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Collagen regulation of *let-7* in pancreatic cancer involves TGF-β1-mediated membrane type 1-matrix metalloproteinase expression

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is associated with a pronounced collagen-rich fibrosis known as desmoplastic reaction; however the role of fibrosis in PDAC is poorly understood. In this report we show that collagen can regulate the tumor suppressive *let-7* family of microRNAs in pancreatic cancer cells. PDAC cells growing in 3D collagen gels repress mature *let-7* without affecting the precursor form of *let-7* in part via increased expression of membrane type 1-matrix metalloproteinase (MT1-MMP, MMP-14) and ERK1/2 activation. PDAC cells in collagen also demonstrate increased TGF-β1 signaling, and blocking TGF-β1 signaling attenuated collagen-induced MT1-MMP expression, ERK1/2 activation, and repression of *let-7* levels. Although MT1-MMP overexpression was not sufficient to inhibit *let-7* on 2D tissue culture plastic, overexpression of MT1-MMP in PDAC cells embedded in 3D collagen gels or grown *in vivo* repressed *let-7* levels. Importantly, MT1-MMP expression significantly correlated with decreased levels of *let-7* in human PDAC tumor specimens. Overall, our study emphasizes the interplay between the key proteinase MT1-MMP and its substrate type I collagen in modulating microRNA expression, and identifies an additional mechanism by which fibrosis may contribute to PDAC progression.

Keywords

TGF-β1; collagen; ERK1/2; MT1-MMP; let-7; fibrosis

Human pancreatic ductal adenocarcinoma (PDAC) is frequently associated with an intense area of fibrosis known as the desmoplastic reaction (Jemal and Siegel *et al*., 2007; Mahadevan and Von Hoff *et al*., 2007). Desmoplastic reaction is composed of bands of fibrous stroma surrounding the malignant cells, while areas of relatively normal pancreas exhibit minimal fibrosis (Hezel and Kimmelman *et al*., 2006; Ottaviano and Sun *et al*.,

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2006). Human PDAC tumors exhibit increased amounts of type I collagen compared to normal pancreas and, with loss of basement membrane, the malignant cells are directly exposed to type I collagen (Imamura and Iguchi *et al.*, 1995). In human pancreatic tumor tissue, there is a strong correlation between the expression of TGF-β1 and type I collagen (Aoyagi and Oda *et al.*, 2004), and TGF-β1 overexpression in pancreatic cancer cells can induce desmoplatic reaction in an experimental model of human pancreatic carcinoma (Lohr and Schmidt *et al.*, 2001).

Previously, we had shown that type I collagen modulated the behavior of PDAC cells by enhancing cell motility and increasing expression of membrane type 1-matrix metalloproteinase (MT1-MMP, MMP-14), a key collagenase that allows cells to invade through the collagen-rich extracellular matrix (Ottaviano and Sun *et al.*, 2006). MT1-MMP is overexpressed in pancreatic tumors mainly in areas of fibrosis, and expression of MT1-MMP is increased in metastatic PDAC lesions compared to the primary tumors. Animal studies support MT1-MMP as a primary regulator of interstitial collagenolysis, as mice genetically deficient in MT1-MMP have severe growth defects as a result of failure to process interstitial collagens during bone and soft tissue formation (Holmbeck and Bianco *et al.*, 1999; Zhou and Apte *et al.*, 2000). This conclusion is supported by *in vitro* studies using organotypic and 3D culture systems which demonstrate that only MT1-MMP confers a 3D growth advantage by removing matrix confines and allowing changes in cellular geometry necessary for proliferation (Hotary and Allen *et al.*, 2003; Sabeh and Ota *et al.*, 2004).

