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J Clin Invest. 2002;110(9):1349-1358. <https://doi.org/10.1172/JCI15614>.

Article

Cell biology

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P311 induces a TGF- β 1-independent, nonfibrogenic myofibroblast phenotype

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P311, also called *PTZ17*, was identified by suppressive subtraction hybridization as potentially involved in smooth muscle (SM) myogenesis. P311 is an 8-kDa protein with several PEST-like motifs found in neurons and muscle. P311 transfection into two fibroblast cell lines, NIH 3T3 and C3H10 T1/2, induced phenotypic changes consistent with myofibroblast transformation, including upregulation of SM α -actin and SM22, induction of FGF-2, VEGF, PDGF, and PDGF receptors, upregulation of integrins α 3 and α 5, and increased proliferation rate. The P311-mediated changes differed, however, from the well-characterized myofibroblast in that P311 inhibited TGF- β 1, TGF- β receptor 2, and TGF- β 1-activating MMP-2 and MMP-9, with the resultant decrease in collagen 1 and 3 expression. The effect of P311 on collagen was overcome by exogenous TGF- β 1, indicating that the cells were responsive to TGF- β 1 paracrine stimulus. In support of a role for P311 *in vivo*, immunohistochemical examination of human wounds showed P311 only in myofibroblasts and their activated precursors. To our knowledge, these studies are the first to implicate P311 in myofibroblast transformation, to demonstrate that transformation may occur independently of TGF- β 1, and to suggest that P311 may prevent fibrosis.

J. Clin. Invest. 110:1349–1358 (2002). doi:10.1172/JCI200215614.

Introduction

Undifferentiated mouse embryonic mesenchymal cells undergo spontaneous smooth muscle (SM) differentiation upon spreading/elongation in culture (1–3). Using these cells, we generated cDNA libraries by suppressive subtraction hybridization (4) to identify genes potentially involved in SM myogenesis. One of these genes coded for P311, an 8-kDa protein originally found in the developing mouse brain (5). The 68-amino acid/long sequence of P311 is highly conserved across human, mouse, and chicken species. P311 does not belong to any known family of proteins; therefore, it does not provide clues suggesting function. Murine P311 has three PEST-like domains, but only one of them, located at the N-terminus, is conserved across the three species. PEST domains are targeted by the ubiquitin/proteasome pathway (6) and are mainly found in short-lived proteins such as transcription factors, cytokines, and signaling molecules (6).

Besides its initial report by Studler in 1993 (5), *P311* was identified by few other groups in the process of screening for differentially expressed genes (7–12). These studies demonstrated the presence of P311 in neural

cells and glioblastomas (5, 7–10, 12), in SM cells (12), and in chondrocytes subjected to hydrostatic pressure (11). Furthermore, these studies showed that P311 is downregulated by HGF (12), stimulates glioblastoma cell migration (8), and is either downregulated or upregulated in the brain during chemically or metabolically induced seizures (7, 10). The agent used to induce chemical seizures in these studies was pentylenetetrazol (PTZ); therefore, P311 is also referred to as PTZ17 (7, 9, 10).

Myofibroblasts are contractile cells with features intermediate between fibroblasts and SM cells, which are characterized by the production of SM α -actin (13–15). Myofibroblasts are generated during normal wound healing, a process in which they play a critical function, in part by their production of collagen and other ECM proteins as well as ECM-degrading enzymes (13–16). Besides their role in wound healing, myofibroblasts have been implicated in a variety of pathological conditions involving fibrosis and tissue remodeling, including lung idiopathic fibrosis, liver cirrhosis, chronic glomerulonephritis, chronic pancreatitis, atherosclerosis, soft tissue fibromatosis, healing of myocardial infarction, and stromal reaction to tumors (13–15, 17). SM-like cells, also referred to as myofibroblasts, have been described in normal tissue locations where a certain degree of tension is needed to function, such as the alveolar septae (18) and the intestinal pericyptal cells (19).

Myofibroblasts participating in wound repair and chronic fibrosis are originated at least in part by transformation of local fibroblasts (13, 20). There is a large body of evidence indicating that these myofibroblasts

Received for publication April 5, 2002, and accepted in revised form September 3, 2002.

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Conflict of interest: No conflict of interest has been declared.

Nonstandard abbreviations used: smooth muscle (SM); pentylenetetrazol (PTZ); striated muscle (STM).

are induced and maintained by paracrine/autocrine TGF- β 1 stimulation (13–15, 20, 21). Although SM α -actin is the most significant marker of myofibroblasts (13–15, 20), many myofibroblasts synthesize one or two additional muscle-specific proteins. Among the latter are desmin, SM-myosin, SM22, and caldesmon (22–24). Myofibroblasts are also characterized by increased expression of TGF- β and PDGF receptors (14, 25–27), production of FGF-2 (13–15, 17) and PDGF (15, 27), increased expression of integrins α 3 and α 5 (14, 28), and increased proliferation rate (14). Some recent publications demonstrate that myofibroblasts are also able to produce VEGF (29–31).

Here we show that expression of P311 in two fibroblasts cell lines induces a change in phenotype consistent with that of a myofibroblast. Supporting such a role in vivo, immunohistochemical studies of human wounds demonstrated the presence of P311 in myofibroblasts and their precursors but not in other cells. In contrast to what we expected, however, P311 decreased TGF- β 1 signaling and caused an inhibition in collagen expression, suggesting that P311 may be involved in reducing the amount of scarring produced during wound repair.

These findings therefore demonstrate a novel role for P311 in inducing TGF- β 1-independent myofibroblast transformation and suggest that myofibroblasts may have a more complex control over fibrogenesis than what was thought previously.

