulation of embryonic mechanisms in the artery wall. We hypothesized that expression of homeobox genes is triggered in atherogenesis and that these regulate proliferation and differentiation of multipotential progenitor cells along one or more specific lineages.

We identified expression of HOXB7 in multipotent clones of bovine aortic medial cells, and in human atherosclerotic lesions, adjacent to calcified areas and scattered in the media and neointima. These locations in the diseased artery wall suggested involvement in differentiation of either osteogenic or myogenic cells. Therefore, we overexpressed HOXB7 in a multipotent mouse mesenchymal cell line, C3H10T1/2 cells, able to differentiate into SMC as well as osteoblasts and chondroblasts. Overexpression of HOXB7 resulted in SMC morphology, increased proliferation and increased expression of the early SMC markers calponin and SM22α. The lack of osteogenic or chondrogenic induction was confirmed in a second cell type, the M2 marrow stromal cell line [Parhami et al., 1999], a marrow stromal cell line that undergoes osteogenic differentiation and calcification in vitro.

These results are consistent with those of Miano et al. [Miano et al., 1996] who detected

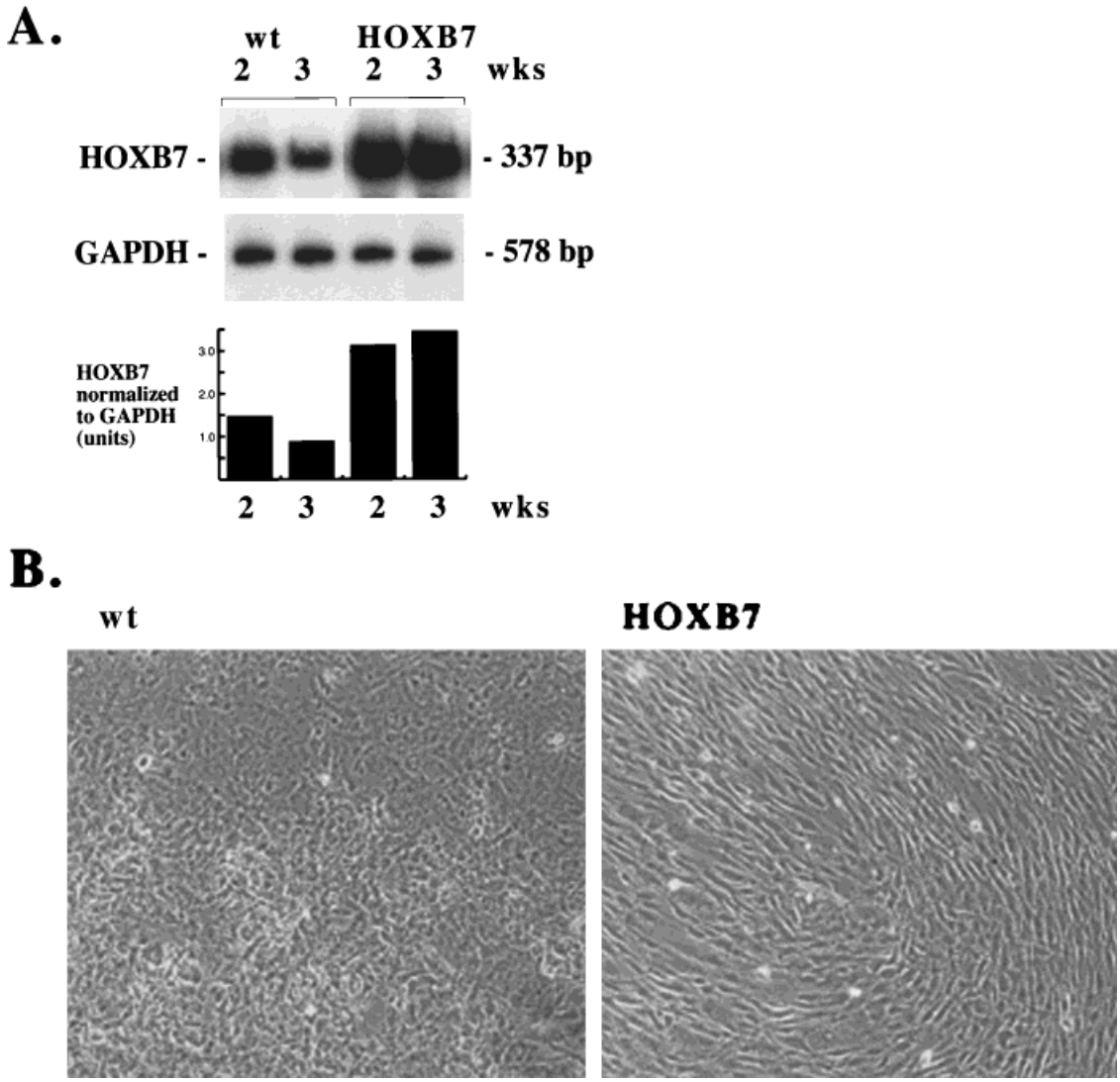

Fig. 4. **A**: HOXB7 expression in C3H10T1/2 cells, a multipotent mesenchymal cell line, transfected with bovine HOXB7 using lipofection. Total RNA and cDNA was prepared from cells after 2 and 3 weeks of culture, and expression of HOXB7 was detected by PCR. GAPDH is shown for comparison. The bottom panel shows expression of HOXB7 normalized to that of GAPDH. (wt = wild type). **B**: Morphology of C3H10T1/2 cells without (left) and with (right) overexpression of HOXB7 after 10 days of culture, by phase contrast microscopy. Original magnification 40×.