MicroRNAs are small single-stranded, non-coding RNAs that bind to target mRNA and inhibit translation or promote degradation of the transcript (Lewis and Burge *et al.*, 2005; Lim and Lau *et al.*, 2005). A recent study showed that members of the *let-7* family of microRNAs, initially identified as key regulators of embryonic development in *C.elegans* (Reinhart and Slack *et al.*, 2000), were decreased in human PDACs (Torrisani and Bournet *et al.*, 2009). *Let-7* expression increases with differentiation and is present in high levels in mature tissue (Erkan and Kleeff *et al.*, 2007), where it inhibits cell proliferation by monitoring DNA replication, mitosis and cytokinesis (Johnson and Esquela-Kerscher *et al.*, 2007). Overexpression of *let-7* in mutant K-Ras-positive lung cancer cells inhibits tumor growth *in vivo*, while overexpression of *let-7* in breast tumor-initiating cells inhibits tumor formation and metastasis *in vivo* (Yu and Yao *et al.*, 2007). Interestingly, K-Ras, which is mutated in > 90% of PDAC, is also a target of *let-7* microRNA (Johnson and Grosshans *et al.*, 2005; Kumar and Erkeland *et al.*, 2008; Morris and McManus *et al.*, 2005).

Although the collagen microenvironment has been shown to regulate gene expression, the extent to which the effect is mediated through modulation of microRNAs has not been previously examined. Therefore, in this study we examined the effect of collagen on *let-7* family microRNAs. Initially, we examined the relative expression of the various members of the *let-7* family of microRNAs in pancreatic cancer cells (Panc1) grown on tissue culture plastic. As shown in Supplemental Fig. 1, there was differing expression of the various members of the *let-7* family. Expression of *let-7a* and *let-7e* was most pronounced, *let-7g* and *let-7i* were in the intermediate range, while the relative levels of *let-7c, let-7d, let-7f* and miR-98 were significantly reduced. We next examined the effect of collagen on 3 members of *let-7* family based on varying expression levels (*let-7a, let-7d* and *let-7g*). Panc1 cells were grown on 2D tissue culture plastic or embedded in 3D type I collagen gels for 24 hours, and then processed for microRNA and mRNA expression. Compared to Panc1 cells grown on tissue culture plastic, Panc1 cells growing in 3D type I collagen gels show 80% reduction in the mature *let-7* levels (Fig. 1A) without affecting the expression of the precursor primary form of *let-7* (Supplemental Fig. 2), indicating that collagen regulates the post-transcriptional processing of *let-7* from the precursor form to the mature form. Since type I collagen induces MT1-MMP expression in PDAC cells [(Ottaviano and Sun *et al.*,

2006) and Fig. 1B], we examined whether MT1-MMP may be involved in collagen-repression of *let-7*. Initially, we examined the effect of the broad-spectrum MMP inhibitor GM6001 on *let-7* levels. As shown in Fig. 1C, GM6001 partially rescued the effect of collagen on *let-7* levels. Moreover, siRNA knockdown of MT1-MMP also partially rescued *let-7* in 3D collagen (Fig. 1D), suggesting that collagen repression of *let-7* is mediated in part via increased MT1-MMP expression.

Since MT1-MMP has previously been shown to promote MEK/ERK1/2 activation (Sounni and Rozanov *et al.*, 2009), we examined the effect of MT1-MMP siRNA on collagen-induced ERK1/2 phosphorylation. As shown in Fig. 2A, decreasing MT1-MMP levels in Panc1 cells grown in 3D type I collagen attenuated ERK1/2 phosphorylation (Fig. 2A). Moreover, collagen-induced ERK1/2 phosphorylation was mediated by MEK1/2 since U0126 completely blocked ERK1/2 activation (Fig. 2B). However, the activation of ERK1/2 was independent of the EGF receptor (EGFR). Treatment with EGFR inhibitors AG1478 or PD153035 did not inhibit collagen mediated ERK1/2 activation (Fig. 2C). As ERK1/2 can repress *let-7* levels (Dangi-Garimella and Yun *et al.*, 2009) we examined the effect of ERK1/2 on collagen regulation of *let-7*. Panc1 cells growing on plastic or in 3D type I collagen were treated with vehicle control (DMSO) or U0126 for 24 hours. Treatment with U0126 significantly rescued the levels of mature *let-7* in 3D collagen (Fig. 2D) without any effect on the precursor form of *let-7* (Supplemental Fig. 3), demonstrating that collagen activates the MEK1/2-ERK1/2 signaling pathway to inhibit processing of *let-7* to the mature form in pancreatic cancer cells. Our findings are consistent with a recently published study that showed a role for ERK in stabilizing the protein complex that regulates processing of precursor microRNAs (Paroo and Ye *et al.*, 2009). Specifically, mitogenic signaling mediated ERK activation was shown to increase levels of growth-promoting microRNAs while decreasing expression of the tumor suppressor microRNAs *let-7*.

