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Pod1 Induces Myofibroblast Differentiation in Mesenchymal Progenitor Cells From Mouse Kidney

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Abstract The class II basic helix-loop-helix (bHLH) transcription factor Pod1 is expressed in mesenchymal cells including smooth muscle progenitors during development and in interstitial cells in adult organs. To determine the role of Pod1 in mesenchymal cell smooth muscle and myofibroblast differentiation, we examined a kidney progenitor cell line (4E) that endogenously expresses Pod1 and its class I bHLH partner E2A. In vitro-translated Pod1 co-immunoprecipitated E2A and increased E2A binding to a calponin promoter E-box sequence as determined by an electrophoresis mobility shift assay (EMSA). Overexpression of Pod1 and E2A resulted in increased smooth muscle and myofibroblast gene expression including calponin, SM22α, αSMA, fibronectin, and connective tissue growth factor (CTGF) compared with overexpression of E2A alone. Suppression of Pod1 by siRNA resulted in increased cell proliferation and reduced expression of αSMA, fibronectin, and CTGF, and myofibroblast secreted proteins including pro-fibrotic cytokines and inhibitors of matrix metalloproteinases. Examination of the signaling pathways for myofibroblast differentiation including Rho/Rho kinase and p38 MAPK showed that inhibition of actin polymerization by Rho kinase inhibitors decreased nuclear Pod1 levels while inhibition of p38 MAPK decreased Pod1 expression. These results indicate that Pod1 increases myofibroblast differentiation in combination with E2A and promotes a myofibroblast phenotype in mesenchymal progenitor cells. J. Cell. Biochem. 103: 675–690, 2008.

Key words: Pod1; mesenchymal cell; myofibroblast

The basic helix-loop-helix (bHLH) family of transcription factors has been shown to have an important role in regulating cell proliferation and differentiation in many cell types including lymphocytes, myocytes, and neurons [Massari and Murre, 2000]. The large number of identified proteins has been subdivided into seven classes based on DNA-binding properties, dimerization characteristics, and sequence homology. bHLH dimers bind specifically to a promoter nucleotide sequence known as an E-box (CANNTG). Class I bHLH factors including E12 and E47, products of the E2A gene, are expressed in many tissues where they control gene transcription promoting cellular differentiation and growth inhibition as homodimers in lymphocytes or heterodimers with other classes of HLH proteins in other cell types. Class II bHLH factors including MyoD, NeuroD/BETA 2, and myogenin have tissue-restricted expression where they activate or repress cell differentiation by controlling cell-specific gene expression by forming heterodimers with class I factors or with other class II factors. Pod1 (also called capsulin, epicardin, or TCF21) is a class II bHLH factor shown to be expressed in embryonic mesodermal cells including proepicardial cells of the heart, mesenchymal cells in areas of tubular epithelial development in the kidney, lung and gastrointestinal tract, visceral smooth muscle, and pericyte progenitors in areas of vasculogenesis and skeletal myoblasts within branchial arches [Hidai et al., 1998; Lu et al., 1998; Quaggin et al., 1998]. Pod1 transcripts are expressed at the highest levels during embryonic development. Pod1 levels rapidly decrease in postnatal tissues including differentiated smooth muscle cells with the exception of a subset of interstitial cells in organs including the kidney, lung, and intestine. Pod1 knockout

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mice die in the perinatal period from lung, kidney, and cardiac defects and also display gonadal, gastric, splenic, and facial muscle dysgenesis, suggesting a complex role in embryogenesis [Quaggin et al., 1999; Lu et al., 2000; Andersson et al., 2001; Tamura et al., 2001; Cui et al., 2004]. Subsequent studies have shown that Pod1 can function as a transcriptional repressor through heterodimer formation with E12 and, in selected cell types, may also promote transcription by an activation domain located in its C-terminus [Miyagishi et al., 2000a,b]. Recent microarray studies using RNA from developing glomeruli isolated from embryonic Pod1 knockout mice identified numerous genes upregulated or downregulated by the absence of this transcription factor including genes involved in cell proliferation, migration, and differentiation, though the biological relevance of the expression profile obtained remains unknown [Cui et al., 2005].

In the developing kidney, Pod1 is expressed in the condensing mesenchyme of the stroma that forms the adult interstitium and in glomeruli. Gene deletion studies using chimeric mice have demonstrated that the absence of Pod1 results in decreased tubulogenesis and glomerulogenesis with defects in formation of the interstitium including pericytes surrounding interstitial capillaries [Cui et al., 2003]. In the adult kidney, Pod1 expression was detected in a subset of interstitial cells and in podocytes, a highly differentiated glomerular epithelial cell derived from mesenchyme. The expression pattern and developmental defects described in the kidney suggest an important role for Pod1 in both interstitial cell autonomous differentiation as well as paracrine effects on adjacent tubular cells that may also be relevant in the adult kidney following injury. Based on studies demonstrating the importance of epithelial–mesenchymal interactions in the development of the lung, GI tract, and kidney, and organ fibrosis following injury, it would be of interest to determine the role of Pod1 in fibroblast, and in particular, myofibroblast differentiation since these cells have a key role in both processes [Powell et al., 1999].

In this study, we have used a multipotent mesenchymal cell line (4E) derived from adult mouse kidney [Plotkin and Goligorsky, 2006] that endogenously expresses Pod1 and E2A to examine the function of these transcription factors in mesenchymal cell proliferation, cytoskeletal arrangement, myofibroblast differentiation, and production of pro-inflammatory and fibrotic cytokines. The phenotypic plasticity and gene expression profile of 4E cells is similar to interstitial fibroblasts that have an important role in tissue repair and remodeling, thereby providing an in vitro model for studying the role of these transcription factors in regulating these processes. The myofibroblast phenotype can be induced by various factors including cytokines and extracellular matrix and this cell type has an important role in tissue fibrosis including the interstitial fibrosis that occurs during chronic renal disease. In vitro study of 4E cells therefore provides an opportunity to determine the role of Pod1 and E2A in transducing external signals for myofibroblast formation. Our results demonstrate that Pod1 in combination with E2A increases 4E smooth muscle-associated gene expression characteristic of activated myofibroblasts and induces these cells to acquire a myofibroblast-like phenotype. These effects appear to be linked to regulators of myofibroblast gene expression and morphology including the RhoA GTPase [Heusinger-Ribeiro et al., 2001] and p38 MAPK signaling pathways [Meyer-Ter-Vehn et al., 2006], and their effectors including connective tissue growth factor (CTGF) and fibronectin [Leask and Abraham, 2004]. These results indicate that regulation of Pod1 and E2A may be important for kidney interstitial cell activation including myofibroblast differentiation following injury.

MATERIALS AND METHODS

Antibodies and Reagents

Primary antibodies against Pod-1, E2A, p21, CTGF, and goat anti-rabbit, donkey anti-goat secondary antibodies used in Western blots were from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against Calponin and β-actin were from Sigma–Aldrich (St.Louis, MO). Primary antibody against α-smooth muscle actin was from Dako (Carpinteria, CA). Primary antibody against Sm22α was from Abcam, Inc. (Cambridge, MA). Primary antibody against Paxillin and phospho-p38 was from Upstate Cell Signaling Solutions (Lake Placid, NY). Texas Red Phalloidin and donkey anti-goat and anti-rabbit secondary antibodies were from Molecular probes (Invitrogen, Carlsbad, CA). Secondary mouse monoclonal antibody was from

Amersham Biosciences (UK). Goat anti-mouse and goat anti-rabbit secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Recombinant TGFβ1, FGF2, VEGF, and TNFα were purchased from Peprotech (Rocky Hill, NJ), BMP-4 and BMP-7 were from R & D Systems (Minneapolis, MN). SB202190 was purchased from Upstate, and Y-27632 and latrunculin A were from Calbiochem (San Diego, CA).

Nuclear and Cytoplasmic Protein Extracts

Nuclear and cytoplasmic protein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). The extracts were prepared according to the manufacturer's instructions.

