iScience, Volume *24*

Supplemental information

Extracellular PKM2 facilitates organ-tissue fibrosis progression

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Extracellular PKM2 Facilitates Organ-tissue Fibrosis Progression

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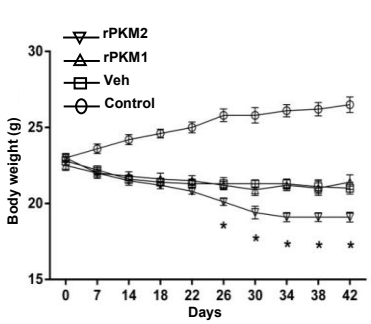
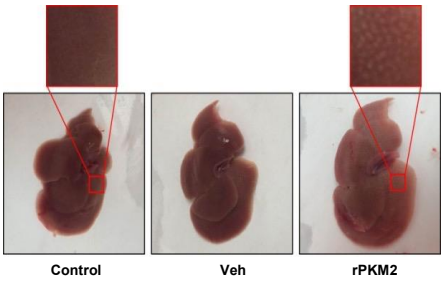
Supplementary Figure S1

A

Serum PKM2 levels in liver disease patients

Serum PKM2 $_{ng/ml}$	Range	Mean
Normal (n=10)	0.08 – 0.35	0.24
Viral hepatitis (n=10)	0.89 – 2.07	1.59
Cirrhosis (n=10)	1.8 – 7.6	3.9

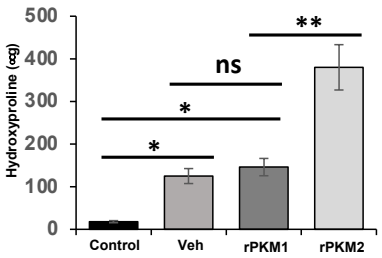


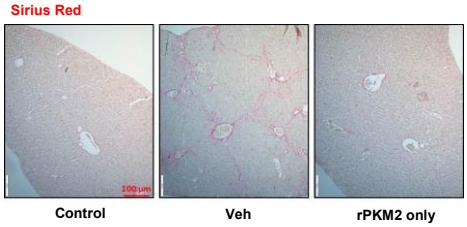


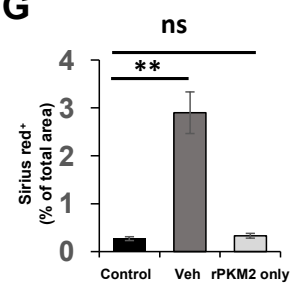
D

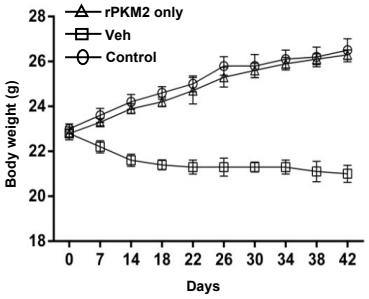
Serum markers in TAA fibrosis mice

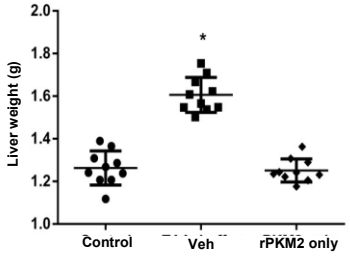
	ALT (U/L)	AST (U/L)
Control	34.2±7.3	29.7±6.4
Veh	93.5±27	97.7±44
rPKM1	92.4±23	98.4±36
rPKM2	169±22.8	207.9±51







