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# Identification and Characterization of Novel Smoothelin Isoforms in Vascular Smooth Muscle

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**Key Words**
Smoothelin · Arteries · Developmental biology · Smooth muscle · Cytoskeleton

**Abstract**
Smoothelin is a cytoskeletal protein specifically expressed in differentiated smooth muscle cells and has been shown to colocalize with smooth muscle alpha actin. In addition to the small smoothelin isoform of 59 kD, we recently identified a large smoothelin isoform of 117 kD. The aim of this study was to identify and characterize novel smoothelin isoforms. The genomic structure and sequence of the smoothelin gene were determined by genomic PCR, RT-PCR and DNA sequencing. Comparison of the cDNA and genomic sequences shows that the small smoothelin isoform is generated by transcription initiation 10 kb downstream of the start site of the large isoform. In addition to the known smoothelin cDNA (c1 isoform) we identified two novel cDNA variants (c2 and c3 isoform) that are generated by alternative splicing within a region, which shows similarity to the spectrin family of F-actin cross-linking proteins. Visceral organs express the c1 form, while the c2 form prevails in well-vascularized tissue as analyzed by RT-PCR. We then generated specific antibodies against the major smoothelin isoforms and could show by Western blotting and immunohistochemistry that the large isoform is specifically expressed in vascular smooth muscle cells, while the small isoform is abundant in visceral smooth muscle. These results strongly suggest that the smoothelin gene contains a vascular and a visceral smooth muscle promoter. The cell-type-specific expression of smoothelin isoforms that are associated with actin filaments may play a role in the modulation of the contractile properties of different smooth muscle cell types.



## Introduction

Vascular smooth muscle cell differentiation as a key event in vascular development and the regulation of smooth muscle cell dedifferentiation in vascular remodeling, atherosclerosis and restenosis remain incompletely understood, which is partly due to the lack of specific markers and defined in vitro differentiation systems. The armentarium of smooth muscle proteins has recently been

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expanded and now includes smooth muscle myosin heavy chain (SM MHC) isoforms 1 and 2, SM22α, α1 integrin, 1E12, SmLIM proteins, caldesmon and calponin, in addition to more general proteins like smooth muscle alpha actin (SMαA) and vinculin/metavinculin [1–7]. However, none is able to detect both the differences between visceral and vascular as well as between proliferating (synthetic) and differentiated (contractile) smooth muscle cells [7, 8]. It remains to be clarified whether the lineage diversity of smooth muscle cells has an impact on functional differences and phenotypic modulation [9–11].

Also the regulation of Ca-dependent contraction and relaxation of smooth muscle is much less understood than that of skeletal or cardiac muscle. Smooth muscles are responsible for the long-lasting movements characteristic of enteric organs and may display a highly contractile type with fast contraction velocities or a less contractile type with slow contraction [12]. Nearly all proteins involved in smooth muscle cells force generation are expressed as alternatively spliced variants. There are isoforms of tropomyosin (TM), actin, MHC, myosin light chain, myosin light chain kinase, caldesmon and calponin specific to smooth muscle cells, which partly show different functions and expression patterns [for reviews, see ref. 7, 13].

Recently, a cytoskeletal protein of about 59 kD was identified that is specifically expressed in differentiated smooth muscle cells and was consequently termed smoothelin [14]. This study also demonstrated an association of smoothelin with filamentous stress fibers. Later, a confocal microscopic analysis showed colocalization of smoothelin with SMαA [15]. The same monoclonal antibody against smoothelin used in the above-mentioned studies was shown to cross-react with a larger protein identified in vascular smooth muscle cells [16]. In fact, a second longer smoothelin transcript was identified by Northern blot analysis, and the corresponding mouse and human cDNAs were cloned and shown to code for a large smoothelin isoform of 921 amino acids [17].We have now generated specific antibodies to smoothelin and shown that the large smoothelin isoform has an apparent molecular weight (MW) of about 117 kD and is specifically expressed in vascular smooth muscle cells. In addition, we identified two novel variants with different C-terminal spectrin family similarity (SFS) domains. Cloning and sequencing of the entire mouse smoothelin gene revealed that the small and large smoothelin isoforms are generated by smooth muscle cell-type specific alternative transcriptional start sites, whereas the SFS domain variants are generated by alternative splicing. This specific expression of smoothelin in smooth muscle cells, its different isoforms in vascular and visceral smooth muscle cells and the colocalization with SMαA suggest that smoothelin isoforms might in part account for the diverse properties of different types of smooth muscle.

## Methods

*Cloning of the Mouse and Human Smoothelin cDNAs*

The full-length mouse smoothelin cDNAs were obtained by 5′ RACE and the primers used for cloning of mouse and human smoothelin cDNAs were described previously [17]. Briefly, for cDNA amplifications, embryonic, heart and intestinal cDNA pools (Clontech Laboratories) and a polymerase mix, consisting of Klen-Taq and a proofreading polymerase (Clontech Laboratories) were used following the manufacturer's instructions. PCR products were then directly cloned into the pCR II-TOPO vector (Invitrogen), according to the manufacturer's protocol and recombinants checked by restriction enzyme analysis. Positive clones were double strand sequenced by the dye terminator method on an automated DNA sequencer (Applied Biosystems Inc.).

For sequence assembling, editing and alignments, we used the LASERGENE software programs EditSeq, SeqMan and MegAlign (DNASTAR Inc.). Databank searches for nucleotide and protein homologies were performed with the programs BLAST, PROFILE and PROSITE [18].

For final full-length mouse smoothelin cDNA amplification of the different alternatively spliced variants, we used 5′-ACGTTGCTGAACCGGCCTGGGCTCT-3′ for the large, and 5′-AGGGGGCAGTATGAAGACTAC-3′ for the small isoforms as forward (upstream) primers and for both isoforms 5′-GTCAAAACACCTCTCCCCTTT-3′ as well as 5′-CACTCTGCTCACACCGCCTGCGCTGCG-3′ as reverse (downstream) primers.

For the cloning of human smoothelin cDNAs, the upstream primer 5′-AGAATCCAGGGGACGGTTGCTGA-3′, and the downstream primer 5′-CCCACATACACACGCAGCGTTTTGAT-3′ were used.

*Cloning of the Mouse Smoothelin Gene*

Genomic PCR reactions using mouse DNA were performed with primer pairs designed from the smoothelin cDNA sequence. In cases of large introns as well as for terminal 3′ and 5′ genomic sequence information, we used the genome walking method (Mouse Genome Walker kit, Clontech Laboratories). PCR were performed with gene-specific and adaptor primers using the long template PCR polymerase mix (Roche Molecular Biochemicals). The primary products were subcloned into the pCR II-TOPO cloning vector and the positive clones were sequenced as described. Restriction analysis with *Eco*RI, *Xba*I and *Bam*HI were conducted to further test the mouse genomic sequence.

*RT-PCR Analysis of Isoform Expression Patterns*

Total RNAs (2 μg) from different mouse organs and tissues were isolated as described previously [17]. RNA samples were transcribed by AMV-RT and a smoothelin gene-specific primer (5′-CGCAGCGCAGGCGGTTGTGAGCAGAGTG-3′) located in exon 22 downstream of the alternatively spliced region (fig. 1, 4). Annealing to the RNA template at 25°C for 10 min was followed by elongation at 42°C for 60 min. Then the AMV-RT reaction products were dena-

