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cFKBP/SMAP; a novel molecule involved in the regulation of smooth muscle differentiation

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SUMMARY

During embryogenesis, smooth muscle cells of the gut differentiate from mesenchymal cells derived from splanchnic mesoderm. We have isolated a gene involved in the differentiation of smooth muscle cells in the gut using differential display between the chicken proventriculus in which the smooth muscle layer develops poorly and the gizzard in which smooth muscles develop abundantly. The protein encoded by this gene showed highest similarity to mouse FK506 binding protein, FKBP65, and from the function of this protein it was designated chicken FKBP/smooth muscle activating protein (cFKBP/SMAP).

cFKBP/SMAP was first expressed in smooth muscle precursor cells of the gut and, after smooth muscles differentiate, expression was restricted to smooth muscle cells. In organ culture of the gizzard, the differentiation of smooth muscle cells was inhibited by the addition of FK506, the inhibitor of FKBPs. Moreover, overexpression of cFKBP/SMAP in lung and gizzard mesenchymal cells induced smooth muscle differentiation. In addition, cFKBP/SMAP-induced smooth muscle differentiation was inhibited by FK506. We postulate therefore that cFKBP/SMAP plays a crucial role in smooth muscle differentiation in the gut and provides a powerful tool to study smooth muscle differentiation mechanisms, which have been poorly analyzed so far.

Key words: Smooth muscle, Gut, Chicken embryo, FKBP, FK506, Differential display

INTRODUCTION

Smooth muscles exist in vascular and digestive systems in vertebrates and bring about involuntary movement of these systems. Although the smooth muscle and the skeletal muscle share common muscular characteristics, they also possess quite different structure, function and developmental origin.

The regulatory systems of skeletal muscle differentiation have been well analyzed and it is known that the myoD family of basic-helix-loop-helix (bHLH) transcription factors are involved as 'master regulatory genes' (Weintraub et al., 1991; Rudnicki et al., 1993; Firlli et al., 1997). In contrast, little is known about the molecular mechanisms that regulate the differentiation of smooth muscle cells. As for smooth muscle differentiation in the gut, only regulation of production of some fiber proteins and related proteins involved in the smooth muscle contraction, such as α-smooth muscle actin, caldesmons, desmin and collagens, has been reported (Hirai and Hirabayashi, 1983; Ueki et al., 1987; Kedinger et al., 1990; Perr et al., 1992).

The chicken gizzard (muscular stomach) has been used as a source of smooth muscle proteins because of the thick layer of smooth muscle which develops in it. The differentiation of smooth muscle in the gizzard becomes apparent on day 5-6 of incubation as evidenced by the production of myosin heavy chain, desmin and β-tropomyosin in smooth muscle cells (Hirai and Hirabayashi, 1983; Rong et al., 1987). Eventually, smooth muscle makes up more than 4/5 of the connective tissue and facilitates mechanical digestion. The proventriculus (glandular stomach), which develops rostral to the gizzard, has only a very thin smooth muscle layer (Romanoff, 1960; Hodges, 1974). Other digestive organs of the chicken (esophagus and small and large intestines) show modest development of smooth muscle layers at the outermost part of the organs (Romanoff, 1960; Hodges, 1974). The lung has a very thin smooth muscle layer beneath the epithelium (Romanoff, 1960; Cook and King, 1970; Hodges, 1974). Thus, the gizzard and proventriculus of the chicken embryo offer the ideal models for the elucidation of mechanisms of smooth muscle differentiation.

To identify molecules that regulate the determination or differentiation of smooth muscle cells in the gizzard, we isolated cDNAs expressed specifically in the gizzard mesenchyme, but not in the proventricular mesenchyme, using the differential display method.

One of the isolated cDNAs encoded a protein with a high level of similarity to mouse FK506-binding protein, FKBP65 (Coss et al., 1995), and was involved in smooth muscle

differentiation (see below). It was, therefore, designated chicken FKBP/smooth muscle activating protein (cFKBP/SMAP).

FK506-binding proteins (FKBPs) are a family of intracellular receptors of the immunosuppressant macrolides, FK506 and rapamycin (Tropschug et al., 1990; Schreiber 1991; Peattie et al., 1992). They constitute an evolutionarily related protein family ranging in size from 12 to 65 kDa (Siekierka et al., 1989; Jin et al., 1991, 1992; Lebeau et al., 1992; Galat et al., 1992; Smith et al., 1993; Alnemri et al., 1993, 1994; Lam et al., 1995; Coss et al., 1995). A characteristic domain shared by FKBPs is the peptidylpropyl *cis-trans*-isomerase (PPIase) domain (Standaert et al., 1990), and activity of the domain is inhibited by binding of FK506 (Harding et al., 1989). Unlike the other ubiquitously expressed FKBPs, FKBP65 mRNA shows restricted expression in lung, spleen, heart and brain in the mouse (Coss et al., 1995). The functions of FKBPs in vivo, except those of FKBP12, are poorly understood. FKBP12 binds to the ligand-free TGFβ type I receptor with its PPIase domain and may block TGFβ family-mediated signaling (Wang et al., 1994, 1996; Okadome et al., 1996). FKBP12 interacts also with inositol 1,4,5-triphosphate receptor in skeletal muscle cells and regulates Ca^{2+} influx (Cameron et al., 1995). These functions are completely inhibited by FK506 (Cameron et al., 1995; Wang et al., 1996).

Here we describe smooth muscle-specific expression of cFKBP/SMAP in embryonic chicken gut. We also present evidence that smooth muscle differentiation was affected by functional blocking of cFKBP/SMAP by FK506 and overexpression of cFKBP/SMAP. These experiments strongly suggest that cFKBP/SMAP is essential for the determination and/or differentiation of smooth muscle cells in the gut.

MATERIALS AND METHODS

Cloning of cFKBP/SMAP

Gizzard-specific cDNA fragments were isolated by differential display as reported by Ito et al. (1994). Total RNA was isolated from 7.5-day chicken embryonic proventriculus and gizzard using Trizol (Gibco BRL, MD). cDNA was synthesized from 5 μg of total RNA by SuperScript II reverse transcriptase (Gibco BRL) using fluorescein isothiocyanate (FITC)-labeled 3′-anchored oligo-dT primer and was amplified between this oligo-dT primer and arbitrary 10 mer (Operon, CA). After electrophoresis and scanning by a FluoroImager 575 (Molecular Dynamics, CA), gizzard-specific bands were subcloned into pCRII (Invitrogen, CA) and were confirmed as gizzard-specific by reverse transcription-polymerase chain reaction (RT-PCR) and whole-mount in situ hybridization. One of the isolated cDNA fragments (using GT15V(A/C/G)T and TGCCGAGCTG as primers) was 1300 bp in length and was expressed in gizzard mesenchyme but not in proventricular mesenchyme.