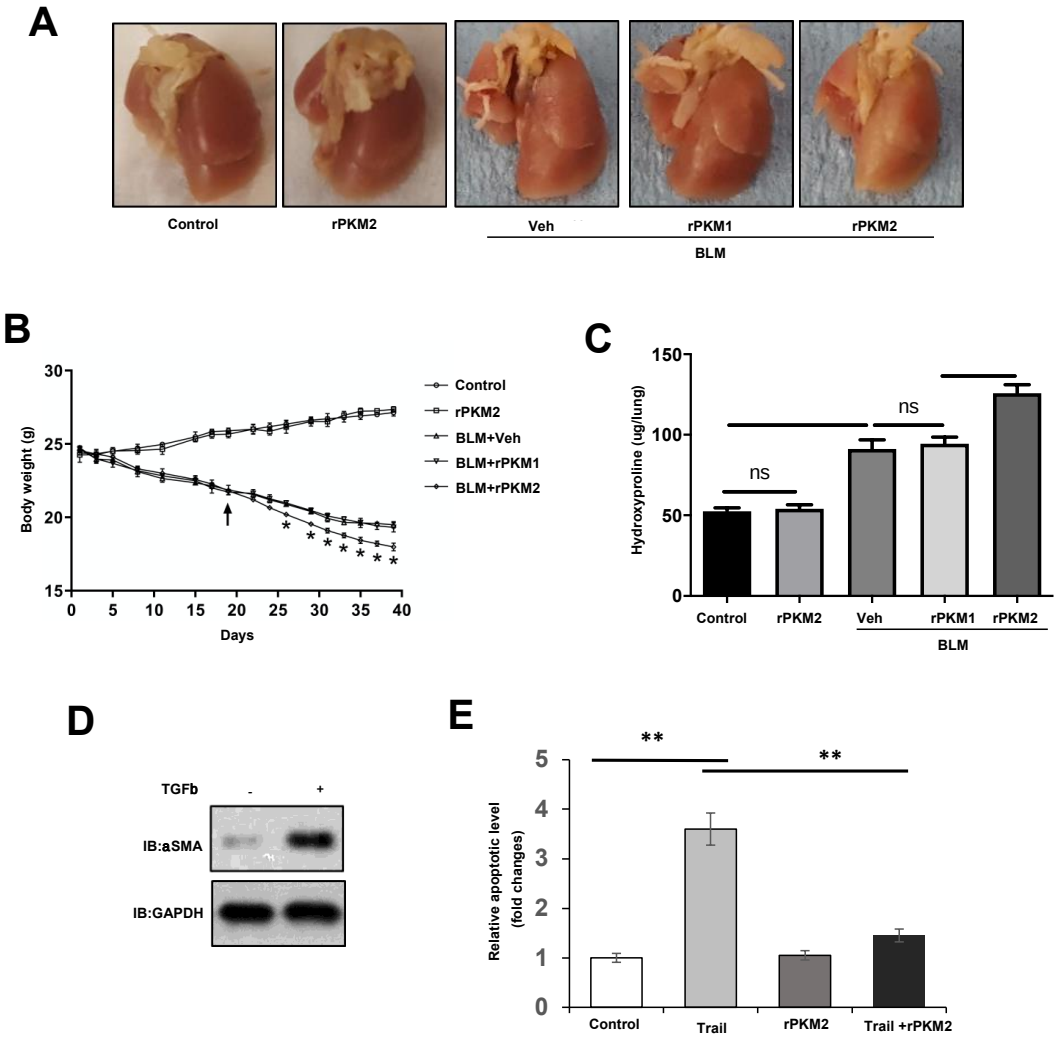




Supplementary Figure 1 EcPKM2 facilitates liver fibrosis progression. Related to Figure 2.

(A) PKM2 levels (ng/ml) in serum of healthy individuals and liver disease patients were measured by ELISA. The range is low and high PKM2 levels in serum. Means is the mean value of PKM2 levels calculated from 10 healthy individuals and patients (n=10). Each patient sample was independently measured five times in the ELISA assay. **(B) - (E),** liver fibrosis was induced by TAA/alcohol and the animals were subsequently treated with addition of rPKM1, rPKM2 or vehicle into TAA as shown in Fig. 2A. (B) Body weight changes during treatment course. Arrow indicates addition of rPKM2/rPKM1/vehicle to the TAA fibrosis induction. (C) Images of representative collected livers with enlarged call-out to show surface features. (D) Serum levels of ALT and AST in mice treated with indicated agents were measured by a commercial service (CPath). (E) Collagen levels in fibrotic livers were measured by hydroxyproline assay and presented as hydroxyproline (μg). **(F) – (I)** Mice were treated with TAA/alcohol plus vehicle as shown in Fig. 2A (Veh) or rPKM2 without TAA/alcohol (rPKM2 only). Control is the animal without treatment. (F), (G) Representative images (F) and Quantitation (G) of Sirius red staining of liver sections from mice with indicated treatment. (H) Body weight changes during treatment course. (I) Liver weight of the animals at end point of the experiments. Error bars in (B), (E), (G), and (H) represent mean ± S.E.M. Scale bars, 100 μm. *P<0.05, **P<0.01. ns: statistically no significance.

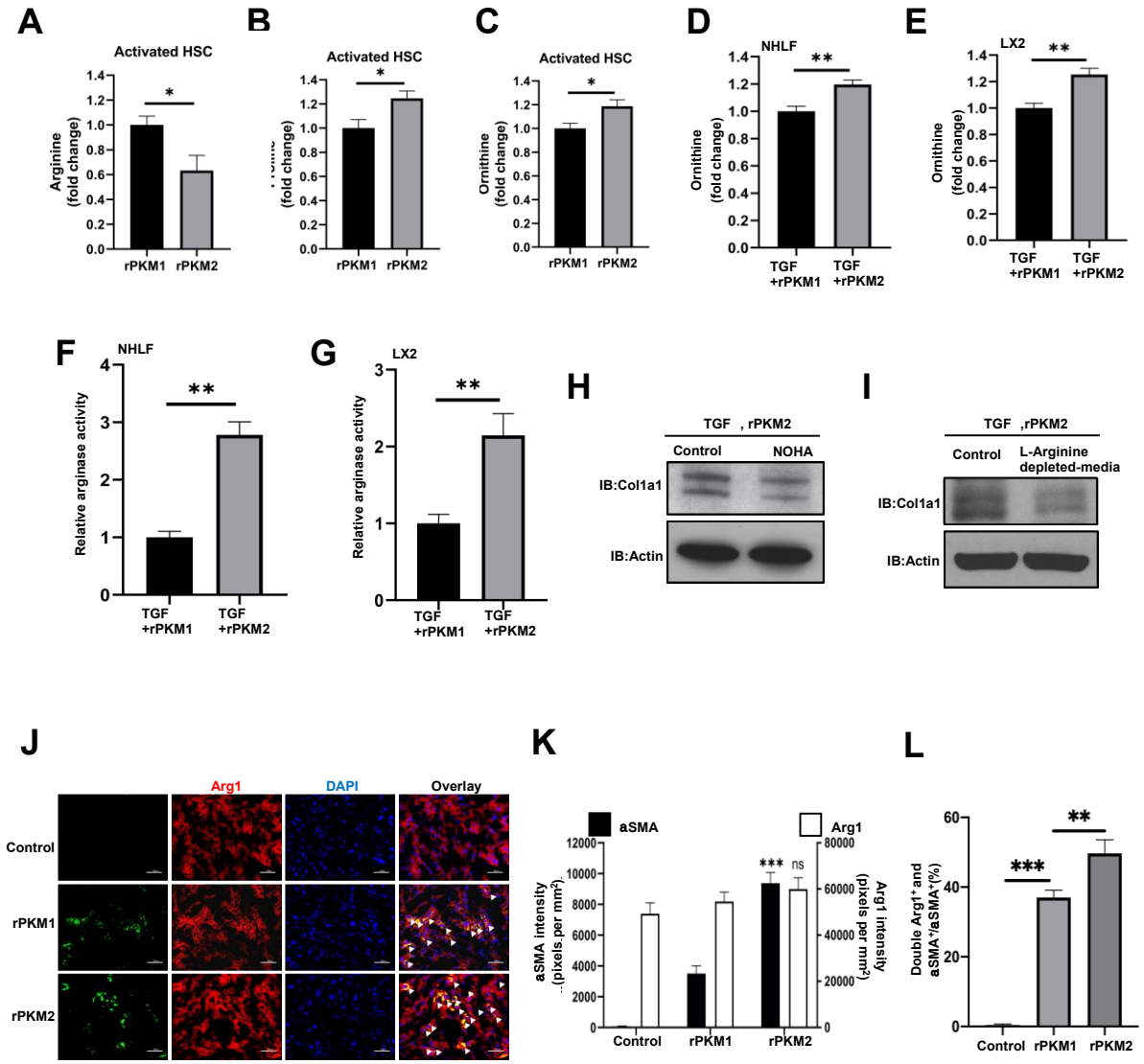
Supplementary Figure S2



Supplementary Figure 2 EcPKM2 facilitates liver fibrosis progression. Related to Figure 2.