As we had previously shown that MT1-MMP expression involves TGF-β1 signaling in pancreatic cancer cells (Ottaviano and Sun *et al*., 2006), we also examined whether TGF-β1 may play a role in collagen repression of *let-7*. Initially, we evaluated the effect of collagen on TGF-β1 signaling. As shown in Figs. 3A and 3B, Panc1 cells growing in 3D type I collagen demonstrate a 2-fold increase in TGF-β1 mRNA and increased Smad2 phosphorylation, which was blocked using a highly specific TGF-β type I receptor (TβRI) inhibitor SB431542. Significantly, treatment with SB431542 attenuated the effect of collagen on MT1-MMP, *let-7*, and ERK1/2 phosphorylation (Figs. 3C and 3D). To directly demonstrate that TGF-β1 regulates *let-7* expression, Panc1 cells grown on plastic were treated with recombinant human TGF-β1 for 24 hours and *let-7* expression was analyzed. As seen in Fig. 3E, TGF-β1 treatment resulted in approximately 40% inhibition of *let-7* expression. These results demonstrate that collagen, in part by enhancing TβRI activity, promotes MT1-MMP expression and ERK1/2 phosphorylation to repress *let-7* in PDAC cells.

We further evaluated the role of MT1-MMP in the regulation of *let-7* expression by generating Panc1 cells expressing full-length or tail-less (ΔC) MT1-MMP protein. Since the MT1-MMP protein without the tail is retained on the surface longer, there was increased MMP-2 activation by cells expressing the tail-less MT1-MMP protein compared to the wild-type MT1-MMP protein (Fig. 4A). Although overexpression of MT1-MMP was not sufficient to repress *let-7* levels on plastic (data not shown), overexpression of MT1-MMP protein in PDAC cells grown in collagen further repressed *let-7* levels compared to control PDAC cells (Fig. 4B). These results indicate that MT1-MMP repression of *let-7* requires the collagen-milieu.

We next injected Panc1 cells into nude mice to examine whether MT1-MMP can modulate *let-7 in vivo*. Panc1-tet-V and Panc1-tet-ΔC cells were subcutaneously injected into the left and right flanks of nude mice respectively, and the mice were maintained on doxycycline-containing water for 3 weeks to induce gene expression. The tumors were then collected and processed for mRNA and microRNA expression. For each mouse, the *let-7* levels in the Panc1-tet-ΔC tumors were normalized to the matched Panc1-tet-V tumors, arbitrarily setting the *let-7* in the Panc1-tet-V tumors to 1.0. As seen in Fig. 4C, each ΔC-expressing tumor had lower levels of *let-7* relative to the corresponding control tumor, indicating that MT1-MMP is also able to repress *let-7 in vivo*. Additionally, even though the effect was less pronounced compared to the ΔC-expressing tumors, Panc1 tumors expressing the wild-type MT1-MMP showed similar results, with lower levels of at least one of the *let-7* family members in the MT1-MMP expressing tumors relative to control tumors (Supplemental Fig. 4). To provide additional support for our hypothesis that MT1-MMP can repress *let-7* levels in the *in vivo* microenvironment, we examined the relationship between MT1-MMP and *let-7* in human PDAC tumors (Fig. 4D). RNA and microRNA isolated from human PDAC tumor samples were analyzed for expression of MT1-MMP and *let-7* and normalized to the adjacent matched normal pancreas. We found a statistically significant inverse relationship between MT1-MMP and *let-7* ($r = -0.7091$, $p = 0.0182$).