A 7.5-day embryonic gizzard cDNA library was constructed using λZap II (Stratagene, CA) and screened under high stringency with a 1300 bp fragment to obtain a 3.8 kb cDNA clone. By database searching, this cDNA was found to have highest homology to mouse FKBP65, and was designated cFKBP/SMAP.

Northern blot hybridization

Total RNA was extracted from various tissues of day 9 and 13 embryonic chicken and hatched chicken using Trizol. 10 μg of total RNA was electrophoresed and transferred to a nylon membrane (Hybond N+, Amersham, UK). ^{32}P-labeled 3.8 kb cFKBP/SMAP cDNA was used as a probe. Probe was subsequently stripped from the membrane and the membrane was rehybridized with a chicken EF1α probe (Wang et al., 1994), which was a kind gift from Dr Morais.

In situ hybridization

In situ hybridization with a digoxigenin-labeled probe was performed on 10 μm cryosections, paraffin sections or cultured cells as described by Ishii et al. (1997). 1300 bp cDNA fragment of cFKBP/SMAP 3′ UTR was used as a probe.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde for 3 hours and 10 μm frozen sections were treated with antibody. Monoclonal antibody 13F4, which recognizes muscle-specific protein (Rong et al., 1987) was a kind gift from Prof. N. M. Le Douarin. Polyclonal anti-caldesmon antibody was a kind gift from Prof. T. Hirabayashi. Antibody binding was visualized using HRP-conjugated goat anti-mouse IgM or anti-rabbit IgG and 3, 3′-diaminobenzidine(DAB) solution.

Cells were fixed in 4% paraformaldehyde for 20 minutes. Anti-FLAG M2 mouse monoclonal antibody was purchased from Eastman Kodak Company (New Heaven, CT). FITC-conjugated goat anti-mouse antibody and FITC- or Rodamin isothiocyanate (RITC)-conjugated goat anti-rabbit antibody were used for detection of primary antibody. After staining with antibodies, nuclei were labeled with DAPI.

Organ culture

Isolated gizzard from a 6-day chicken embryo was placed on a porous membrane (Nuclepore SN11049, 8 μm pore size, Costar), rested on a stainless-steel grid. Medium (50% embryo extract in Earl's 199; Takiguchi et al., 1988) was added to reach the filter, and the culture dishes were incubated for 4 days at 37°C in 5% CO_2.

FK506 was a kind gift from Fujisawa Pharmaceutical Co. (Japan). 1 mM FK506 in ethanol was diluted with culture medium to a final concentration of 10 μM. An equivalent amount of ethanol was added to the medium of control cultures.

Cell culture and transfection

The N-terminal end of the *Xba*I fragment containing the full coding region of cFKBP/SMAP was tagged with FLAG epitope from pyDF30. The FLAG-tagged cFKBP/SMAP cDNA was inserted in pmiwSV (Suemori et al., 1990; Wakamatsu et al., 1996) and designated pmiwFLAG-SMAP.

The gizzard and lung were isolated from 4-day embryo and divided into epithelium and mesenchyme with 0.03% collagenase in Tyrode's solution. Mesenchymes were dissociated into single cells by treatment with 0.25% trypsin in PBS. The cells were placed on a chamber slide (Nunc) with OPTI-MEM medium (Gibco BRL). Then, pmiwFLAG-SMAP or pmiwFLAG-GFP (Wakamatsu and Weston, 1997) containing FLAG epitope subcloned from pyDF30 and mutant green fluorescent protein gene was transfected into these cells using Lipofectin reagent (Gibco BRL) as indicated by the manufacturer. Transfected cells were cultured for 2 days in DMEM and 10% fetal calf serum at 37°C in 5% CO_2 with 0, 5 or 20 μM FK506. Smooth muscle cells from 9-day embryonic gizzard were cultured as a control.

RESULTS

The isolation and sequence analysis of cFKBP/SMAP

We isolated 18 independent cDNA fragments expressed in the 7.5-day embryonic chicken gizzard mesenchyme, but not in the proventricular mesenchyme, by the differential display method. A cDNA library of 7.5-day embryonic gizzard was

then screened with these fragments as probes to obtain longer cDNAs.

The predicted amino acid sequence of one of the isolated genes showed high similarity to the mouse FK506 binding protein, FKBP65 (59 % of amino acid residues are identical) and the cDNA encodes a member of FKBPs, and we designated it cFKBP/SMAP. This 3.8 kb clone of cFKBP/SMAP possessed an ORF encoding 580 amino acids (Fig. 1) with predicted molecular mass of 65 kDa. Four repeats of the PPIase domain, characteristic of FKBPs, were found in cFKBP/SMAP as in mouse FKBP65 (Coss et al., 1995).

The expression pattern of cFKBP/SMAP

The spatiotemporal distribution of cFKBP/SMAP mRNA was first determined by northern hybridization. The 3.8 kb message was detected in all embryonic digestive organs (Fig. 2). The strongest signal was detected in the gizzard. The proventriculus and lung showed very weak expression. The expression patterns in digestive organs were similar between the 9-day and 13-day embryos (Fig. 2A and B), but slightly different in the adult. In hatched chicken the intensity was almost the same between the esophagus, gizzard and intestine. The transcript was never detected in brain, heart, lung, liver, kidney and spleen in hatched chicken. The only organ expressing cFKBP/SMAP in the hatched chicken other than digestive organs was testis (Fig. 2C).

The distribution of the cFKBP/SMAP transcripts was analyzed in detail by in situ hybridization to tissue sections of chicken embryos (Fig. 3).

cFKBP/SMAP mRNA was first detected in fibroblasts in the gizzard mesenchyme of 4-day embryos. In 6-day embryonic gizzard the mRNA was clearly restricted to the presumptive smooth muscle layer of the mesenchyme at a distance from the epithelium (Fig. 3A,B). As morphological differentiation of smooth muscle cells became evident, the expression of the gene was restricted to elongated smooth muscle cells which occupy most of the mesenchyme, except a narrow area adjacent to the epithelium (Fig. 3C-E). The intensity of cFKBP/SMAP signals was uniform throughout the positive area at this stage.

In 12-day embryonic gizzard, smooth muscle cells are well organized and stained with 13F4 antibody, one of the earliest markers of all types of muscle cells (Rong et al., 1987). The cFKBP/SMAP-positive cells coincided with the 13F4-immunoreactive cells (Fig. 3E,F). The cFKBP/SMAP-positive cells were further confirmed to be smooth muscle cells by the fact that they coexpressed caldesmon, which is expressed in differentiated smooth muscle cells (data not shown).