five nonparalogous homeobox genes, HOXA5, HOXA11, HOXB1, HOXB7, and HOXC9, in a fetal human smooth muscle cell library. Of these, HOXB7 and HOXC9 showed expression in fetal human smooth muscle cells but not in adult human smooth muscle cell lines or adult aorta. Thus HOXB7 expression is associated with the immature SMC phenotype, possibly present during development when calponin and SM22α expression is initially induced.

HOXB7 is an Antennapedia-like homeobox gene [Simeone et a., 1987; Meijlink et al., 1987; Vogels et al., 1990; Deschamps et al., 1987] which was first described in *Drosophila*, where its expression was linked to the establishment of a correct body plan. In human and mouse embryos, HOXB7 is expressed in vertebral rudiments, limb buds, spinal cord, heart, and skin. On a cellular level, HOXB7 expression has been shown to increase proliferation of melanoma cells by transactivation of the basic fibroblast growth factor (bFGF) gene [Care et al., 1996; Care et al., 1998], and to modulate the proliferation/differentiation program of hematopoietic progenitor/stem cells [Care et al., 1999]. In particular, a discrete population of

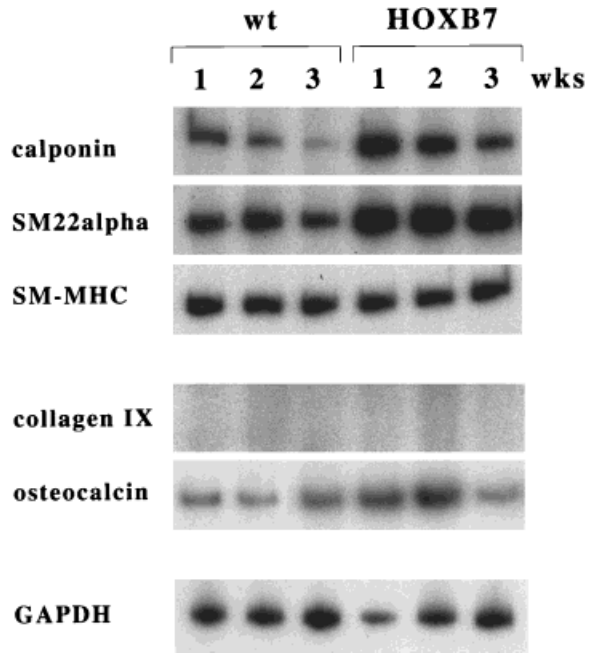

Fig. 5. Expression of cell specific differentiation markers at one, two and three weeks in control cells and cells overexpressing HOXB7. Total RNA and cDNA was prepared from cells from each time point, and expression of the SMC-markers calponin, SM22α, and SM-MHC was detected by PCR (upper part of panel). Expression of the markers for chondrogenic (collagen IX) and osteogenic differentiation (osteocalcin) are shown in the lower part of the panel. GAPDH is shown for comparison, and the bottom panel shows expression of the calponin and SM22α normalized to GAPDH expression. The results show that expression of calponin and SM22α increase in HOXB7-overexpressing cells.

