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PAI-1 mediates TGF-β1 induced myofibroblast activation in tenocytes via mTOR signaling

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Abstract

Transforming growth factor-beta (TGF-β1) induces Plasminogen Activator Inhibitor 1 (PAI-1) to effect fibrotic pathologies in several organs including tendon. Recent data implicated PAI-1 with inhibition of PTEN suggesting that PAI-1 induced adhesions involves PI3K/AKT/mTOR signaling. Ergo, we investigated effects of TGF-β1, PAI-1 and mTOR signaling crosstalk on myofibroblast activation, senescence, and proliferation in primary flexor tenocytes from wildtype (WT) and PAI-1 knockout (KO) mice. PAI-1 deletion blunted TGF-β1 induced myofibroblast activation in murine flexor tenocytes and increased the gene expression of *Mmp2* to confer protective effects against fibrosis. While TGF-β1 significantly reduced phosphorylation of PTEN in WT cells, PAI-1 deletion rescued the activation of PTEN. Despite that, there were no differences in TGF-β1 induced activation of mTOR signaling (AKT, 4EBP1 and P70S6K) in WT or KO tenocytes. Phenotypic changes in distinct populations of WT or KO tenocytes exhibiting high or low mTOR activity were then examined. TGF-β1 increased αSMA abundance in WT cells exhibiting high mTOR activity, but this increase was blunted in KO cells exhibiting high 4EBP1 activity but not in cells exhibiting high S6 activity. DNA damage (γH2AX) was increased with TGF-β1 treatment in WT tenocytes but was blunted in KO cells exhibiting high mTOR activity. Increased mTOR activity enhanced proliferation (Ki67) in both WT and KO tenocytes. These findings point to a complex nexus of TGF-β1, PAI-1, and mTOR signaling in regulating proliferation, myofibroblast differentiation, and senescence in tenocytes, which could define therapeutic targets for chronic tendon adhesions and other fibrotic pathologies.

Graphical Abstract

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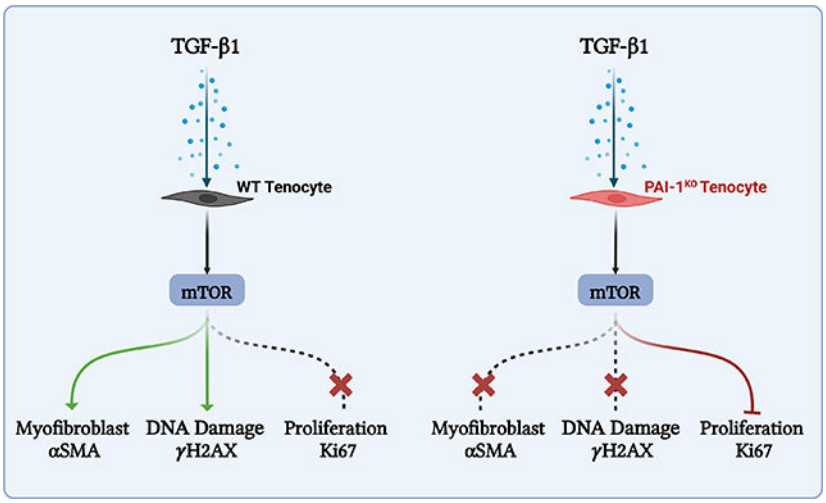

Keywords

Flexor Tendon; TGF-β1; PAI-1; mTOR; Fibrosis

INTRODUCTION

Tendons play the crucial and adaptable role of transmitting mechanical forces and facilitating joint movement by connecting muscles to bones. However, work, sport, trauma, aging and overuse in tendons lead to acute or degenerative injury and morbidity with the associated pain and prolonged rehabilitation. As a result, tendon pathologies account for nearly 50% of all self-reported medical conditions in the USA,[1; 2] while existing medical and surgical treatments often only restore only up to 70% of preinjury tendon strength, leaving the tissue susceptible to recurring injury.[2; 3] Scarring or fibrosis, which encompass a complex set of cellular and molecular responses, represents a clinical challenge. When a tissue is damaged, it undergoes a thrombin-mediated coagulation cascade to provide an impermanent fibrin extracellular matrix to stage the repair process. Plasmin is then activated to breakdown the fibrin clot through fibrinolysis, where the clot is replaced by an immature, often vascular collagenous matrix. Dysregulation of this healing response results in the abundant deposition of extracellular matrix and activation of degenerative inflammatory pathways that suppress tissue remodeling and lead to scar formation. Through complex and multifactorial processes, it is generally accepted that fibrogenesis is a key driver of major organ dysfunction[4], cancers[5], inflammatory diseases[6], and metabolic disorders[7], as well as flexor tendon adhesions[8]. These processes are regulated by various cytokines and growth factors including Transforming Growth Factor beta (TGF-β), which upregulates the protease inhibitor Plasminogen Activator Inhibitor-1 (PAI-1).

Interestingly, while TGF-β signaling is indispensable for the formation of tendons[9–12] it is also implicated in conferring the fibrotic pathology during injury healing.[13; 14] TGF-βs are associated with fibrosis in renal, cardiac, lung, and skin through downstream myofibroblast activation, excessive ECM production and inhibition of ECM turnover.[15–21] While TGF-β2 and TGF-β3 isoforms have also been shown to be elevated in fibrotic diseases, TGF-β1

is recognized as a putative driver of tissue fibrosis.[21–24] Numerous studies have correlated endogenous or exogenous TGF-β1 to promoting myofibroblast differentiation, abundant extracellular matrix deposition and the recruitment of inflammatory cells.[18–21] Furthermore, inhibition of TGF-β1 significantly reduced peritendinous fibrotic adhesions following flexor tendon injury, resulting in improved flexion range of motion.[18; 25; 26]

Our group and others have demonstrated that TGF-β1 upregulates tendon matrix synthesis and alpha smooth muscle actin (α-SMA) gene (*Acta2*) expression in mouse tenocytes *in vitro* and inhibits the activity of matrix metalloproteases (MMPs) by upregulating PAI-1. *In vivo*, PAI-1, a suppressor of fibrinolysis and protease activity, is ubiquitously secreted downstream of TGF-β1 and highly upregulated in injured flexor tendons.[3] Furthermore, PAI-1 knockout (PAI-1^{KO}) mice exhibit reduced adhesion formation and accelerated recovery of injured tendon mechanical properties through reduced plasmin-mediated MMP activity compared to wild type littermates.[3; 16; 27]