Methods

Construction of libraries and subtracted probe. Undifferentiated mesenchymal cells were isolated from E11 mouse lungs by differential plating as described previously (2, 32, 33). The cells were cultured for either 1 or 18 hours, the first time point representing undifferentiated embryonic mesenchymal cells and the second representing cells undergoing SM differentiation. The mRNA from the two cultures was amplified using the SMART cDNA synthesis kit (CLONTECH Laboratories Inc., Palo Alto, California, USA), and PCR-Select (CLONTECH Laboratories Inc.) was then used for suppressive subtraction hybridization. Briefly, two pools of RsaI-digested cDNA from either undifferentiated or SM-differentiated cells were used as testers and ligated to two different oligonucleotide adapters. RsaI-digested cDNAs from either undifferentiated or differentiated SM cells were used as drivers without adapters. Two hybridizations were performed between the tester population and excess driver. Only the cDNAs with different adapters at both ends were PCR amplified and produced a pool of cDNA fragments more abundant in the undifferentiated or in the differentiated cells. The subtracted cDNAs were cloned into a pGEM-T vector (Promega Corp., Madison, Wisconsin, USA) and transformed into *Escherichia coli*. Three hundred transformed colonies were randomly selected for screening. Dot-blot hybridization was performed with [32 P]-labeled forward- and reverse-subtracted cDNAs as probes. The reverse-subtracted probe was made by subtractive

hybridization performed with the original tester cDNA as a driver and the driver cDNA as a tester.

Cells. NIH 3T3 and C3H10 T1/2 fibroblast cell lines were obtained from American Type Culture Collection (Manassas, Virginia, USA). Cells were maintained in DMEM supplied with 10% FBS, 100 U/ml penicillin G, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (all from Life Technologies Inc., Grand Island, New York, USA).

Plasmid construct. cDNA representing the entire coding region of mouse P311 was amplified using modified primers (sense: 5'-TGTGGATCCGGTGTAGAAAGCAGAGT-3'; antisense: 5'-GACGATATCAAAGGGTGGAGGTA-3'), digested with BamHI and EcoRV, and then cloned into pcDNA3.1/Myc-His A, B, and C (+) expression vector (Invitrogen Corp., Carlsbad, California, USA). The recombinant protein produced by this construct is the full-length murine P311 with myc and his epitope tags at the C-terminus.

Transfections. The plasmid construct and control empty vector were transfected into NIH 3T3 and C3H10 T1/2 cells using Effectene Transfection Reagent (QIAGEN Inc., Santa Clarita, California, USA) following the manufacturer's instructions. Briefly, the plasmid was incubated for 5 minutes with enhancer solution at a 1:8 ratio. Effectene transfection reagent was then added to the DNA-enhancer mixture, and the samples were incubated for another 10 minutes to allow complex formation. Cells were transfected overnight in complete medium with this mixture added to obtain a final plasmid concentration of 0.16 μ g/ml. The transfection mixture was then removed and replaced with fresh medium. For stable transfections, 500 μ g/ml G418 (Life Technologies Inc.) was added into the culture medium, and the cells were cultured for at least a month before using them for studies. Periodic estimations of P311 levels in P311- and mock-transfected cells were done by immunoprecipitation using previously characterized anti-P311 Ab's (12).

Immunoprecipitation. Immunoprecipitation was done following a previous protocol (12). NIH 3T3 and C3H10 T1/2 cells transfected with P311 construct or vector control were plated on 60-mm plates and allowed to reach confluence. Cells were lysed and incubated with 1:1,000 dilution of anti-myc or anti-his tag Ab (Invitrogen Corp.) or 1:500 dilution of rabbit polyclonal Ab generated against the synthetic peptide KGRLPVPKEVNRKK, which represents a P311 sequence conserved between human and mouse species (12). This was followed by immunoprecipitation with anti-IgG Ab-conjugated agarose beads (Sigma-Aldrich, St. Louis, Missouri, USA). The recovered samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-myc or anti-his tag Ab's at 1:1,000 dilution (both from Invitrogen Corp.). Then, 10 μ M lactacystin and 1.26 mM *o*-phenanthroline (Sigma-Aldrich) were added to the cell cultures 30 minutes before lysis and during the immunoprecipitation process.

Semiquantitative RT-PCR. Cell cultures were washed with PBS, and total RNA was extracted with TRIzol

(Life Technologies Inc.). RT-PCR was performed with the GeneAmp RNA PCR kit (Perkin Elmer Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. All amplifications shown here represent the product of 25 to 30 cycles. A primer pair (Ambion Inc., Austin, Texas, USA) was used to produce a GAPDH amplicon as an internal control. Under the conditions used in these studies, a plateau of internal control and most amplicons were reached at 35 cycles. PCR amplicons were selected at different cycles and compared to ensure that amplifications were within linear range. The primers used in this study were as follows: P311, sense: 5'-GCTACCAAGAGTGTGAGAGG-3', antisense: 5'-CTTTGGGAAAGGGATAATTT-3'; desmin, sense: 5'-TCAGCCTCAGAGATGTTCTT-3', antisense: 5'-ACAACCTGATAGACGACCTG-3'; SM α -actin, sense: 5'-TCTGGCACCCTCTTCTAT-3', antisense: 5'-CATGAGGTAGTCGGTGAGAT-3'; MEF2, sense: 5'-CATGTACCAGGAAGCCTTA-3', antisense: 5'-TAACACCAACCAGAA-CATCA-3'; SRF, sense: 5'-GACAGCAGCACAGACCTCAC-3', antisense: 5'-CCATACACAGCCATCTGGT-3'; myogenin, sense: 5'-CAATACAAAAGCACTGGAA-3', antisense: 5'-TCTGAGGAGAGAAAGATGGA-3'; myf5, sense: 5'-GAACCCATTATTGCAAATGT-3', antisense: 5'-ATCACGTCTACAGGCCATC-3'; myoD, sense: 5'-CTCCACATCCTTTGTTGT-3', antisense: 5'-AGCGTCTTTATTTCAACAC-3'; TGF- β 1, sense: 5'-TGAGTGGCTGTCTTTGACG-3', antisense: 5'-TTCTCTGTGGAGTGAA-GCA-3'; MMP-2, sense: 5'-ACCAGAACACCATCGAGACC-3', antisense: 5'-CAAAGTGAGAATCTCCCCA-3'; MMP-9, sense: 5'-GACTTTTGTGGTCTTCCCCA-3', antisense: 5'-TTGAAGGTTTGAATCGACC-3'; MMP-1, sense: 5'-ATTTCTCGGCTTTTCAACTT-3', antisense: 5'-ATGCACAGCTTCTCCACT-3'; collagen α 1 type 1, sense: 5'-CTGGTGAACAGGGTGTTCCT-3', antisense: 5'-AATGGGACCAGTCAGACCAC-3'; collagen α 1 type 3, sense: 5'-TGGACCTCCTGGAAAAGATG-3', antisense: 5'-TTTTCGAGAACCATCAGGG-3'; VEGF, sense: 5'-TTACTGCTGTACCTCCACC-3', antisense: 5'-ACAGGACGGCTTGAAGATG-3'; GAPDH, sense: 5'-ATCACCATCTCCAGGAGCGA-3', antisense: 5'-GCCAGTGAGCTTCCCGT-TCA-3'; FGF-2, sense: 5'-GCCAGCGGCATCACCTCGCT-3', antisense: 5'-TATGGCCTTCTGTCCAGGTCCCGT-3'. The identity of P311, TGF- β 1, VEGF, and collagen amplicons was confirmed by sequencing.