Overall, in this report we show that PDAC cells growing in 3D type I collagen gels repress *let-7* microRNA, in part via increased TGF-β1-mediated expression of MT1-MMP and ERK1/2 activation. It was recently shown that TGF-β1 can downregulate expression of miR-200 family members, and upregulate the oncogenic microRNA miR-21 (Qian and Katsaros *et al.*, 2009). We now provide evidence that TGF-β1 signaling can also regulate *let-7* family members. *Let-7* expression can inhibit pancreatic cancer cell proliferation *in vitro*; however, *let-7* did not affect progression of the primary tumor *in vivo* (Torrisani and Bournet *et al*., 2009). Interestingly, work done by Oh et al suggests that *let-7* could have therapeutic potential in the treatment of pancreatic cancer (Oh and Kim *et al.*). Expression of *let-7* in A549 (lung cancer) and AsPC1 (pancreatic cancer) cells decreased K-Ras levels and potentiated the effect of radiation.

By gene signature analysis, additional microRNAs have also been shown to be differentially regulated between pancreatic cancer and normal pancreatic tissue or chronic pancreatitis (Bloomston and Frankel *et al.*, 2007). Seven microRNAs (miR-21, miR-221, miR-222, miR-181a, b and d and miR-155) were confirmed to be overexpressed in the tumor samples when compared to benign pancreatic tissue. Significantly, miR-155 can inhibit the pro-apoptotic p53 target protein TP53INP1, thus promoting PDAC progression (Gironella and Seux *et al.*, 2007). FISH analysis on tumor tissue microarrays also demonstrated that miR-21, which targets phosphatase and tensin homologue (PTEN), is overexpressed in pancreatic cancer tissue relative to benign pancreatic lesions or chronic pancreatitis and is associated with poor outcome (Dillhoff and Liu *et al.*, 2008). Additionally, antisense-mediated inhibition of miR-21 along with miR-221 in pancreatic cancer cells reduced proliferation, increased apoptosis and sensitized cells to gemcitabine treatment (Park and Lee *et al.*, 2009). Despite the importance of these microRNAs in pancreatic cancer progression, neither collagen nor MT1-MMP affected the levels of miR-21, miR-155, miR-221 or miR-375 in our model system.

Finally, even though microRNAs can regulate MMP expression and function (Gabriely and Wurdinger *et al.*, 2008; Roy and Khanna *et al.*, 2009), it was not heretofore shown that MMPs could in turn modulate microRNAs. Significantly, we show that MT1-MMP, which removes matrix barriers to allow cells to proliferate in the 3D microenvironment (Hotary and Allen *et al*., 2003; Sabeh and Ota *et al*., 2004), may also contribute to PDAC

progression by modulating a microRNA program in the collagen-rich tumor microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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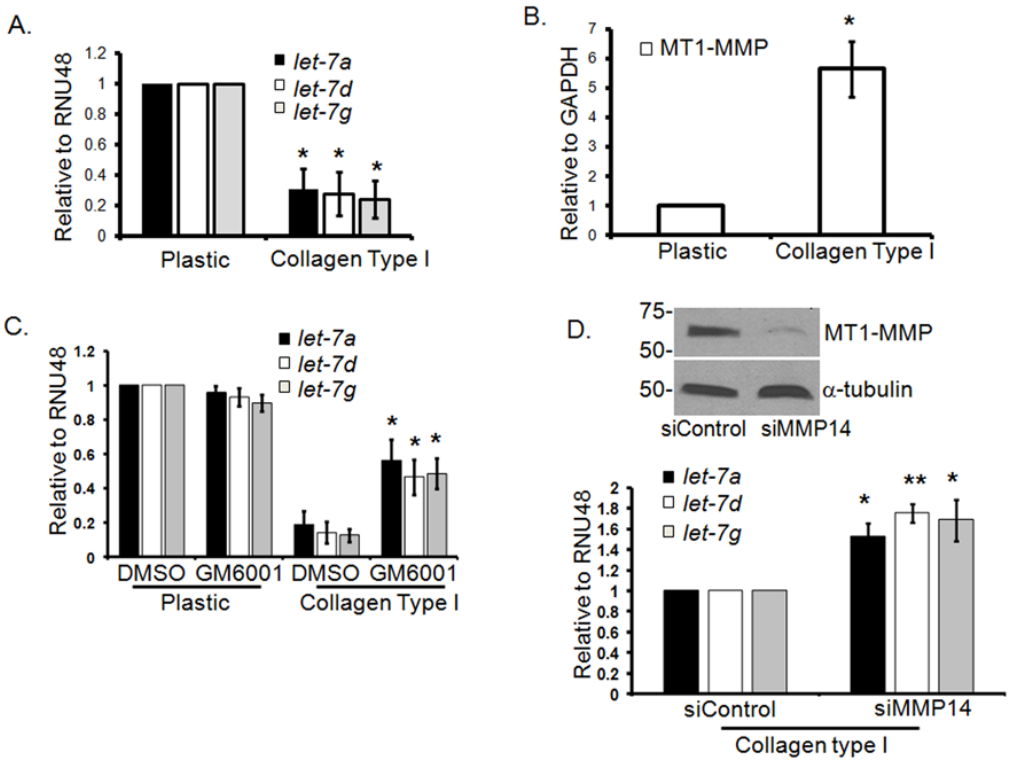