In addition to its functions as fibrinolysis and protease inhibitor, PAI-1 plays a central role in age-related and fibrotic pathologies through vascular inflammatory pathways.[28; 29] In a previous study, we demonstrated that genetic knockdown (PAI-1^{KO}) and therapeutic siRNA inhibition of PAI-1 reduces flexor tendon adhesions without compromising the tensile properties in mice.[27] Subsequently, bulk tissue RNA sequencing of injured wild type (C57Bl/6J) and PAI-1^{KO} mouse flexor tendons identified not only a plethora of inflammatory pathways that were downregulated in PAI-1^{KO} injured tendons, but also the activation of phosphatase and tensin homolog (PTEN) signaling and inhibition of FOXO-associated biological processes in PAI-1^{KO} injured tendons compared to wild type littermates.[30] PTEN, a negative regulator of the PI3K/AKT/mTOR pathway, functions to regulate fundamental cellular processes, including protein synthesis, cell cycle progression, proliferation, growth, autophagy, and senescence. [31; 32] Suppression of PTEN activity has also been coupled with induced fibrosis and senescence in disease models of cancers, organ fibrosis and respiratory distress syndromes.[3; 30] Furthermore, mTOR signaling has been implicated in the progression of pulmonary, cardiac, renal and tendon fibrogenesis through the induction of TGF-β1.[33–35] These observations suggest a link between PAI-1 and mTOR signaling in tendon fibrosis. Thus, we set out to test the hypothesis that *PAI-1 mediates TGF-β1-induced myofibroblast activation in murine tenocytes through regulation of mTOR signaling.*

MATERIALS AND METHODS

Cell Isolation, Culture, and Treatment

Primary murine tenocytes were isolated from digital flexor tendons (DFT) from WT (C57Bl/6J) or PAI-1^{KO} (B6.129S2-Serpine1^{tm1Mlg}/J) mice (male, 20–30 weeks, Jackson Laboratory) in compliance with protocols approved by the University of Rochester Committee on Animal Research (UCAR). The isolated tendon tissues were immersed in alpha minimum essential medium (αMEM) supplemented with 10% Pen-Strep (10,000 U/mL penicillin/10 mg/mL streptomycin) and cut into 1-mm^3 pieces using ophthalmic scissors. After mechanical digestion, the tissue was transferred to an enzyme solution consisting of 2.5mg/ml of Collagenase D (Millipore Sigma), 3mg/ml of Dispase II

(Millipore Sigma), and 1mg/ml of DNase (New England Biolabs) dissolved in αMEM. Tissues in enzyme solution were placed in a Roto-Therm™ Plus Incubated Rotator (Benchmark Scientific) at 37°C while rotating in combination with oscillations for an hour. αMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% Pen-Strep was added to enzyme solution at a 2:1 ratio to inactivate proteases. The digested tissue solution was strained through a 70-micron filter, centrifuged, and cells resuspended in αMEM supplemented with 10% FBS, 1% Pen-Strep, and 55 μM 2-Mercaptoethanol (ThermoFisher Scientific). The isolated tendon cells were seeded in a T-75 flask (Corning) coated with 1 μg/cm^2 Fibronectin (Sigma-Aldrich). Cell culture was performed under standard conditions (37°C, 5% CO_2, 95% humidity) with a media change 4 days post seeding and then, every other day until ~80% confluent. Cells were passaged at a 1:4 split using 0.25% trypsin/0.02% EDTA solution (Sigma-Aldrich) and after passage 1, the cells were cryopreserved in Recovery™ Cell Culture Freezing Medium (ThermoFisher Scientific) for future experiments.

Unless otherwise stated, all cells used in this study and the following assays were used at passage 2. At the start of P2, tenocytes were seeded at ~10,000 cells/cm^2 on day 0 in α-MEM media supplemented with 10% FBS. On day 1, the media was replaced with α-MEM media supplemented with 0.1% FBS to induce serum starvation for 6 hours. The media was then switched to 1% FBS with or without TGF-β1 (10 ng/ml) for 24 hours. A commercial Mouse PAI-1 total antigen assay ELISA kit (Molecular Innovations) was used to quantify secreted PAI-1 protein in the culture media.

Cell Proliferation

The Celigo imaging cytometer (Nexcelom Bioscience) was used to track cell growth between WT and PAI-1^{KO} tenocytes. Cells were seeded at 2000 cells/well of a 96-well flat-bottom microplate and allowed to grow for 10 days. The cells were stained with a NUCLEAR-ID Red DNA stain (Enzo Life Sciences) and imaged daily. The data was analyzed in Prism for growth curve analysis under each treatment condition. The Population doubling time (PDT) was calculated using the formula $PDT = \Delta t \times \ln(2)/(\ln N_t - \ln N_0)$, where Δt is the duration of exponential phase in hours, and N_0 and N_t are the respective numbers of cells at the beginning and end (144 hrs) of the culture period.

Immunofluorescence

Tenocytes were fixed with 4% paraformaldehyde in PBS for 15 mins RT followed by permeabilization for 10 min with 0.5% Triton X-100. Nonspecific binding was blocked in 1% BSA with TBST for 1-hour 4°C shaking. The fixed cells were incubated with the following antibodies overnight at 4°C: anti-Actin, α-Smooth Muscle-FITC (Millipore Sigma), phospho-Histone H2A.X (Ser139) (CST), and anti-Ki67 (Abcam). The cells were then washed in PBS before being incubated with Goat anti-Rabbit IgG (H+L) secondary antibodies (ThermoFisher Scientific) for 2-hours at room temperature. The cells were washed again, counterstained with Hoechst 33342 (ThermoFisher Scientific) nuclear stain and photographed with a fluorescence microscope (EVOS M5000).

RNA Extraction and RT-PCR analysis

Tenocytes were lysed in TRIzol reagent (Invitrogen) for RNA isolation and purified using Zymo Research Direct-Zol™ mini columns (Zymo Research). Quantitative real-time PCR analysis (qPCR) was performed via standard methods using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) and PerfeCTa SYBR® Green SuperMix (Quantabio). qPCR was carried out with specific primers for genes of interest (Table 1), and expression was normalized to β-actin. All experiments were done in biological triplicates at minimum.

Protein Extraction and Western Blotting

Tenocytes were lysed in ice-cold Cell Lysis Buffer (Cell Signaling Technology) supplemented with the non-specific serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Cell Signaling Technology). Protein concentrations were determined and normalized using a BCA protein assay kit (ThermoFisher Scientific). Following SDS PAGE with 4X Laemmli Sample Buffer (Bio-Rad) and gel to membrane transfer, the membrane was blocked for 1-hour 4°C in Blotting-Grade Blocker (Bio-Rad) and tris-buffered saline with 0.1% Tween (TBST). Primary antibodies were incubated overnight in Blotting-Grade Blocker with TBST or 5% BSA (Cell Signaling Technology) with TBST for total antibodies or phosphorylated antibodies, respectively (Table 2). Reactions were detected using the appropriate HRP-conjugated secondary antibodies and the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific). Visualization of membranes was performed using Bio-Rad Image Lab software and normalized to β-actin. All quantifications are represented as fold change against untreated WT.