In other digestive organs such as esophagus, small intestine and large intestine, cFKBP/SMAP was also expressed in presumptive and differentiated smooth muscle layers (Fig. 4A-C). These cells expressing cFKBP/SMAP were also marked by 13F4 and anti-caldesmon antibodies (data not shown). In the proventriculus cFKBP/SMAP mRNA was not detected at early stages (4- to 6-day embryo; Fig. 4D) and only weak expression could be detected in poorly developed smooth muscles at a later stage (12-day embryo; data not shown). In the pancreas, skeletal muscle, and cardiac muscle, the transcripts of this gene could not be detected, while, it was detected abundantly in aortic smooth muscles (data not shown). In summary, the expression of cFKBP/SMAP coincides well with smooth muscle differentiation. These results prompted us to examine the function of cFKBP/SMAP in smooth muscle differentiation.

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MFL-SLGATL LHRIRVIPVL LILTTQEKAL GRAVPSAGKL ADVHVERRFV  50
MFLVGSSSHT LHRVRILPLL LLLQTLERGL GRASPAGAPL EDVVIERYHI

PERCPRAVRR GDFVRYHYLG SFPDGTRFDS SYDRGSTFNV FVGKGQLIAG 100
PRACPREVQM GDFVRYHYNG TFEDGKKFDS SYDRSTLVAI VVGVGRLITG

MDKALVGMCV NERRFVKIPP KLAYGSEGVS GVIPPNAVLH FDVLLIDLWN 150
MDRGLMGMCV NERRRLIVPP HLGYGSIGVA GLIPPDATLY FDVVLLDVWN

SEDEVQVETY FKPEKCTRRV QVSDFVRYHY NGTFLDGTLF DSSHNRMRTY 200
KADTVQSTIL LRPPYCPRMV QNSDFVRYHY NGTLLDGTGF DNSYSRGGTY

DTYVGIGWLI PGMDQGLLGM CIGEKRIITI PPFLAYGEEG DGKEIPGQAS 250
DTYIGSGWLI KGMDQGLLGM CPGEKRKIII PPFLAYGEKG YGTVIPPQAS

LVFDVVLLDL HNPKDGITIE NQLVPESCER RTQTGDFIRY HYNGTLLDGT 300
LVFYVLLLDV HNPKDTVQLE TLELPQGCVR RAVAGDFMRY HYNGSLMDGT

LFDSSYSRNR TYDTYVGKGY VIAGMDEGLL GVCTGERRRI IIPPHLGYGE 350
LFDSSYSRNH TYNTYVGQGY IIPGMDQGLQ GACIGERRRI TVPPHLAYGE

EGRG-KIPGS AVLVFDIHVA DFHNPSDSVS I-TVNYKPSN CSLLSKKGDY 400
NGTGDKIPGS AVLIFDVHVI DFHNPSDPVE IKTLSRPPEN CNETSKIGDF

LKYHNYASLL GWHSASLDTQ SWQDLQHSSG SGQVVIGMDM GLQDMCVGER 450
IRYHYNCSLL DGTRFLSSHD YEAPQEITLG ANKVIEGLDR GLQGMCVGER

RTVVIPPHLG YGEDGVEGEV PGSAVLVFDI ELLELVSGLP EGYMFVWNGE 500
RQLIVPPHLA HGENGARG-V PGSAVLLFEV ELVSREDGLP TGYLFVWYQD

VSPNLFEEID QNHDGEVLLE EFSEYIQAQV DSGKGKLAPG FDFEKIVKNM 550
PSTSLFEDMD LNKDGEVPPE EFSSFIKAQV NEGKGRLMPG QDPDKTISDM

FTNQDRDGNG KVTAEEFKLK DQEAKEGHDE L-
FQNQDRNQDG KITAEELKLK SDEDQERVHE EL
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Fig. 1. Amino acid sequence comparison between mouse FKBP65 and cFKBP/SMAP. Lower line indicates amino acid sequence of mouse FKBP65 and upper line that of FKBP/SMAP. Amino acid residues identical between two genes are shaded. PPIase domains are underlined. These domains show high similarity between mouse FKBP65 and cFKBP/SMAP. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with accession number AB008675.

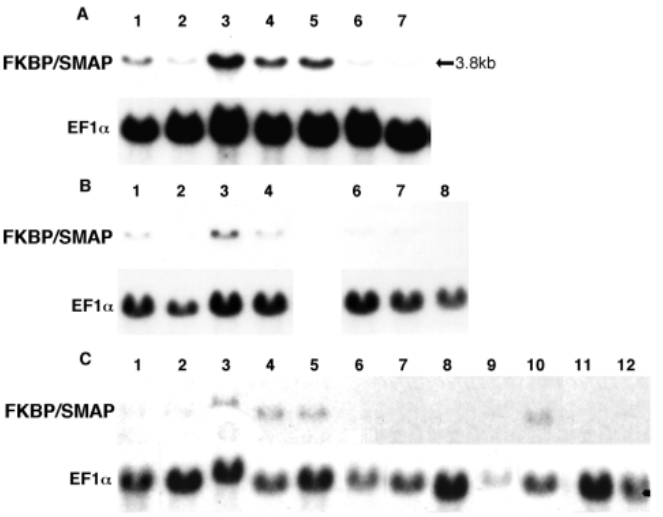

Fig. 2. Expression of cFKBP/SMAP in various organs during development examined by northern hybridization. (A) 9-day embryo. (B) 13-day embryo. (C) Hatched chicken. The single 3.8 kb transcript is indicated by an arrow. EF1α was used as a control. Lane 1, esophagus; 2, proventriculus; 3, gizzard; 4, small intestine; 5, large intestine; 6, lung; 7, liver; 8, heart; 9, kidney; 10, testis; 11, spleen; 12, brain.

Inhibition of smooth muscle differentiation by FK506

We first cultured isolated gizzard in the presence of FK506, a common inhibitor of the activities of FKBPs (Bitter et al., 1990; Jin et al., 1991; Lam et al., 1995).