cDNA microarray analysis. cDNA microarray analysis was done to complement the semiquantitative RT-PCR. Two different cDNA expression arrays were required to test all the mRNAs included in this study (nos. 7741 and 7858; CLONTECH Laboratories Inc.). [α - 32 P]-labeled cDNAs were generated by random primer extension from 5 μ g of Dnase I-treated RNA obtained from control and P311-expressing NIH 3T3 cells. The cDNAs were purified by column chromatography, and an aliquot of the eluted product was used to determine [α - 32 P] incorporation by scintillation counting. Equal counts of labeled probes from control and P311-transfected cells (> 100,000 counts/minute) were added, respectively, onto duplicate cDNA microarray

membranes, which included several housekeeping cDNAs as positive controls. The arrays were exposed to x-ray film (Kodak BioMax MR, Kodak, Rochester, New York, USA) for 18–72 hours in the presence of an intensifying screen at -70°C . Exposure time for each membrane was adjusted until the signals for the housekeeping genes were the same for P311-positive and P311-negative cells. The autoradiographs were scanned with a MultiImager-Max system (Bio-Rad Laboratories Inc., Hercules, California, USA) and analyzed using Quantity One software (Bio-Rad Laboratories Inc.).

Western blot analysis. Western blot analysis was done on cell lysates, except for TGF- β 1, which was immunodetected in the culture medium. The standard amount of protein loaded per lane was 15 μ g, with the exception of TGF- β 1, for which 30 μ g was loaded after concentrating the samples tenfold with consecutive centrifugal filter devices (Millipore, Bedford, Massachusetts, USA) to exclude proteins with molecular weight higher than 50 kDa and lower than 10 kDa. Rabbit polyclonal Ab's against myogenin, MEF2, myoD, SRF, TGF- β 1, TGF- β R1, and TGF- β R2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) and used at a concentration of 1 μ g/ml. Rabbit polyclonal Ab's against myoglobin were obtained from DAKO Corp. (Carpinteria, California, USA) and used at 1:200 dilution. Mouse mAb's against striated muscle (STM) α -actin (Sigma-Aldrich) were used at 1:200 dilution. As in previous studies (1–3), the following Ab's were used for detection of SM-specific proteins: mouse mAb to SM α -actin (Boehringer Mannheim Biochemicals Inc., Indianapolis, Indiana, USA) at a concentration of 0.25 μ g/ml, a mouse mAb to desmin (DAKO Corp.) at a concentration of 1.125 μ g/ml, rabbit polyclonal Ab's to SM-myosin (Biomedical Technologies Inc., Stoughton, Massachusetts, USA) at a concentration of 10 μ g/ml, rabbit polyclonal Ab's to SM22 (kindly provided by Rodrigo Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey, USA) at a concentration of 0.2 μ g/ml. Mouse mAb's to myc and his tags were purchased from Invitrogen Corp. and used at a concentration of 0.125 μ g/ml. A mouse mAb to GAPDH (Research Diagnostics Inc., Flanders, New Jersey, USA), at a concentration of 0.25 μ g/ml, was used for control purposes in a few experiments. Primary Ab's were detected with horseradish peroxidase-conjugated secondary Ab diluted 1:3,000 (Bio-Rad Laboratories Inc.). The bands were visualized by chemiluminescence using a commercial kit (Amersham Life Sciences, Arlington Heights, Illinois, USA) according to the manufacturer's instructions.

Cell proliferation assay. NIH 3T3 cells stably transfected with P311 plasmid construct or empty vector were trypsinized, resuspended in complete medium, and seeded in six-well plates at 10^4 cells/well. In additional studies, cells were cultured overnight in complete medium, washed, and then cultured in the serum-free conditions. After 24–48 hours, the cells were trypsinized and counted.

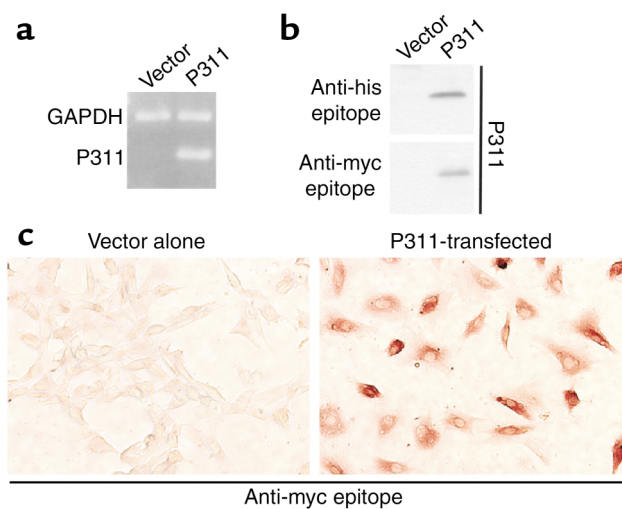


Figure 1
 Transfection of myc-his-tagged P311 into NIH-3T3 cells. (a and b) RT-PCR and Western blot showing no P311 expression in cells transfected with vector alone and synthesis of P311 mRNA and protein upon P311 transfection. (c) Immunostaining with anti-tagging epitope showing that NIH-3T3 cells transfected with vector alone are negative (left panel), whereas P311-transfected cells (right panel) show rather uniform synthesis of P311 after a month of G418 treatment for selection of transfected cells.