(A) - (C), lung fibrosis was induced by bleomycin and the animals were subsequently treated with addition of rPKM1, rPKM2 or vehicle into bleomycin as shown in Fig. 2G. (A) Representative images of collected lungs from animals that underwent indicated treatment. (B) Body weight changes during treatment course. Arrow indicates addition of rPKM2/rPKM1/buffer to the bleomycin fibrosis induction. (C) Collagen levels in fibrotic lungs were measured by hydroxyproline assay and presented as hydroxyproline (μg). **(D)** Cellular levels of αSMA in human primary HSC cells that were treated (+) or untreated (-) with TGFβ were analyzed by immunoblot (IB:αSMA). Immunoblot of GAPDH (IB:GAPDH) is a loading control. **(E)** Apoptosis of TGFβ treated LX-2 cells that underwent additional indicated treatment was measured by Annexin v-FACS assay. The apoptosis is presented as relative apoptosis by defining the untreated LX-2 cells as reference 1. Error bars in (B), (C), and (E) represent mean ± S.E.M. **P*<0.05, ***P*<0.01. ns: statistically no significance.

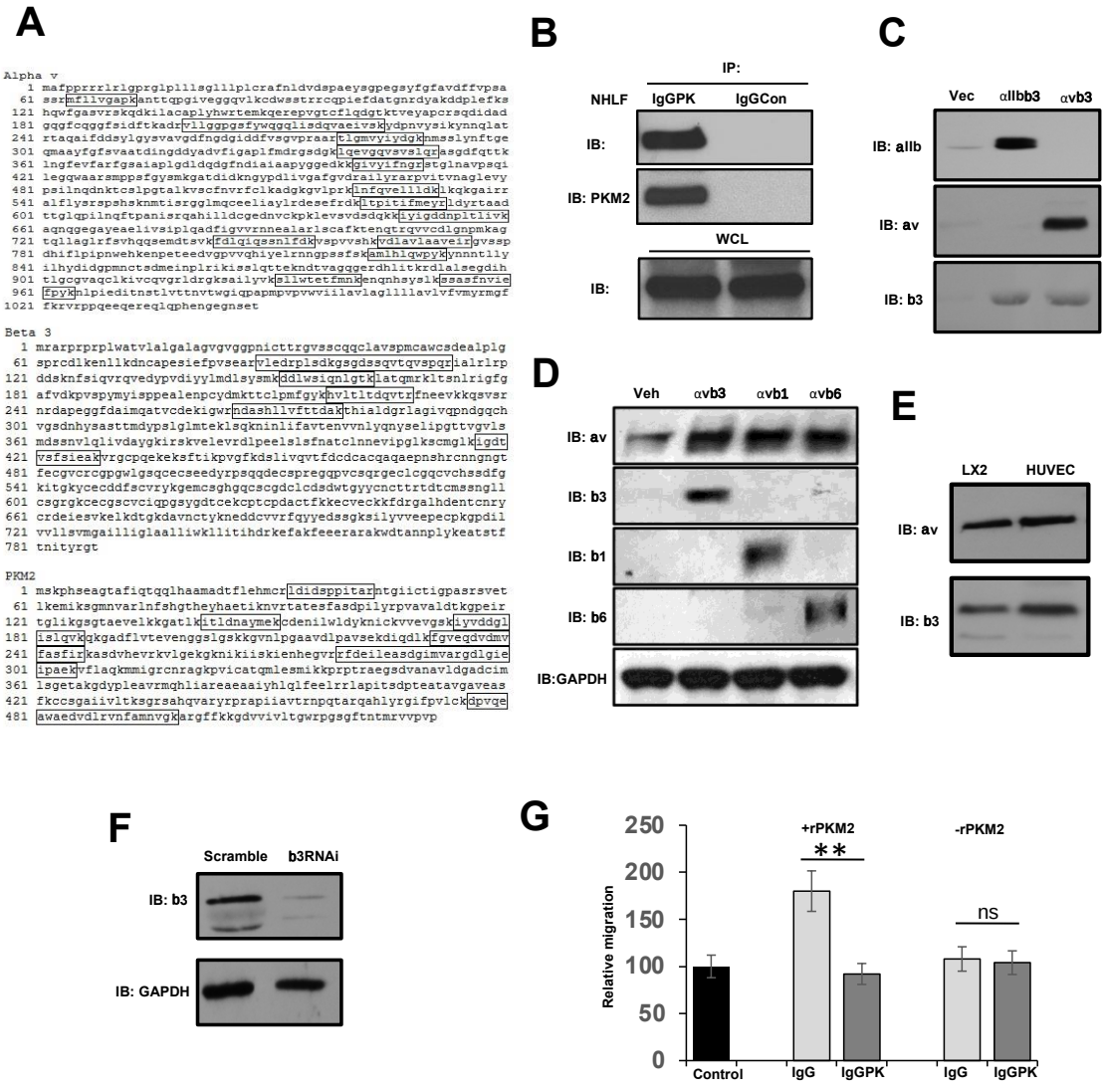
Supplementary Figure S3



Supplementary Figure 3 EcPKM2 facilitates collagen synthesis in myofibroblasts by upregulating Arg-1. Related to Figure 4.