Western Blotting

Cells cultures were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer (1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1%SDS, 1mM EDTA, 0.5% Triton 100 in PBS) containing Complete Mini protease inhibitor (Roche, Indianapolis, IN) and 2 mM PMSF. Protein concentrations were measured using a Bradford assay (Bio-Rad, Hercules, CA). The protein samples were denatured by adding sodium-dodecyl-sulfate (SDS) sample buffer and heating at 90°C for 5 min. Sample (10–40 μg) containing β-mercaptoethanol were resolved on 4–20% Tris Glycine polyacrylamide gels. Gels were transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) and blocked with 5% non-fat dry milk in PBS-T (0.1% Tween-20 in PBS). The membranes were then incubated overnight at 4°C with primary antibody in blocking buffer. This was followed by washing and 1 h incubation with an appropriate horseradish peroxidase-conjugated secondary antibody. Protein bands were detected by chemiluminiscence using a commercial kit (Pierce) according to the manufacturer's instructions. For quantification of protein levels, autoradiographs were scanned with the Scion Image densitometry program and results were corrected for variations in the amount of protein loaded on each lane using corresponding β-actin levels.

Electrophoretic Mobility Shift Assays (EMSA) and In Vitro Dimerization Assay

Gel shift assays were performed using a Lightshift Chemiluminiscent EMSA kit (Pierce). Briefly, biotin-labeled oligonucleotide probes containing an E-box sequence from the calponin promoter: 5′-AAATAGGGGGTGTGCCAGCTG TTACTGCCAGGCTCAGTGC-3′ were incubated with or without unlabeled competitor oligonucleotides in the presence of 2–3 μg 4E cell nuclear extracts or in vitro-translated transcription factors prepared using a TnT Quick-Coupled Transcription/Translation system (Promega, Madison, WI) using plasmid DNA, and binding buffer (50 mM KCl, 1 mM DTT, 5% Glycerol, 0.1% Nonidet P-40, 10 mM MgCl2; pH 7.5) at room temperature for 30 min. Resulting products were resolved on 6% non-denaturing polyacrylamide gels, transferred to PVDF membranes, and visualized with a chemiluminiscent detection kit (Pierce) according to the manufacturer's instructions. For supershift assays, 0.1 μg antibody was added to the mixture following protein binding and incubated at room temperature for 30 min.

For evaluation of in vitro Pod1-E2A binding, biotinylated Pod1 was prepared using a TNT Quick-Coupled Transcription/Translation System with Transcend Biotin-Lysyl-tRNA (Promega). Translated protein was bound to streptavidin–agarose beads (Upstate) followed by washes with PBS. Pod1–streptavidin beads were incubated with 4E cell nuclear extracts for 4 h at 4°C, followed by washes with PBS. Proteins were boiled for 5 min in sample buffer and loaded on 4–20% Tris Glycine polyacrylamide gels. Gels were transferred to PVDF membranes and Western blots were performed as described above.

Cytokine Antibody Array

Protein extracts were analyzed using a RayBiotech Mouse Atherosclerosis Antibody Array I (RayBiotech, Inc., Norcross, GA) according to the manufacturer's instructions. The array simultaneously detects 22 mouse cytokines in duplicate. Briefly, cytokine array membranes were blocked in 2 ml of 1× blocking buffer for 60 min, and then incubated with 1 ml (100 μg) of sample at 4°C overnight. Following washes, membranes were incubated with biotin-conjugated primary antibodies at room temperature for 1–2 h and washed before incubation in 1:1000 diluted HRP-conjugated streptavidin. After incubation in HRP-conjugated streptavidin for 60 min, membranes were washed thoroughly, and exposed to a peroxide substrate for 1 min before imaging. Membranes

were exposed to X-ray film (HyBlot CL, Denville Scientific, Inc., Denville, PA). Autoradiographs were scanned and the Scion Image densitometry program was used to determine signal intensity. Biotin-conjugated IgG served as a positive control at six spots, where it was used to identify membrane orientation and to normalize the results from different membranes that were being compared. For each spot, the net optical density level was determined by subtracting the background optical density from the total raw optical density and the level of each cytokine was represented as a percentage of the positive control.

Gelatinase/Collagenase Assay

Assays for collagenase inhibitors were done with an EnzChek Gelatinase/Collagenase assay kit (Molecular Probes) including collagenase substrate DQ gelatin and its inhibitor 1, 10 phenanthroline according to the manufacturer's instructions. Briefly, the supernatants of transfected cells grown on Vitrogen type 1-collagen gels (Cohesion, Palo Alto, CA) for 24 h were incubated with FITC-gelatin and collagenase for 4–6 h. The fluorescence intensity of digested gelatin was detected using a microplate reader at different time points.

Cells and Transfections

4E cells were grown in EMEM (Invitrogen) supplemented with 10% horse serum on 1% gelatin-coated dishes. siRNA was transfected in 4E cells resuspended in siPORT Electroporation Buffer (Ambion, Austin, TX) using a Bio-Rad Gene Pulser II at 250 V. Plasmids were transfected using an Amaxa Nucleofector with human MSC transfection solution (Qiagen). Pod1 (TCF 21) siRNA was from Dharmacon siGENOME SMART pool reagents (Lafayette, CO) and consisted of a mixture of four targeting oligonucleotides. The expression plasmid pcDNA3.1 encoding Pod1 was a gift from Dr. Susan Quaggin (University of Toronto). E2A cloned in pCMV-SPORT6 was purchased from Invitrogen. Calponin promoter luciferase reporter (SM-Calp I-1) and SM22 505 promoter luciferase reporter plasmids cloned in pGL3 were gifts from Dr. Joseph Miano (University of Rochester). The SM α-actin promoter–reporter ($-2{,}560$ to 2,784) plasmid was a gift from Dr. Gary Owens (University of Virginia). RhoA G14V and RhoA T19N plasmids were purchased from UMR cDNA Resource Center (Rolla, MO) and pRL-TK Renilla and pGL3 control plasmids were purchased from Promega. Luciferase assays were performed using a DLR Dual Luciferase assay (Promega) with cell lysates obtained from cells transfected with promoter–reporter and internal control Renilla luciferase plasmids. Luciferase activity was measured using a Berthold Mithras luminometer equipped with dual injectors.

BrdU ELISA

4E cells were grown to 50–70% confluence in 96-well plates. Following treatment with cytokines for 24 h, cells were labeled with BrdU overnight. BrdU incorporation was measured using a colorimetric BrdU ELISA kit (Roche) according to the manufacturer's instructions.

Statistics

All values within mean $\pm$ 2 SD were used for statistical analysis. To compare results between different groups, an F-test followed by a t-test was done and the difference was considered significant with $P < 0.05$.

RESULTS

Pod1 Binds E2A in a Pull-Down Assay and Increases E2A Binding to a Calponin Promoter E-Box-Binding Site

To determine the effects of E2A and Pod1 on 4E cell gene expression and differentiation, cells were transiently transfected with plasmids encoding full-length E2A (E12) and Pod1 to provide constitutive overexpression of these transcription factors. In vitro translation of biotin-labeled proteins using these plasmids resulted in protein products migrating near the predicted molecular weights of 68 kDa for E2A [Jacobs et al., 1993] and 19 kDa for Pod1 (Fig. 1A). In vitro-translated biotin-labeled Pod1 was incubated with streptavidin–agarose beads followed by 4E cell nuclear extracts and the resulting products were precipitated and separated by PAGE. Western blot analysis of these proteins using an E2A antibody demonstrated a band at approximately 75 kDa not present in control lanes (Fig. 1B, lane 3, arrowhead) corresponding to the predicted molecular weight of an E2A monomer on SDS–PAGE [Loveys et al., 1996], demonstrating that Pod1 binds E2A in vitro. To evaluate the effect of Pod1 heterodimer formation on E2A DNA binding, electrophoretic mobility shift assays

(EMSA) using a calponin promoter E-box sequence was performed. The specificity of binding of in vitro translated and 4E cell nuclear lysate-derived E2A and supershift of E2A bound oligonucleotides using E2A antibodies are shown in Figure 1C. EMSA using in vitro-translated Pod1 resulted in non-specific products (data not shown), suggesting that Pod1 homodimers are unable to bind the calponin E-box sequence. E2A binding (Fig. 1D lanes 1–2) was increased by addition of increasing concentrations of in vitro-translated Pod1 (Fig. 1D lanes 3–5), indicating that Pod1-E2A heterodimers bind to this specific E-box sequence with increased affinity compared with E2A alone.