Figure 1. Collagen repression of *let-7* microRNA involves MT1-MMP

Panc1 cells were grown on tissue culture plastic or in 3D type I collagen gels (5 mg/ml) in serum containing medium for 24 hours. *A.* MicroRNA were isolated using mirVana kit (Applied Biosystems, Foster City, CA) and analyzed for expression of the mature *let-7a*, *let-7d* and *let-7g* by qRT-PCR, with RNU48 as the endogenous control, using gene specific Taqman probes (Applied Biosystems, Foster City, CA). The data were quantified by the comparative C_T method for relative gene expression from 3 experiments. *, $p < 0.05$ relative to cells grown on plastic. *B.* Similarly, MT1-MMP and GAPDH (endogenous control) mRNA were analyzed by qRT-PCR. *, $p < 0.05$ relative to cells grown on plastic. Data are an average from 3 separate experiments. *C.* Panc1 cells were grown on plastic or in 3D type I collagen gels (5 mg/ml) and treated with either DMSO or GM6001 (10 μM; EMD, Gibbstown, NJ) for 24 hours. MicroRNA were isolated and analyzed for *let-7* and RNU48 (endogenous control) expression by qRT-PCR. *, $p < 0.05$ relative to DMSO-treated cells grown in collagen. Data are an average of 3 separate experiments. *D.* Panc1 cells were transiently transfected with siRNA against MT1-MMP or control siRNA (50 nmoles) using Nucleofector Kit R (Lonza, Walkersville, MD). Cells were allowed to recover overnight and then plated on plastic or in collagen gels for 24 hours. MT1-MMP knockdown was analyzed by immunoblotting for cells grown on plastic (upper panel), while microRNA were analyzed for *let-7* and RNU48 (endogenous control) expression by qRT-PCR. * $p < 0.05$; **, $p < 0.01$ relative to siControl transfected cells grown in collagen. Data are an average of 3 separate experiments.