(A), (B) Relative abundance of Arginine (A) and Proline (B) in activated hHSC cells were analyzed by HPLC-MS. The cells were treated by TGFβ + rPKM1 (black bars) or TGFβ + rPKM2 (grey bars). The amino acid levels in the cells that were treated with TGFβ + rPKM1 are set as reference 1. **(C) - (E)** Cellular ornithine levels in activated hHSC (C), NHFL (D) and LX-2 (E) cells were analyzed using a commercially available ornithine kit. **(F**), **(G)** Arginase activities in extracts of NHFL (F) and LX-2 (G) cells were measured using a commercially available arginase activity assay kit. **(H**), **(I)** Cellular levels of collagen 1A1 in NHFL cells were analyzed by immunoblot (IB:Col1a1). In (A) – (I), the cells were treated with TGFβ plus rPKM1 or rPKM2. In (H), the cells were treated with Arg-1 inhibitor (MOHA). In (I), the cells were cultured in L-Arginine-depleted media. In (H) and (I), control is the cells without MOHA treatment (in H) and without L-Arg depletion (in I). Immunoblot of Actin (IB:Actin) is a loading control. **(J) – (L)** Representative images of immunofluorescence (IF) staining (J) and quantitation of αSMA and Arg1 staining (K) and αSMA and Arg1 double staining over αSMA staining (%) (L) in sections from liver of mice that were treated by the indicated agents. In (J), green is IF staining of αSMA. red is Arg1 staining. Blue is DAPI staining. Arrows indicate αSMA and Arg1 double staining. In (K), quantities of αSMA and Arg1 are presented as staining intensity pixels per mm^2. Error bars in (A), (B), (C), (D), (E), (F), (G), (K), and (L) represent mean ± S.E.M. Scale bars, 100 μm. **P*<0.05, ***P*<0.01, ****P*<0.001. ns: statistically no significance.

Supplementary Figure S4

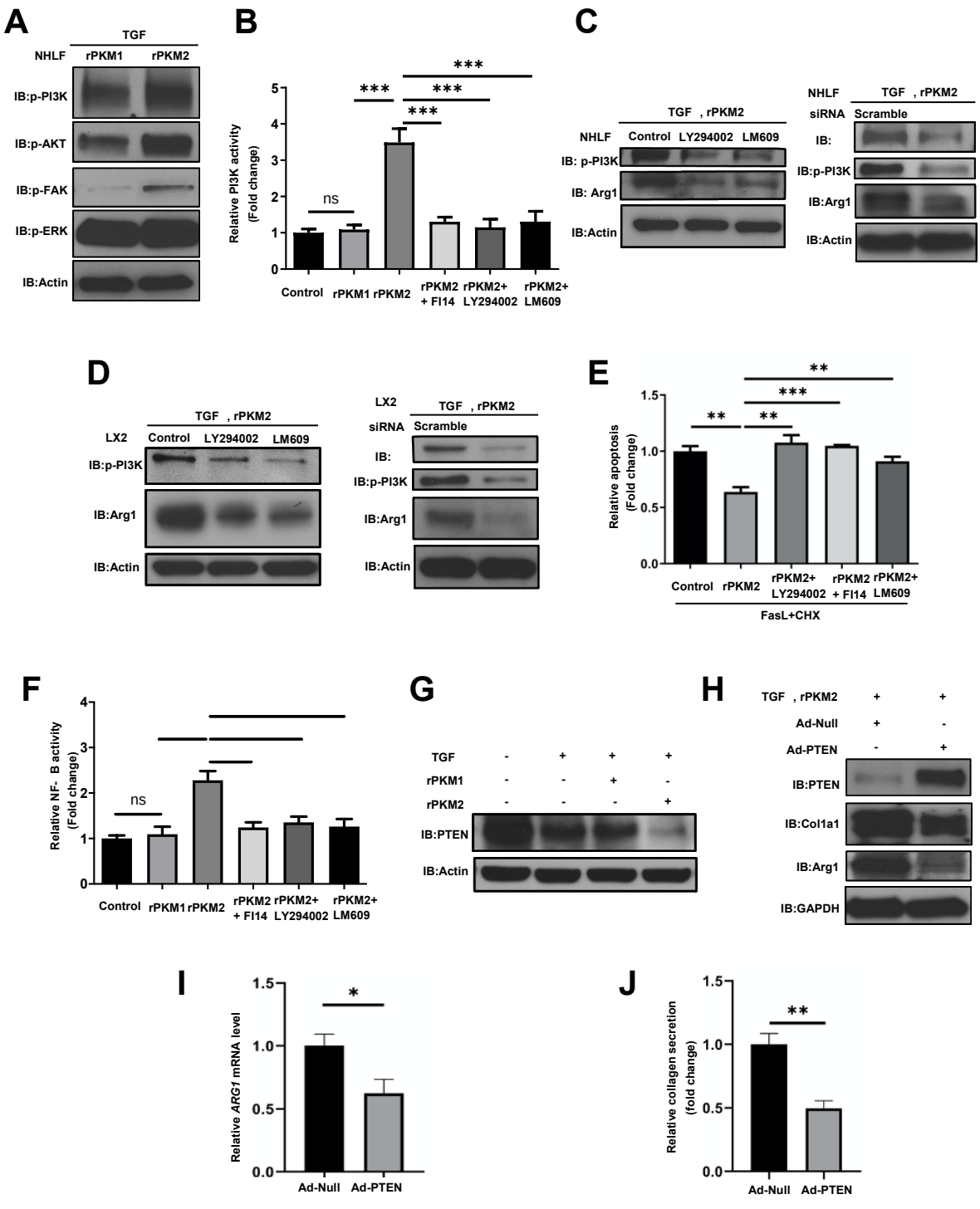


Supplementary Figure 4 EcPKM2 interacts with integrin $\alpha_v\beta_3$ and activates the integrin signaling. Related to Figure 5.