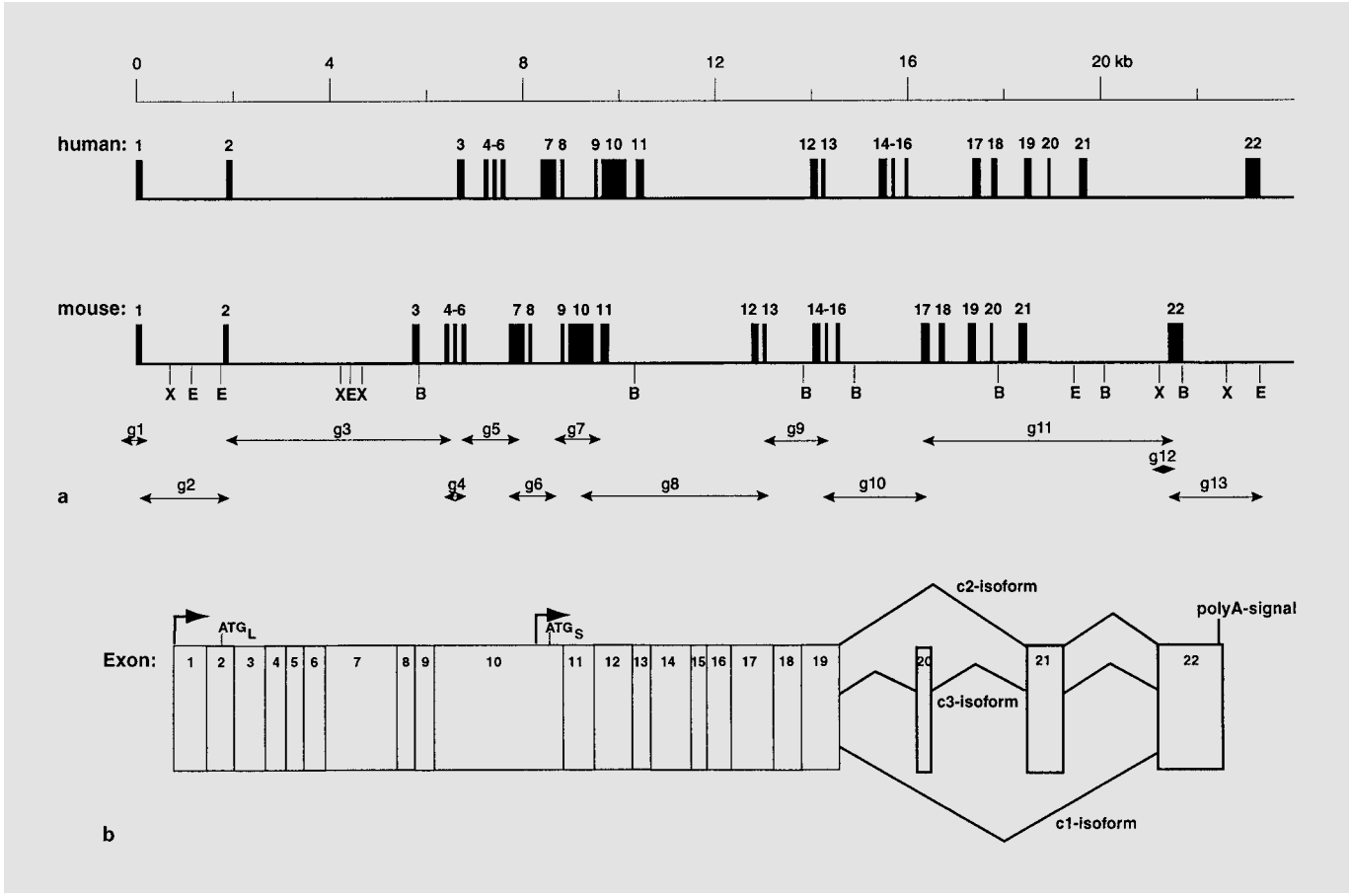



**Fig. 1.** Genomic structure of the mouse and human smoothelin genes. **a** Illustrates the exon-intron structures of the mouse and human smoothelin genes. The upper scale indicates the sequence length in kilobases. X, B, and E denote the restriction enzyme sites *Xba*I, *Bam*HI, and *Eco*RI, respectively. The overlapping mouse genomic clones, obtained by internal primer design and 5′ and 3′ genome walking, are shown underneath. The full mouse sequence is deposited in GenBank (accession No. AF132449). The human sequence is included in the GenBank sequence for the PAC clone RP3-412A9 (accession No. AC005005). **b** Exon composition of the splice variants. Exons are drawn to scale. Start codons of the small ($ATG_S$) and large ($ATG_L$) isoform are indicated. Transcription start sites are indicated by bent arrows.

tured at 99°C for 5 min and finally cooled on ice. Subsequent PCR amplification was performed with the gene specific primer 5′-AAGGCCATGATTGAGAAGCTAGAGAAAGAA-3′ positioned upstream of the alternatively spliced region (fig. 4) and the downstream primer used for RT. The cycling conditions were: 30 s denaturation at 94°C, and then 35 cycles consisting of 94°C for 20 s, 62°C for 45 s, and 70°C for 2 min. Aliquots of 8 µl were diluted 1:1 with distilled water and loaded onto an 1.8% agarose gel to analyze the RT-PCR products. The extra fragment indicated by an asterisk in figure 4 is a heteroduplex between the c1 and the c2 splice variant sequences.

*Smoothelin Expression Constructs*

The smoothelin cDNA fragments were cloned in frame by PCR into the pBAD-TOPO vector (Invitrogen) for arabinose-inducible overexpression in *Escherichia coli*. Amino acids included from smoothelin are 1–921 for large c2 form (Lc2), 457–923 for small c1 form (Sc1) or 3–466 according to the small protein, respectively, 1–511 for the large N-terminal domain (LN).

*Generation and Purification of Antibodies*

For the detection of mouse smoothelin, the polyclonal antisera anti-smo1 and anti-smo2 were raised against KLH-conjugated peptides: smo1 (KRFRAERQNKENWC) which corresponds to amino acids 52–65, and smo2 (RQRKRDQRDKERERC) which corresponds to amino acids 614–627 of the large mouse smoothelin isoform both with an additional C at the C-terminal end. Synthesis of peptides and rabbit immunization were performed by Eurogentec. For affinity purification of the antibodies, the synthetic peptides were coupled to Affi Gel 10 (BioRad) following the anhydrous coupling instructions of the manufacturer. The antisera were diluted 1:2 in TBS (100 mmol/l Tris-HCl pH 7.4, 100 mmol/l NaCl) and run

through the column. Elution was carried out with 100 mmol/l glycine-HCl pH 2.3 with immediate change of the pH to 7.4 with 1 mol/l Tris-HCl pH 8.0. Then, the eluted antibodies were concentrated to about 1 mg/ml.

*Cell Extracts and Western Blotting*

Cell pellets from smoothelin overexpression in *E. coli* were washed in PBS and boiled directly in Laemmli sample buffer. For tissue extracts, porcine bladder and porcine coronary arteries dissected from fresh hearts were directly frozen in liquid nitrogen, pulverized in a mortar and extracted with RIPA buffer. To determine protein concentration, we used the *DC* Protein Assay (BioRad) following the manufacturer's instructions.

Protein extracts were analyzed on a gradient (7–20%) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon™-P membranes (Millipore) using a semi-dry transblotter (Hoefer Scientific Instruments). Nonspecific binding was blocked with 5% fat-free milk powder in PBS/0.05% Tween 20. Membranes were incubated with the primary antibodies: anti-smo1, 1:10,000; anti-smo2, 1:5,000; anti-V5 (Invitrogen), 1:5,000, in 1% fat-free milk powder in PBS/0.05% Tween 20 overnight at 4°C or 2 h at room temperature. Blots were washed with PBS/0.05% Tween 20 followed by incubation for 1 h at room temperature with the secondary antibodies: HRP-conjugated goat anti-rabbit IgG (Sigma), 1:10,000 or HRP-conjugated sheep anti-mouse IgG (Amersham), 1:5,000. After a final washing step, blots were developed with ECL-Plus (Amersham) and signals were recorded on a luminescent imaging system (Fuji). Stripping of blots was achieved by incubation at 50°C for 30 min in 100 mmol/l Tris pH 6.8, 200 mmol/L β-mercaptoethanol , 2% SDS, followed by extensive washing in PBS.

*Immunostaining and Microscopy*

All procedures were carried out at room temperature, unless otherwise specified. Freshly prepared mouse tissue was fixed for 24 h with Bouin's solution (70% picric acid/6.75% formalin/5% acetic acid) and then embedded in paraffin. Serial tissue sections of 5 µm thickness were placed on Superfrost-Plus slides (Menzel-Gläser). Sections were deparaffinized, rehydrated through a descending ethanol series and placed in 10 mmol/l citrate buffer pH 6.0 for 10 min. After boiling for 2 min in a microwave (for anti-SMαA) or autoclaving for 20 min at 121°C (for anti-smo) in the same buffer, samples were placed in PBS for 10 min. To inhibit any endogenous peroxidase activity, sections were incubated in 0.3% $H_2O_2$ in PBS for 20 min. Then, sections were blocked in 10% goat serum in PBS for 30 min to minimize nonspecific binding. The incubation with the primary antibodies (anti-smo1 and 2, 1:500; anti-SMαA (Clone 1A4, Sigma), 1:1,000) was done in 5% goat serum/PBS overnight at 4°C in a humidified chamber. Sections were washed with PBS and incubated for 1 h with the secondary antibodies: HRP-conjugated goat anti-rabbit IgG, 1:1,000 or HRP-conjugated sheep anti-mouse IgG, 1:1,000, in 5% goat serum/PBS. For detection of SMαA, sections were washed again with PBS and color development was then carried out with DAB-substrate tablets (Sigma). For detection of smoothelin with anti-smo1 and anti-smo2, an amplification step was used. After washing in TBS (100 mmol/l Tris-HCl pH 7.4, 100 mmol/l NaCl)/0.05% Triton X-100, sections were incubated for 10 min with TSA amplification reagent (TSA-Indirect, NEN Life Science Products), according to the manufacturer's instructions. After one more wash, incubation for 30 min with HRP-conjugated streptavidin (NEN Life Science Products) diluted 1:100 in TBS was performed. After a final washing step, color development was carried out with DAB substrate tablets, monitored on a light microscope and stopped by incubation in distilled water. All sections were counterstained with Mayer's hematoxylin solution (Sigma), dehydrated with an ascending ethanol series, rinsed in xylene and mounted with Entellan (Merck). Stained sections were screened on a Zeiss Axiolab microscope and images were taken with a CCD camera (ProgRes, Kontron Electronic) and Adobe Photoshop software.