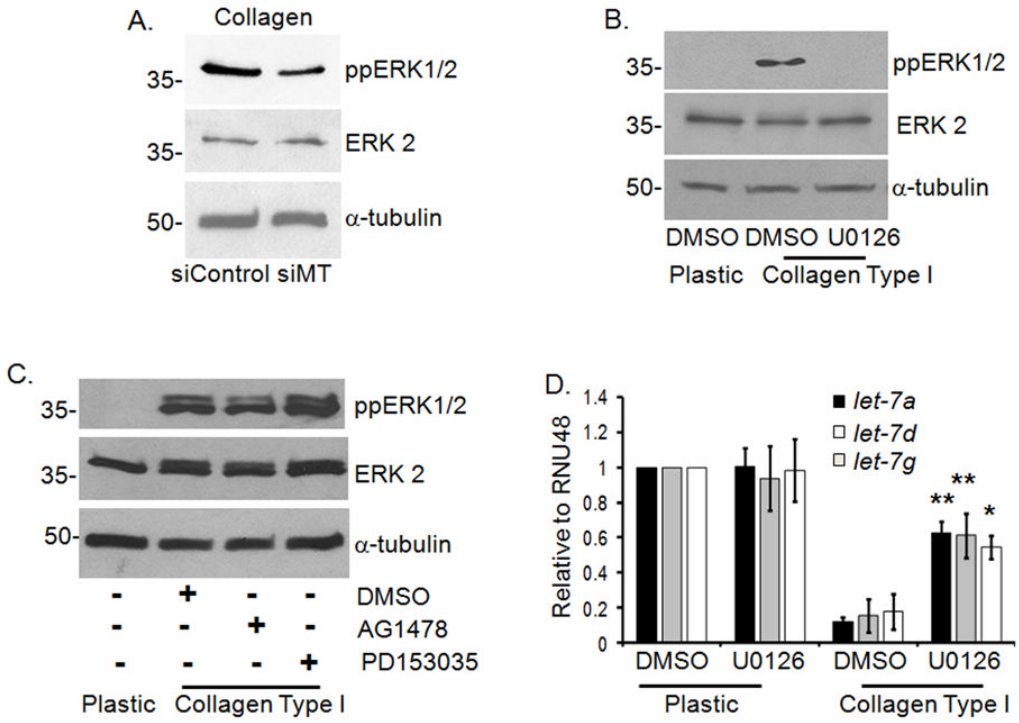

Figure 2. ERK1/2 mediates collagen repression of *let-7* in PDAC cells

A. Panc1 cells were transiently transfected with siRNA against MT1-MMP or control siRNA (50 nmoles) as detailed in Fig. 1D. Cells were allowed to recover overnight and then plated in 3D type I collagen gels (5 mg/ml) for 24 hours, the matrix was dissolved in collagenase (Worthington Biologicals, Lakewood, NJ) and then lysed in m-RIPA buffer. Lysates were immunoblotted for ppERK1/2, ERK/12 and α-tubulin (loading control). Data is representative of 3 separate experiments. *B.* Panc1 cells were grown on tissue culture plastic or in 3D type I collagen gels (5 mg/ml) for 24 hours and treated with vehicle control (DMSO) or U0126 (10 μM; Cell Signaling, Danvers, MA) for 24 hours. Cells grown in collagen were recovered with collagenase treatment and then lysed in m-RIPA buffer, prior to immunoblotting for ppERK1/2 and α-tubulin (loading control). Data is representative of 4 separate experiments. *C.* Panc1 cells were grown on plastic or in 3D type I collagen gels (5 mg/ml) for 24 hours and then treated with DMSO, AG1478 (10 μM; Calbiochem, Gibbstown, NJ) or PD153035 (10 μM; Calbiochem, Gibbstown, NJ) for an additional 24 hours. Cells were lysed as before and then immunoblotted for ppERK1/2, ERK2 and α-tubulin (loading control). Data is representative of 3 separate experiments. *D.* Panc1 cells grown on plastic or in collagen gels were treated with DMSO or U0126 for 24 hours. MicroRNA were isolated using mirVana kit and analyzed for expression of *let-7a*, *let-7d* and *let-7g* by qRT-PCR, with RNU48 as the endogenous control, using gene specific Taqman probes. The data were quantified by the comparative C_T method for relative gene expression from 4 separate experiments. *, $p < 0.05$; **, $p < 0.01$ relative to DMSO-treated cells grown in collagen.