Pod1 and E2A Induce Smooth Muscle Gene Expression and Promote Myofibroblast Activation of 4E Cells

4E and other mesenchymal progenitor cells express smooth muscle genes following cell adhesion and in response to inducing cytokines and in vivo studies have shown smooth muscle progenitor cell Pod1 expression. Because many smooth muscle gene promoters contain E-box-binding sites [Kawai-Kowase et al., 2005], we examined the effect of changes in 4E cell Pod1 expression on smooth muscle gene expression. Endogenous Pod1 expression was decreased $66 \pm 17\%$ ($n = 3$) by transfection of Pod1 siRNA (Fig. 2A). Pod1 and E2A overexpression were induced by transient transfection of full-length Pod1 and E2A expression plasmids and changes in Pod1 expression were examined by RT-PCR (Fig. 2B) and Western analysis (Figs. 2C and 3A). Transfection of Pod1 resulted in Pod1 mRNA levels equivalent to whole embryo levels by RT-PCR and a 3.6 ± 1.5-fold ($n = 4$) increase in Pod1 protein levels. In all transfection experiments, overexpression of E2A and Pod1 resulted in a nearly twofold increase in E2A levels compared with overexpression of Pod1 alone (Fig. 3A). The resulting changes in the smooth muscle-specific actin-binding proteins calponin and SM22α, the myofibroblast and smooth muscle actin isoform alpha-smooth muscle actin (αSMA), the myofibroblast inducing cytokine CTGF and fibronectin, an extracellular matrix protein that promotes myofibroblast differentiation, were examined by Western analysis (Fig. 3A and E). Overexpression of E2A for 72 h in 4E cells resulted in a twofold increase in calponin, SM 22α and αSMA levels. Transient overexpression of Pod1 alone resulted in no changes in these smooth muscle proteins and increased levels of CTGF and fibronectin. Overexpression of Pod1 and E2A resulted in increased expression of calponin, SM22α, αSMA, and CTGF compared with E2A alone, consistent with an activating effect of Pod1 on E2A-induced gene expression.

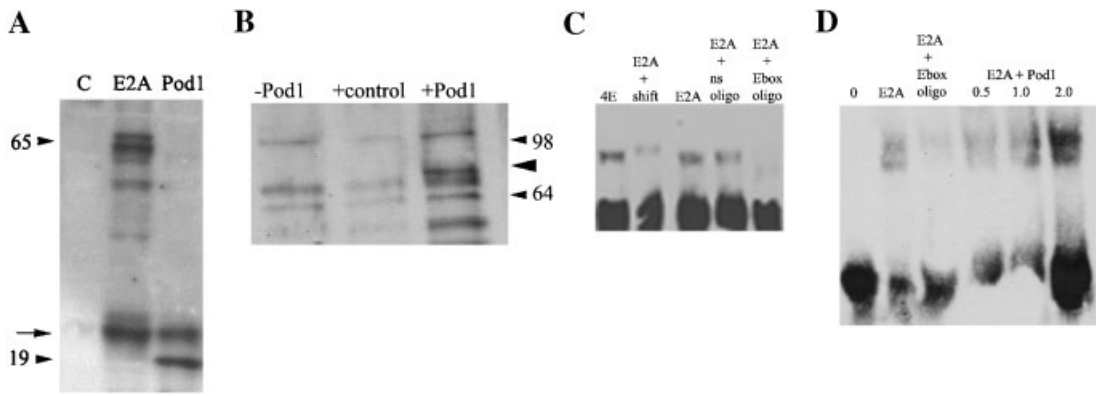

Fig. 1. In vitro-translated Pod1 binds to E2A and inhibits E2A binding to a calponin E-box by EMSA. **A**: Biotinylated, in vitro-translated proteins from Pod1, E2A, and control plasmid without insert were detected by streptavidin-HRP (arrowheads with kDa size markers). Arrow indicates non-specific 25 kDa aminoacyl tRNA band. **B**: Biotinylated Pod1 bound to streptavidin–agarose beads was used to precipitate proteins from 10 μg 4E nuclear extracts (**lane 3**) and proteins were analyzed by Western blot using an anti-E2A antibody. A band corresponding to the predicted size of E2A (arrowhead) was not present in extracts incubated without biotinylated Pod1 (**lane 1**), or biotinylated products using control plasmid without a Pod1 insert (**lane 2**), arrowheads with kDa size markers. **C**: EMSA using biotin-labeled oligonucleotides containing a calponin E-box sequence. **Lane 1**: 4E nuclear extract, **lane 2**: anti-E2A supershift, **lane 3**: in vitro-translated E2A, **lane 4**: E2A plus non-specific unlabeled oligo, **lane 5**: E2A plus unlabeled calponin E-box oligo. **D**: EMSA with calponin E-box oligo and in vitro-translated E2A or E2A plus Pod1. **Lane 1**: no protein, **lane 2**: in vitro-translated E2A, **lane 3**: E2A plus unlabeled E-box oligo, **lanes 4–6**: E2A plus 0.5, 1, and 2.0 μl Pod1 protein.

Because CTGF is known to be a potent inducer of myofibroblast differentiation [Heusinger-Ribeiro et al., 2001; Leask and Abraham, 2004], changes in its expression were also evaluated. As shown in Figure 3A, transfection with Pod1 siRNA resulted in decreased CTGF levels. 4E cell CTGF expression in response to exogenous cytokines showed increased levels following TGFβ as previously reported [Leask and Abraham, 2004] and complete suppression following BMP7, an inducer of the differentiated smooth muscle phenotype in progenitor cells [Dorai et al., 2000; Dorai and Sampath, 2001].

Changes in Pod1 expression in response to 48 h exposure to exogenous cytokines known to either increase or decrease myofibroblast transdifferentiation were also examined (Fig. 3A). Basic fibroblast growth factor (FGF), an inducer of the differentiated fibroblast phenotype [Papetti et al., 2003], resulted in decreased Pod1 protein levels while TNFα and TGFβ, inducers of the myofibroblast phenotype, resulted in increased Pod1 expression.

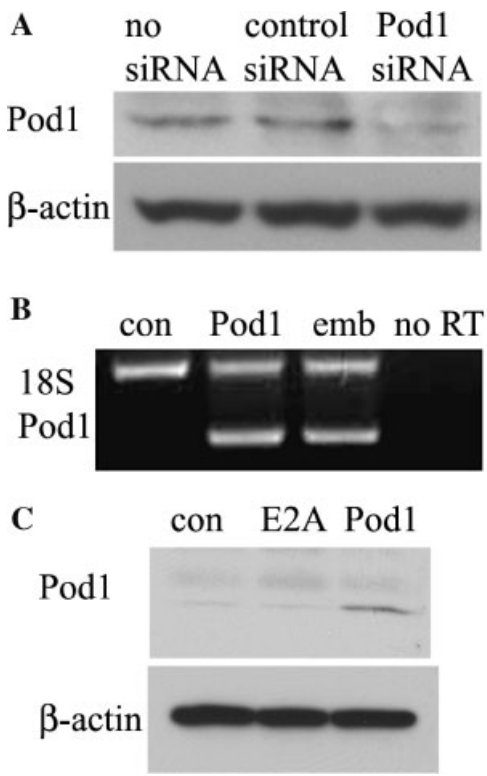

Fig. 2. Changes in Pod1 levels by siRNA and an overexpression plasmid. 4E cells were transfected with siRNA-targeting Pod1 and changes in Pod1 protein levels were evaluated after 72 h by Western blot. **A**: Control cells were transfected with non-targeting siRNA. Pod1 overexpression was evaluated by RT-PCR and compared with control cells transfected with expression plasmid without insert (con) and whole embryo cDNA (emb). **B**: 18S RNA amplification was used as an internal control. **C**: Western blot analysis of Pod1 levels in control, E2A, and Pod1 expression plasmid-transfected cells.

The effect of E2A and Pod1 overexpression and Pod1 depletion by siRNA on smooth muscle promoter activity was examined using transfection of promoter–luciferase reporter plasmids containing at least 1 E-box-binding site [Miano and Olson, 1996; Kumar et al., 2003]. E2A transfection resulted in significantly increased SM22α (Fig. 3B), and αSMA (Fig. 3C) promoter activity as expected based on its activating function. Pod1 overexpression alone had no significant effect on promoter activity. Co-expression of Pod1 and E2A resulted in no significant change in SM22α and αSMA activity compared with E2A alone. Treatment with Pod1 siRNA to reduce endogenous Pod1 levels for 24 h prior to reporter plasmid transfection, however, resulted in decreased E2A-induced promoter activity for αSMA and SM22α compared with cells transfected with control siRNA.