Flow Cytometry

Tenocytes were trypsinized and suspended in a FACS buffer and stained using the Cyto-Fast™ Fix/Perm Buffer Set (BioLegend) intracellular antigen detection kit. The permeabilization kit was used according to the recommendations of the manufacturer. The intracellular antibody panel included Anti-AKT (T308) AF647 (BD Biosciences), phospho-4EBP1 (Thr36, Thr45) PE (Invitrogen), phospho-S6 (Ser235, Ser236) PerCP-eFluor 710 (Thermo), anti-Ki-67 V450 (BD Biosciences), anti-H2AX (pS139) PE/Cy7 (BioLegend), and anti-αSMA AF488 (Novus Biologicals). For each sample, at least 20,000 events were recorded, run on the BD LSR II flow cytometer and analyzed using FCS Express.

Statistical Analysis

All data were graphed as mean ± SEM, and statistically analyzed with GraphPad Prism 7.0. Significant differences ($p<0.05$) for all data were determined using a 2-way ANOVA and Bonferroni-corrected multiple comparison post-tests.

RESULTS

PAI-1 deletion suppresses TGF-β1 activated myofibroblast differentiation

We set out to investigate whether TGF-β1 induced PAI-1 regulates mTOR signaling and downstream effectors of fibrosis in primary tenocytes from WT and PAI-1^{KO} mice. First, we investigated if there are differences in the morphology or proliferation rates of tenocytes from WT and PAI-1^{KO} mice. We observed no genotype differences in the yields of tenocytes from the explant outgrowth assays, and further observed the cell morphology to become increasingly more uniform across passages between the WT and PAI-1^{KO} cells (Fig 1A). We measured the proliferative behavior of the primary tenocytes during the logistic growth period (Days 1–4) and observed a slight increase in doubling time of the PAI-1^{KO} cells (128.04 ± 8.29 hrs) relative to WT (118.96 ± 2.04 hrs), but these differences were not statistically significant (Fig 1B). As expected, treatment with 10 ng/ml TGF-β1 for 24 hours significantly upregulated secreted PAI-1 in the culture media of WT tenocytes, whereas PAI-1 was undetectable in culture media of PAI-1^{KO} tenocytes (Fig. 1C).

We then investigated the role of PAI-1 in conferring the myofibroblast phenotype in primary tenocytes using immunofluorescence and qPCR. In WT tenocytes, treatment with 10ng/ml TGF-β1 upregulated αSMA protein levels by nearly 30%, albeit these increases were not significant due to the high basal levels of αSMA in the absence of TGF-β1 (Fig. 2A,B). Similarly, TGF-β1 almost doubled *Acta2* gene expression in WT tenocytes (Fig. 2C). In contrast, TGF-β1 treatment did not significantly increase cytoplasmic αSMA protein or *Acta2* gene expression in PAI-1^{KO} tenocytes. Moreover, the loss of PAI-1 significantly blunted αSMA protein and gene expression regardless of TGF-β1 treatment (Fig 2B,C).

PAI-1 deletion modulates TGF-β1 effects on ECM synthesis, remodeling, and cell cycle

Next, we sought to elucidate the effects TGF-β1 and PAI-1 on gene expression of collagenous ECM, matrix metalloproteinases, and cell cycle regulators. With respect to ECM synthesis (Fig. 3A), we observed that TGF-β1 upregulated gene expression of *Col1a1* in WT and PAI-1^{KO} tenocytes. However, while there were no significant differences in *Col3a1* expression in WT tenocytes in response to TGF-β1, loss of PAI-1 decreased *Col3a1* expression in untreated PAI-1^{KO} cells, which was significantly increased in response to TGF-β1 treatment. Similar trends were observed for *Serpinh1,* which encodes the gene for heat shock protein 47 (HSP47) but the TGF-β1 effects were not significant in both WT and PAI-1^{KO} cells. As for ECM remodeling (Fig. 3B), TGF-β1 treatment resulted in a significant decrease in gene expression of *Mmp-2*, *-3*, and *-9* in WT tenocytes. Similar trends of TGF-β1 effects were observed in the gene expression of *Mmp3* and *Mmp9* in PAI-1^{KO} cells, although these were not significant. In contrast, gene expression of *Mmp-2* in untreated PAI-1^{KO} cells was significantly lower than untreated WT cells, and treatment with TGF-β1 resulted in a slight increase in PAI-1^{KO} cells. Moreover, TGF-β1 treatment downregulated expression of cell cycle genes (*Tp53*, *Cdkn2a*, and *Rb*) in WT cells (Fig. 3C). However, loss of PAI-1 resulted in lower baseline level of expression of these genes in untreated PAI-1^{KO} cells, and further blunted the effects of TGF-β1 treatment in PAI-1^{KO} cells.

PAI-1 deletion blunts TGF-β1 attenuation of PTEN activity

Since we have shown in previous work that transcriptional pathways associated with activation of Phosphatase and Tensin Homolog (PTEN) were enriched in PAI-1^{KO} injured tendons,[30] we sought to examine differences in mTOR signaling in WT and PAI-1^{KO} tenocytes in response to TGF-β1 treatment. PTEN is a tumor suppressor and master negative regulator of the PI3K/AKT/mTOR pathway and mediator of protection against fibrosis [36]. We first investigated the temporal kinetics of mTOR signaling in response to TGF-β1 treatment. Western blot analysis of WT tendon cells showed the most notable upregulation of phosphorylated mTOR proteins, AKT and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) in cultures treated with TGF-β1 for 24 hours (data not shown), which was consistent with published reports.[32]

WT and PAI-1^{KO} tenocytes cultured in 1% FBS with 0 or 10 ng/ml TGF-β1 (10 ng/ml) for 24 hours were lysed to extract cellular proteins and perform Western blot analysis. Canonical TGF-β1 signaling was active at 24 hrs as evident by elevated phosphorylated SMAD3 (pSMAD3) proteins in both WT and PAI-1^{KO} tenocytes (Fig. 4B,C). We observed phosphorylated PTEN (pPTEN) to be significantly decreased upon TGF-β1 treatment in WT tenocytes. Interestingly, TGF-β1-induced inhibition of pPTEN is significantly attenuated in PAI-1^{KO} when compared to WT. The results confirm that in primary tenocytes, PAI-1 plays a pivotal role in negatively regulating PTEN activation. We next investigated the mTOR signaling nodes in response to TGF-β1 treatment. Western blot analysis indicated TGF-β1 treatment increased phosphorylation of AKT (Thr308 and Ser473), 4EBP1 and P70S6K proteins in both WT and PAI-1^{KO} tenocytes (Fig. 4B,C). Together, these data indicate that loss of PAI-1 did not significantly influence TGF-β1 induced mTORC1 signaling despite rescuing phosphorylated PTEN activation.