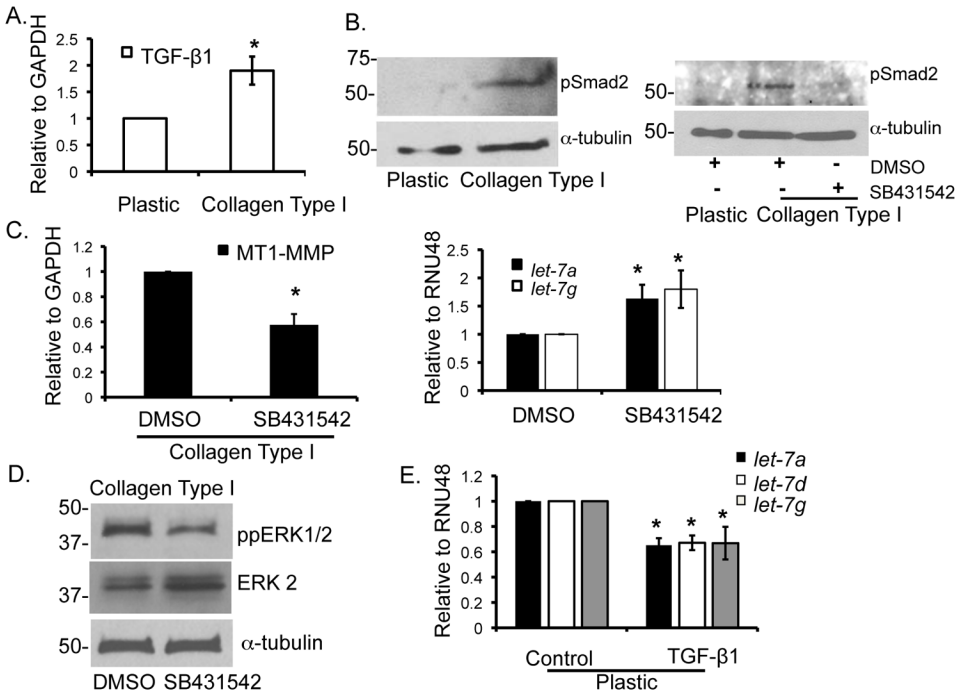

Figure 3. TGF-β receptor type I (TβRI) mediates collagen repression of *let-7*

A. The mRNA isolated from Panc1 cells grown on plastic or in 3D type I collagen gels for 24 hours was analyzed by qRT-PCR for TGF-β1 expression with GAPDH as the endogenous control. *, $p < 0.05$ relative to cells grown on plastic. Data are an average of 3 separate experiments. *B.* The lysates were analyzed for pSmad2 and α-tubulin (loading control) by immunoblotting (left panel). Panc1 cells grown in 3D type I collagen gels were treated with DMSO (control) or TGF-β type I receptor (TβRI) inhibitor SB431542 (10 μM, TOCRIS, Ellisville, MO) for 24 hours and cell lysates immunoblotted for pSmad2 and α-tubulin (right panel). Data is representative of at least 2 separate experiments. *C.* Panc1 cells growing in 3D type I collagen gels were treated with DMSO or SB431542 and changes in MT1-MMP and *let-7* monitored by qRT-PCR. *, $p < 0.05$ relative to DMSO-treated cells grown in collagen. Data are an average of 4 separate experiments. *D.* The protein lysates were immunoblotted for phospho-ERK1/2, ERK2 and α-tubulin (loading control). Data are representative of at least 3 separate experiments. *E.* Panc1 cells grown on plastic were induced with recombinant human TGF-β1 (10 ng/ml) for 24 hours; microRNA isolated from the cells was analyzed for *let-7* by qRT-PCR from 3 separate experiments.