TGF- β 1 treatment. NIH 3T3 cells stably transfected with P311 plasmid construct or empty vector were plated in six-well plates and treated for 6, 18, and 48 hours with recombinant human TGF- β 1 (R&D Systems Inc., Minneapolis, Minnesota, USA). The concentrations ranged from 1 to 10 ng/ml. TGF- β 1 was added at time zero and allowed to remain in the culture medium until the end of the experiment, in which the cells were lysed and analyzed for collagen 1 expression by RT-PCR.

Tissues. Formalin-fixed, paraffin-embedded histological sections from seven different human wounds were selected from our archival material. All the wounds involved skin, soft tissues, and peritoneum and were produced by abdominal trauma or surgery. The tissues were obtained during re-excision of the abdominal wall due to reasons unrelated to the tissues under study. Two of the wounds were 2 and 3 days old. The third and fourth wounds were 1 week old. One of them was infected and treated. A fifth wound was 2 weeks old. The last two wounds were 4 weeks old; one was completely healed, and the other, which was extensive, showed all the stages of wound healing, including a well-developed scar at the periphery and granulation tissue in the center. Control tissues consisted of normal subcutaneous and subperitoneal fibroblasts, intestine SM, and muscular blood vessels.

Immunohistochemistry. Five-micrometer-thick, formalin-fixed, paraffin-embedded sections from human wounds were double immunostained with Ab's against P311 and SM α -actin following published protocols (33). Rabbit polyclonal Ab to P311 at a 1:50 dilution was used first. The slides were incubated with the Ab

overnight at 4°C, followed by 1-hour incubation with a 1:50 dilution of biotin-conjugated goat anti-rabbit IgG (Sigma-Aldrich). Immunostaining was completed using a commercial peroxidase-anti-peroxidase kit following the manufacturer's instructions (ABC Kit; Vector Laboratories, Burlington, California, USA). The samples were then treated with mouse mAb to SM α -actin (Boehringer Mannheim Biochemicals Inc.) at a concentration of 1 μ g/ml, followed by FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) at 1:60 dilution. The slides were counter stained with hematoxylin. Cells transiently transfected with P311 were fixed for 5 minutes in 100% ethanol and then double immunostained as described above using anti-his Ab's at 1:400 dilution for immunoperoxidase and Ab's to muscle-related transcription factors at 1:60 dilution for immunofluorescence. Stably transfected cells were periodically immunostained with anti-myc or anti-his Ab's to confirm their uniform expression of P311.

Northern blot. Since the Ab against P311 is not well suited for Western blotting (12) and immunoprecipitation of untagged P311 requires metabolic radiolabeling (impossible in tissues), we used Northern blot analysis to determine whether levels of P311 expression in transfected cells were similar to those normally found in vivo in myofibroblast-rich lesions. The latter were microdissected from 10- μ m-thick frozen sections of bleomycin-treated mouse lungs (a single intratracheal instillation of 1 U/kg weight) after identifying the lesions by hematoxylin staining. Northern blots were performed as done previously (4) using the same P311 cDNA fragment as for library screens. A control [³²P]-cDNA fragment complementary to GAPDH was used to reprobe the membranes. The autoradiograms were quantified using the software Quantity One (Bio-Rad Laboratories Inc.).

Results

All the experiments conducted during this project were repeated at least three times, and most of them were done multiple times to ensure that the results were highly consistent.

P311 stimulated SM-specific protein expression in a pattern consistent with a myofibroblast phenotype. RT-PCR, immunoprecipitation, and immunohistochemistry analysis demonstrated that NIH 3T3 cells did not produce endogenous P311, but they were able to express P311 upon transfection (Figure 1, a-c). Since we identified P311 to be differentially expressed in embryonic mesenchymal cells undergoing SM myogenesis, and since it is normally present in adult muscle, we determined the effect of P311 in muscle gene expression by NIH 3T3 cells. P311 stimulated expression of critical muscle-related transcription factors involved in SM and striated muscle (STM) differentiation with exception of myf5, which was downregulated (Figure 2, a and c). Double immunostaining of transiently transfected NIH 3T3 cells confirmed increased levels of muscle-related transcription factors in P311 expressing cells (Figure 2b).

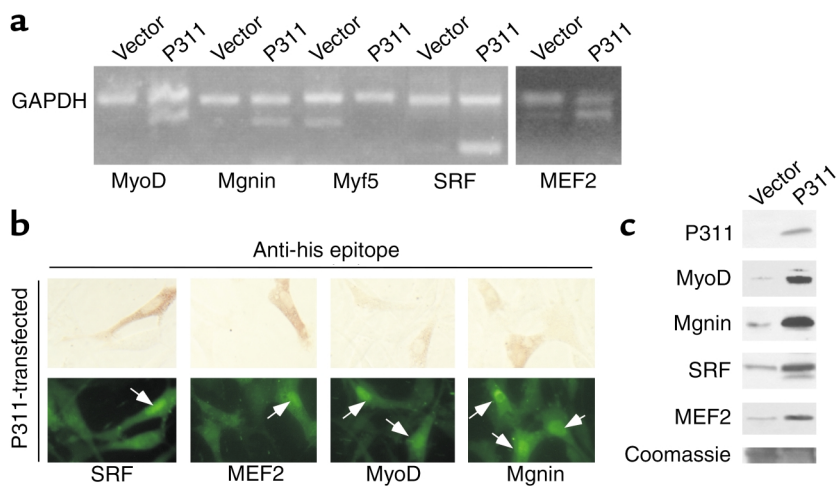


Figure 2
Transfection of P311 into NIH-3T3 cells stimulates muscle-specific transcription factors. (a) RT-PCR showing mRNA for muscle-related transcription factors in cells transfected with empty vector or transfected with P311. Cells expressing P311 show higher levels of muscle transcription factors, whether related to striated or SM differentiation, except for Myf5, which was downregulated. (b) NIH 3T3 cells transiently transfected with P311 and double immunostained for P311 (immunoperoxidase, brown in the upper panel) and various myogenic transcription factors (green immunofluorescence in lower panel). Notice upregulation of muscle transcription factors in the nucleus of P311-transfected cells (white arrows). (c) Immunoprecipitation (P311) and immunoblots (others) demonstrating upregulation of muscle transcription factors by transfected P311 compared with control cells transfected with empty vector. The same membrane used for detection of SRF and reprobing for MEF2 was stained blue with Coomassie to show protein loading. Mgnin, myogenin.