(A) The peptide fragments (boxed) from trypsin digestion of the His-tag pulled-down crosslinking band of His-rPKM2-LX-2 crosslinks (see Figure 5A) and analyzed by MALDI-tof/tof that match the aa sequence of integrin α_v, β_3, and PKM2. **(B)** Co-immunoprecipitation of integrin β_3 with PKM2 (IP:IgGPK) in extracts of NHLF cells treated with TGFβ was examined by immunoblot of integrin β_3 (IB:β3). Immunoblot of PKM2 (IB:PKM2) indicates amounts of PKM2 that are precipitated down. Immunoprecipitation using IgG purified from pre-immune serum (IP:IgGCon) is control immunoprecipitation. Immunoblot of integrin β3 (IB:β3) in whole cell lysate (WCL) is a control indicating equal amounts of cell extracts used in the IPs. **(C)** Expression of integrin αIIb, αv, and β3 (IB:α_v, IB:α_{IIb}, IB:β_3) in CHO cells that exogenously express integrin $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ pairs was probed by immunoblot analyses. **(D)** Expression of integrin αv, β1, β3, and β6 (IB:α_v, IB:β_3, IB:β_1, IB:β_6) in CHO cells that exogenously express integrin $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_v\beta_6$ pairs was probed by immunoblot analyses. In (C) and (D), Veh the cells were transfected with empty vector. **(E)** Expression of integrin αv (IB:α_v) and β3 (IB:β_3) in LX-2 and HUVEC cells was probed by immunoblot analyses. **(F)** Expression of integrin β3 (IB:β3) in LX-2 cells in which integrin β3 was knocked down by β3 RNAi or scrambled RNAi was probed by immunoblot analyses. Immunoblot of GAPDH in (D) and (F) is a loading control. **(G)** Migration of LX-2 cells treated with TGFβ in the presence of rPKM2 (+rPKM2) absence of rPKM2 (-rPKM2) and with addition of antibodies IgGPK or IgGCon in the culture media were analyzed. The migration was assayed in plats with vitronectin (VN) coating. The cell migrations are presented as relative migration by defining the cell migration of control in the absence of rPKM2 and without antibody treatment as

100. Error bars in (A), (B), (C), (D), (H) and (I) represent mean ± S.E.M. Scale bars, 100 μm. **P<0.01, ***P<0.001. ns: statistically no significance.

Supplementary Figure S5

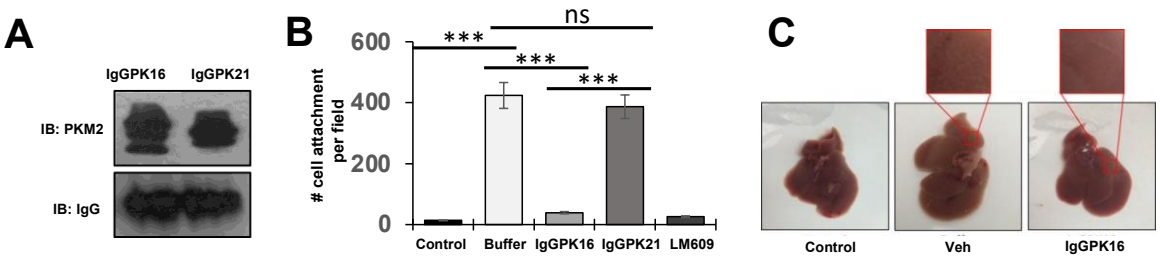


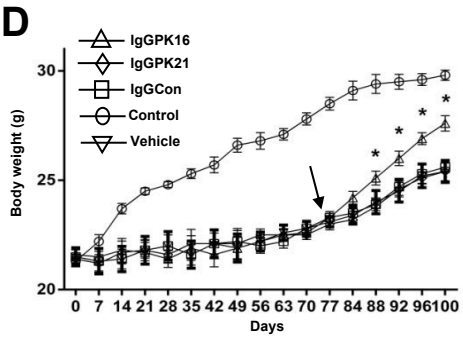
Supplementary Figure 5 EcPKM2 Activates FAK-PI3K signaling axis. Related to Figure 6.

(A) Cellular levels of phospho-PI3K (IB:p-PI3K), phospho-AKT (IB:p-AKT), phospho-FAK (IB:p-FAK), and phospho-ERK (IB:p-ERK) in NHLF cells were analyzed by immunoblot. The cells were treated by TGFβ plus rPKM1 or rPKM2. **(B)** Activation of PI3K in NHLF cells in the presence of indicated agents was measured by ELISA PI3K kit. The PI3K activities are presented as relative activity by defining the activities of PI3K in buffer treated cells as 1 (Control). **(C), (D)** Cellular levels of phospho-PI3K (IB:p-PI3K) and Arg-1 (IB:Arg1) in NHLF (C) and LX-2 (D) cells were analyzed by immunoblot. The cells were treated by TGFβ and rPKM2. (Left) The TGFβ and rPKM2 treated cells were further treated by PI3K inhibitor (LY294002), antibody LM609, or no treatment (Control). (Right) Integrin β_3 was knocked down (β3) or knockdown by scrambled siRNA (Scremble). Immunoblot of integrin β_3 (IB:β3) indicate cellular level of the integrin. **(E)** Apoptosis of NHLF cells under treatment of indicated agents were measured by apoptosis kit. Cell apoptosis is presented as Relative apoptosis by defining apoptosis of cell treated with buffer (control) as 1. **(F)** NF-kB activity in NHLF cells in the presence of indicated agents was measured using transcription assay kit. The NF-kB activity is present as Relative NF-kB activity by defining the measured NF-kB activity in buffer treated NHLF cells as 1. **(G)** Cellular levels of PTEN (IB:PTEN) in NHLF cells treated with indicated agents were analyzed by immunoblot. **(H)** Cellular levels of PTEN (IB:PTEN), collagen 1A1 (IB:collagen1a1), and Arg-1 (IB:Arg1) in NHLF cells were analyzed by immunoblot. The cells were treated with TGFβ and rPKM2. PTEN was exogenously expressed in the cells using adenoviral vector (Ad-PTEN). Ad-Null is empty viral vector. **(I)** Cellular levels of Arg-1 mRNA in NHLF cells were analyzed by qRT-PCR. **(J)** Secreted collagen in culture medium of NHLF cells were analyzed by precipitation (as in Fig. 4 C&D). The cells were treated with TGFβ and rPKM2. PTEN was exogenously expressed in the

cells using adenoviral vector. Immunoblots of Actin (IB:Actin) in (A), (C), (D), and (G) and GAPDH (IB:GAPDH) in (H) and are loading controls. Error bars in (B), (E), (F), (I), and (J) represent mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001. ns: statistically no significance.