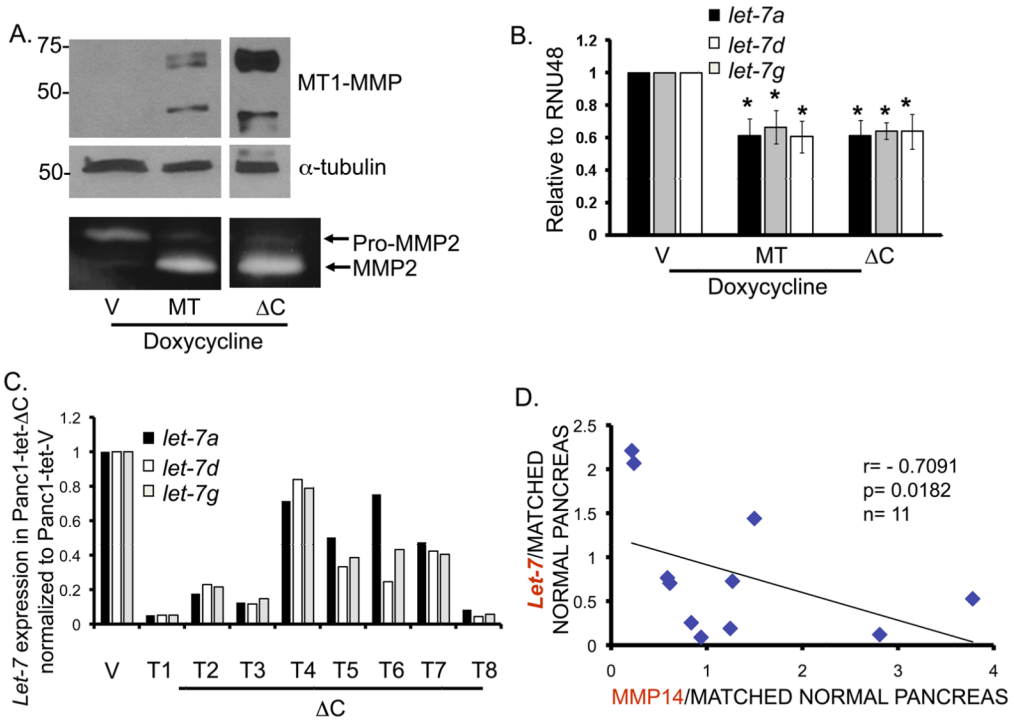

Figure 4. MT1-MMP represses *let-7* microRNA *in vivo*

A. Full-length MT1-MMP and ΔC mutant were subcloned into pRetroX-Tight-Pur vector (Clontech, Mountainview, CA). Panc1 cells inducibly expressing MT1-MMP were then generated as previously described (Dangi-Garimella and Redig *et al*., 2010) to create Panc1-tet-V, Panc1-tet-MT and Panc1-tet-ΔC. These cells were grown on plastic, induced with doxycycline (2 μg/ml) for 24 hours, and then immunoblotted for MT1-MMP and α-tubulin (loading control) (upper panel). To examine MT1-MMP activity, the conditioned medium was analyzed for MMP-2 activation using gelatin zymography (lower panel). Data are representative of at least 4 separate experiments. *B.* Panc1-tet-V, Panc1-tet-MT and Panc1-tet-ΔC cells were grown on tissue culture plastic or in 3D collagen gels (5 mg/ml), induced with doxycycline for 64 hours and changes in *let-7* and RNU48 (endogenous control) expression were analyzed by qRT-PCR from 3 separate experiments. *, $p < 0.05$ relative to control Panc1-tet-V cells. *C*. Mice were housed, fed and treated in accordance with the guidelines approved by the Northwestern University IACUC. Eight-week old athymic nu/nu animals (n=8) were subcutaneously injected with 5×10^6 Panc1-tet-V cells in the left flank and Panc1-tet-ΔC cells in the right flank of the same animal. Twenty-four hours later, doxycycline was added to the drinking water to induce MT1-MMP expression, and monitored twice a week for the development of skin tumors. The mice were euthanized, following which the tumors were dissected and placed in RNAlater (Qiagen, Valencia, CA). The RNAlater-preserved tumors were processed and analyzed for human MT1-MMP and human GAPDH mRNA or human *let-7* and human sno135 microRNA expression by quantitative real-time PCR. For each animal the relative expression of *let-7a*, *let-7d* and *let-7g* was calculated in Panc1-tet-ΔC tumors and normalized to the levels present in the Panc1-tet-V tumors, with the *let-7* expression in the control animals arbitrarily set at 1.0. Relative *let-7* values < 1.0 indicate repression by MT1-MMP. *D.* Pancreatic tissue was obtained from patients with pancreatic adenocarcinoma and de-identified on an IRB-approved protocol. The cancerous and adjacent normal tissue samples were dissected and processed for RNA extraction using Trizol. The RNA quality was checked using Bioanalyzer-309 and samples with RNA Integrity Number (RIN) greater than 6 were used

for RNA and microRNA studies. The *let-7* levels relative to RNU48 and MT1-MMP expression relative to GAPDH in PDAC tumors (n=11) were normalized to the levels present in the matched adjacent normal pancreas, which was arbitrarily set at 1.0. Spearman's correlation (GraphPad Instat) was used to determine significance between MT1-MMP and *let-7* levels in these tumors.