Freshly prepared porcine coronary arteries were directly frozen in liquid nitrogen. Frozen sections of 12 µm thickness were fixed with methanol for 5 min at –20°C and then with acetone for 30 s at –20°C. Staining was carried out in the same way as for the mouse sections with the exception that blocking was done with 0.4% fish skin gelatin (Sigma)/PBS and incubation with the different antibodies was done in 0.2% fish skin gelatin/PBS. The secondary antibody used was an anti-rabbit-IgG Texas Red conjugated (Amersham), 1:200. Stained sections were counterstained with 1 µg/ml Hoechst/PBS for 5 min and mounted with Moviol. Samples were screened on a Zeiss Axioplan 2 microscope and images were taken with a CCD camera (SensiCam) and Zeiss Axiovision 2 software.

## Results and Discussion

*Organization of the Smoothelin Gene*

Smoothelin promises to be a useful tool to investigate smooth muscle cell differentiation due to its specific expression in smooth muscle. To study the regulation of the smoothelin gene and the expression of potential isoforms, we set out to determine the genomic structure of the smoothelin gene. Overlapping mouse genomic DNA fragments designated g1–g13 (fig. 1a) were cloned by PCR using the sequence information from the mouse smoothelin cDNA [17] for primer design. The 5′ and 3′ ends of the smoothelin gene as well as large introns were obtained by genomic walking. The complete genomic sequence was established by double-strand sequencing and was submitted to GenBank (accession No. AF132449). The human genomic DNA sequence is included in the GenBank sequence for the PAC clone RP3-412A9 (accession No. AC005005). The mouse and human smoothelin gene structures were determined by comparison with the respective cDNA sequences, leading to the identification of 22 exons spanning 22 and 23 kb of genomic DNA, respectively. Parts of the mouse smoothelin gene were published during the preparation of this article [19]. The location and size of exons are shown in figure 1a together with the utilized mouse genomic clones. The intron-exon boundaries obtained with their splicing acceptor and donor site sequences are listed in table 1 and fit the mammalian consensus well [20]. All mouse and human smoothelin gene exons except for exons 10 and 16 are of identical size and most introns (excluding introns 2,

**Table 1.** Exon-intron boundaries of mouse and human smoothelin gene

| EXON | | | INTRON | | EXON |
|---|---|---|---|---|---|
| # | Size | 5' Donor Site | Size | 3' Acceptor Site | |
| | | | | | ACGTTG |
| | | | | | CAGAAT |
| 1m | 128 | TGAGAG gtgggt | 1674 | ttgcag | AATTCT |
| 1h | 138 | TGAGAG gtgggt | 1722 | ctgcag | AATTCT |
| 2m | 114 | AAACTG gtaata | 3798 | ttgcag | CTGGAG |
| 2h | 131 | AAACTG gtaagt | 4655 | ctgcag | CTGGAG |
| 3m | 149 | GCTACA gtgagt | 520 | ctgcag | CTCTCA |
| 3h | 149 | GCTGCA gtgagt | 399 | ctgcag | CTCTCA |
| 4m | 94 | ACACTG gtaagg | 84 | ccacag | CTTCGG |
| 4h | 94 | GCACTG gtgagg | 92 | ccacag | TTGCGA |
| 5m | 79 | TTAAGG gtaggt | 89 | ttccag | CTGCCA |
| 5h | 79 | TTGAGG gtatgt | 94 | ttccag | CTGCCA |
| 6m | 98 | GGTAAG gtaagg | 888 | ctgcag | GTGCCA |
| 6h | 98 | TGTGAG gtaagg | 729 | ttacag | GTGCCA |
| 7m | 321 | AGCAAG gcgagt | 84 | ttgcag | CCCCTG |
| 7h | 321 | AACAAG gtgagt | 88 | ttgcag | CTTCTG |
| 8m | 73 | GAGCAG gtgagg | 603 | tgccag | ACCCGT |
| 8h | 73 | GAGCAG gtgagg | 627 | tgccag | ACGTGG |
| 9m | 72 | ATCGAG gtactg | 87 | tgccag | AGCCAA |
| 9h | 72 | ACCGAG gtacta | 81 | ttctag | AGTCCA |
| 10m | 516 | GAGCAG gtaggt | 154 | ctccag | AATTGA |
| 10h | 522 | GGGCAG gtaggc | 192 | actcag | AACTGA |
| 11m | 173 | TTTAAG gtgagc | 2959 | ctgtag | ATGGAG |
| 11h | 173 | ATCAAG gtgagc | 3455 | ctgcag | ATGGAA |
| 12m | 153 | AAGATG gtatgt | 87 | ctacag | CTGGAC |
| 12h | 153 | AAGATG gtatag | 78 | atgcag | CTGGAT |
| 13m | 76 | AGAGAG gtagag | 957 | gctcag | ACCAGA |
| 13h | 76 | AGAGAG gtagag | 1123 | gcccag | ACCAGC |
| 14m | 165 | ACTCCA gtaagg | 96 | ttccag | ATGATG |
| 14h | 165 | ACTCCA gtaagg | 100 | ctgcag | ATGATG |
| 15m | 63 | TGGAGA gtaagg | 155 | ccacag | ATGGCA |
| 15h | 63 | CGGAGA gtaagg | 208 | ttgcag | ATGGCA |
| 16m | 94 | GGGCAG gtgagc | 1681 | ctccag | TATCTT |
| 16h | 70 | GGGCAG gtgagc | 1328 | ccctag | CATCTT |
| 17m | 174 | TGCAGG gtcagt | 196 | ccacag | TGGTCC |
| 17h | 174 | GGCCGG gtgagc | 221 | ctacag | CAGCCC |
| 18m | 118 | TACGAG gtgagc | 491 | ccaaag | CACGTG |
| 18h | 118 | TACGAG gtgagc | 565 | ttatag | CACGTC |
| 19m | 152 | CGCTGA gtaagt | 302 | taccag | AGCTTC |
| 19h | 152 | TGCGGA gtaagt | 336 | caccag | AGCTTC |
| 20m | 58 | GCCCAG gcaccg | 538 | ccccag | GACCCA |
| 20h | 58 | GCCCGG gcaccg | 594 | ccccag | GACCCA |
| 21m | 165 | CCCACG gtgaga | 2924 | ttgcag | GATGCT |
| 21h | 165 | CCCACG gtgaga | 3266 | ctgcag | GATGCT |
| 22m | 308 | until poly(A) addition site | | | |
| 22h | 309 | | | | |
| | | **A G** **g t** r a g t | | y y n c **a g** | N |
| mouse smoothelin consensus | 52 81 | 100 86 95 67 81 38 | | 95 86 95 100 100 | |
| human smoothelin consensus | 48 81 | 100 95 100 67 81 29 | | 86 90 86 100 100 | |
| mammalian consensus | 63 87 | 100 100 96 74 81 47 | | 88 87 76 100 100 | |

Tabular representation of the 3′ acceptor and 5′ donor splice sites of the 22 mouse (m) and human (h; shaded) smoothelin gene exons. Exact exon and intron lengths are indicated in base pairs. The percent sequence conservation at the splice site junction is indicated for both smoothelin genes and compared with the consensus for mammals.

11, 16 and 21) are very similar, giving rise to an almost perfectly conserved gene structure.