TGF-β1/PAI-1 regulates distinct mTOR-active signaling populations to confer fibrosis

To further explore the fibrotic sequelae of the TGF-β1, PAI-1, and mTOR signaling nexus, flow cytometry was used to quantitatively assess differences in myofibroblast differentiation (αSMA), proliferation (Ki67), and senescence (γH2AX) as a function of mTOR signaling dynamics. WT and PAI-1^{KO} tenocytes cultured in 1% FBS with 0 or 10 ng/ml TGF-β1 for 24 hours were fixed, permeabilized and stained with a panel of 6 antibodies (αSMA, γH2AX, Ki67, pAKT, p4EBP1, pS6). On average, TGF-β1 confers a senescent myofibroblast phenotype (increased αSMA and γH2AX) that is slightly amplified in WT cells compared PAI-1^{KO} cells (Fig. 5A and B), but there were no discernable effects of TGF-β1 on cell proliferation (Ki67) in either WT or PAI-1^{KO} cells (Fig. 5C). We next applied a gating strategy to define two populations of tenocytes, after excluding debris and aggregates, based on the phosphorylation state of AKT & 4EBP1 or AKT & S6, which we reasoned to signify mTOR activity (Fig. 5D). Downstream of AKT, 4EBP1 and S6 were analyzed separately for their differential downstream effects on the fibrotic phenotype. We identified 2 populations to signify high mTOR activity based on the highest intensities (> median fluorescence intensity or MFI), pAKT^{Hi}/p4EBP1^{Hi} and pAKT^{Hi}/pS6^{Hi}. We identified a second set of populations to define cells with low activity of mTOR signaling based on the lowest intensities (< MFI), pAKT^{Lo}/p4EBP1^{Lo} and pAKT^{Lo}/pS6^{Lo}. (Fig. 5D)

We then evaluated the fibrotic traits of these subsets of WT and PAI-1^{KO} tenocytes in response to TGF-β1 treatment based on the MFI of the distributions of αSMA, γH2AX, and Ki67 within each population. In WT cells, treatment with TGF-β1 increased αSMA and γH2AX but did not affect Ki67 regardless of pAKT/p4EBP1 activity, but the effect on αSMA was significantly elevated in $pAKT^{Hi}$/$p4EBP1^{Hi}$ cells (Fig. 5E–G). While TGF-β1 treatment of PAI-1^{KO} cells increased αSMA and γH2AX in $pAKT^{Lo}$/$p4EBP1^{Lo}$ cells, it decreased Ki67 regardless of mTOR activity. While TGF-β1 treatment induced minimal effects of Ki67, PAI-1^{KO} cells demonstrate a markedly inhibitory effect in response to TGF-β1 (Fig. 5J).

Similarly, treatment of WT cells with TGF-β1 increased αSMA, γH2AX, and Ki67 in but did not affect Ki67 in $pAKT^{Hi}$/$pS6^{Hi}$ cells (Fig. 5H–J). The effects of TGF-β1 treatment in PAI-1^{KO} cells were similar to WT cells in terms of αSMA and Ki67 abundance in $pAKT^{Hi}$/$pS6^{Hi}$ cells. However, loss of PAI-1 attenuated γH2AX in $pAKT^{Hi}$/$p4EBP1^{Hi}$ cells regardless of TGF-β1 treatment.

DISCUSSION

We previously reported that TGF-β1 precipitates peritendinous fibrovascular adhesions following flexor tendon injuries in mice by upregulating PAI-1[27]; both of which are putative senescence markers. Using transgenic mouse models, we have shown macrophage infiltration and TGF-β1 secretion to the injury site [37], which is associated with the differentiation of tenocytes to an α-SMA^{+} myofibroblast phenotype. We also identified the activation of PTEN signaling and the inhibition of FOXO as unique transcriptional signatures in the PAI-1^{KO} mice that distinguishes their accelerated tendon healing response from wild type mice.[30] PTEN, a tumor suppressor gene, is a potent inhibitor of the phosphoinositide 3-kinase (PI3K) and of the mammalian target of rapamycin (mTOR) pathway, which has been linked to major organ fibrosis and senescence.[38; 39] These findings suggested a mechanistic nexus between TGF-β1, PAI-1 and mTOR signaling, which remains largely unexplored in peritendinous adhesions in mice.[30] Therefore, in this study we set out to investigate the crosstalk between TGF-β1, PAI-1 and mTOR signaling on myofibroblast activation and senescence in flexor tenocytes. Here, we demonstrated that PAI-1 deletion attenuates TGF-β1 induced myofibroblast activation in murine flexor tenocytes, which is consistent with prior reports in lung fibroblasts.[40] We also demonstrated for the first time that PAI-1 deletion rescues activation of PTEN in tenocytes, even upon treatment with TGF-β1. However, contrary to our hypothesis, we didn't observe global differences in TGF-β1 induced activation of upstream (AKT) and downstream (4EBP1 and P70S6K) mTOR signaling nodes in WT and PAI-1^{KO} tenocytes. This might suggest unknown compensatory mechanisms involving other isoforms of plasminogen activator inhibitor, as it was reported that PAI-2 overexpression supports bladder cancer development in PAI-1 knockout mice.[41] However, we observed high mTOR activity in WT tenocytes to be associated with increased myofibroblast activation, proliferation and DNA damage. Furthermore, PAI-1 deletion blunted the mTOR activity associated proliferative myofibroblast phenotype.

Scar-mediated tendon healing leads to excessive accumulation of collagen and aberrant ECM turnover. As tenocytes function as collagen-producing cells that make up the majority

of cells in a healthy tendon, we probed for the ECM proteins and proteinase components associated with fibrosis. It is unsurprising that our results demonstrated significantly higher expression of *Col1a1* mRNA with TGF-β1. In contrast, we did not observe a TGF-β1-induced increase with *Col3a1* or *Serpinh1* mRNA levels in WT tenocytes. This is consistent with reports of unaltered *Col3a1* mRNA levels in TGF-β1 treated human skin and lung fibroblasts.[42; 43] *Serpinh1* encodes Hsp47 protein, which functions as a collagen-specific chaperone that facilitates collagen folding and synthesis, and is recognized as a fibrotic marker. HSP47 protein or *Serpinh1* mRNA inhibition has been proven successful to reduce fibrosis in preclinical models of fibrosis.[44] Interestingly, *Col3a1* and *Serpinh1* mRNA levels in PAI-1^{KO} cells are lower compared to WT, but both were increased by TGF-β1 treatment.