The effects of Pod1 and E2A overexpression on stress fiber formation were also examined by double immunofluorescent staining of 4E cells for F-actin using labeled phalloidin and αSMA, the actin isoform incorporated into myofibroblast stress fibers. As shown in Figure 3D, increased F-actin stress fiber formation that was double-labeled for αSMA was detected in cells overexpressing E2A and, more frequently, E2A and Pod1. In contrast, Pod1 siRNA resulted in decreased stress fiber-αSMA expression compared with control siRNA transfection (Fig. 3D). Pod1 and E2A overexpression resulted in increased fibronectin expression by Western analysis and Pod1 siRNA resulted in decreased fibronectin expression by immunocytochemistry and Western analysis (Fig. 3E). Together, these results suggest that Pod1 increases smooth muscle gene expression in combination with E2A and induces a myofibroblast phenotype in mesenchymal progenitor cells.

TGFβ1 and BMP7 Increase Nuclear Expression of E2A and Pod1

The differentiation of mesenchymal cells to myofibroblasts following injury and in fibrotic diseases is mediated, in part, by cytokines including TGFβ1. This cytokine is also capable of inhibiting cell growth and inducing smooth

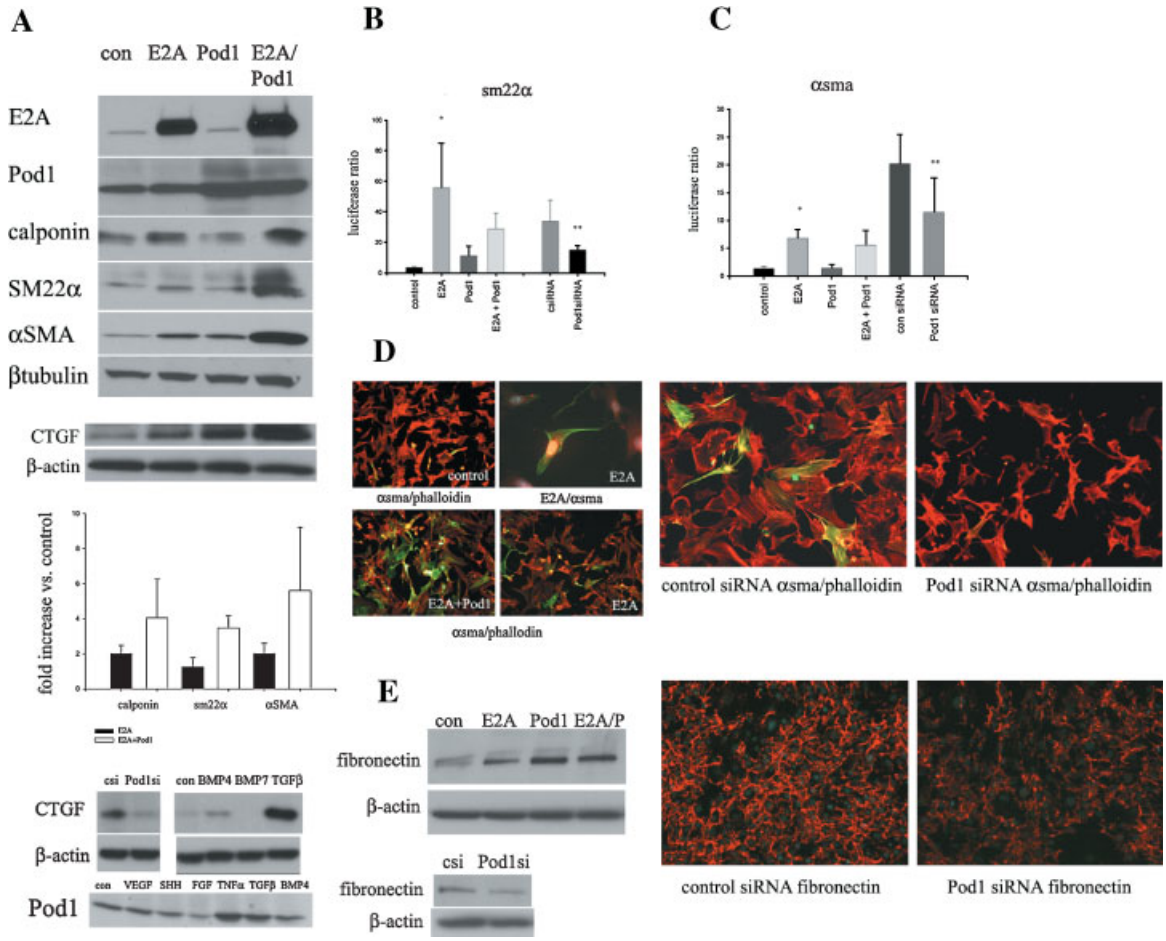

Fig. 3. Pod1 overexpression increases smooth muscle gene expression and induces a myofibroblast-like phenotype. 4E cells were transfected with control, E2A, Pod1, and E2A plus Pod1 expression plasmids, Pod1 siRNA or treated with cytokines including BMP4 (50 ng/ml), BMP7 (50 ng/ml), TGFβ1 (5 ng/ml), VEGF (10 ng/ml), Sonic Hedgehog (10 ng/ml), FGF2 (50 ng/ml), and TNFα (5 ng/ml), and expression of protein levels after 72 h were evaluated by Western blot analysis **A**: Bar graph shows mean plus standard deviation of densitometry results of Western blots normalized with β-tubulin from three separate experiments ($P < 0.01$ for all groups). **B**, **C**: Dual luciferase assays using 4E cells transfected with overexpression, promoter–reporter plasmids (B, SM22α and C, αSMA) and Renilla luciferase plasmid as internal control. Pod1 siRNA was transfected as indicated 48 h prior to plasmid transfection. Control cells were transfected with non-targeting siRNA or pGL2 luciferase plasmid without promoter insert. Luciferase activity was measured 48 h following transfection. Results are mean values plus standard deviation from three experiments, $^{*}P < 0.001$, $^{**}P < 0.05$. **D**: Immunofluorescence labeling of 4E cells transfected as indicated with Texas Red phalloidin (red), anti-αSMA (green), or anti-E2A (red). Double-labeled cells appear yellow. (Original magnification, 100×). **E**: Western blot analysis and immunofluorescence images of fibronectin expression of 4E cells transfected as indicated. Cell nuclei labeled with Hoescht (blue). Original magnification, 100×.

muscle differentiation including expression of contractile proteins such as calponin and sm22α by regulation of multiple genes including those encoding transcription factors, matrix and cytoskeletal proteins, and signaling molecules [Parmacek, 2001]. Because Pod1 and E2A overexpression increased calponin expression in unstimulated 4E cells, we further evaluated the effect of TGFβ1 on Pod1 and E2A nuclear expression and the effect of increased Pod1 expression on TGFβ1-induced calponin promoter activation. Immunofluorescence labeling of untreated 4E cells (Fig. 4A) revealed that Pod1 was predominately located in the cytoplasm while E2A was located in the nucleus. Western blot analysis of protein isolated from cytoplasmic and nuclear fractions of 4E cells treated with TGFβ1 (Fig. 4A) for 48 h demonstrated both an increase in the overall level and nuclear localization of Pod1 and E2A expression. In contrast, BMP-7, a member of the TGFβ superfamily that promotes smooth muscle differentiation [Dorai et al., 2000; Dorai and Sampath, 2001] but appears to inhibit

myofibroblast differentiation in models of organ fibrosis [Lund et al., 2002], increased E2A expression and had only a small effect on Pod1 nuclear expression. Evaluation of the effect of TGFβ on 4E cells transfected with a calponin promoter luciferase reporter demonstrated a twofold increase in promoter activity in cells overexpressing Pod1 compared with control cells (Fig. 4B).