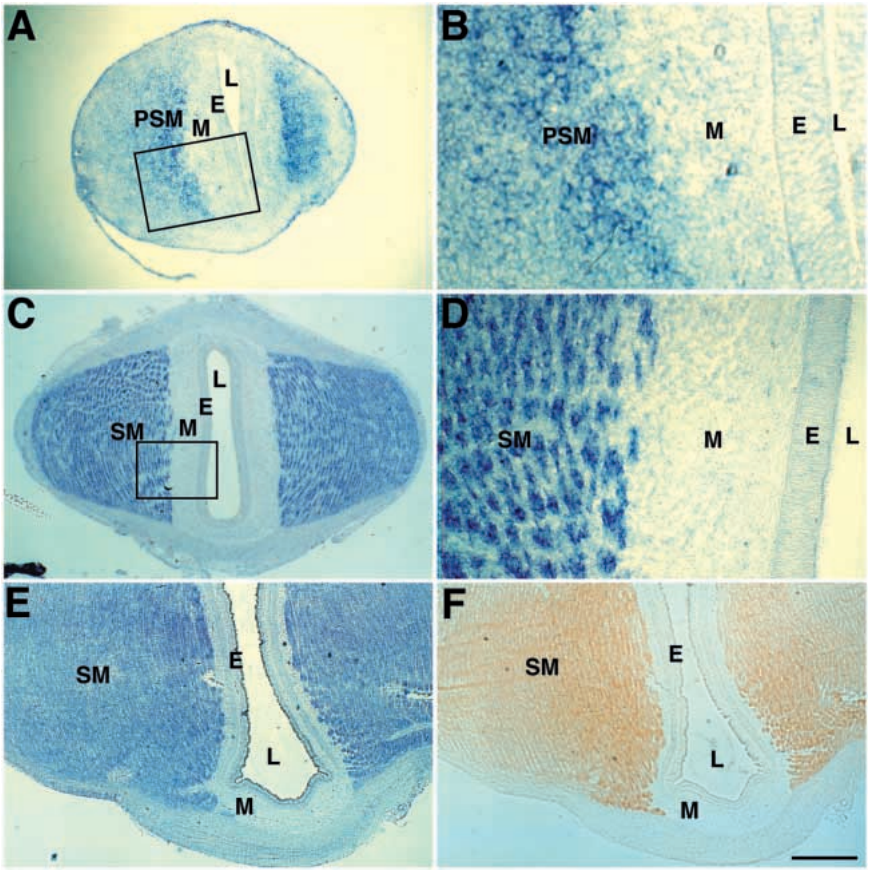

Fig. 3. Expression pattern of cFKBP/SMAP in the developing gizzard demonstrated by in situ hybridization. (A) cFKBP/SMAP mRNA expression in the 6-day embryonic gizzard. Strong expression is detected in the presumptive smooth muscle cells in mesenchyme. (C) cFKBP/SMAP expression in the 9-day embryonic gizzard. The expression is restricted to smooth muscle cells. (B,D) Higher magnification of the areas outlined in A and C, respectively. (E) cFKBP/SMAP expression; (F) immunoreactivity to 13F4 monoclonal antibody in the 12-day embryonic gizzard. Cells expressing cFKBP/SMAP are immunoreactive to 13F4. E, epithelium; L, lumen; M, mesenchyme; PSM, presumptive smooth muscle layer; SM, smooth muscle layer. Scale bar, 200 μm (A); 50 μm (B); 500 μm (C,E,F); 100 μm (D).

In mesenchyme of 6-day embryonic gizzard cultured without FK506, differentiated smooth muscle cells were stained with the 13F4 antibody at a distance from epithelium similar to the in vivo situation (Fig. 5A). On the contrary, in the gizzard cultured with 10 μM of FK506, there was no 13F4-positive smooth muscle cells (Fig. 5B). The differentiation of smooth muscle cells was thus inhibited by FK506.

Overexpression of cFKBP/SMAP in mesenchymal cells

To assess directly the role of cFKBP/SMAP in smooth muscle differentiation, cFKBP/SMAP was overexpressed in cultured mesenchymal cells. It is known that dissociated gizzard mesenchymal cells isolated from embryos younger than day 7 of incubation do not differentiate into smooth muscle cells (Ko et al., 1996).

We examined the relationship between smooth muscle differentiation and cFKBP/SMAP expression in cultured mesenchymal cells. In mesenchymal cells of 4-day embryonic gizzard, cFKBP/SMAP is already expressed, while dissociated mesenchymal cells from 4-day embryonic gizzard, did not express cFKBP/SMAP after 2 days in culture (Fig. 6A). In this case cultured mesenchymal cells were caldesmon-negative (Fig. 6B). Smooth muscle cells of 9-day embryonic gizzard expressed cFKBP/SMAP as described previously (Fig. 3D). Cultured cells continued to express cFKBP/SMAP (Fig. 6C) and caldesmon after 2 days in culture (Fig. 6D).

We therefore transfected and overexpressed cFKBP/SMAP in dissociated mesenchymal cells from 4-day embryonic gizzard.

Dissociated mesenchymal cells plated on culture dishes were transfected with cFKBP/SMAP expression vector, pmiwFLAG-SMAP (see Materials and Methods) and cultured

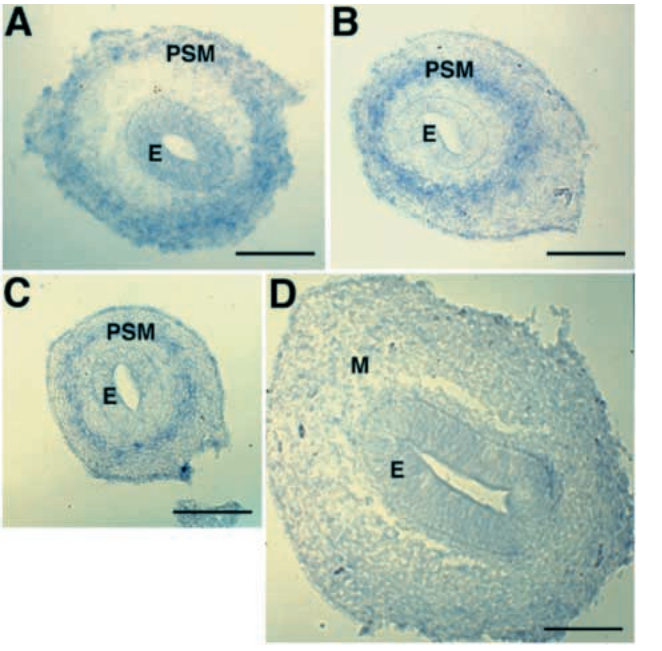

Fig. 4. cFKBP/SMAP expression in 6-day embryonic esophagus (A), small intestine (B), large intestine (C) and proventriculus (D), respectively. Abbreviations as in Fig. 3. Scale bars, 10 μm.

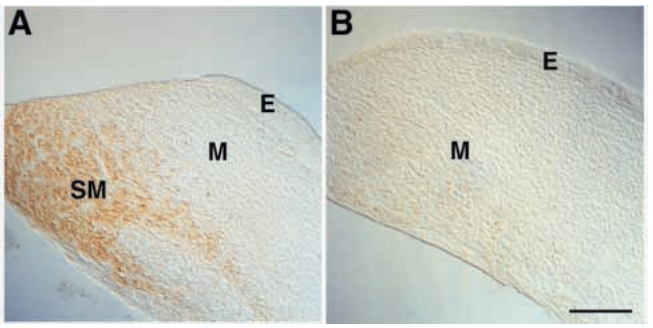

Fig. 5. Effect of FK506 on differentiation of smooth muscle in the gizzard. (A) In gizzard cultured without FK506, 13F4-positive smooth muscle cells differentiated in the mesenchyme away from the epithelium, as in normal gut. (B) In gizzard cultured with 10 μM FK506, no smooth muscle cells differentiated. Abbreviations as in Fig. 3. Scale bar, 100 μm.

for 2 days. pmiwFLAG-GFP was transfected into cells as a negative control.