TGF-β1 induced PAI-1 acts as a master regulator of plasmin activity, thereby regulating fibrinolysis and plasmin-mediated activation of matrix metalloproteinases.[45] MMP expression is also regulated by various cytokines and signaling molecules. Not surprisingly, the expression levels of *Mmp2*, *Mmp3* and *Mmp9* were significantly reduced in WT tenocytes in response to TGF-β1. Similar reductions were observed for *Mmp3* and *Mmp9* in PAI-1^{KO} cells upon treatment with TGF-β1, but *Mmp2* expression was increased in TGF-β1 treated PAI-1^{KO} cells. Therefore, MMP2 likely acts as a critical proteinase influenced by the TGF-β1/PAI-1 axis to regulate tendon matrix turnover.

Reduced PTEN signaling has been associated with mTOR-dependent induction of cellular senescence and fibrosis.[38; 39] shRNA inhibition of PAI-1 was reported to rescue the PTEN loss-associated renal fibrosis.[46] Our results demonstrated phosphorylated PTEN to be significantly downregulated in response to TGF-β1 treatment in WT cells but to a lesser extent in PAI-1^{KO} cells. While the mechanism of action is not fully understood, PAI-1, SMAD4, PKCα, and NF-κB have all been shown to mediate TGF-β1 induced transcriptional downregulation of PTEN.[47–49] Since PAI-1^{KO} cells only partially mitigated the PTEN response, the aforementioned signaling proteins hypothetically utilize PAI-1 independent or dependent pathways to regulate on PTEN.

PTEN exert its fibrosis-protective effects through the PI3K-AKT-mTOR signaling pathway to influence fibroblast proliferative responses, induce myofibroblast differentiation and regulate collagen production.[31; 32; 50; 51] However, genetic deletion of PAI-1 did not induce any changes in phosphorylated mTOR proteins (AKT, 4EBP1 and S6). By employing flow cytometry to examine distinct populations of cells exhibiting high or low mTOR activity through the signaling targets, 4EBP1 and S6, we demonstrated that TGF-β1 increases αSMA protein abundance in WT cells particularly exhibiting high mTOR activity. In contrast to $pAKT^{Hi}/pS6^{Hi}$ cells, TGF-β1 effects on αSMA protein abundance were blunted in in $pAKT^{Hi}/p4EBP1^{Hi}$ PAI-1^{KO} cells. These results suggest that the effects of TGF-β1 on myofibroblast activation are dependent on PAI-1 and 4EBP1. Furthermore, TGF-β1 likely utilizes alternate mechanisms to PAI-1 to induce S6-mediated myofibroblast differentiation. PAI-1^{KO} cells appear to host higher levels of baseline DNA damage, and as such, the increase in γH2AX DNA damage in response to TGF-β1 in PAI-1^{KO} cells could be lower than WT cells. As we observe $pAKT^{Lo}/pS6^{Lo}$ cells to confer almost no shifts in γH2AX expression while TGF-β1 induced $pAKT^{Hi}/pS6^{Hi}$ WT cells exhibit high γH2AX, the TGF-β1, PAI-1, mTOR nexus through S6 likely plays a significant role in regulating the

DNA damage response. As for proliferation, high 4EBP1 activity increased the abundance of Ki67, nuclear marker of active cell proliferation, in WT cells independent of TGF-β1. However, genetic deletion of PAI-1 decreased proliferation of tendon cells in response to treatment with TGF-β1 regardless of 4EBP1 activity, which is consistent with reports of PAI-1 promoting the proliferation of lung fibroblasts.[52] In contrast, Ki67 abundance in both WT and PAI-1^{KO} cells was increased in cells exhibiting high S6 activity, which implicates divergent roles for 4EBP1 and S6 in regulating proliferation.

While studies of inhibition of TGF-β1 report reduced fibrosis, this also leads to the formation of more weak tendons due to its multifactorial effects [18; 25]. PAI-1, a downstream target of TGF-β1, plays a crucial role in regulating MMPs, which break down collagen and other ECM components. By increasing MMP activity and reducing fibrosis, PAI-1 inhibition may provide a more specific therapeutic target while avoiding the formation of weaker tendons. We have previously shown that delivering siRNA targeting PAI-1 in injured flexor tendons using nanoparticles led rescue of MMP activity, which suggests that targeting PAI-1 could be an effective approach to treat adhesions and speed up the healing process of flexor tendon injuries [27]. Alternatively, since mTOR inhibitors (e.g. rapamycin and its analogs) are in clinical trials for other fibrotic conditions, including idiopathic pulmonary fibrosis, our findings merit further investigation of the effects mTOR inhibitors on tenocytes in vitro and tendon healing in vivo.

Our study is not without limitations. While in vitro studies in monolayer cultures are commonplace, it is known that fibroblasts grown on rigid polystyrene surfaces acquire a myofibroblast phenotype with extensive passaging.[53] Therefore, we limited the use of our tenocytes to those only expanded through two passages. Our analysis was based on a 24 hour treatment with TGF-β1. However, we performed a temporal analysis of mTOR where we observed mTOR activation in the first 3 hours, which was sustained out to 48 hours post-treatment. As we expanded our scope to analyze mTOR signaling in PAI-1^{KO} cells, we observed minimal effects on TGF-β1-induced mTOR activation at 3-hours, which became evident by increased phosphorylation of Akt (S473) and 4EBP1 (S65) at 24-hours. Woodcock et al. has shown TGF-β1 to induce peak phosphorylation of Akt and 4EBP1 in primary human lung fibroblasts between 12–24 hours.[32] He et al. demonstrated TGF-β1 to induce PAI-1 expression in human proximal tubular epithelial cells in a time-dependent manner with peak expression between 12 and 48 hours.[54] Aligned with the literature, our *in vitro* model using 24 hours of TGF-β1 treatment showed significant activation of myofibroblasts and DNA damage associated with high mTOR activity, which were mitigated with deletion of PAI-1.