Pod1 Inhibits 4E Cell Proliferation and Decreases Cell Migration in an In Vitro Wound Assay

E2A induction of muscle progenitor cell differentiation has been shown to depend on cell cycle arrest due to increased expression of the cyclin inhibitor $p21^{WAF1/CIP}$ [Peverali et al., 1994]. To determine the effect of Pod1 on 4E cell proliferation, a BrdU incorporation assay was used to measure S-phase activity in control and Pod1 siRNA-transfected cells treated with cytokines previously shown to increase mesenchymal cell proliferation. Results shown in Figure 5A demonstrate that cells transfected with Pod1 siRNA had a twofold increase in baseline BrdU incorporation compared with control cells. Treatment with FGF2, VEGF, and the BMP antagonist Noggin resulted in significantly increased 4E cell proliferation while siRNA-transfected cells displayed no additional increase in BrdU incorporation. To further evaluate the effect of Pod1 on 4E cell proliferation, cells were transfected with E2A and Pod1 expression plasmids, and resulting changes in p21 expression were quantified by nuclear labeling with p21 antibody. As expected from previous studies [Funato et al., 2003], E2A overexpression resulted in a 14-fold increase in cells expressing strong nuclear p21 signal while Pod1 overexpression resulted in an eightfold increase. Together, these results suggest that Pod1 has an inhibitory effect on 4E cell proliferation.

In order to determine the effect of Pod1 and E2A on 4E cell migration, an in vitro wound-healing assay was used on confluent monolayers of 4E cells transfected with E2A and Pod1 expression plasmids. Wound healing in this assay is associated with matrix remodeling and cell migration without the need for cell proliferation to cover the scratched area [Nobes and Hall, 1999]. Examination of scratched monolayers at 0 and 12 h demonstrated that overexpression of E2A or Pod1 resulted in a small decrease in cell migration at 12 h (Fig. 6).

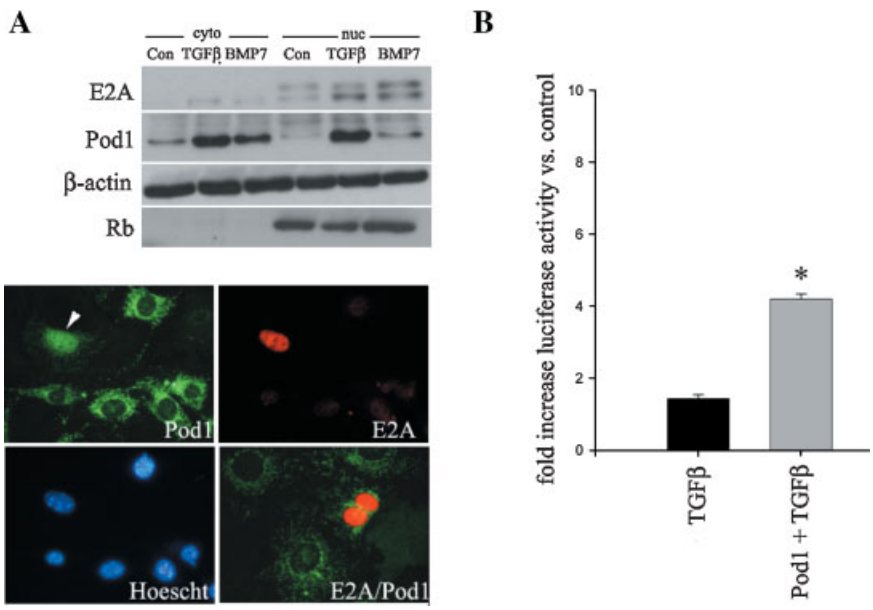

Fig. 4. TGFβ and BMP7 increase nuclear levels of Pod1 and E2A. **A**: Western blot analysis of nuclear and cytoplasmic extracts prepared from 4E cells treated with the indicated cytokines for 72 h. β-Actin used as loading control and Retinoblastoma (Rb) used to control for separation of nuclear and cytoplasmic fractions. Immunofluorescent labeling of 4E cells with anti-Pod1 (green), and E2A antibodies (red), and Hoescht nuclear stain (blue) demonstrating predominately cytoplasmic localization of Pod1 with occasional double-labeled nucleus (arrowhead). **B**: Dual luciferase assays using 4E cells transfected with Pod1 overexpression, calponin promoter–reporter and Renilla luciferase plasmids as internal control. Cells were incubated with TGFβ (5 ng/ml) and luciferase activity was measured 48 h following transfection. Results are mean values plus standard deviation from three experiments, $^{*}P < 0.05$.

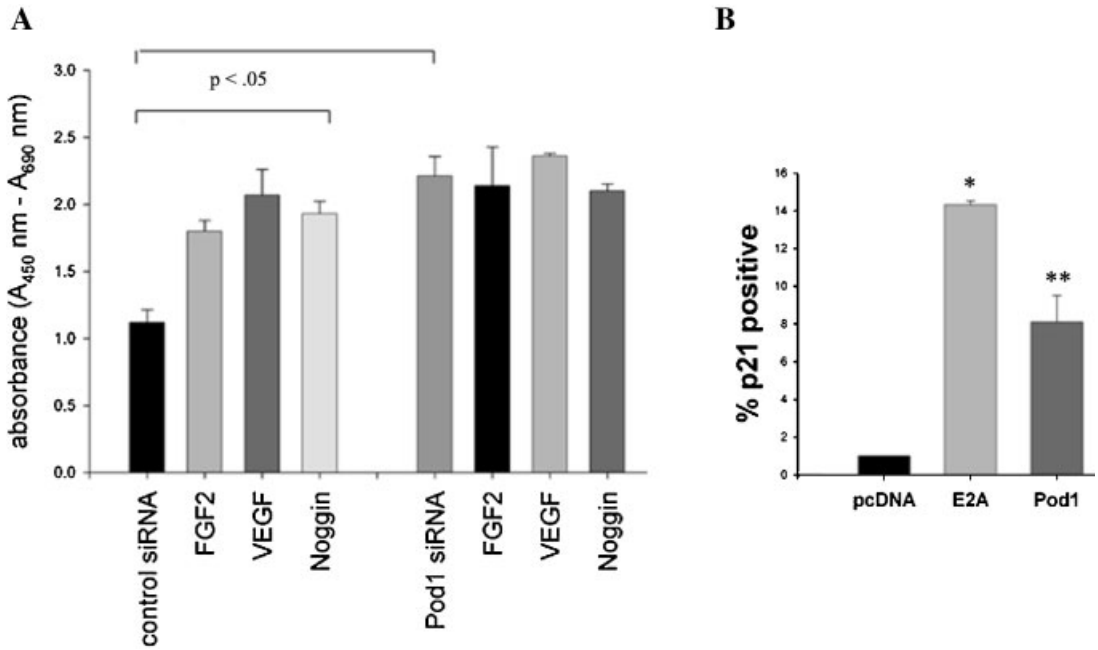

Fig. 5. Pod1 decreases 4E cell proliferation and increases p21 expression. **A**: 4E cells were transfected with control and Pod1 siRNA followed by treatment with the indicated mitogenic cytokines for 24–48 h. Cell proliferation was measured by BrdU ELISA. Results are means with standard deviation from six samples from two separate experiments. **B**: Nuclear p21 expression was detected by double immunofluorescence labeling with anti-p21 antibody and Hoescht. Positive nuclei were counted from 200 to 300 cells in each group from three separate experiments, $^{*}P < 0.001$, $^{**}P < 0.05$.

Overexpression of E2A and Pod1 appeared to have an additive effect on cell migration at 12 h with a twofold increase in exposed area compared with cells transfected with either factor alone. Examination of scratched monolayers from control-transfected cells at 12 h with immunofluorescence labeling for Pod1 and E2A revealed decreased expression of both transcription factors in migrating cells located within the scratched area while the surrounding monolayer expressed detectable levels of both proteins. E2A and Pod1-transfected cells displayed both increased stress fiber formation by phalloidin labeling and paxillin-positive focal adhesion formation compared with cells transfected with control plasmid, indicating that the resulting contractile myofibroblast-like phenotype is associated with decreased cell migration and increased focal adhesion formation in this model of wound healing.