Gizzard mesenchymal cells transfected with pmiwFLAG-GFP remained unchanged in their fibroblastic appearance and did not express caldesmon (Fig. 7A-C). On the other hand gizzard mesenchymal cells transfected with pmiwFLAG-SMAP differentiated into elongated, spindle-like cells and expressed caldesmon (Fig. 7D-F) like cultured smooth muscle cells from 9-day embryonic gizzard (Fig. 7G). These cells also showed 13F4 immunoreactivity (data not shown).

Similar results were obtained when lung mesenchymal cells were transfected with pmiwFLAG-SMAP. All of the pmiwFLAG-SMAP-transfected cells became caldesmon-positive (Fig. 8D-F), while lung mesenchymal cells transfected with pmiwFLAG-GFP grew as fibroblastic cells not expressing caldesmon (Fig. 8A-C).

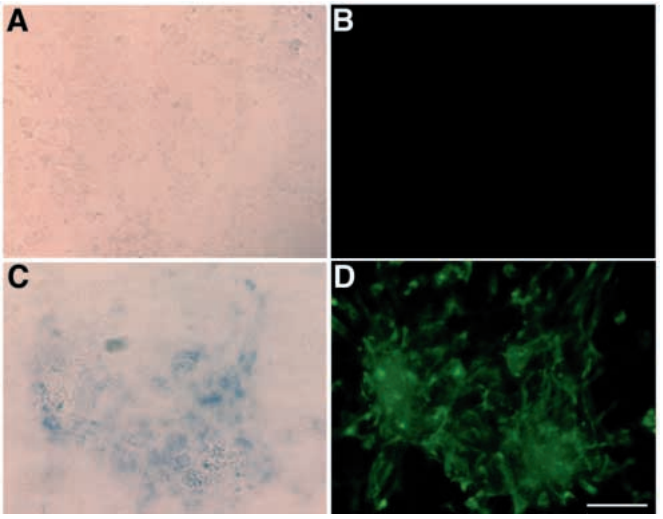

Fig. 6. Relationship between cFKBP/SMAP expression and smooth muscle differentiation in dissociated mesenchymal cells. (A) cFKBP/SMAP expression ceased in mesenchymal cells from 4-day embryonic gizzard during 2 days in culture and (B) cells were caldesmon-negative. (C) cFKBP/SMAP transcript was detected in cultured smooth muscle cells from 9-day embryonic gizzard after 2 days in culture and (D) cells were still caldesmon-positive. Scale bar is 10 μm.

Inhibition of cFKBP/SMAP-induced smooth muscle differentiation by FK506

To show the direct interaction between cFKBP/SMAP and FK506, we examined whether FK506 inhibits smooth muscle differentiation in the cells transfected with cFKBP/SMAP. When the cells transfected with pmiwFLAG-SMAP were cultured with 5 μM FK506, some cells expressed the smooth muscle marker, caldesmon (Fig. 9E,F, arrowhead), but other cells did not express it. There was no expression of caldesmon in transfected cells cultured with 20 μM FK506 (Fig. 9G,H), and cells were fibroblastic as were those transfected with the control vector, pmiwFLAG-GFP (Fig. 9A). There was no effect of 20 μM FK506 on the cells transfected with control vector (Fig. 9A,B), but of those treated with 50 μM FK506, almost all cells died (data not shown).

DISCUSSION

The origin of gut smooth muscle cells and their phenotypic characteristics are well known. However, little is understood about the regulatory mechanism of smooth muscle differentiation in the gut.

In the well developed gizzard mesenchyme a smooth muscle layer differentiates, whereas smooth muscle differentiation is poorer and later in the proventriculus. Taking advantage of this feature, we tried to isolate cDNAs encoding molecules necessary for the differentiation of smooth muscle cells by the differential display method using mRNA panels from the proventriculus and gizzard of the chicken embryo at the onset of smooth muscle differentiation. As a consequence, we identified a new gene, cFKBP/SMAP, which is involved in the differentiation of smooth muscle.

cFKBP/SMAP is a new member of the FKBPs

Though cFKBP/SMAP shows highest homology to mouse FKBP65 and their proteins have similar predicted molecular masses (Coss et al., 1995), the expression patterns of these two genes are quite different. cFKBP/SMAP was expressed throughout the digestive tract during development and in the testis, but was detected only weakly or never detected in the brain, heart, liver, kidney, pancreas and spleen (Fig. 2), whereas in adult mouse FKBP65 is expressed specifically in the heart, lung, brain, testis, spleen and pancreas (Coss et al., 1995). cFKBP/SMAP may therefore be a new member of FKBPs.

Functions of FKBPs are totally unknown except that FKBP12, which was originally discovered as an intracellular binding protein for FK506, is an immunosuppressant (Siekierka et al., 1989). The present paper identifies a new function of the FKBPs (see below).

cFKBP/SMAP regulates the differentiation of the smooth muscles

Northern hybridization and in situ hybridization data revealed that cFKBP/SMAP expression is related to smooth muscle differentiation. In situ hybridization clearly demonstrated that cFKBP/SMAP expression coincides with presumptive and differentiated smooth muscle in organs such as esophagus,