Conclusion

Our findings and evidence from the literature suggest that PAI-1 or its deficiency interfere with signaling pathways, including the canonical TGF-β1 and the PI3K/AKT/mTOR pathways, to exert pathogenic or protective effects and is involved in regulating cell survival and death[55], senescence[56], and fibrosis[29; 57]. Our novel findings point to a complex nexus of TGF-β1/PAI-1/mTOR signaling in regulating proliferation and myofibroblast differentiation and senescence in tenocytes *in vitro*, which remains to be explored *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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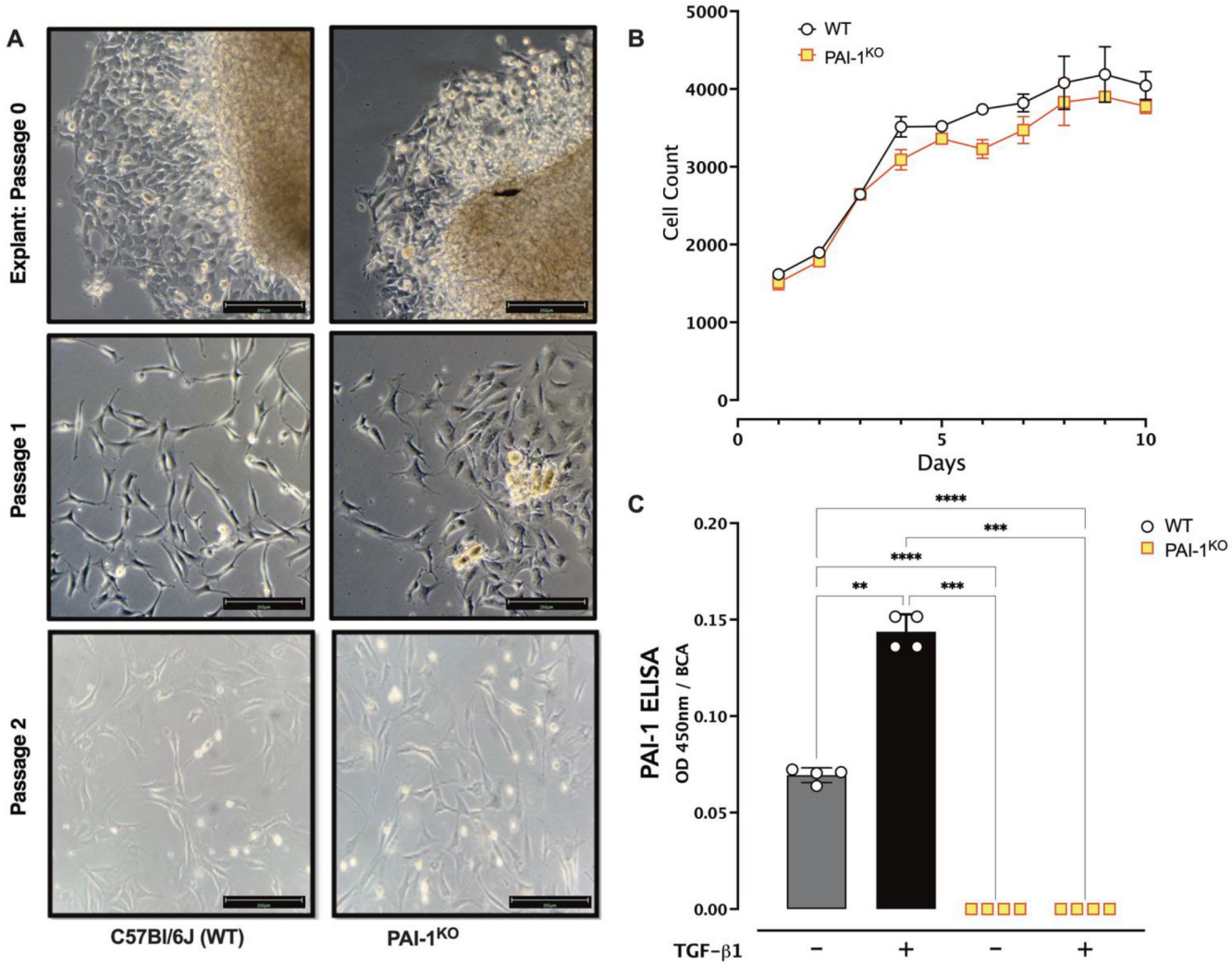

Figure 1. Loss of PAI-1 does not alter morphology and proliferation of murine digital flexor tenocytes.

A) Bright field micrographs of primary tenocyte outgrowth from tendon explants and subsequent passaging reveals no qualitative differences in cell morphology. B) Cell proliferation curves for the WT (black) and PAI-1KO (red) tenocytes plotted over 10 days under 1% FBS low serum conditions (Mean±SD for triplicates per genotype). C) ELISA quantification of PAI-1 protein levels in primary tenocytes without or with TGF-β1 (Mean±SD for quadruplicates per genotype).

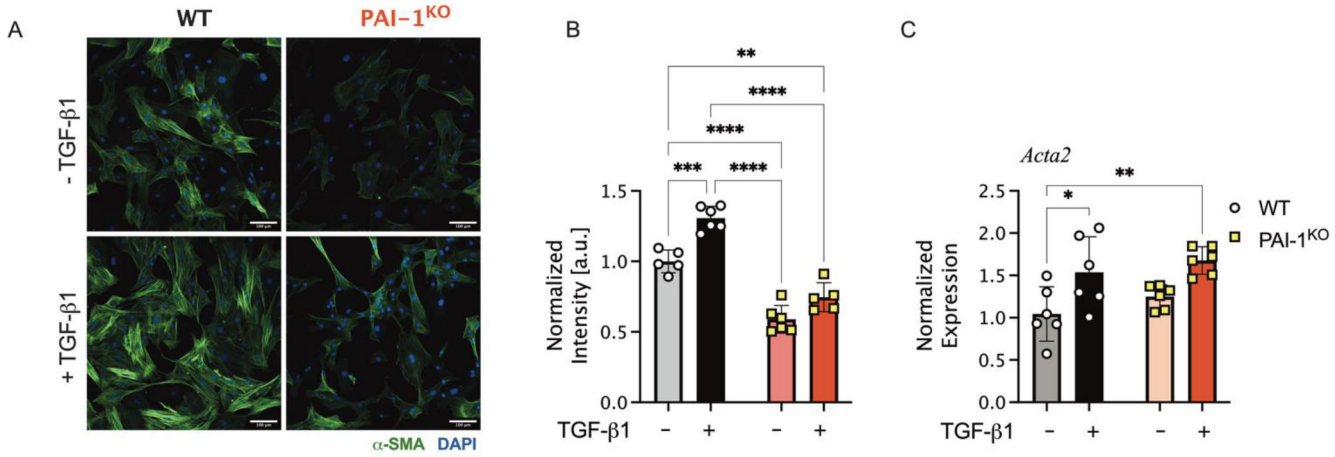

Figure 2: Loss of PAI-1 blunts TGF-β1 activation of α-SMA+ myofibroblasts in murine digital flexor tenocytes.