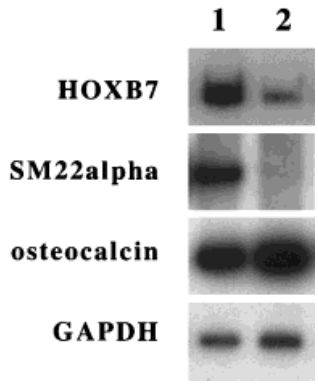

Fig. 6. Expression of SM22α and osteocalcin in control-transfected cells after spontaneous loss of HOXB7-expression during selection. Total RNA and cDNA was prepared from each cell-type, and expression of SM22a and osteocalcin was detected by PCR. The results show that SM22α expression decreased in parallel with HOXB7 expression. GAPDH expression was used to control for cDNA quality. (1 = wild type; 2 = HOXB7-deficient).

blast cells and a large pool of differentiated myeloid precursors were seen to proliferate, suggesting a role for HOXB7 in expansion and self-renewal of multipotent progenitor cells. It is possible that HOXB7 has a similar function in promoting progenitor cells in the artery wall. Our finding of a simultaneous increase in both proliferation and expression of SMC markers as a result of HOXB7 overexpression, is consistent with results from Lee et al. [Lee et al., 1997]. They found that early proliferation of aortic SMC precursors occurs concomitantly with the progressive accumulation of SMC contractile proteins including SM-α-actin, calponin, and SM-MHC, during vessel formation in mice.

There are known DNA target sequences to which homeodomain transcription factors preferentially bind [Kalionis and O'Farrell, 1993]. The 5′ flanking regions for SM22α and calponin genes [Yamamura et al., 1997; Miano and Olson, 1996; Takahashi et al., 1996] contain sequences that partially match these consensus sequences. HOXB7 has also been shown to physically interact in vitro with IκB-α [Chariot et al., 1999], which increases HOXB7-dependent transcription, and thus, functions as a positive regulator of transcriptional activation by HOXB7. IκB-α inhibits the proinflammatory transcription factor nuclear factor-κB (NF-κB), which plays a central role in the inflammatory response in vascular SMC [Berliner et al., 1995]. The positive regulatory effect on HOXB7 may be part of the anti-inflammatory effect of IκB-α, and may promote regeneration of SMC in the artery wall. Srebrow et al. [Srebrow et al., 1998] showed HOXB7 expression to be suppressed by intact, endogenous basement membrane matrix in mammary epithelial cells. It is possible that HOXB7 is induced by the fragmentation of the internal elastic lamina in atherogenesis, again for the purpose of SMC regeneration.

In summary, our results suggest that HOXB7 plays a role in expansion of immature cells or alternatively, dedifferentiation of mature cells. Thus, homeobox genes may be involved in adult vascular pathology, and may serve as potential targets in disease prevention.

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REFERENCES

Abe N, Yoshioka H, Inoue H, Ninomoya Y. 1994. The complete primary structure of the long form of mouse alpha 1(IX) collagen chain and its expression during limb development. Biochim Biophys Acta 1204:61–67.

Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. 1995. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation 91:2488–2496.

Bodner M, Castrillo J, Theill LE, Deerinck T, Ellisman M, Karin M.1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55:505–518.

Boström K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL.1993. Bone morphogenetic protein expression in human atherosclerotic lesions. J Clin Invest 91:1800–1809.

Buerger L, Oppenheimer A.1908. Bone formation in sclerotic arteries. J Exp Med 10:354–367.

Bunting CH.1906. The formation of true bone with cellular (red) marrow in a sclerotic aorta. J Exp Med 8:365–376.