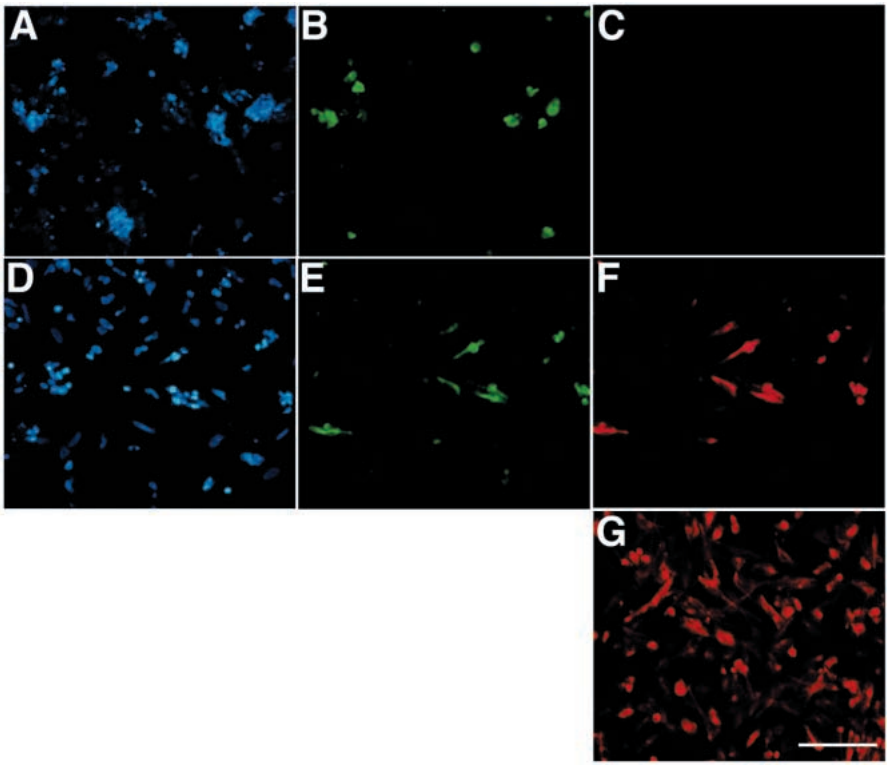

Fig. 7. Induction of smooth muscle differentiation by FKBP/SMAP expression in gizzard mesenchymal cells. FLAG-GFP (A-C) and FLAG-SMAP (D-F) expression vectors were transfected to mesenchymal cells isolated from 4-day embryonic gizzard. Cells were stained with DAPI (A,D), anti-FLAG antibody (B,E) and anti-caldesmon antibody (C,F). FLAG-SMAP-transfected cells expressing FLAG were caldesmon-positive and elongated. (G) Cultured smooth muscle cells from 12-day embryonic gizzard. Smooth muscle cells are stained with anti-caldesmon antibody. Scale bar, 10 μm.

gizzard and small and large intestines in which smooth muscle develops intensively or moderately. In contrast, in the proventriculus, lung, liver and pancreas, in which little or no smooth muscle is formed, the expression was very weak or totally absent.

Addition of FK506 to the culture medium inhibited completely the differentiation of smooth muscle cells in the gizzard mesenchyme. Since FK506 is known to specifically bind and block the action of FKBPs, it is possible to conclude that the effect of FK506 on smooth muscle differentiation is via blocking of cFKBP/SMAP activity. However, since FK506 binds all members of FKBP family, it is still possible that FKBPs other than cFKBP/SMAP are involved in smooth muscle differentiation. To confirm the participation of cFKBP/SMAP in the differentiation, the effect of overexpression of cFKBP/SMAP in mesenchymal cells was examined. Dissociated mesenchymal cells from 4-day embryonic gizzard did not maintain expression of cFKBP/SMAP and did not differentiate into smooth muscle in culture, while both gizzard and lung mesenchymal cells of 4-day embryonic chicken transfected with a cFKBP/SMAP expression vector expressed the smooth muscle-specific marker, caldesmon, after 2 days in culture. This result suggested that continuous expression of FKBP/SMAP was needed for smooth muscle differentiation. In the normal course of development caldesmon appears in mesenchymal cells from 9-day of development both in the gizzard and lung. Therefore, cFKBP/SMAP overexpression accelerated the differentiation of smooth muscle cells in culture. These cFKBP/SMAP-induced smooth muscle differentiation was completely inhibited by the addition of FK506. These results indicate that FK506 interacts with cFKBP/SMAP directly and inhibits smooth muscle differentiation by blocking of cFKBP/SMAP activity.

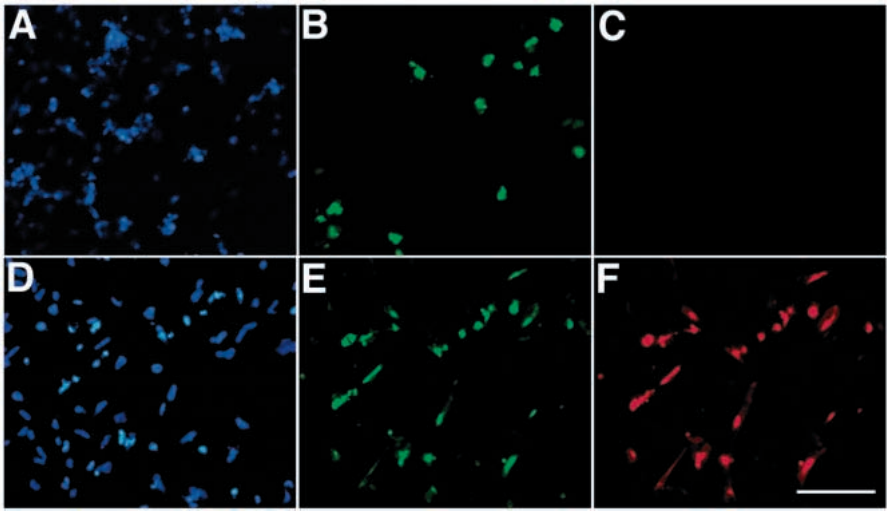

Fig. 8. Induction of smooth muscle differentiation by FKBP/SMAP expression in lung mesenchymal cells. FLAG-GFP (A-C) and FLAG-SMAP (D-F) expression vectors were transfected to mesenchymal cells isolated from 4-day embryonic lung. Cultured cells were stained with DAPI (A,D), anti-FLAG antibody (B,E) and anti-caldesmon antibody (C,F). Scale bar, 10 μm.

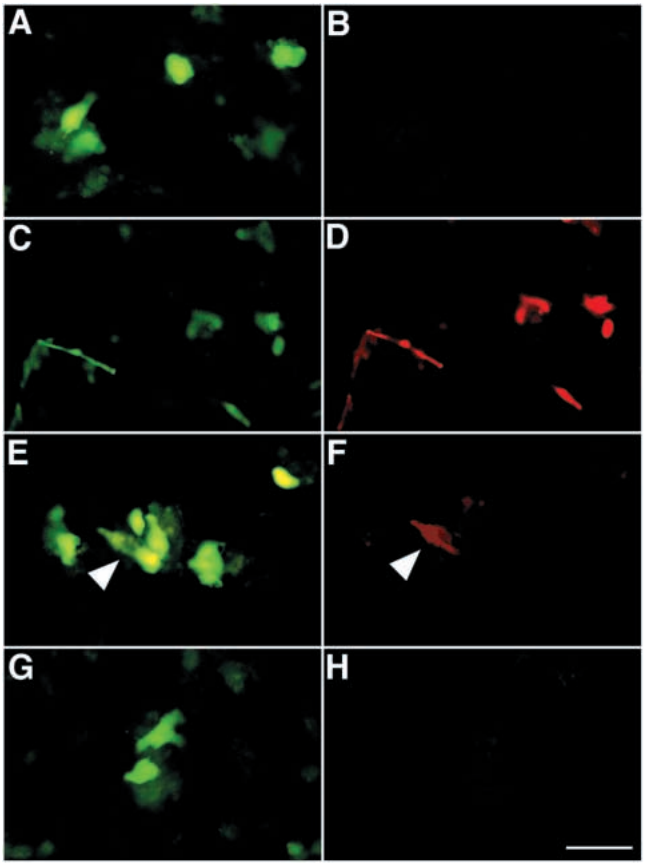

Fig. 9. The inhibition of cFKBP/SMAP-induced smooth muscle differentiation by FK506. (A,B) FLAG-GFP and (C-H) FLAG-SMAP expression vectors were transfected to mesenchymal cells from 4-day embryonic gizzard. The transfected cells were cultured without (C,D) or with 5 μM (E,F) or (A,B,G,H) 20 μM FK506. Cultured cells were stained with anti-FLAG antibody (A,C,E,G) and anti-caldesmon antibody (B,D,F and H). Scale bar is 5 μm.