(A) Representative micrographs of α-SMA-immunostained (WT and PAI-1KO) primary flexor tendon cells treated with TGF-β1 (10 ng/ml) for 24 hours. (B) Quantified α-SMA mean fluorescence normalized to cell count (arbitrary unit). Data represent means and error bars represent standard deviations, n=5–6 replicates. (C) Quantitative real-time RT-PCR analysis of Acta2 gene expression normalized to ActB gen. Data represent means and error bars represent standard deviations, n=6 replicates. Asterisks indicate significant differences inferred from Bonferroni-corrected multiple comparisons following a two-way ANOVA (*=p<0.05, **p<0.005, ***p<0.001, and ****=p<0.0001)

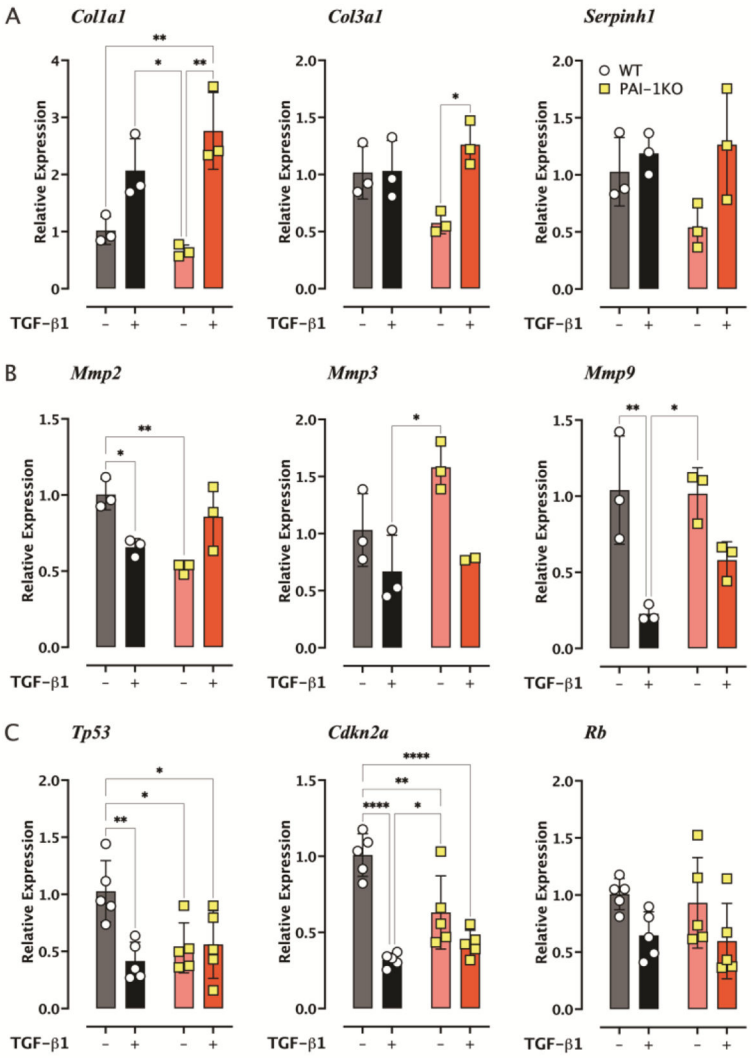

Figure 3: Loss of PAI-1 modulates ECM synthesis and remodeling and cell cycle genes in murine digital flexor tenocytes.

Quantitative real-time RT-PCR analysis of relative expression of: (A) ECM synthesis genes (Col1a1, Col3a1, and Serpinh1). (B) ECM remodeling genes (Mmp2, Mmp3, and Mmp9). (C) cell cycle genes (Tp53, dkn2a, and Rb). Gene expression was normalized to normalized to β actin gene (Actb) and arbitrarily normalized to untreated WT cells. Data represent means and error bars represent standard deviations, n=3–5 pooled replicates. Asterisks indicate significant differences inferred from Bonferroni-corrected multiple comparisons following a two-way ANOVA (*=p<0.05, **=p<0.01, and ****=p<0.0001)

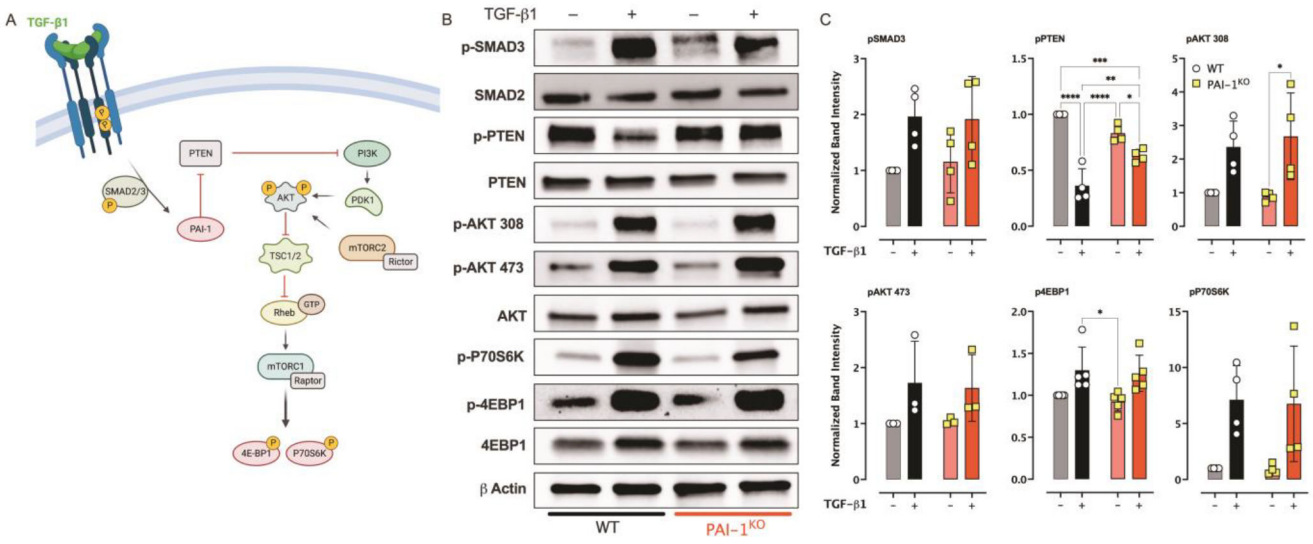

Figure 4: Loss of PAI-1 rescues PTEN activation but does not affect mTORC1 signaling in murine digital flexor tenocytes treated with TGF-β1.