Although P311 stimulated expression of transcription factors critical for SM and STM lineages, only two SM-related proteins were synthesized, SM α -actin and SM22 (Figure 3). P311 did not induce synthesis of other SM markers studied here, even though the cells expressed some of the corresponding mRNAs, such as was the case for SM-myosin (Figure 3a). Similarly, P311 did not induce expression of STM α -actin or myoglobin, two STM-specific proteins (Figure 3, b and c), and neither showed changes in morphology consistent with STM differentiation, such as cell fusion or myotube formation (Figure 1c).

We next expressed P311 in C3H10 T1/2 cells to determine whether the observed effects were specific to NIH 3T3 cells or could be reproduced in other fibroblast cell lines. We selected C3H10 T1/2 cells because, unlike NIH 3T3 cells, these cells have the potential to differentiate into multiple mesenchymal cell lineages (34, 35). C3H10 T1/2 cells showed a pattern of response similar to the one seen in NIH 3T3 cells. P311 broadly stimulated muscle gene expression at the transcription factor level (Figure 4, a and b), but only induced synthesis of SM α -actin (Figure 4, c and d).

whether NIH 3T3 or C3H10 T1/2, had a significant decrease in the production of TGF- β 1 (Figure 6a). This was accompanied by a decrease in TGF- β 2 levels (Figure 6b) and also in MMP-2 and MMP-9, two matrix metalloproteinases involved in TGF- β 1 activation (39)

P311 induced additional cellular changes characteristic of myofibroblast transformation. Since expression of SM α -actin plus one or two additional SM markers is typical of myofibroblasts (13–15), we sought to determine whether P311 induces other molecular changes characteristic of myofibroblast transformation. Our studies confirmed that P311 induced most of these changes. Specifically, semiquantitative RT-PCR and microarray analysis demonstrated marked induction of VEGF, PDGF, and PDGF receptors, and integrins α 3 and α 5 (Figure 5, a–d). Also consistent with a myofibroblast phenotype in vivo (36–38), P311-transfected cells showed increased migration rate (Figure 5e). In FBS-free medium the cells did not proliferate; however, P311-transfected cells were more tolerant to serum deprivation (Figure 5e).

P311 decreased TGF- β 1 and collagen expression. In contrast to what is presently expected from myofibroblasts, RT-PCR and immunoblotting showed that P311-expressing cells,

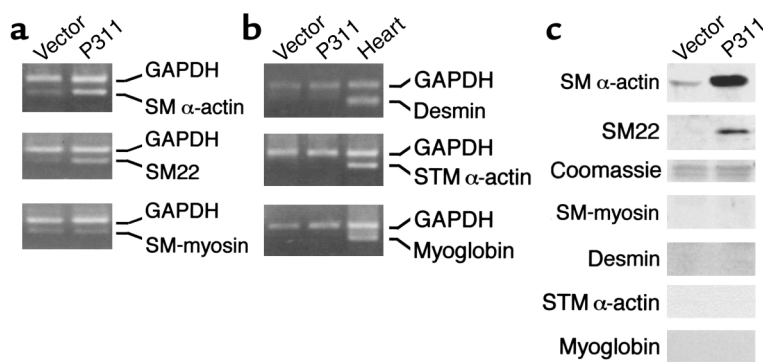


Figure 3
Transfection of P311 into NIH 3T3 cells stimulates expression of SM α -actin and SM22. (a) RT-PCR showing mRNA for three SM-specific markers in cells transfected with empty vector or transfected with P311. Notice that P311 upregulated SM α -actin message, induced SM22 message and did not alter significantly the mRNA for SM-myosin, present in both control and P311-expressing cells. (b) Cells expressing P311 do not show either STM-specific markers such as STM α -actin and myoglobin or desmin, a protein present in both STM and SM. (c) Immunoblot demonstrating upregulation of SM α -actin and induction of SM22 in P311-transfected cells and absence of other SM and STM markers. The membrane used for SM22 detection was stained with Coomassie blue to show equal protein loading.

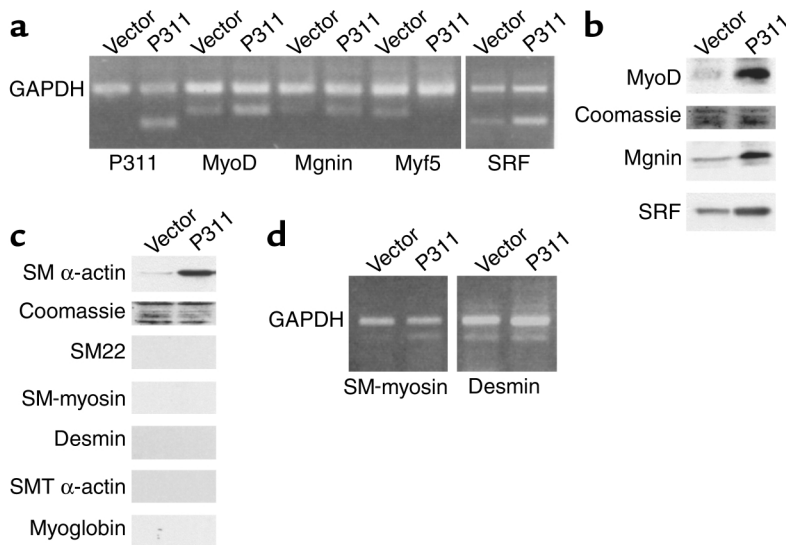


Figure 4 Transfection of P311 into C310 T1/2 cells stimulates muscle-specific transcription factors and induces SM α -actin synthesis. (a) RT-PCR showing mRNA for P311 and muscle-specific transcription factors in cells transfected with empty vector or transfected with P311. P311-expressing cells have higher levels of muscle-specific transcription factors than their control counterparts, with the exception of Myf5, which is downregulated. (b) Immunoblots showing increase in the synthesis of muscle-related transcription factors in P311-transfected cells compared with controls. The membrane used for detection of MyoD was stained with Coomassie blue to show equal protein loading. (c) Immunoblots demonstrating P311-induced upregulation of SM α -actin but absence of other SM and STM markers. (d) RT-PCR showing presence of mRNA for desmin and SM-myosin in control and P311-expressing cells, with some increment in SM-myosin message, which was consistent among experiments.