cFKBP/SMAP is thus the first molecule shown to be involved in regulation of smooth muscle differentiation. In the differentiation of skeletal muscle, MyoD, and related member of MyoD family, which encode bHLH-transcription factors, act at least in part, via the direct activation of a number of muscle-specific genes. In a recent study, expression of some smooth muscle genes such as smooth muscle actin, myosin heavy chain, telokin, SM-22 and h-caldesmon was shown to be regulated by a common *cis*-element, CArG element (Blank et al., 1992; Shimizu et al., 1995; Yano et al., 1995; Herring and Smith, 1996; Li et al., 1996; Madesen et al., 1996) Thus it is likely that these genes are controlled through a common transcriptional regulatory pathway. Cells overexpressing cFKBP/SMAP showed expression of at least two different proteins, caldesmon and 13F4 antigen, suggesting that cFKBP/SMAP may be involved in such a common control pathway.

The role of cFKBP/SMAP in the differentiation of smooth muscles remains to be elucidated. FKBP12 can bind to various TGFβ type I receptors by its PPIase domain and may block TGFβ-mediated signaling pathways (Wang et al., 1996). cFKBP/SMAP also has four PPIase domains (Fig. 1). So, it is possible that cFKBP/SMAP also binds to type I receptors of the TGFβ superfamily and blocks signaling in smooth muscle precursor cells. A new member of the TGFβ superfamily, GDF-8, was recently cloned and in knockout mice showed overgrowth of skeletal muscle cells (McPherron et al., 1997), suggesting that the TGFβ superfamily negatively regulates the differentiation of skeletal muscle cells. TGFβ itself activates the differentiation of vascular smooth muscle in vitro (Arciniegas et al., 1992). Also, bone morphogenic proteins (BMPs), members of the TGFβ superfamily, repress the differentiation of skeletal muscle in limb bud (Duprez et al., 1996). BMPs are expressed adjacent to the smooth muscle layer in embryonic chicken gut (our unpublished data). We are currently investigating if cFKBP/SMAP participates in the signal transduction pathway mediated by the TGFβ superfamily, by transfecting cDNAs of BMP-2, BMP-4 or BMP-7 into mesenchymal cells and examining the expression of cFKBP/SMAP and smooth muscle-specific proteins.

In conclusion, our present study provides a first clue in the analysis of the molecular mechanisms of differentiation of smooth muscles and also offers a useful system with which we can identify molecules involved in this process.

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REFERENCES

Alnemri, E. S., Fernandes-Alnemri, T., Nelki, D. S., Dudley, K., Dubois, G. C. and Litwack, G. (1993). Overexpression, characterization, and purification of a recombinant mouse immnophilin FKBP-52 and identification of an associated phosphoprotein. *Proc. Natl. Acad. Sci. USA* **90**, 6839-6843.

Alnemri, E. S., Fernandes-Alnemri, T., Pomerenke, K., Robertson, N. M., Dudley, K., Dubois, G. C. and Litwach, G. (1994). FKBP46, a novel sf9 insect cell nuclear immunophilin that forms a protein-kinase complex. *J. Biol. Chem.* **269**, 30828-30834.

Arciniegas, E., Sutton, A. B., Allen, T. D. and Schor, A. M. (1992). Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J. Cell Sci.* **103**, 521-529.

Bitter, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G. and Schreiber, S. L. (1990). Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc. Natl. Acad. Sci. USA* **87**, 9231-9235.

Blank, R. S., McQuinn, T. C., Yin, K, C., Thompson, M. M., Takeyasu, K., Schwartz, R. J. and Owens, G. K. (1992). Elements of the smooth muscle α-actin promoter required in cis for transcriptional activation in smooth muscle cells: evidence for cell type-specific regilation. *J. Biol. Chem.* **267**, 984-989.

Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V. and Snyder, S. H. (1995). Calcineurin associated with the inositol 1,4,5-triphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. *Cell* **83**, 463-472.

Cook, R. D. and King, A. S. (1970). Observations on the ultrastructure of the smooth muscle and its innervation in the avian lung. *J. Anat.* **106**, 273-283.

Coss, M. C, Winterstein, D., Sowder II, R. C. and Simek, S. L. (1995). Molecular cloning, DNA sequence analysis, and biochemical

characterization of a novel 65-kDa FK506-binding protein (FKBP65). *J. Biol. Chem.* **270**, 29336-29341.

Duprez, D. M., Coltey, M., Amthor, H., Brickell, P. M. and Tickle, C. (1996). Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev. Biol.* **174**, 448-452.

Firulli, A. B. and Olson, E. N. (1997). Molecular regulation of muscle gene transcription: a mechanism for muscle cell diversity. *Trends Genet.* **13**, 364-369.

Galat, A., Lane, W. S., Standaert, R. F. and Schreiber, S. L. (1992). A rapamycin-selective 25-kDa immunophilin. *Biochemistry* **31**, 2427-2434.

Harding, M. W. Galat, A., Uehling, D. E. and Schreiber, S. L. (1989). A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-pyolyl isomerase. *Nature* **341**, 758-760.

Herring, B. P. and Smith, A. F. (1996). Telokin expression is mediated by a smooth muscle cell-specific promoter. *Am. J. Physiol.* **270**, c1656-c1665.

Hirai, S. and Hirabayashi, T. (1983). Developmental change of protein constituents in chicken gizzards. *Dev. Biol.* **97**, 438-493.

Hodges, R. D. (1974). *The Histology of the Fowl.* New York, USA: Academic Press.

Ishii, Y., Fukuda, K., Saiga, H., Matsushita, S. and Yasugi, S. (1997). Early specification of intestinal epithelium in the chicken embryo: a study on the localization and regulation of *CdxA* expression. *Dev. Growth Differ.* **39**, 643-653.

Ito, T., Kito, K., Adati, N., Mitsui, Y., Hagiwara, H. and Sakaki, Y. (1994). Fluorescent differential display: arbitary primed RT-PCR fingerprinting on an automated DNA sequencer. *FEBS Letters* **351**, 231-236.

Jin, Y., Burakoff, S. J. and Biere, B. E. (1992). Molecular cloning of a 25-kDa high affinity rapamycin binding protein, FKBP25. *J. Biol. Chem.* **267**, 10942-10945.