The alignment of the genomic and the cDNA sequences of the small and large isoforms showed that the 5′ end of the shorter transcript is located in the 10th exon about 10 kb downstream of the start site of the long transcript (fig. 1b). These results indicate that the small and large smoothelin isoforms are generated via two different transcriptional start sites. The translational start of the large isoform is located in exon 2 with an open reading frame (ORF) coding for a protein of 921 amino acids in the mouse. For the small isoform, the previously described translational start [14] was at the start of exon 12, resulting in a protein with a predicted MW of 40 kD. This start codon assignment was based on the existence of an upstream stop codon in exon 11, which turned out to be a mistake in the originally published sequence. The corrected sequence now shows an ORF extending further upstream into exon 10, corresponding to an 88-amino-acid longer protein with a predicted MW of 50 kD (also see fig. 1b). The latter fits well with the size obtained after neuramidase treatment of human colon tissue extracts reported in the original publication [14], indicating the presence of glycosylated residues in smoothelin.

*Identification of Tissue-Specific Alternative Splice Variants of the Smoothelin Gene*

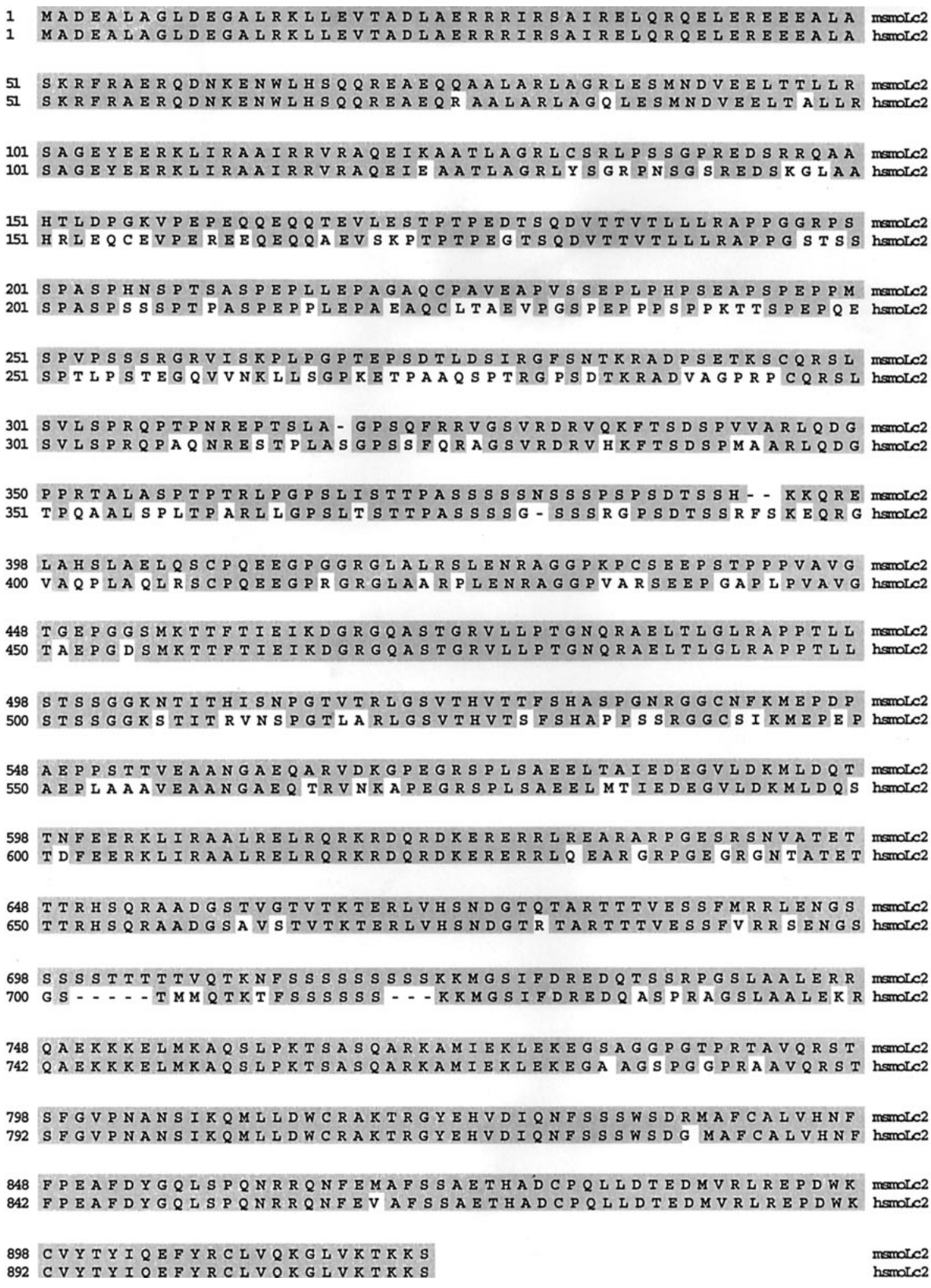
In parallel with the elucidation of the genomic structure, cDNAs from various mouse tissues were screened by PCR for further potential smoothelin variants. Altogether three novel splicing variants were identified that are generated by alternative 3′-acceptor splice site selection. Alternative splicing occurs after exon 19 and produces three different splice variants, termed c1–3 (fig. 1b). The previously described c1 smoothelin isoform [17] skips exons 20 and 21 and finishes with exon 22. The two novel variants continue either with exons 21 and 22 (c2 isoform) or with exons 20, 21 and 22 (c3 isoform). Interestingly, the three alternatively spliced transcripts are of unequal size but nevertheless encode proteins of almost identical size differing merely by 2 amino acids. The alternative 165-bp exon 21 of the c2 form continues in a peptide sequence similar to the downstream exon 22 present in the c1 form whereas the additional 58-bp exon 20 of the c3 form causes a frame shift (fig. 3, 4).

Since the smoothelin gene is highly conserved in the mouse and in humans [17], we screened human cDNA to test whether these novel splice variants are conserved as well. In addition to the known c1 splice variant, we could isolate an alternative splice variant from human fetus cDNA corresponding to the mouse c2 form, which has an identical amino acid sequence and 98% homology in the 165-bp cDNA sequence (GenBank accession No. AF064238). Figure 2 illustrates the protein alignment between mouse and human smoothelin c2 isoforms, showing an 82% overall identity. The c3 variant could not be detected in this screen but DNA sequence corresponding to the c3 isoform specific mouse exon 20 is present in the human smoothelin gene and shows 96.5% DNA sequence identity (GenBank accession No. AC005005; fig. 1a). These results show that the smoothelin splice variants between exon 20 and 22 are conserved as well in mouse and humans.

Interestingly, this alternative splice site is located in the C-terminal domain of smoothelin and falls right into a domain that has striking similarity with an SFS domain. This domain is conserved in a number of F-actin cross-linking cytoskeletal proteins (spectrin family), and some examples are shown in figure 3. Surprisingly, not only the known c1 isoform but also the new c2 isoform fits well into this alignment despite the fact that the coding sequence stems from two different exons (exons 22 and 21, respectively; fig. 3). In fact, most residues shown to be important for the structure of this domain in the spectrin family members [21] are conserved in mouse and human smoothelin as well as in the alternative exons in the c1 and c2 isoforms. Only the third variant (c3) does not show any discernible similarity in the alternative second half. As presented above, all three splice variants encode proteins of almost identical size, differing only in their last 46 or 48 amino acids. Thus the alternative splicing generates three smoothelin isoforms with different SFS domains.

These results raised the possibility that specialized smoothelin isoforms are expressed in different tissues and smooth muscle cell types. We therefore analyzed the expression of these three different splice forms in mouse tissues by RT-PCR with common primers flanking the alternative exons (fig. 4). Both c1 and c2 forms are expressed in heart samples, which include the coronary artery system. In visceral organs, the smaller c1 form is the predominant splice form, whereas kidney and brain show almost only the c2 splice form. The c3 form, which was originally isolated from mouse heart cDNA, was not detected with this assay, suggesting that it is weakly expressed and/or confined to rare smooth muscle cell types.

Our data show that smoothelin is expressed not only as a large and a small isoform in vascular and visceral smooth muscle cells, respectively, but also in alternatively spliced variants.