Pod1 Expression is Associated With Increased Production of Myofibroblast Proteins Including Pro-Fibrotic Cytokines and Inhibitors of Matrix Metalloproteinases

Myofibroblasts play a major role in the inflammatory response through secretion of chemokines and cytokines and production of extracellular matrix [Powell et al., 1999]. Since Pod1 expression appears to control expression of the myofibroblast phenotype, we examined the effect of decreased Pod1 levels on 4E cell cytokine expression using a commercially available semi-quantitative cytokine antibody array. Following transfection with Pod1 siRNA, 4E cell lysates contained between 25% and 50% lower levels of IL-1β, IL-2, IL-3, L-selectin, TNFα, and VEGF compared with control-transfected cells, consistent with a decreased pro-inflammatory phenotype (Fig. 7A). To examine production of inhibitors of matrix metalloproteinases, another key product of myofibroblasts responsible for wound healing and tissue fibrosis [Powell et al., 1999], 4E cells were grown on type 1-collagen gels and culture supernatants were evaluated for the presence of collagenase inhibitors using a fluorescence-based assay. As shown in Figure 7B, 4E cell supernatants from cells transfected with both E2A and Pod1 resulted in collagenase inhibition equal to medium containing 0.25 mM 1,10-phenanthroline. Transfection with E2A alone resulted in no significant increase in inhibitor levels compared with control cells. Together, these results

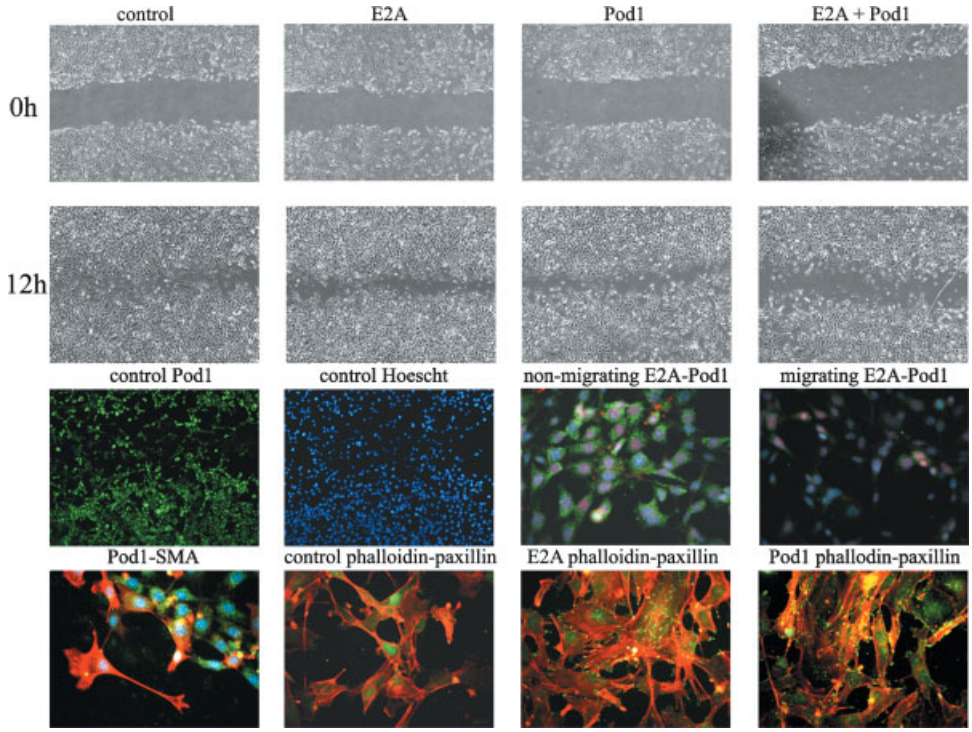

Fig. 6. E2A and Pod1 overexpression decreases 4E cell migration in a scratch-wound assay and increases expression of focal adhesion complexes. Scratch-wound migration assays were performed using confluent layers of 4E cells transfected with expression plasmids as indicated. Representative phase contrast images at 0 and 12 h from three separate experiments are shown. Original magnification, 40×. Migrating and non-migrating cells from control-transfected monolayers at 12 h following injury were labeled by immunofluorescence with the indicated antibodies: anti-Pod1 (green), anti-E2A (red), anti-αSMA (red), and Hoescht nuclear stain (blue). Original magnification 40× (control Pod1) and 100× (non-migrating and migrating E2A and Pod1). Control, E2A, and Pod1-transfected cells were labeled with Texas Red Phalloidin (red) and Paxillin (green). Original magnification, 400×.

support the role of Pod1 in 4E cell myofibroblast differentiation through interaction with E2A.

Pod1 Expression is Regulated by the p38 MAP Kinase Pathway and Nuclear Expression is Decreased by Disassembly of the Actin Cytoskeleton

The roles of p38 MAP kinase signal transduction, Rho GTPase activity, and actin cytoskeleton organization in mesenchymal cell smooth muscle and myofibroblast differentiation have been extensively examined [Heusinger-Ribeiro et al., 2001; Kobayashi et al., 2001; Mack et al., 2001; Beqaj et al., 2002; Deaton et al., 2005]. These studies have demonstrated the importance of RhoA signaling in serum response element (SRE)-dependent smooth muscle gene expression and of the p38 pathway in TGFβ and CTGF-induced myofibroblast differentiation and tissue fibrosis. To determine if these pathways also operate in regulating Pod1 and E2A expression, the effect of constitutively active RhoA and dominant negative RhoA expression and inhibition of p38 MAP kinase in control and TGFβ-treated 4E cells was investigated. Expression of activated RhoA resulted in increased stress fiber formation while dominant negative RhoA transfection with RhoA-N19 resulted in expression of predominately cortical stress fibers with extension of elongated cellular processes (Fig. 8A). By Western analysis (Fig. 8B), no changes in Pod1 or E2A expression were detected following RhoA activation. Transfection with dominant negative RhoA resulted in a $39 \pm 2\%$ $(n = 2)$ decrease in nuclear E2A levels with a corresponding small increase in the lower molecular weight E2A band in the cytoplasmic fraction, suggesting decreased nuclear translocation of E2A. Following treatment with the Rho kinase inhibitor Y-27632 (10 μM), marked morphological changes occurred including the absence of stress fibers and growth of elongated cell processes (Fig. 8A). An approximately 50% decrease in the nuclear to cytoplasmic ratio of Pod1 was detected. A similar effect on nuclear

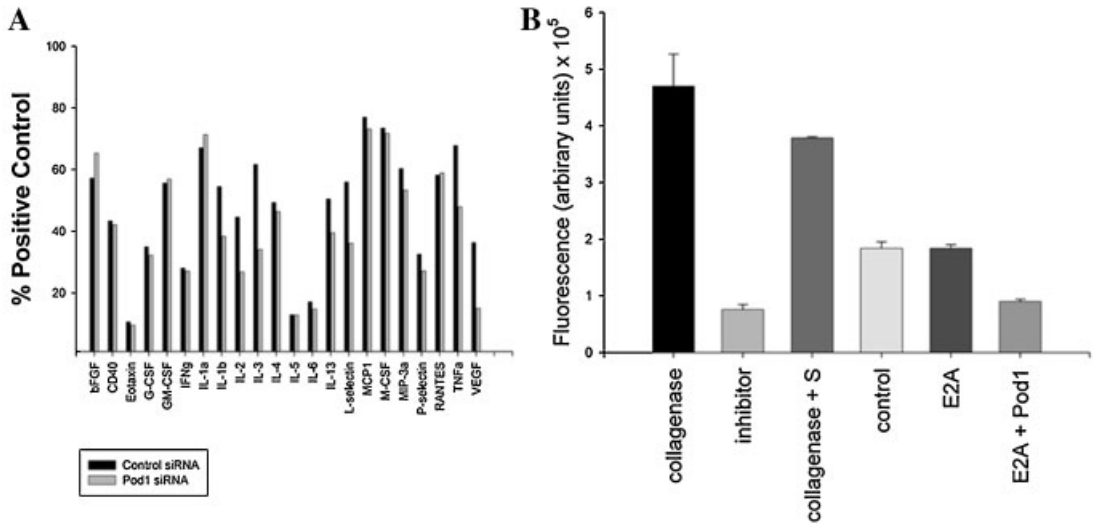

Fig. 7. Pod1 expression is associated with expression of myofibroblast-secreted proteins including pro-fibrotic cytokines and inhibitors of matrix metalloproteinases. **A**: Cell lysates from 4E cells transfected with control and Pod1 siRNA were hybridized with a cytokine antibody array to detect relative levels of inflammatory cytokines. Results expressed as percentage of positive control standard and are means of duplicate signals from two separate hybridization experiments. **B**: Production of inhibitors of metalloproteinases (TIMP) was measured using a fluorescence-based gelatinase assay. Cell culture supernatants from 4E cells grown on type 1-collagen gels (Vitrogen) 48 h after transfection with indicated plasmids were incubated with FITC-labeled gelatin. Control samples included: collagenase, collagenase plus inhibitor (0.25 mM 1,10-phenanthroline), and collagenase plus medium with serum (S). Results are means plus standard deviations from three separate experiments.