Supplementary Figure S6

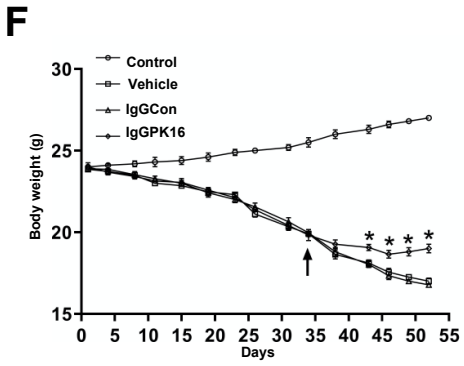


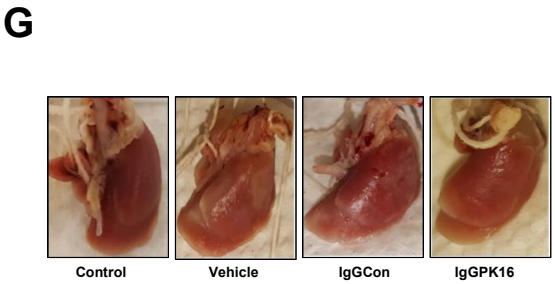


E

Serum markers in TAA fibrosis mice

	ALT (U/L)	AST (U/L)
Control	31.38±7.15	35.43±8.18
Veh	198.88±40.19	195.86±47.84
IgGPK16	82.75±22.21	118.4±32.39
IgGPK21	180.63±37.83	174.25±23.74
IgGCon	195.25±46.49	189.12±31.71

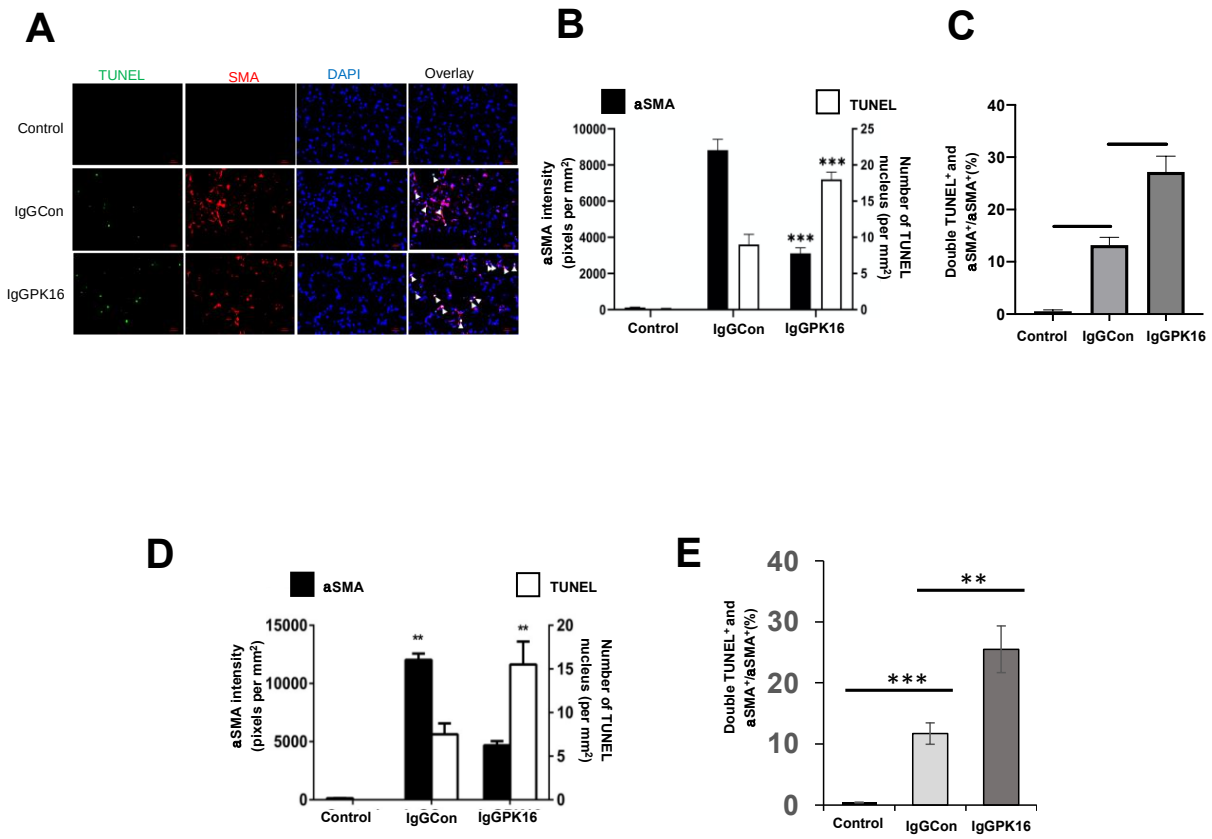




Supplementary Figure 6 Antibody against PKM2 reversed organ/tissue fibrosis. Related to Figure 7.

(A) Recognition of rPKM2 (IB:PKM2) by two clones of rabbit monoclonal antibody IgGPK21 and IgGPK16 is illustrated by immunoblot. IB:IgG is a loading controls to show amount of purified IgG used in each immunoblot. **(B)** Attachment of LX-2 cells to rPKM2 coated plate in the presence of buffer, PKM2 antibody clone 16 (IgGPK16), PKM2 antibody clone 21 (IgGPK21), and LM609 (αvβ3Ab). The cells were treated with TGFβ prior to attachment assay. The attachment is presented as total number of cells attached to the plate per view field. Control is the cell attachment to the plate without rPKM2 coat. **(C) - (E)** Liver fibrosis is induced by TAA/alcohol and the animals were subsequently treated with indicated agents as shown in figure 7A. (C) Representative images of collected livers with enlarged call-out to show surface features. (D) Body weight changes during treatment course. Arrow indicates starting time point of antibody or vehicle treatment. (E) Serum levels of ALT and AST in blood of mice treated with indicated agents were measured by a commercial service (CPath). **(F), (G)** Lung fibrosis was induced by bleomycin and the animals were subsequently treated with IgGPK16, IgGCon, or vehicle as shown in Fig. 7G. (F) Body weight changes during treatment course. Arrow indicates starting time point of antibody or vehicle treatment. (G) Representative images of collected lungs from animals that underwent indicated treatment. Control in (C) – (G) is animals without fibrosis induction and subsequent treatment. Error bars in (B), (D), and (E) represent mean ± S.E.M. *P<0.05, ***P<0.001. ns: statistically no significance.