1 MADEALAGLDEGALRKLLEVTADLAERRRIRSAIRELQRQELEREEEALA msmoLc2
1 MADEALAGLDEGALRKLLEVTADLAERRRIRSAIRELQRQELEREEEALA hsmoLc2
51 SKRFRAERQDNKENWLHSQQREAEQQAALARLAGRLESMNDVEELTTLLR msmoLc2
51 SKRFRAERQDNKENWLHSQQREAEQR AALARLAGQLESMNDVEELT ALLR hsmoLc2
101 SAGEYEERKLIRAAIRRVRAQEIKAATLAGRLCSRLPSSGPREDSRRQAA msmoLc2
101 SAGEYEERKLIRAAIRRVRAQEIE AATLAGRLY SGR PNSG SREDSKGLAA hsmoLc2
151 HTLDPGKVPEPEQQEQQTEVLESTPTPEDTSQDVTTVTLLLRAPPGGRPS msmoLc2
151 HRLEQCEVPE REEQEQQA EV SKPTPTPE G TSQDVTTVTLLLRAPPG STSS hsmoLc2
201 SPASPHNSPTSASPEPLLEPAGAQCPAVEAPVSSEPLPHPSEAPSPEPPM msmoLc2
201 SPASPSSSPT PASPEPPLEPA EAQCLTAEVPGSPEP PPSPPKTTSPEPQE hsmoLc2
251 SPVPSSSRGRVISKPLPGPTEPSDTLDSIRGFSNTKRADPSETKSCQRSL msmoLc2
251 SPTLPSTEGQVVNKLLSGPKETPAAQSPTRG PSDTKRADVAGPRPCQRSL hsmoLc2
301 SVLSPRQPTPNREPTSLA- GPSQFRRVGSVRDRVQKFTSDSPVVARLQDG msmoLc2
301 SVLSPRQPAQ NRESTPLAS GPSSFQRAGSVRDRV HKFTSDSPMA ARLQDG hsmoLc2
350 PPRTALASPTPTRLPGPSLISTTPASSSSSNSSSPSPSDTSSH- - KKQRE msmoLc2
351 TPQAALSPLTPARLLGPSLTSTTPASSSSG- SSSRGPSDTSSRFSKEQRG hsmoLc2
398 LAHSLAELQSCPQEEGPGGRGLALRSLENRAGGPKPCSEEPSTPPPVAVG msmoLc2
400 VAQPLAQLRSCPQEEGPR GRGLAA RPLENRAGGPVARSEEP GAPLPVAVG hsmoLc2
448 TGEPGGSMKTTFTIEIKDGRGQASTGRVLLPTGNQRAELTLGLRAPPTLL msmoLc2
450 TAEPGDSMKTTFTIEIKDGRGQASTGRVLLPTGNQRAELTLGLRAPPTLL hsmoLc2
498 STSSGGKNTITHISNPGTVTRLGSVTHVTTFSHASPGNRGGCNFKMEPDP msmoLc2
500 STSSGGKSTIT RVNSPGTLA RLGSVTHVTS FSHAP PSSRGGCSIKMEPEP hsmoLc2
548 AEPPSTTVEAANGAEQARVDKGPEGRSPLSAEELTAIEDEGVLDKMLDQT msmoLc2
550 AEPLAAAVEAANGAEQ TRVNKAPEGRSPLSAEEL MT IEDEGVLDKMLDQS hsmoLc2
598 TNFEERKLIRAALRELRQRKRDQRDKERERRLREARARPGESRSNVATET msmoLc2
600 TDFEERKLIRAALRELRQRKRDQRDKERERRLQ EAR GRPGE GRGNTATET hsmoLc2
648 TTRHSQRAADGSTVGTVTKTERLVHSNDGTQTARTTTVESSFMRRLENGS msmoLc2
650 TTRHSQRAADGSAVSTVTKTERLVHSNDGTR TARTTTVESSFVRR SENGS hsmoLc2
698 SSSSTTTTTVQTKNFSSSSSSSSSKKMGSIFDREDQTSSRPGSLAALERR msmoLc2
700 GS- - - - - TMMQTKTFSSSSSS - - - KKMGSIFDREDQ ASPRAGSLAALEKR hsmoLc2
748 QAEKKKELMKAQSLPKTSASQARKAMIEKLEKEGSAGGPGTPRTAVQRST msmoLc2
742 QAEKKKELMKAQSLPKTSASQARKAMIEKLEKEGA AGSPGGPRAAVQRST hsmoLc2
798 SFGVPNANSIKQMLLDWCRAKTRGYEHVDIQNFSSSWSDRMAFCALVHNF msmoLc2
792 SFGVPNANSIKQMLLDWCRAKTRGYEHVDIQNFSSSWSDG MAFCALVHNF hsmoLc2
848 FPEAFDYGQLSPQNRRQNFEMAFSSAETHADCPQLLDTEDMVRLREPDWK msmoLc2
842 FPEAFDYGQLSPQNRRQNFEV AFSSAETHADCPQLLDTEDMVRLREPDWK hsmoLc2
898 CVYTYIQEFYRCLVQKGLVKTKKS msmoLc2
892 CVYTYIQEFYRCLVQKGLVKTKKS hsmoLc2

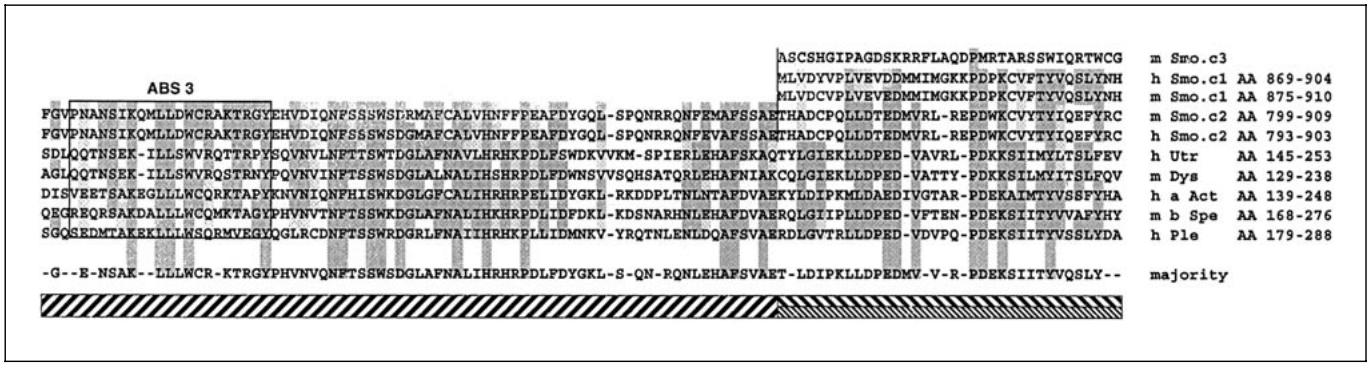



**Fig. 3.** Comparison of mouse smoothelin isoforms with members of the spectrin family. The C termini of the mouse smoothelin alternative splice variants c1–3 and the respective human c1 and c2 forms were aligned with the spectrin protein family. The vertical line indicates the site of alternative splicing and thus the beginning of the different C termini. Notice that the C termini of c1 and c2, derived from different, alternatively spliced exons, both show a high similarity to the second half of the SFS domain, whereas the c3 specific exon lacks this similarity. The c3 specific exon is present in the human smoothelin gene but could not be detected in human cDNA and was therefore not included in this alignment. Dark gray indicates identical amino acids and light grey similar amino acids. Sequences in this alignment were identified with BLAST database searches and downloaded from GenBank (accession No. are: X15804 for α-actinin, α-Act; U53204 for plectin, Ple; S66283 for α-spectrin, α-Spe; X69086 for utrophin, Utr; and M68859 for dystrophin, Dis). The alignment of the sequences corresponding to the actin-binding site 3 (ABS 3) of utrophin is boxed. Small letters 'm' and 'h' denote the origin from mouse and human, respectively. The hatched pattern bar indicates the homology to the SFS domain corresponding to the bars in figure 4.

*Analysis of the Tissue-Specific Expression of Smoothelin Isoforms*

The identification of different smoothelin isoforms raises the possibility that the tissue and development specific expression of these isoforms could contribute to the diversity of smooth muscle cells. We therefore generated specific antibodies against the small and the large isoforms of smoothelin to investigate their expression pattern in vivo. Polyclonal rabbit antibodies were raised against potentially antigenic peptide sequences. For the N-terminus, amino acids 52–65 (smo1, located in exon 3) were chosen that are specific for the large smoothelin isoform. Since the entire sequence of the short isoform is also present in the large isoform, antibodies against the small isoform automatically also recognize the large isoform. Therefore, antibodies raised against the C-terminal amino acids 614–627 (smo2, located in exon 14) react with both, small and large smoothelin isoforms. Both peptide sequences are identical in the mouse and in humans. The relative position of these antigenic peptide sequences is outlined in figure 5a. Both antisera were affinity purified to reduce unspecific reactions.

To test the specificity of the anti-smo1 and anti-smo2 antibodies, we subcloned different parts of the smoothelin cDNA in a bacterial expression vector (fig. 5a). Bacterial protein extracts were used for Western blots and probed with anti-smo1 and anti-smo2 antibodies. All three smoothelin constructs are correctly expressed as shown with an antibody against their common C-terminal V5 epitope tag (fig. 5a). The full-length construct (Lc2) corresponding to the large smoothelin isoform is recognized by both antisera. In contrast, the N-terminal domain (LN) is recognized only by the anti-smo1 antibodies, while the C-terminal domain (Sc1), which corresponds to the small isoform, is recognized only by the anti-smo2 antibodies (fig. 5a). These results clearly show that the antibodies specifically react with smoothelin and that anti-smo1 reacts only with the large isoform.