Jin. Y, Albers, M. W., Lane, W. S., Bierer, B. E., Schreiber, S. L. and Burakoff, S. J. (1991). Molecular cloning of a membrane-associated human FK506- and rapamycin- binding protein, FKBP-13. *Proc. Natl. Acad. Sci. USA* **88**, 6677-6681.

Kedinger, M., Simon-Assmann, P., Bouziges, F., Arnold, C., Alexandre, E. and Haffen, K. (1990). Smooth muscle actin expression during rat gut development and induction in fetal skin fibroblastic cells associated with intestinal embryonic epithlelium.. *Differentiation* **43**, 87-97.

Ko, J-A., Murahashi, S., Arata, T. and Inoue, A. (1996). Differentiation of smooth muscle cells from undifferentiated cells of chicken gizzard occurs on the layer of fibriblast-like cells. *Cell Tissue Res.* **285**, 395-401.

Lam, E., Martin, M. M., Timerman, A. P,. Sabers, C., Fleischer, S., Lukas, T., Abraham, R. T., O'Keefe, S. J., O'Neill, E. A. and Wiederrecht, G. J. (1955). A novel FK506 binding protein can mediate the immunosuppressive effects of FK506 and is associated with the cardiac ryanodine receptor. *J. Biol. Chem.* **270**, 26511-26522.

Lebeau, M C., Massol, N., Herrick, J., Faber, L. E., Renoir, J. M., Radanyi, C. and Beulieu, E. E. (1992). P59, an hsp 90-binding protein. Cloning and sequencing of its cDNA and preparation of a peptide-directed polyclonal antibody. *J. Biol. Chem.* **267**, 4281-4284.

Li, L., Miano, J. M., Mercer, B. and Olson, E. N. (1996). Expression of the SM22α promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J. Cell Biol.* **132**, 849-859.

Madsen, C. S., Hershey, J. C. and Owens, G. K. (1996). Identification of a positive cis element in the rat smooth muscle myosin heavy chain promoter. *FASEB J.* **10**, A343.

McPherron, A. C., Lawler, A. M. and Lee, S. (1997). Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature* **387**, 83-90.

Okadome, T., Oeda, E., Saitoh, M., Ichijo, H., Moses, H, L., Miyazono, K. and Kawabata, M. (1996). Characterization of the interaction of FKBP12 with transforming growth factor-β type I receptor in vivo. *J. Biol. Chem.* **271**, 21687-21690.

Peattie, D. A., Harding, M. W., Fleming, M. A., DeCenzo, M. T., Lippke, J. A., Livingston, D. J. and Benasutti, M. (1992). Expression and chracterization of human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complexes. *Proc. Natl. Acad. Sci. USA* **89**, 10974-10978.

Perr, H. A., Grider, J. R., Mills, A. S., Kornstein, M., Turner, D. A., Diegelmann, R. F. and Graham, M. F. (1992). Collagen production by human smooth muscle cells isolated during intestinal organogenesis. *Anat. Embryol.* **185**, 517-527.

Romanoff, A. L. (1960). *The Avian Embryo. Structural and functional development.* New York, USA: The Macmillan Company.

Rong, P.-M., Ziller, C., Pena-Melian, A. and Le Douarin, N. (1987). A monoclonal antibody specific for avian early myogenic cells and differentiated muscle. *Dev. Biol.* **122**, 338-353.

Rudnicki, M. A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H. and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Schreiber, S. L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science* **251**, 283-287.

Shimizu, R. T., Blank, R. S., Jervis, R., Lawrenz-Smith, S. C. and Owens, G. K. (1995). The smooth muscle α-actin gene promoter is differentially regulated in smooth muscle versus non-smooth muscle cells. *J. Biol. Chem.* **270**, 7631-7643.

Siekierka, J. J., Hung, S. H. Y. Poe, M., Lin, C. S. and Sigal, N. H. (1989). A cytosolic binding protein for the immunosuppressant FK506 has a peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341**, 755-757.

Smith, D. F., Albers, M. W., Schreiber, S. L., Leach, K. L. and Deibel, Jr. M. R. (1993). FKBP54, a novel FK506-binding protein in avian progesteron receptor complexes and HeLa extract. *J. Biol. Chem.* **268**, 24270-24273.

Standaert, R. F., Galat, A., Verdine, G. L. and Schreiber, S. (1990). Molecular cloning and overexpression of the human FK506-binding protein FKBP. *Nature* **346**, 671-674.

Suemori, H., Kadonaga. Y., Goto, K., Araki, I., Kondoh, H. and Nakatsuji, N. (1990). A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous β-galactosidase expression. *Cell Differ. Dev.* **29**, 181-186.

Takiguchi, K., Yasugi, S. and Mizuno, T. (1988). Pepsinogen induction in chick stomach epithelia by reaggregated proventricular mesemchymal cells in vitro. *Dev. Growth Differ.* **30**, 241-250.

Tropschug, M., Wachter, E., Mayer, S., Schoenbrunner, E. R. and Schmid, F. X. (1990). Isolation and sequence of an FK506-binding protein from *N. crassa* which catalyses protein folding. *Nature* **346**, 674-676.

Ueki, N., Sobue, K., Kanda, K., Hada, T.,and Higashino, K. (1987). Expression of high and low molecular weight caldesmons during phenotypic modulation of smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **84**, 9049-9053.

Wakamatsu, Y., Watanabe, Y., Nakamura, H. and Kondoh, H. (1997). Regulation of the neural crest cell fate by N-myc: promotion of ventral migration and neuronal differentiation. *Development* **124**, 1953-1962.

Wakamatsu, Y. and Weston, J. A. (1997). Sequential expression and role of Hu RNA-binding proteins during neurogenesis. *Development* **124**, 3449-3460.

Wang, T., Donahoe, P. K. and Zervos, A. S. (1994). Specific interaction of type I receptors of the TGF-β family with the Immunophilin FKBP-12. *Science* **265**, 674-676.

Wang, T., Li, B.-Y., Danielson, P. D., Shah, P. C., Rockwell, S., Lechleider, R. J., Martin, J., Manganaro, T. and Donahoe, P. K. (1996). The immunophilin FKBP12 functons as a common inhibitor of the TGFβ family type I receptors. *Cell* **86**, 435-444.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Hollenberg, S., et al. (1991). The MyoD gene family: nodal point during specification of the muscle cell linage. *Science* **251**, 761-766.

Yano, H., Hayashi, K., Momiyama, T., Saga, H., Haruna, M. and Sobue, K. (1995). Transcriptional regulation of the chicken caldesmon gene: activation of the gizzard-type caldesmon promoter requires a CArG box-like motif. *J. Biol. Chem.* **270**, 23661-23666.