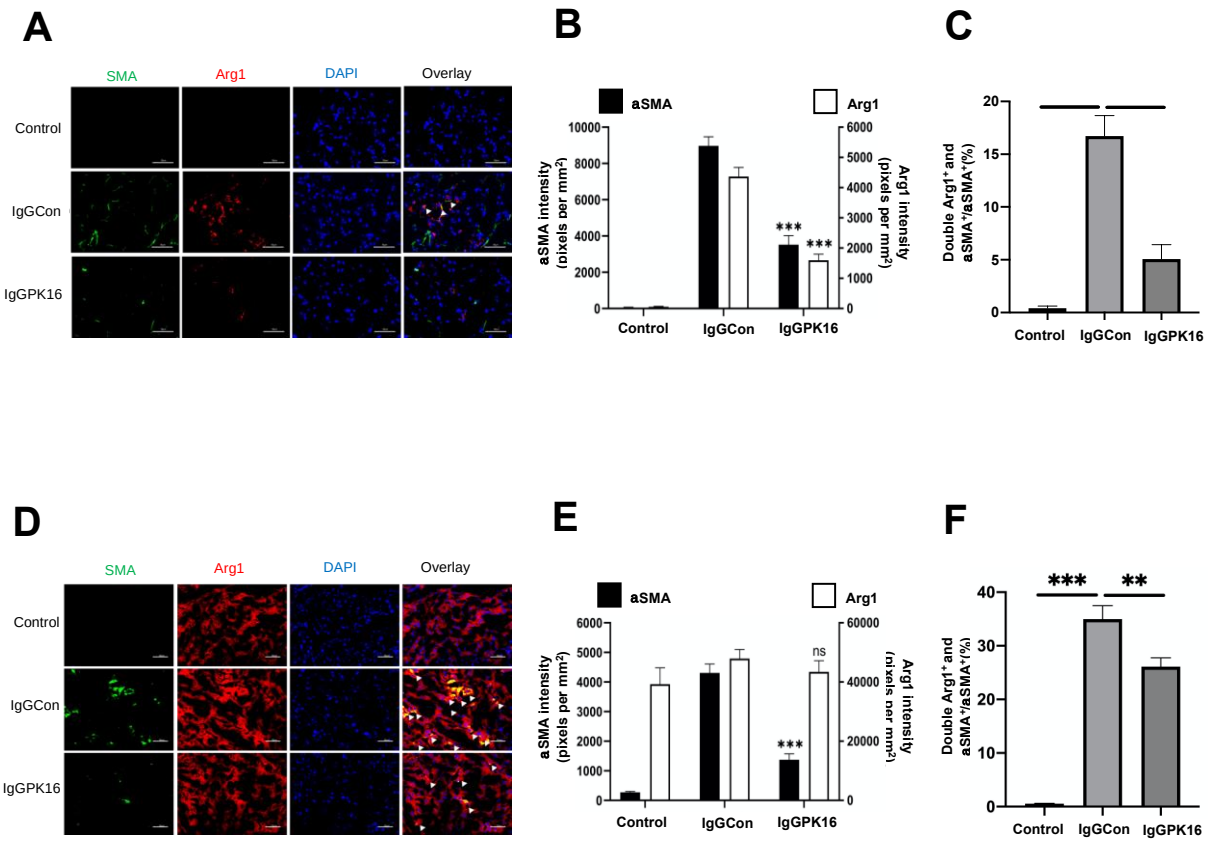
Supplementary Figure S7



Supplementary Figure 7 PKM2 antibody increases myofibroblasts apoptosis in fibrotic lung and liver. Related to Figure 3 and Figure 7.

(A) – (C) Representative images of immunofluorescence (IF) staining (A) and quantitation of αSMA and TUNEL staining (B) and αSMA and TUNEL double staining over αSMA staining (%) (C) in sections from lung of mice that were treated by the indicated agents. In (A), Red is IF staining of αSMA. Green is TUNEL staining. Blue is DAPI staining. Arrows in overlay indicate examples of αSMA and TUNEL double staining. **(D), (E)** Quantitation of αSMA and TUNEL staining (D) and αSMA and TUNEL double staining over αSMA staining (%) (E) in sections from liver of mice that were treated by the indicated agents. In (B) and (D), quantity of αSMA is presented as staining intensity pixels per mm^2, quantity of TUNEL staining is presented as number of positive nucleus per mm^2. Control in (A) – (E) is animals without fibrosis induction and subsequent treatment. Error bars in (B), (D), and (E) represent mean ± S.E.M. $^{**}P<0.01$, $^{***}P<0.001$.

Supplementary Figure S8



Supplementary Figure 8 PKM2 antibody decreases Arg-1 expression in myofibroblasts in fibrotic lung and liver. Related to Figure 4 and Figure 7.

(A) – (C) Representative images of immunofluorescence (IF) staining (A) and quantitation of αSMA and Arg1 staining (B) and αSMA and Arg1 double staining over αSMA staining (%) (C) in sections from lung of mice that were treated by the indicated agents. **(D) – (F)** Representative images of immunofluorescence (IF) staining (D) and quantitation of αSMA and Arg1 staining (E) and αSMA and Arg1 double staining over αSMA staining (%) (F) in sections from liver of mice that were treated by the indicated agents. In (A) and (D), green is IF staining of αSMA. red is Arg1 staining. Blue is DAPI staining. In (B) and (E), quantities of αSMA and Arg1 are presented as staining intensity pixels per mm^2. Control in (A) – (F) is animals without fibrosis induction and subsequent treatment. Error bars in (B), (C), (E), and (F) represent mean ± S.E.M. $^{**}P<0.01$, $^{***}P<0.001$. ns: statistically no significance.