(Figure 6c). Expression of TGF- β R1 was not altered, as indicated by RT-PCR, immunoblotting (Figure 6b), and microarray analysis (not shown). As expected, the decrease in TGF- β 1 signaling was accompanied by a marked inhibition in the expression of collagen 1 and collagen 3 (Figure 6c). MMP-1, a protein upregulated by TGF- β 1 (40), was also decreased (Figure 6c). P311-induced collagen expression was overcome by exogenous TGF- β 1, indicating that the cells were still responsive to the growth factor (Figure 6d).

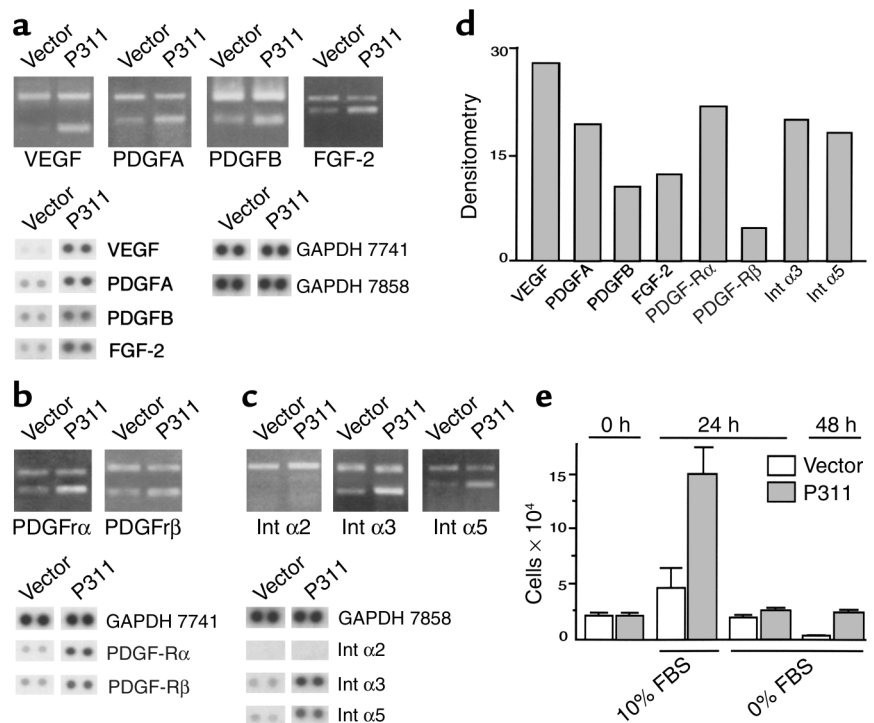
P311 is expressed by myofibroblasts and their precursors in vivo. Immunostaining of normal blood vessels, intestinal SM, and connective tissue with Ab's against SM

α -actin and P311, confirmed that P311 is present in adult SM and is absent in fibroblasts (Figure 7a). Double immunostaining also demonstrated that P311 is present in myofibroblasts (Figure 7, a and b). P311 polypeptide levels could not be determined in vivo because our Ab is not effective for Western blotting (12) and radiolabeling/immunoprecipitation requires living cells. Northern blot analysis, however, showed that P311 mRNA levels in transfected cells were similar to those of myofibroblasts in vivo (not shown).

We found that at early phases of repair (1–3 days), wound fibroblasts, also referred to as activated fibroblasts or promyofibroblasts (undistinguishable cyto-

Figure 5

P311 induces cellular changes in NIH 3T3 cells characteristic of myofibroblast transformation. (a–c) RT-PCR and microarray analysis demonstrating stimulation of VEGF, PDGF, and FGF-2 (a), PDGF receptors α (PDGF-R α) and β (PDGF-R β) (b), and integrins α 3 (Int α 3) and α 5 (Int α 5) (c) in P311-transfected cells compared with controls transfected with vector alone. Integrin α 2 (Int α 2) was not expressed in either of them. There were two different cDNA microarray membranes; therefore the GAPDH internal control for each was included in the figure. (d) Histogram showing the increase in each respective mRNA in P311-expressing cells as established by densitometry of microarray dots. (e) Histogram showing increased proliferation rate for P311-expressing cells when cultured in the presence of serum and better survival in serumless conditions.