Carè A, Silvani A, Meccia E, Mattia G, Stoppacciaro A, Parmiani G, Peschle C, Colombo MP.1996. HOXB7 constitutively activates basic fibroblast growth factor in melanomas. Mol Cell Biol 16:4842–4851.

Caré A, Silvani A, Meccia E, Matiia G, Peschle C, Colombo MP.1998. Transduction of the SkBr3 breast carcinoma cell line with the HOXB7 gene induces bFGF expression, increases cell proliferation and reduces growth factor dependence. Oncogene 16:3285–3289.

Caré A, Valtieri M, Mattia G, Meccia E, Masella B, Luchetti L, Felicetti F, Colombo MP, Peschle C.1999. Enforced expression of HOXB7 promotes hematopoietic stem cell proliferation and myeoloid-restricted progenitor differentiation. Oncogene 18:1993–2001.

Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA, Wozney JM.1986. Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. EMBO J 5:1885–1890.

Chariot A, Princen F, Gielen J, Merville M-P, Franzoso G, Brown K, Siebenlist U, Bours V. 1999. IkappaB-alpha enhances transactivation by the HOXB7 homeodomain-containing protein. J Biol Chem 274:5318–5325.

Cserjesi P, Lilly B, Bryson L, Wang Y, Sassoon DA, Olson EN.1992. Mhox: a mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. Development 115:1087–1101.

Deschamps J, de Laaf R, Verrijzer P, de Gouw M, Destrée O, Meijlink F.1987. The mouse Hox2.3 homeobox-containing gene: regulation in differentiating pluripotent stem cells and expression pattern in embryos. Differentiation 35:21–30.

Dorn A, Affolter M, Gehring WJ, Leupin W.1994. Homeodomain proteins in development and therapy. Pharmac Ther 61:155–183.

Frohman MA, Boyle M, Martin GR.1990. Isolation of the mouse Hox-2.9 gene; analysis of embryonic expression suggests that positional information along the anterior–posterior axis is specified by mesoderm. Development 110:589–607.

Gehrig WJ, Affolter M, Bürglin T.1994. Homeodomain proteins. Annu Rev Biochem 63:487–526.

Gorski DH, LePage DF, Patel CV, Copeland NG, Jenkins NA, Walsh K.1993. Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G0/G1 transition in vascular smooth muscle cells. Mol Cell Biol 13:3722–3733.

Haust MD, Geer JC.1970. Mechanism of calcification in spontaneous aortic arteriosclerotic lesions of the rabbit: an electron microscopic study. Am J Pathol 60:330–346.

Haust MD, Moore RH.1965. Spontaneous lesions of the aorta in the rabbit. In: Roberts JC, Straus R, editors. Comparative atherosclerosis. New York; Harper and Row. p 268–270.

Hirschi KK, Rohovsky SA, D'Amore PA.1998. PDGF, TGFβ, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. J Cell Biol 141:805–814.

Ingraham HA, Chen R, Mangalam HJ, Elsholtz HP, Flynn SE, Lin CR, Simmons DM, Swanson L, Rosenfeld MG.1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell 55:519–529.

Kalionis B, O'Farrell PH.1993. A universal target sequence is bound in vitro by diverse homeodomains. Mech Develop 43:57–70.

Kolettas E, Buluwela L, Bayliss MT, Muir HI.1995. Expression of cartilage-specific molecules is retained on long-term cultures of human articular chondrocytes. J Cell Science 108:1991–1999.

Komuro I, Izumo S.1993. Csx: a murine homeobox-containing gene specifically expressed in the developing heart. Proc Natl Acad Sci 90:8145–8149.

Lee SH, Hungerford JE, Little CD, Iruela-Arispe ML.1997. Proliferation and differentiation of smooth msucle cell precursors occurs simultaneously during the development of the vessel wall. Develop Dynam 209:342–352.

Li L, Miano JM, Cserjesi P, Olson EN.1996. SM22α, a marker of adult smooth msucle, is expressed in multiple myogenic lineages during embryogenesis. Circ Res 78: 188–195.

Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP.1993. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119:419–431.

Majesky MW, Giachelli CM, Reidy MA, Schwartz SM.1992. Rat carotid neointimal smooth muscle cells reexpress a developmentally regulated mRNA phenotype during repair of arterial injury. Circ Res 71:759–768.