We then used these new antibody tools to analyze smoothelin expression in different smooth muscle cells. Since the antigenic peptide sequences used are identical in the mouse and in humans, they are likely to be conserved in other species as well. Protein extracts from porcine coronary arteries and porcine bladder were assayed

**Fig. 2.** Human and mouse smoothelin sequence alignment. The amino acid alignment of the human and mouse smoothelin large c2 isoform shows the highly conserved protein sequence in both species. 'msmoLc2' denotes the mouse smoothelin large c2 isoform and 'hsmoLc2' the human homolog.

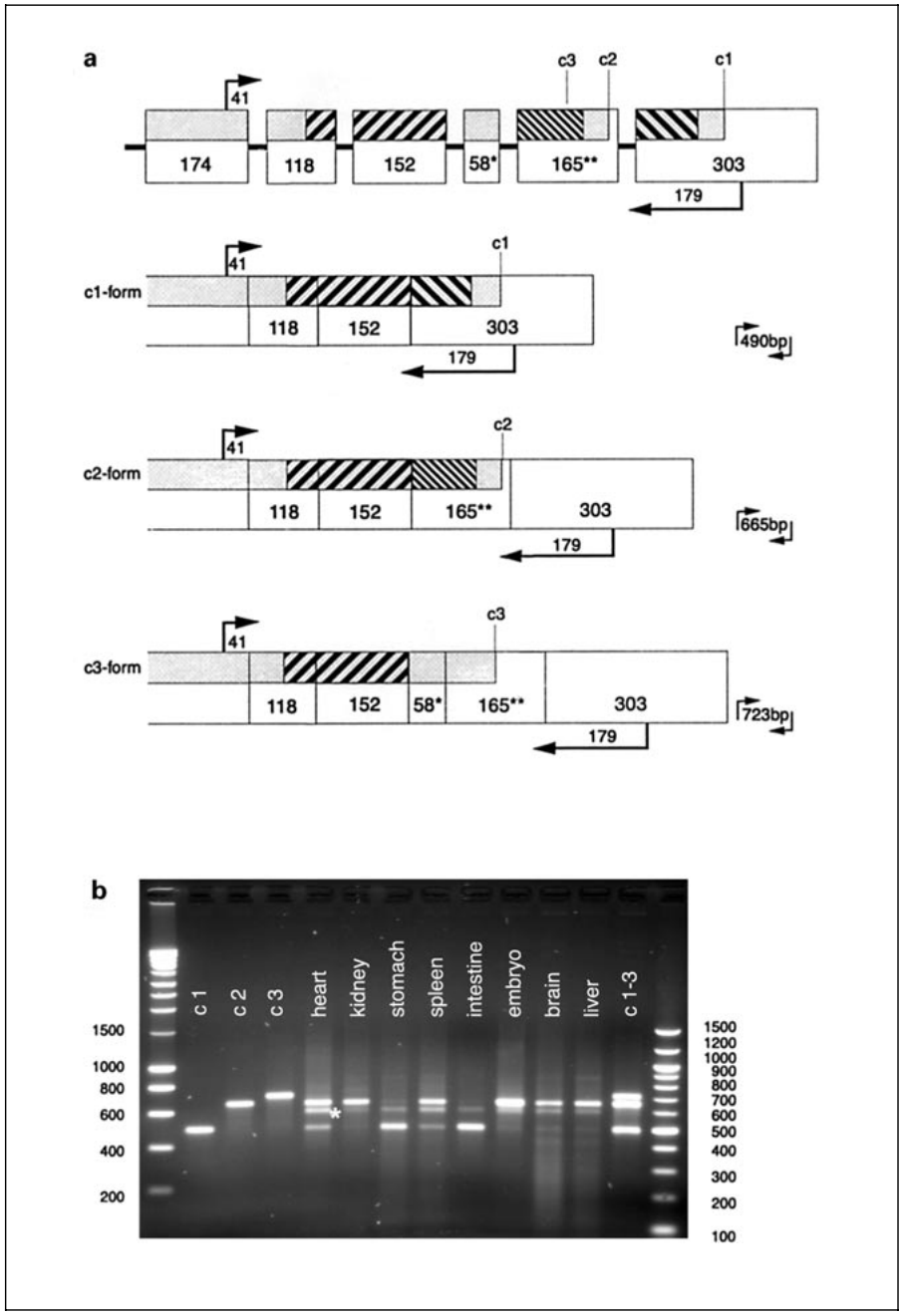



**Fig. 4.** Structure and tissue-specific expression of smoothelin splice variants. **a** Exon structure of the mouse smoothelin C-terminal region. Exons are depicted as open boxes with their size indicated in base pairs. Bent arrows indicate the position of primers used for amplification of the alternatively spliced region and numbers reflect the size of the fragments expected from the different isoforms. Gray bars indicate the ORF, and the position of the stop codon of each alternatively spliced form is marked by a vertical bar and the name of the isoform. Regions corresponding to the SFS domain are marked with hatched pattern as in figure 3. The common first half and the two conserved second halves that are generated by alternative splicing are distinguished with different patterns. **b** RT-PCR products obtained from various tissues using the primer pair shown above in **a**. For comparison, products from control reactions with subcloned cDNAs containing the three possible splice variants c1, c2 and c3 were loaded. Samples were analyzed on a 1.8% agarose gel and the sizes of DNA molecular weight marker bands are indicated on both sides. The additional band marked with a white asterisk does not fit in size with any of the known splice forms. Control experiments showed that this band is a PCR artifact and corresponds to a heteroduplex between the c1 and c2 forms. This artifact does not appear in the control c1–3, since those reactions were carried out separately from each other.

for smoothelin expression by Western blot. Both antibodies, anti-smo1 and anti-smo2, recognized a single protein band at about 117 kD in coronary artery extracts (fig. 6a). In bladder extract, however only the anti-smo2 recognizes a band at about 60 kD (Fig. 6a). The results show a specific expression of the large isoform in coronary arteries and of the small isoform in visceral tissue. These results are in good agreement with an apparent MW of about 115 kD recently reported for the large smoothelin isoform [15] and for the short isoform shown by van der Loop et al. [14]. This large isoform is clearly larger than the 95-kD protein previously identified on Western blots of chicken vascular smooth muscle extracts with a smoothelin-specific monoclonal antibody [22] raising the possibility that an avian-specific isoform or another cross-reacting protein may exist in chicken.

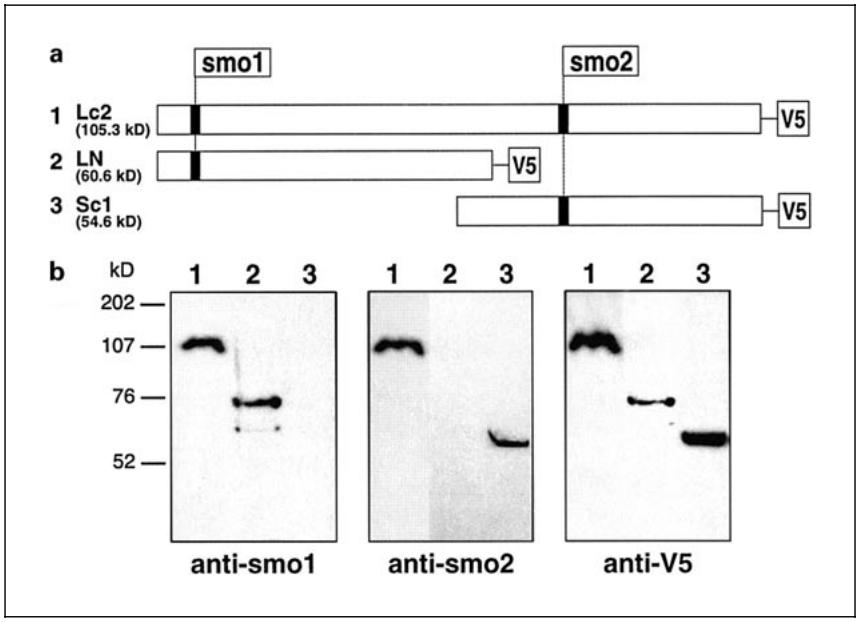



**Fig. 5.** Characterization of anti-smoothelin antibodies. **a** Structure of the three epitope tagged, bacterially produced smoothelin proteins (Lc2, LN and Sc1). The location of the V5 epitope tag as well as the smo1 and smo2 peptides used to raise antibodies, one directed against a common part in the C-terminal end and the other against a large isoform specific peptide sequence in the N-terminus, are indicated. The predicted molecular weight of the fusion proteins is also displayed. **b** Western blots of extracts from bacteria overexpressing each of the three epitope-tagged smoothelin constructs were probed with anti-V5, anti-smo1 and anti-smo2 antibodies. As expected, all of the fusion proteins were detected with the V5-specific antibody (lower panel, right). The anti-smo1 antibody reacted only with the fusions proteins containing the large N-terminal sequence (Lc2 and LN), whereas anti-smo2 antibody recognized only the Lc2 and Sc1 fusion proteins, which contain the C-terminal sequence confirming the specificity of these antibodies.