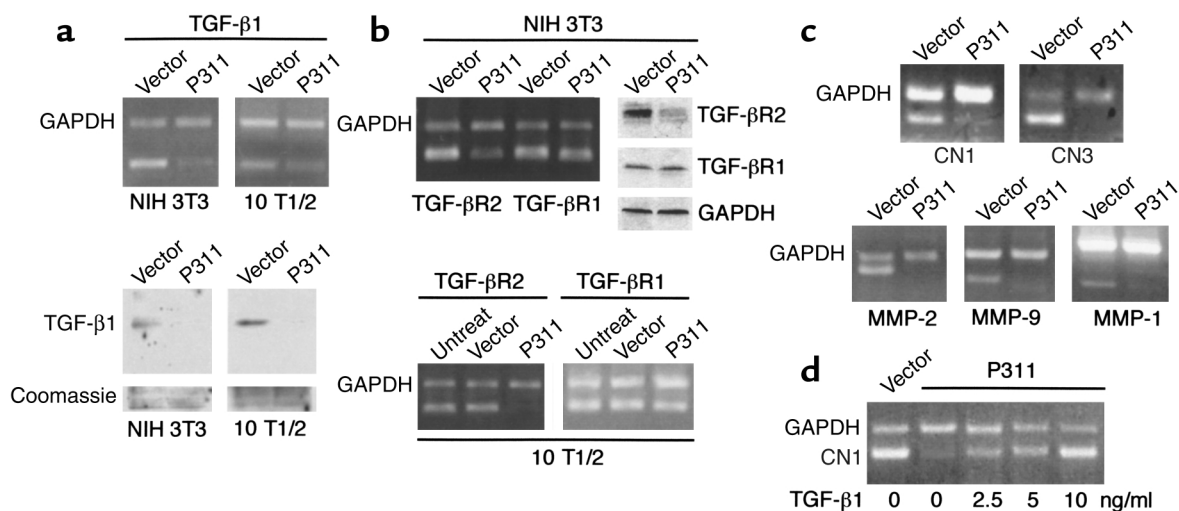


Figure 6

P311 inhibits TGF- β 1 and TGF- β 2 in NIH 3T3 and C3H10 T1/2 cells and decreases collagen 1 and 3 expression. (a) RT-PCR and immunoprecipitation followed by immunoblotting (lower panels) showing downregulation of TGF- β 1 in P311-expressing cells compared with controls. (b) RT-PCR and Western blotting demonstrating P311-induced downregulation of TGF- β 2 but not of TGF- β 1 in NIH 3T3 (upper panels) and C3H10 T1/2 (lower panels). Untreated (untreat) cells were used as an additional control for C3H10 T1/2 cells in the lower panels. (c) RT-PCR showing significant collagen 1 (CN1) and 3 (CN3) downregulation in P311-expressing cells (upper panels) and inhibition of MMP-2 and MMP-9, which participate in TGF- β 1 activation and inhibition of MMP-1 that is upregulated by TGF- β 1. (d) RT-PCR performed on control and P311-expressing NIH 3T3 cells after 48 hours in culture with or without TGF- β 1 treatment demonstrating reversal of P311-induced downregulation in collagen expression by exogenous TGF- β 1.

logically from myofibroblasts, but still SM α -actin negative) (20), synthesized detectable amounts of P311 (upper right and left panels of Figure 7b, arrow). Examination of well-developed granulation tissue (2- to 3-week-old wounds) (Figure 7b, middle panels), showed P311 in myofibroblasts, but not in other mesenchymal cells present at the wound site, for example a regular (not activated) fibroblast (arrowhead). Healed wounds showed fibroblasts negative for P311 (lower panels of Figure 7b).

Discussion

We identified *p311* in the search of a subtracted cDNA library for genes upregulated during SM myogenesis. P311 is an 8-kDa intracellular protein expressed in adult muscle and neurons (5, 8, 12). P311 does not belong to any established protein family; neither does it have specific motifs that can suggest biological activity. P311, however, has been shown to be involved in glioblastoma cell migration (8) and to be regulated in neurons by HGF (12) and PTZ, an agent used to cause chemical seizures (7, 10). To determine whether P311 induces SM differentiation, we transfected P311 into NIH 3T3 and CH310 T1/2, two fibroblast cell lines that do not normally express it. In both cell lines P311 stimulated synthesis of SM and STM-related transcription factors with the exception of *myf5*, which was inhibited. It is noteworthy that it has been demonstrated that cell proliferation downregulates *myf5* (41, 42) without affecting other muscle-related transcription factors. This may explain the decrease in *myf5*

mRNA observed in P311-transfected cells. Transient transfections followed by double immunostaining with anti-his-tag epitope and anti-transcription factor Ab indicated that the P311-induced effect was limited to the transfected cells. This ruled out a potential paracrine-mediated activation of muscle gene expression, such as through the secretion of a growth factor/cytokine. Interestingly, the broad increase in muscle-related transcription factor activity did not result in SM or STM differentiation. On the contrary, only two SM-related proteins were induced in these cells, SM α -actin and SM22 in NIH 3T3 and SM α -actin in CH310 T1/2 cells. This restricted pattern of SM-specific gene expression is a hallmark of myofibroblasts (13–15, 20). It could be argued that the response to P311 was related to an intrinsic limitation of the cells to differentiate. This possibility, however, seems unlikely since NIH 3T3 cells can undergo STM differentiation upon overexpression of specific transcription factors (43) and CH310 T1/2 are multipotential mesenchymal cells that can be induced to differentiate into a variety of cell lineages (34, 35). Because this set of studies indicated that P311 induced an intermediate SM-like phenotype consistent with that of a myofibroblast, we next determined whether P311 induced other cellular features characteristic of myofibroblast transformation and whether P311 was present in human wounds. In vivo and in vitro studies have demonstrated that myofibroblasts participating in wound repair show a significant increment in PDGF receptors (14, 25–27) and integrins α 3 and α 5 (14, 28).

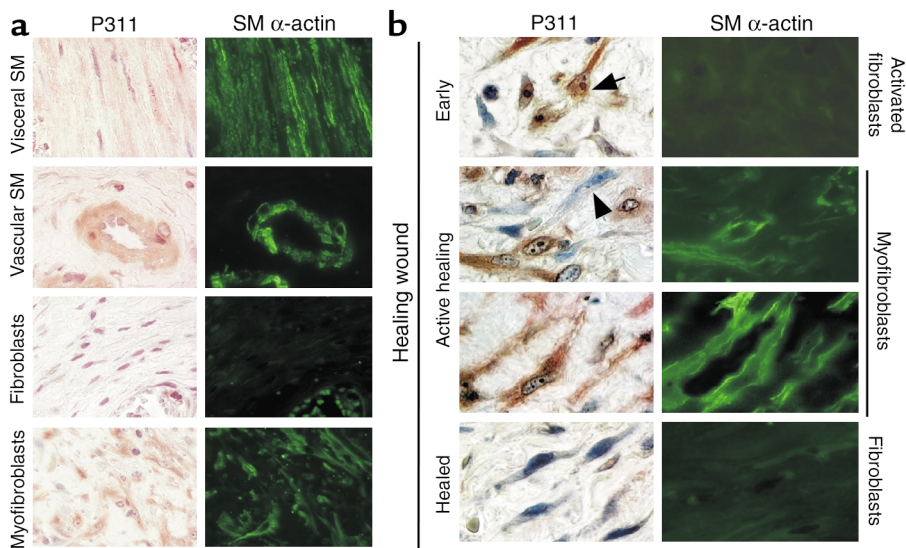


Figure 7

P311 is expressed in human visceral and vascular SM cells and myofibroblasts. All the sections have been double immunostained for P311 and SM α -actin, the latter to identify SM and myofibroblasts. (a) P311 is present in both visceral (intestine) and vascular SM (first and second panels down, respectively). P311 is not synthesized by fibroblasts (third panels), but is produced by myofibroblasts (fourth panels). (b) Immunohistochemical studies performed at an early stage of wound repair demonstrate P311 in most of the activated fibroblasts, also referred to as premyofibroblasts (SM α -actin-negative myofibroblast precursors, arrow in upper left photo). In more advanced stages of wound healing (two middle panels), P311 is detected in myofibroblasts (activated, SM α -actin-positive cells). Arrowhead in the second left photo points to a regular fibroblast present in the same field. Notice its smaller size, lack of discernible nucleoli, and negativity for P311. Once myofibroblasts disappear and the wound contains only regular fibroblasts, P311 is no longer detected (lowest panels).