Pod1 levels occurred following treatment with latrunculin A (1 μM), a cell permeable toxin that disrupts actin microfilaments, suggesting the Rho kinase effect was due to its effect on cytoskeleton disassembly (Fig. 8C).

4E cell phospho-p38 MAPK (p-p38) levels increased in response to TGFβ in a bimodal pattern over 48 h along with increased expression of αSMA, indicating sustained activation of the p38 MAPK pathway (Fig. 8D). To examine the effect of this pathway on Pod1 expression, cells were treated with TGFβ with or without the addition of SB202190, a specific p38 MAPK inhibitor. By Western analysis (Fig. 8E), SB202190 resulted in suppression of both baseline and TGFβ-induced Pod1 expression. Corresponding decreases in αSMA levels were also detected as previously reported in other cell types [Meyer-Ter-Vehn et al., 2006].

The effect of RhoA/ROCK and p38 MAPK inhibition on αSMA promoter activation in 4E cells was subsequently examined using the luciferase reporter construct (Fig. 8F). Transfection of RhoA G14V and T19N plasmids had no effect on promoter activation while inhibition of ROCK and Pod1 expression using siRNA resulted in an additive effect on decreasing promoter activity. As expected based on Western analysis, an eightfold increase in promoter activity by TGFβ was reduced to near control levels by treatment with SB202160.

DISCUSSION

Pod1 is expressed in mesenchymal cells adjacent to developing epithelial structures that serve as progenitors for various cells including smooth muscle and kidney interstitial cells. Analysis of chimeric mouse embryos showed that Pod1 is necessary for kidney mesenchymal cells to differentiate into interstitial cells and pericytes that are responsible for extracellular matrix and cytokine/chemokine production that support adjacent tubules [Cui et al., 2003]. During development, these cells provide the extracellular framework necessary for tubular growth and branching, a function attributed to myofibroblasts [Powell et al., 1999]. The focus of the present study was to determine the role of Pod1 in mesenchymal cell myofibroblast differentiation. The results presented indicate that Pod1: (1) increases E2A-induced smooth muscle-specific gene promoter activation and expression of smooth muscle-specific contractile proteins characteristic of

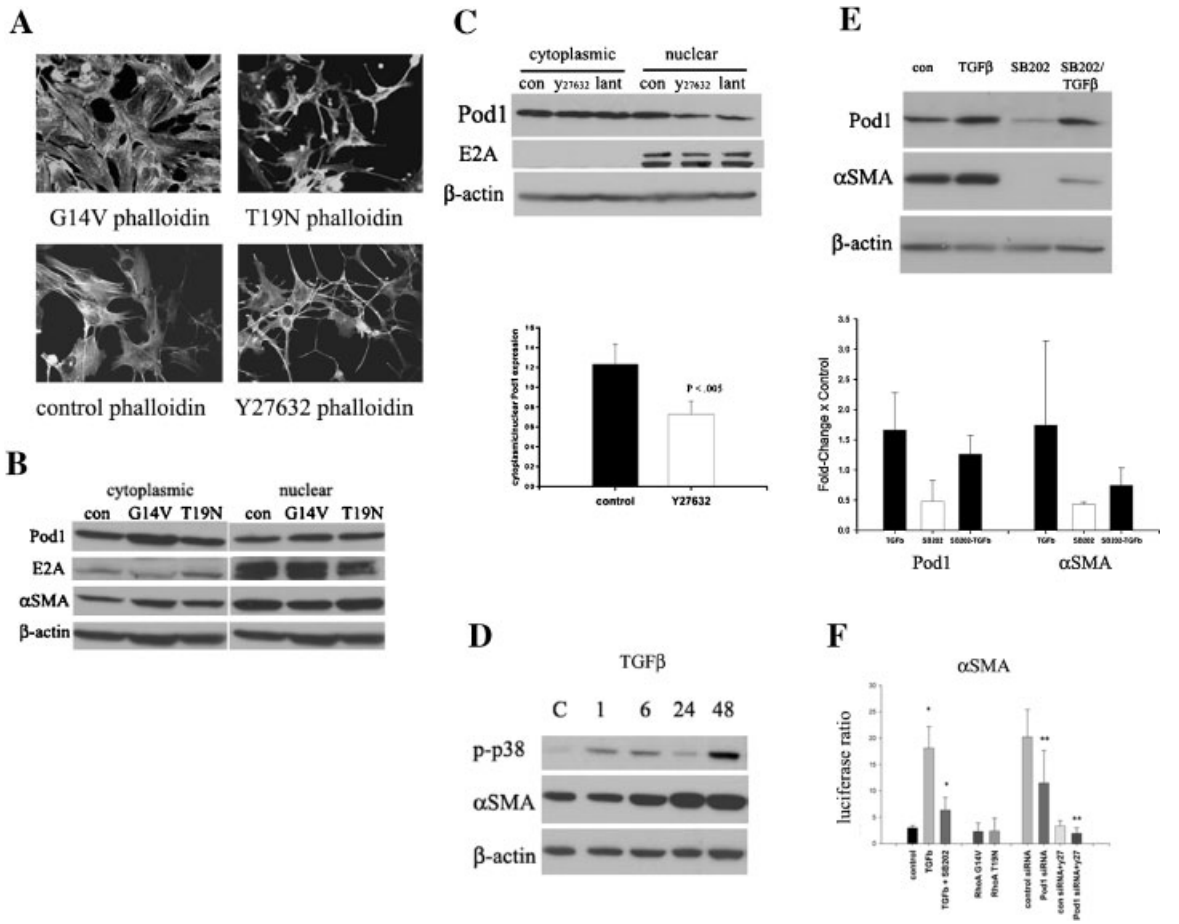

Fig. 8. Pod1 expression is regulated by the p38 MAP kinase pathway and nuclear expression is decreased by disassembly of the actin cytoskeleton. 4E cells were transfected with either constitutively activated RhoA (G14V), dominant negative RhoA (T19N), or control vector minus insert for 48 h and Rho kinase was inhibited with Y27632 (10 μM) for 24 h. **A**: Evaluation of cell morphology and stress fiber formation by phalloidin staining. Original magnification, 400×. **B**: Western blot analysis of E2A, Pod1, and αSMA levels in cytoplasmic and nuclear fractions from transfected cells (con = control vector). **C**: Western blot analysis of Pod1 and E2A levels in cytoplasmic and nuclear fractions from cells treated with Y27632 or latrunculin A (1 μM) for 48 h. Results are mean values plus standard deviation from densitometry scanning of three experiments. **D**: Western blot analysis of phosphorylated-p38 (p-p38) and αSMA expression in cells treated with TGFβ (5 ng/ml) for indicated time points (hours). **E**: Western blot analysis of Pod1 and αSMA levels in control (con), SB202190 (10 μM), TGFβ (5 ng/ml), and TGFβ plus SB202190-treated cells. Bar graph shows mean plus standard deviation of densitometry results of Western blots from three separate experiments. **F**: αSMA promoter–reporter luciferase assay of 4E cells transfected or treated as indicated. Results are mean plus standard deviation from three experiments ($^{*}P < 0.001$, $^{**}P < 0.05$).

activated myofibroblasts; (2) decreases 4E cell proliferation and migration; (3) increases expression of the myofibroblast phenotype including production of contractile stress fibers, extracellular matrix, pro-inflammatory cytokines, and focal adhesions, and (4) expression is regulated by the cytokine-induced p38 MAPK pathway shown to be important for myofibroblast differentiation, suggesting a role in myofibroblast differentiation following activation.

Mesenchymal cells are fibroblast-like cells that arise early in development by transformation of embryonic epithelial cells. These cells proliferate, migrate, secrete growth factors, and produce extracellular matrix that control epithelial growth, differentiation, and morphogenesis including tubular branching. In the adult, myofibroblasts perform a similar function by generation of mechanical force on surrounding matrix and cells by expression of αSMA and secretion of growth factors. Myofibroblasts also secrete various cytokines, chemokines, and inflammatory mediators that are responsible for inflammation and tissue fibrosis

following tissue injury. While myofibroblasts express many of the genes associated with smooth muscle and, to a lesser extent, skeletal muscle differentiation, relatively little is known about the transcriptional mechanisms controlling their differentiation from mesenchymal progenitors.