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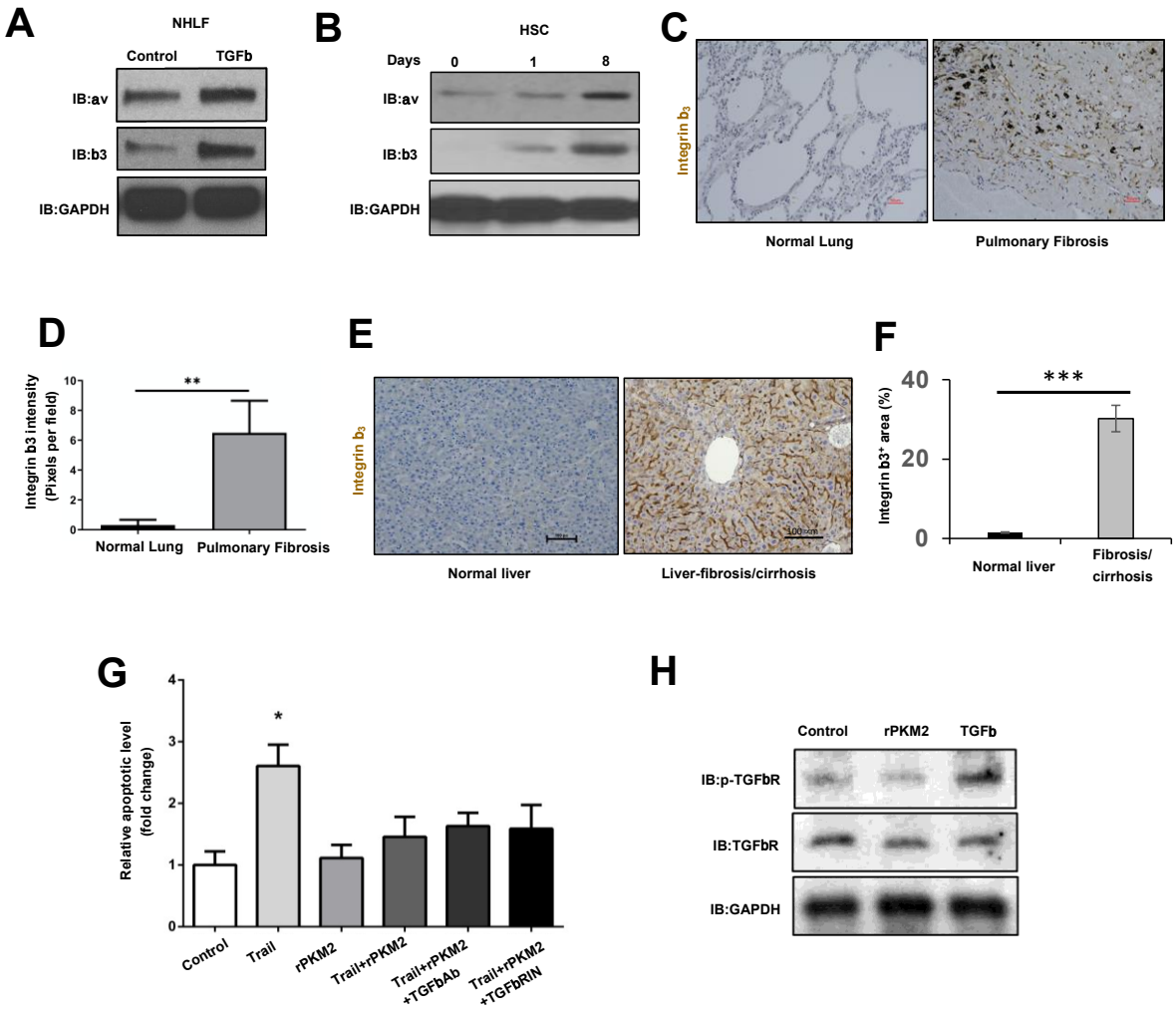
Supplementary Figure S9



Supplementary Figure 9 Myofibroblasts express integrin $\alpha_v\beta_3$. EcPKM2 does not activate TGFβ signaling. Related to Figure 5.

(A), (B) Cellular levels of integrin α_v (IB:α_v) β_3 (IB: β_3) in NHLF (A) and human primary HSC (B) cells were analyzed by immunoblot. The cells were treated with TGFβ for 0 (control in (A)), 1, and 8 days before cell extracts preparation. **(C)** – **(F)** IHC staining of integrin β3 in tissue array sections from lung (C and D) and liver (E and F) fibrosis patients. (C) and (E) Representative images of IHC staining of integrin β3. (D) and (F) Quantitation of integrin β3 staining (integrin β3 stain intensity (pixels per field) in (D) and integrin β_3^+ area (%) in (E)) of lung and liver diseases tissue array. Quantitation were calculated from randomly selected four view fields from each spot of the array that were analyzed. The integrin β3 staining intensity and integrin β_3^+ area were calculation of all tissue spots in the array (Liver disease tissue array: Total 9 normal n=9, 15 viral hepatitis n=15, 40 cirrhosis liver n=40 spots. Lung disease tissue array: Total 7 normal n=7, 25 lung fibrosis n=25 spots). **(G)** Apoptosis of LX-2 cells under treatment of indicated agents were measured by apoptosis kit. TGFβAb is a TGFβ neutralizing antibody. TGFβRIN is an inhibitor that inhibits TGFβ receptor activity. Cell apoptosis is presented as Relative apoptosis by defining apoptosis of cell treated with buffer (control) as 1. **(H)** Cellular levels of phospho-TGFβR in LX-2 cells that were treated by the indicated agents were analyzed by the immunoblots (IB:p-TGFβR). Immunoblot of TGFβR (IB:TGFβR) is a loading control indicates the total TGF receptor levels. Control, the LX-2 cells treated by buffer. Immunoblot of GAPDH (IB:GAPDH) in (A), (B), and (H) is a loading control. Error bars in (D), (F), and (G) represent mean ± S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001