It has also been shown that myofibroblasts characteristically secrete FGF-2 and PDGF (13–15, 17) and that the paracrine/autocrine effects of these growth factors lead to their enhanced proliferation (14). More recently, it has become evident that some myofibroblasts secrete significant amounts of VEGF (29–31) and therefore are likely to contribute to the rich neovascularization seen in granulation tissue. We found that P311-transfected cells, whether NIH 3T3 or C3H10T 1/2, exhibited all these changes. Equally important, immunohistochemical examination of human wounds demonstrated presence of P311 in myofibroblasts and their precursors, but not in other cells.

The P311-induced phenotype differed, however, from the typical myofibroblast in an unexpected and significant aspect; rather than stimulating TGF- β 1 and its receptor TGF- β R2, P311 downregulated them. Binding of TGF- β to the constitutively phosphorylated TGF- β R2 enables the formation and stabilization of TGF- β R1/TGF- β R2 receptor complexes and activates TGF- β R1 (44). P311 also reduced mRNA levels for MMP-2 and MMP-9, two proteolytic enzymes that participate in TGF- β activation (39). The message for MMP-1, which is upregulated by TGF- β 1 (40), was also inhibited. TGF- β 1 is the most potent profibrogenic factor currently identified (13–15, 17, 21, 25, 27, 36, 37, 39) and, accordingly, the P311-induced decrease in

TGF- β 1 was accompanied by significant downregulation of collagen 1 and collagen 3 expression. The inhibition of TGF- β 1 signaling was striking since it is considered the key paracrine/autocrine growth factor in induction and maintenance of the myofibroblast phenotype (13, 14, 17, 20, 36, 45). TGF- β 1 treatment is sufficient to induce SM α -actin gene expression and collagen synthesis, the two main features of myofibroblasts, as they are currently viewed (13–15, 17). TGF- β 1 directly activates transcription of SM α -actin, as well as other SM-specific proteins, by binding to a TGF- β control element present in their promoters (46). In addition, TGF- β 1 paracrine/autocrine signaling initiates a mechanistic cascade involving activation of extracellular matrix synthesis, mainly collagen 1 (13–15, 44).

This process culminates in healing with different

degrees of scarring, which varies from minimal to exuberant, as seen in hypertrophic scars and keloids. The factors that control the amount of fibrosis produced during wound repair are multiple, complex, and only partially understood. In this context, our data are the first to indicate that, in addition to their ability to degrade collagen/extracellular matrix (13–16), myofibroblasts have an intrinsic antifibrogenic mechanism(s) mediated at least in part by P311.

A growing body of evidence indicates that tensile forces determine all essential aspects of wound repair, including TGF- β 1 synthesis and collagen production (20). It is noteworthy that tensile forces also play a capital role during myogenesis, as we demonstrated in a previous study in which stretch alone was sufficient to induce undifferentiated mesenchymal cells to undergo SM differentiation (3). Since tension generated by SM α -actin contraction stimulates TGF- β 1 and collagen synthesis in myofibroblasts (20), it will be of interest to determine whether P311 may exert some of its antifibrogenic effect by promoting SM α -actin relaxation.

Although the molecular pathways whereby P311 stimulates myofibroblast transformation are presently unknown, some of its effects could be explained through the upregulation of SRF, either alone or in combination with PDGF and FGF-2. SRF plays a critical role in SM-gene expression (47, 48) and SM myo-

genesis (3, 49). Like TGF- β 1, SRF binds to the promoter regions of SM α -actin, SM22, and few other SM-related proteins, and directly stimulates their transcription (47, 48). Hence, P311 could induce SM α -actin and SM22 expression without the need for TGF- β 1. In fact, it has been shown that TGF- β does require SRF to initiate SM-specific gene transcription (50, 51). Similarly, the observed increase in cell proliferation and migration can also be related to SRF. The mitogenic effect of SRF is well-established (47), and it was recently shown that *srf*^{-/-} embryonic stem cells migrate poorly (52). P311 may also stimulate myofibroblast transformation through the upregulation of PDGF, because gene ablation studies demonstrated a crucial role for the latter in the development of SM-like cells normally found in tissue locations in which a certain degree of tension is needed. Effects of PDGF deficiency include absence of alveolar myofibroblasts in PDGF A^{-/-} mice (53) and absence of pericytes and mesangial cells in PDGF B^{-/-} mice (54).

Since P311 downregulates collagen expression and increases VEGF, P311 levels may be one of the factors contributing to determine whether a lesion will heal faster or with less fibrosis. It should be stressed, however, that although P311 inhibits TGF- β 1 synthesis, myofibroblasts remain responsive to exogenous TGF- β 1, as indicated by our in vitro studies. In vivo, myofibroblasts are not the only source of TGF- β 1, which is mainly produced by inflammatory cells normally present at the wound site (14, 20). Therefore, depending on the magnitude and duration of the inflammatory response, the autocrine antifibrogenic effect of P311 may be partially counterbalanced or completely offset by the paracrine production of TGF- β 1. In a noncomplicated wound, however, inflammatory cells are present at early stages of repair, and then gradually disappear from the site. Thus, the antifibrogenic effect of P311 may increase over time and become maximal toward the end of the reparative process.

In conclusion, these studies demonstrated a novel role for P311 in inducing TGF- β 1-independent myofibroblast transformation. By preventing collagen expression and stimulating VEGF, P311 and its potential effectors might open new possibilities for therapeutic interventions aimed at facilitating wound healing and/or minimizing scarring.

Acknowledgments

This work has been supported by National Heart, Lung, and Blood Institute grants HL-48730 and HL-67100, a grant from the Children's Research Center of Michigan to Lucia Schuger, and by National Institute on Aging grant AG11268, John A. Hartford Foundation grant 990339, and a Veterans Affairs Merit Review grant to Gregory A. Taylor.

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