Muscle differentiation is associated with exit from the cell cycle and expression of myocyte-specific genes along with increased expression of E2A. E2A regulates growth arrest by activating p21, an inhibitor of cyclin-dependent kinases [Peverali et al., 1994]. No E2A is expressed in undifferentiated myoblasts. Heterodimer formation between E2A and the myocyte-specific class II bHLH factors including MyoD allows for the tissue-specific effect of changes in E2A levels in myoblast differentiation. Similar coordination between cell cycle exit and differentiation under control of E2A has also been shown for cells of the osteoblast lineage [Funato et al., 2001]. Despite the presence of E-boxes in the promoters of many smooth muscle-specific genes including those examined in this study, no role for bHLH factors in smooth muscle differentiation has been found with the exception of αSMA [Kumar et al., 2003]. A key role for the MADS box transcription factor, serum response factor (SRF), in smooth muscle cell gene expression and the contractile phenotype including αSMA, sm22α, and calponin has been clearly shown and studies have suggested that this factor may interact with bHLH proteins on smooth muscle promoters [Parmacek, 2001]. Sequence analysis of the Pod1 5′ UTR demonstrates five potential E-box and one SRF-binding sequences, suggesting that Pod1 expression may be regulated by these factors. Although class II bHLH factors including HAND and Pod1 have been shown to be expressed in smooth muscle cells, this is the first study to demonstrate a role for Pod1 in activation of calponin, sm22α, and αSMA gene expression in combination with E2A. A recent study seeking to identify class I bHLH heterodimer proteins using yeast two hybrid screening of smooth muscle libraries found no class II bHLH proteins and found no evidence that Pod1 had any effect on αSMA promoter activation despite in vitro evidence that Pod1 can form a heterodimer with E2A [Kawai-Kowase et al., 2005]. Differences reported in the present study are likely due to the use of progenitor cells with increased plasticity compared with more fully differentiated smooth muscle cultures. In addition to the in vitro results presented above, a mammalian cell two-hybrid assay by Funato et al. (2003), support a role for Pod1-E2A interaction in vivo.

The transcriptional activity of E2A is repressed by dimerization with HLH negative regulators including the Id (inhibitor of differentiation) proteins that lack a basic amino acid binding sequence, and class II bHLH factors Twist and MyoR resulting in inhibition of differentiation and cell proliferation in multiple tissues including muscle and bone. E2A and SRF activation of the αSMA promoter was inhibited by Id and Twist, HLH factors with increased expression following vascular injury. The adult kidney and 4E cells used in this study express Id proteins (data not shown). By forming heterodimers with Id proteins, overexpression of Pod1 alone with resulting increased levels of unbound E2A may result in increased E2A activity as demonstrated in this study in experiments showing increased p21, CTGF, and fibronectin expression. In addition, our results demonstrate that overexpression of Pod1 and E2A result in higher levels of E2A compared with overexpression of E2A alone, an effect that may be due to decreased E2A degradation as shown with other HLH factors [Lingbeck et al., 2005]. Control of E2A levels has been most clearly shown at the post-translational level by phosphorylation and rapid degradation by the ubiquitin-proteasome pathway [Nie et al., 2003]. Whether myofibroblast-associated gene expression is a direct result of Pod1–E2A promoter activation or an indirect effect of Pod1 on other regulatory proteins including Id's, SRF, CTGF, or Smads remains unknown.

In response to TGFβ and the resulting increase in CTGF expression, fibroblasts exit the cell cycle and express αSMA and increased matrix production characteristic of myofibroblasts, a process that has also been shown to depend on IGF-2 [Grotendorst et al., 2004]. Results from this study suggest that increased Pod1 expression is also involved in myofibroblast differentiation through its control of cell proliferation and expression of CTGF. Since CTGF induces fibronectin and other myofibroblast-associated proteins, it is possible that many of the observed Pod1 effects on 4E cells are due to increased levels of this protein. The mechanisms by which Pod1 regulates CTGF

expression are not known. CTGF transcription in smooth muscle and fibroblasts is activated by TGFβ through a c-jun-NH_2-terminal kinase (JNK) signaling pathway. The CTGF promoter also contains Smad and TGFβ response elements [Utsugi et al., 2003]. The CTGF promoter, however, does not contain E-box sequences and no role for bHLH factors in CTGF expression has been demonstrated, suggesting that Pod1 may regulate CTGF through a potentially complex alternative mechanism. Since a polymerized actin cytoskeleton is necessary for basal and TGFβ-induced CTGF expression [Heusinger-Ribeiro et al., 2001], an effect of Pod1 through its regulation of stress fiber formation remains a possibility.

Regulation of Pod1 expression appears to correlate with the key regulators of myofibroblast differentiation. In vitro studies of human fibroblasts have shown that TGFβ1 induces myofibroblast differentiation through both the p38 MAPK and JNK pathways. As previously reported [Meyer-Ter-Vehn et al., 2006], we found a biphasic activation pattern of p38 by TGFβ with a sustained level of phospho-p38 at 48 h correlating with increased αSMA and Pod1 expression. Inhibition of the p38 pathway with SB202190 resulted in both decreased TGFβ-induced αSMA and Pod1 expression, with no effect on E2A levels (data not shown), compatible with a possible role for Pod1 in this signaling cascade. p38 MAPK has been shown to regulate transcriptional control of skeletal muscle differentiation through phosphorylation of multiple transcription factors including E2A, MEF2, and NF-κB. Analysis of the Pod1 promoter sequence shows the presence of multiple E2A and NF-κB binding sites, suggesting a potential mechanism for regulation of Pod1 levels.

TGFβ signaling has also been linked to activation of the RhoA signaling pathway and its downstream mediator Rho kinase in multiple cell types including smooth muscle cells and fibroblasts. RhoA signaling has been reported to regulate transcription factors involved in smooth muscle differentiation including SRF and MEF2 in part by controlling nuclear localization of these proteins. Our results suggest that the effect of these signaling pathways on myofibroblast differentiation may be through control of E2A and Pod1 expression and subcellular localization. By immunocytochemistry and Western analysis, Pod1 appears to be located primarily in 4E cell cytoplasm with E2A expression confined to the nucleus. This difference in localization raises the possibility that regulation of Pod1-E2A binding may occur through control of Pod1 nuclear translocation or binding of E2A to Pod1 in the cytoplasm. Expression of dominant negative RhoA resulted in decreased nuclear E2A levels but had no effect on Pod1. In contrast, disruption of stress fiber formation resulted in decreased nuclear Pod1 levels with no effect on E2A. Since mechanical stress and resulting stress fiber formation induce myofibroblast differentiation, these results suggest that cytoskeleton-induced Pod1 nuclear translocation may, in part, control this process. The differential regulation of E2A and Pod1 by these pathways may be important for controlling the divergent gene expression of myofibroblasts and smooth muscle cell from a common progenitor cell.

In response to injury, an initial decrease in E2A would promote interstitial cell proliferation and migration. Results from the scratch migration assays demonstrated decreased 4E cell migration with overexpression of E2A. Preliminary results using E2A overexpression have demonstrated decreased filipodia formation that could account for the observed effects on cell migration (data not shown). Increased Pod1 expression would then promote myofibroblast differentiation, and production of extracellular matrix, and pro-fibrotic cytokines. By immunohistochemistry, E2A was detected in human embryonic kidney in developing glomeruli, tubules, and mesenchymal cells within the interstitium [Rutherford and LeBrun, 1998] where it has been thought to regulate cell proliferation and differentiation. The localization and function of E2A in the adult kidney remains unknown but may have the same role following injury as during development. Time course studies of changes in interstitial morphology after various causes of acute kidney injury including severe hypoxia and infarction have all noted increased interstitial cell infiltration at 3 days post injury followed by the persistence of αSMA-positive cells at 7–14 days [Sun et al., 2000; Rosenberger et al., 2003]. Results presented from in vitro studies of 4E cells support this sequence of events. The potential for targeting expression of the transcription factors to inhibit myofibroblast formation to treat or prevent interstitial fibrosis may be of interest for future studies.

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