Meijlink F, De Laaf R, Verrijzer P, Destrée O, Kroezen V, Hilkens J, Deschamps J.1987. A mouse homeobox containing gene on chromosome 11: sequence and tissue-specific expression. Nucl Acids Res 15:6773–6786.

Meijlink F, Verrijzer P, De Laaf R, De Graaff W, Deschamps J.1988. Nucleotide sequence of the Hox2.3 gene region. Nucl Acid Res 16:2729.

Miano JM, Cserjesi P, Ligon KL, Perisamy M, Olson EN.1994. Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis. Circ Res 75:803–812.

Miano JM, Firulli AB, Olsen EN, Hara P, Giachelli CM, Schwartz SM.1996. Restricted expression of homeobox genes distinguishes fetal from adult human smooth muscle cells. Proc Natl Acad Sci USA 93:900–905.

Miano JM, Olson EN.1996. Expression of the smooth muscle cell calponin gene marks the early cardiac and smooth muscle cell lineages during mouse embryogenesis. J Biol Chem 271:7095–7103.

Parhami F, Jackson SM, Tintut Y, Le V, Balucan JP, Territo M, Demer LL.1999. Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. J Bone Min Res (in press).

Patel CV, Gorski DH, LePage DF, Lincecum J, Walsh K.1992. Molecular cloning of a homeobox transcription factor from adult aortic smooth muscle. J Biol Chem 267:26085–26090.

Prockop DJ.1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–4.

Schwartz S.1994. Biology of the neointima. Exp Nephrol 2:63–77.

Scott MP.1992. Vertebrate homeobox gene nomenclature. Cell 71:551–553.

Simeone A, Mavilio F, Acampora D, Giampaolo A, Faiella A, Zappavigna V, D'Esposito M, Pannese M, Russo G, Boncinelli E, Peschle C.1987. Two human homeobox genes, c1 and c8: structure analysis and expression in embryonic development. Proc Natl Acad Sci USA 84:4914–4918.

Srebrow A, Friedmann Y, Ravanpay A, Daniel CW, Bissell MJ.1998. Expression of Hoxa-1 and Hoxb-7 is regulated by extracellular matrix-dependent signals in mammary epithelial cells. J Cell Biochem 69:377–391.

Takahashi K, Tazunoki T, Okada T, Ohgami K, Miwa T, Miki A, Shibata N.1996. The 5′-flanking region of the human smooth muscle cell calponin gene contains a cis-acting domain for interaction with a methylated DNA-binding transcription repressor. J Biochem 120: 18–21.

Tintut Y, Parhami F, Boström K, Jackson SM, Demer LL.1998. cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. J Biol Chem 273:7547–7553.

Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, Sakiyama S.1987. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res 47:5616–5619.

Villaschi S, Nicosia RF, Smith MR.1994. Isolation of a morphologically and functionally distinct smooth muscle cell type from the intimal aspect of the normal rat aorta. Evidence for smooth muscle cell heterogeneity. In Vitro Cell Dev Biol 30A:589–595.

Vogels R, de Graaff W, Deschamps J.1990. Expression of the murine homeobox-containing gene Hox-2.3 suggests multiple time-dependent and tissue-specific roles during development. Development 110:1159–1168.

Wang EA, Israel DI, Kelly S, Luxenberg DP.1993. Bone morphogenetic protein-2 casues commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors 9:57–71.

Watson KE, Boström K, Ravindranath R, Lam T, Norton B, Demer LL.1994. TGF-β1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify. J Clin Invest 93:2106–2113.

Yamamura H, Masuda H, Ikeda W, Tokuyama T, Takagi, M, Shibata N, Tatsuta M, Takahashi K.1997. Structure and expression of the human SM22α gene, assignment of the gene to chromosome 11, and repression of the promoter activity by cytosine DNA methylation. J Biochem 122:157–167.

Zhao GQ, Zhao S, Zhou X, Eberspaecher H, Solursh M, de Crombrugghe B. 1994. rDlx, a novel distal-less-like homeoprotein is expressed in developing cartilages and discrete neuronal tissues. Develop Biol 164:37–51.