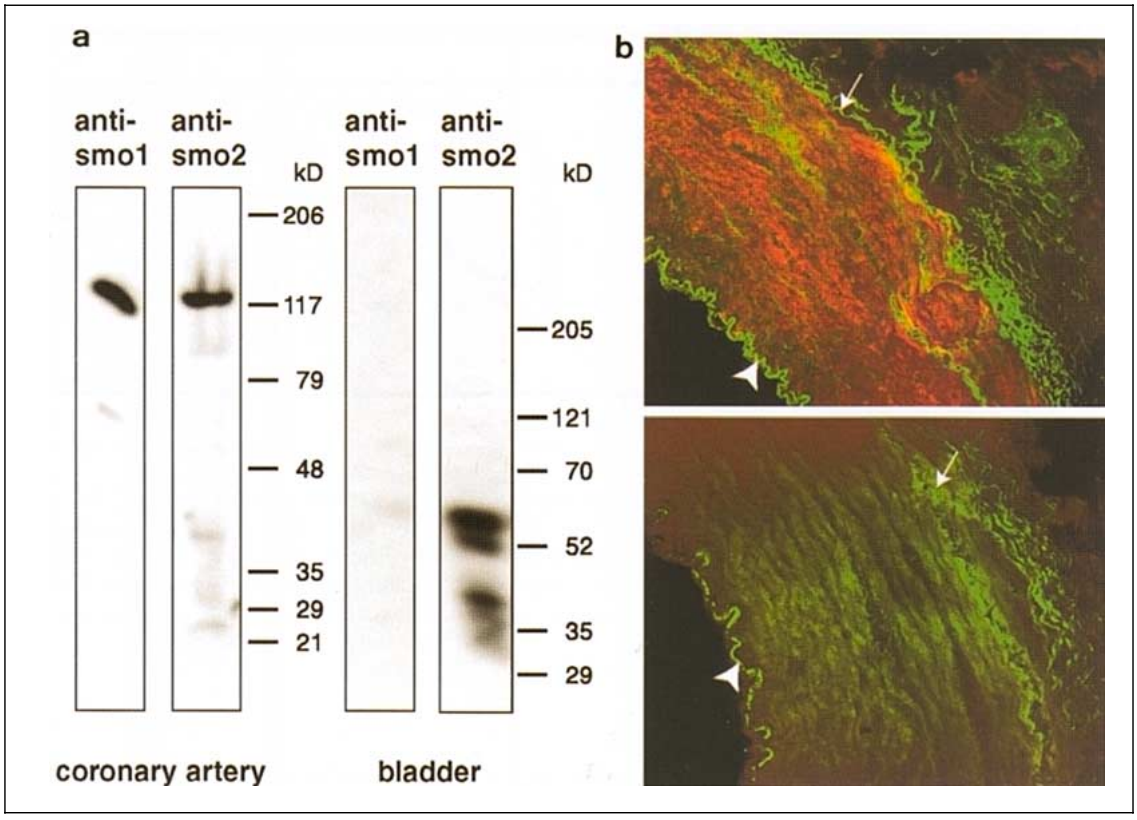



**Fig. 6.** Large smoothelin isoform is expressed in coronary smooth muscle cells. **a** Western blots each containing 40 μg of protein extract from porcine coronary arteries or porcine bladder, incubated with anti-smo1 and anti-smo2 antibodies, as indicated above the blots. Both recognize a single protein species of about 117 kD in coronary arteries, corresponding to the large vascular-specific smoothelin isoform and in bladder only the anti-smo2 recognizes a band at 60 kD whereas the anti-smo1 does not. **b** Sections of porcine coronary arteries stained with anti-smo1 (upper panel) and the control without the antibody (lower panel). The specific staining for the large smoothelin isoform is shown in red. The auto fluorescence of the tissue in the FITC-filter (green) shows the elastic fibers, elastica externa (arrow) and elastica interna (arrowhead).

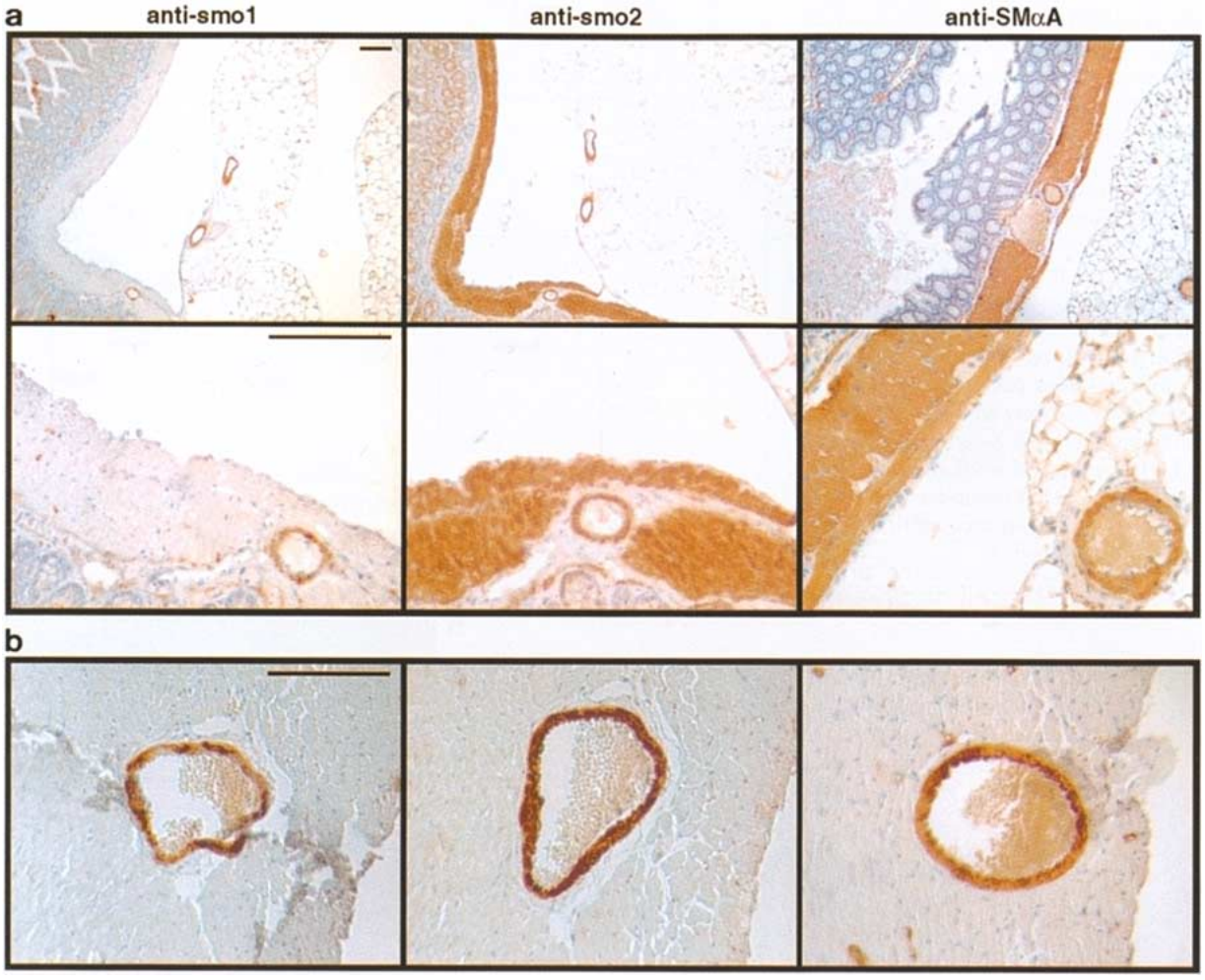



**Fig. 7.** Tissue- and cell-type-specific expression of smoothelin isoforms. **a** Mouse intestinal tissue sections stained (brown color) with anti-smo1 (left) and anti-smo2 (middle) and anti-SMαA (right) antibodies at different magnifications. Vascular and visceral smooth muscle cells are labeled specifically with the anti-SMαA antibody and the anti-smo2 antibody, which recognizes both, large and small, smoothelin isoforms. The images on the left show the specific detection of vascular smooth muscle cells by the anti-smo1 antibody, which reacts only with the large smoothelin isoform. **b** Staining of mouse heart sections with the same antibodies as in **a**. Signals with both, anti-smo1 and anti-smo2, are only detected in vascular smooth muscle cells of the coronary arteries, but not in cardiomyocytes. Scale bars = 100 μm.