A) schematic representation of the TGF-β1/PAI-1/mTOR signaling axis. B) Representative Western blots of primary mouse flexor tendons (WT & PAI-1KO) stimulated without or with TGF-β1 (10 ng/ml) for 24 hours showing relative abundance and phosphorylation for various nodes in the TGF-β1/PAI-1/mTOR signaling axis. C) Densitometry quantification of phosphorylated proteins normalized to β Actin. Data represent means and error bars represent standard deviations, n=3–5 pooled replicates. Asterisks indicate significant differences inferred from Bonferroni-corrected multiple comparisons following a two-way ANOVA (*=p<0.05, **=p<0.01, and ****=p<0.0001)

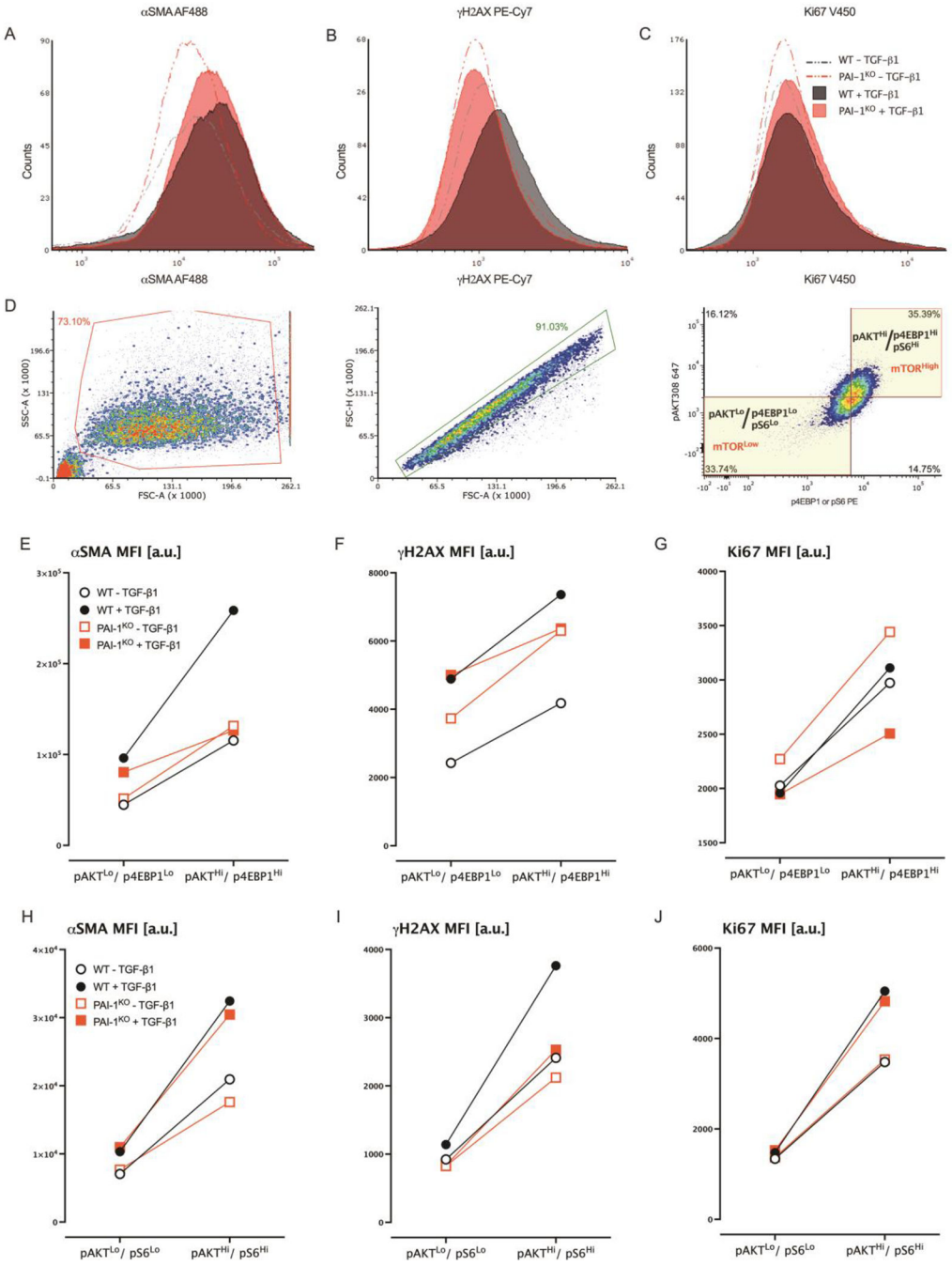

Figure 5: TGF-β1 and mTOR additive effects on increased myofibroblast activation are dependent on PAI-1.

Flow cytometry histogram of A) myofibroblast differentiation (αSMA), B) DNA damage (γH2AX), and C) proliferation (Ki67) in WT and PAI-1KO tenocytes treated with TGF-β1 (10 ng/ml) for 24 hours. D) Gating strategy to define two mTOR activity populations based on the median fluorescence intensity (MFI) for pAKT and p4EBP1 or pS6. Forward and side scatter gating are used to identify the cells of interest with subsequent doublet exclusion. Tenocytes are then gated for either high and low abundance of pAKT/p4EBP1 or pAKT/pS6

cells. MFI values of E,H) αSMA, F,I) γH2AX, and G,J) Ki67 are graphically represented to illustrate the shifts in cellular protein abundance.

Table 1:

Real-Time qPCR Primers

Gene		Sequence (5’ → 3’)
Actb	Fwd	CCTGAGGCTCTTTTCCAGCC
	Rev	TAGAGGTCTTTACGGATGTCAACGT
Col1a1	Fwd	GCTCCTCTTAGGGGCCACT
	Rev	CCACGTCTCACCATTGGGG
Col3a1	Fwd	ACGTAGATGAATTGGGATGCAG
	Rev	GGGTTGGGGCAGTCTAGTG
Serpinh1	Fwd	AAGGCCTGGATGGGAAAGAT
	Rev	CCAGATGTTTCTGCAGGTCATG
Rb	Fwd	AGCAGCCTCAGCCTTCCATACT
	Rev	TGTTCTGGCTCTGGGTGGTCAG
Mmp2	Fwd	CAAGTTCCCCGGCGATGTC
	Rev	TTCTGGTCAAGGTCACCTGTC
Mmp3	Fwd	CAGGAAGATAGCTGAGGACTTT
	Rev	CTGCGAAGATCCACTGAAGAA
Mmp9	Fwd	TGAATCAGCTGGCTTTTGTG
	Rev	ACCTTCCAGTAGGGGCAACT
Bcl-2	Fwd	AGG CTG GGA TGC CTT TGT GG
	Rev	ACT TGT GGC CCA GGT ATG C
Acta2	Fwd	GAGGCACCACTGAACCCTAA
	Rev	CATCTCCAGAGTCCAGCACA

Table 2:

List of Antibodies for Western Blots

Protein	Company	Catalog #
Smad3 (C67H9)	CST	9523S
Smad3 (phospho S423 + S425)	Abcam	Ab52903
Akt (pan) (C67E7)	CST	4691S
Phospho-Akt (Thr308) (C31E5E)	CST	2965S
4EBP1 (53H11)	CST	9644S
Phospho-4EBP1 (Ser65)	CST	9451S
Phospho-P70S6K Ribosomal Protein (Ser235/236)	CST	2211S
β-Actin (13E5)	CST	4970s
GAPDH (14C10)	CST	2118S