The additional bands detected with the anti-smo2 antibody in the protein extract of the bladder are probably due to proteolytic degradation. The discrepancy with the MW deduced from the ORF can be accounted for by the previously described glycosylation [14]. Furthermore, the immunofluorescent staining of porcine coronary arteries with anti-smo1 clearly shows that only the smooth muscle cells of the media possess a strong specific signal while the adventitial and the endothelial cell layers do not (fig. 6b).

To further investigate the expression of different smoothelin isoforms in specific tissues and smooth muscle cell types, we performed immunohistochemical staining of adult mouse organs. Vascular and visceral smooth muscle cells can be compared directly in intestinal tissue sections. For a positive control, the sections were incubated with an antibody against SMαA, which stains all smooth muscle cells (fig. 7a, b, right). The common smoothelin antibody anti-smo2 (fig. 7a, middle) also

stains both the visceral and the vascular smooth muscle cells, whereas anti-smo1 predominantly stains the smooth muscle cells of small arteries without labeling the visceral muscle layer (fig. 7a, left). The arteries in both the surrounding fat tissue and the tunica muscularis are labeled with anti-smo1. These results show a specific expression of the large smoothelin isoform in vascular smooth muscle cells. In addition, there is a weak signal in the cells of the muscularis mucosae, which however remains to be confirmed with independently generated antibodies.

Immunostaining in sections of mouse heart is consistent with the results obtained in intestinal tissue and shows labeling of vascular smooth muscle cells with both anti-smoothelin antibodies, but no reactivity with any other cell type (fig. 7b). The same vascular staining patterns were also found in kidney and brain tissue (data not shown). These results clearly show that different smoothelin isoforms are expressed in different smooth muscle cells, most probably as a large c2 (vascular) and a small c1 (visceral) form, as indicated by our RT-PCR data.

Johansson et al. [23] published a study in which they showed the increase in smoothelin abundance in human arteries from subjects 3 months of age to 1 year and older, thereby supporting the idea of a highly specific marker for differentiated vascular smooth muscle cells. However, they also found smoothelin in cardiomyocytes, which may be due to the lack of specificity of the R4A antibody. In mouse heart, no staining of cardiomyocytes was detected by our anti-smo1 and anti-smo2 antibodies (fig. 7b), and others did not describe these findings in their studies either. Most recently, Gsell et al. [24] might have supplied an explanation by detecting smoothelin with an antibody directed at a G-protein subunit α in pregnant uterine myometrium. Conversely, cross-reaction with the smoothelin antibody R4A may also take place.

In summary, until now all studies on smoothelin expression have been based solely on a single monoclonal antibody that does not differentiate between any of the isoforms nor does it recognize murine smoothelin [14]. The polyclonal antibodies described here are isoform specific and showed that the large smoothelin form is specific to vascular smooth muscle while the short isoform is present only in visceral smooth muscle. We could show that vascular and visceral smoothelin isoforms are generated by two independent transcription start sites. The future characterization of the corresponding promoters should help to elucidate the mechanism of smooth muscle cell differentiation, diversification and dedifferentiation during normal development and in vascular disease.

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## References

1 Duband JL, Gimona M, Scatena M, Sartore S, Small JV: Calponin and SM 22 as differentiation markers of smooth muscle: Spatiotemporal distribution during avian embryonic development. Differentiation 1993;55:1–11.
2 Campbell JH, Kocher O, Skalli O, Gabbiani G, Campbell GR: Cytodifferentiation and expression of alpha-smooth muscle actin mRNA and protein during primary culture of aortic smooth muscle cells. Correlation with cell density and proliferative state. Arteriosclerosis 1989;9:633–643.
3 Frid MG, Shekhonin BV, Koteliansky VE, Glukhova MA: Phenotypic changes of human smooth muscle cells during development: Late expression of heavy caldesmon and calponin. Dev Biol 1992;153:185–193.
4 Henderson JR, Macalma T, Brown D, Richardson JA, Olson EN, Beckerle MC: The LIM protein, CRP1, is a smooth muscle marker. Dev Dyn 1999;214:229–238.
5 Li L, Miano JM, Mercer B, Olson EN: Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cellss. J Cell Biol 1996; 132:849–859.
6 Hungerford JE, Owens GK, Argraves WS, Little CD: Development of the aortic vessel wall as defined by vascular smooth muscle and extracellular matrix markers. Dev Biol 1996;178: 375–392.
7 Sobue K, Hayashi K, Nishida W: Expressional regulation of smooth muscle cells-specific genes in association with phenotypic modulation. Mol Cell Biochem 1999;190:105–118.
8 Ross R: The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 1993;362: 801–809.
9 Topouzis S, Majesky MW: Smooth muscle lineage diversity in the chick embryo. Two types of aortic smooth muscle cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor-beta. Dev Biol 1996;178:430–445.
10 Dettman RW, Denetclaw W Jr, Ordahl CP, Bristow J: Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. Dev Biol 1998;193:169–181.
11 Gittenberger-De Groot AC, Deruiter MC, Bergwerff M, Poelmann RE: Smooth muscle cell origin and its relation to heterogeneity in development and disease. Arterioscler Thromb Vasc Biol 1999;19:1589–1594.
12 Arner A, Malmqvist U: Cross-bridge cycling in smooth muscle: A short review. Acta Physiol Scand 1998;164:363–372.
13 Stull JT, Lin PJ, Krueger JK, Trewhella J, Zhi G: Myosin light chain kinase: Functional domains and structural motifs. Acta Physiol Scand 1998;164:471–482.

14 Van Der Loop FT, Schaart G, Timmer ED, Ramaekers FC, Van Eys GJ: Smoothelin, a novel cytoskeletal protein specific for smooth muscle cells. J Cell Biol 1996;134:401–411.
15 Christen T, Bochaton-Piallat ML, Neuville P, Rensen S, Redard M, Van Eys G, Gabbiani G: Cultured porcine coronary artery smooth muscle cells. A new model with advanced differentiation. Circ Res 1999;85:99–107.
16 Van Eys GJ, Voller MC, Timmer ED, Wehrens XH, Small JV, Schalken JA, Ramaekers FC, Van Der Loop FT: Smoothelin expression characteristics: Development of a smooth muscle cell in vitro system and identification of a vascular variant. Cell Struct Funct 1997;22: 65–72.
17 Krämer J, Aguirre-Arteta AM, Thiel C, Gross CM, Dietz R, Cardoso MC, Leonhardt H: A novel isoform of the smooth muscle cell differentiation marker smoothelin. J Mol Med 1999; 77:294–298.
18 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 1990;215:403–410.
19 Rensen S, Merkx G, Doevendans P, Geurts Van Kessel A, Van Eys G: Structure and chromosome location of Smtn, the mouse smoothelin gene. Cytogenet Cell Genet 2000;89:225–229.
20 Senapathy P, Shapiro MB, Harris NL: Splice junctions, branch point sites, and exons: Sequence statistics, identification, and applications to genome project. Methods Enzymol 1990;183:252–278.
21 Keep NH, Norwood FI, Moores CA, Winder SJ, Kendrick-Jones J: The 2.0 A structure of the second calponin homology domain from the actin-binding region of the dystrophin homologue utrophin. J Mol Biol 1999;285:1257–1264.
22 Wehrens XH, Mies B, Gimona M, Ramaekers FC, Van Eys GJ, Small JV: Localization of smoothelin in avian smooth muscle and identification of a vascular-specific isoform. FEBS Lett 1997;405:315–320.
23 Johansson B, Eriksson A, Ramaekers F, Thornell L: Smoothelin in adult and developing human arteries and myocardium. Histochem Cell Biol 1999;112:291–299.
24 Gsell S, Eschenhagen T, Kaspareit G, Nose M, Scholz H, Behrens O, Wieland T: Apparent upregulation of stimulatory G-protein alpha subunits in the pregnant human myometrium is mimicked by elevated smoothelin expression. FASEB J 2000;14:17–26.