Supporting Information

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Smad3 Promotes Cancer-Associated Fibroblasts Generation via Macrophage-Myofibroblast Transition

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Supplementary Figure S1. MMT exists in human TME. CAF marker (α-SMA) expressing TAM (CD68) in NSCLC, prostate and kidney cancer specimens were detected by (A) confocal imaging and (B) flow cytometry. (C) CAF marker (α-SMA or FAP) expressing TAM (F4/80) were detected in human NSCLC A549 xenograft bearing nude mice. Scale bar, (A, C) 50 µm
Supplementary Figure S2. Clodronate liposome mediated macrophage depletion largely reduced MMT in vivo. (A) Clodronate liposomes (Lip-Clodronate) effectively depleted TAM (F4/80) and reduced MMTs (α-SMA F4/80) in the LLC-tumor in vivo, (B) associated with a markedly reduction of tumor growth compared to the control mice treated with PBS liposomes (Lip-PBS; **p<0.01 vs Lip-PBS, n=4, t-test). Scale bar, (A) 50 μm.
Supplementary Figure S3. Increment of macrophage-lineage CAFs during tumorigenesis. (A) Flow cytometry detected macrophage-lineage derived CAFs (α-SMA+ tdTomato+) during LLC tumor progression. Interestingly, their quantifications showed that (B) the predominate expression of macrophage marker (F4/80, left panel) in tdTomato+ cells was switched to be CAF marker (α-SMA, right panel) at the late stage of the tumorigenesis in vivo, accounting for (C) ~40% of total CAFs on Day 34 (n=3-4).
Supplementary Figure S4. The existence of BMDM-derived CAFs \textit{in vivo}. (A) GFP expressing or (B) Dil tracker dye stained BMDMs were adoptively transferred into the LLC-bearing mice, their derived $\alpha$-SMA$^+$ CAFs were detected in tumors on day 25 by immunofluorescence. Scale bar, (A, B) 50 $\mu$m.
**Supplementary Figure S5.** M2-derived CAF exists in the LLC-tumor *in vivo.* GFP expressing BMDM-generated M2 cells (polarized by IL-4 *in vitro*) were adoptively transferred into the LLC-bearing mice, their derived α-SMA⁺ CAFs were found in the tumors on day 25 by immunofluorescence. Scale bar, 50 µm.
Supplementary Figure S6. RNA velocity analysis recapitulates dynamics of MMT in the LLC-tumor *in vivo*. (A) A unique CAF cluster (α-SMA+) was found from the macrophage-specific 10x scRNA-seq of the LysM-driven tdTomato+ cells sorted from LLC-tumor. (B) Interestingly, we observed a progressive loss of macrophage lineage markers (Csf1r, Cd68, Ccr5, MHC II molecules (H2-Eb1, H2-Ab1, Cd74) in the sorted LysM-driven tdTomato+ cells, from the strongest TAM phenotype (α-SMA-CD68+++ to CAF phenotype (α-SMA+++CD68-) in LLC-tumor on Day 16. (C) RNA velocity analysis recapitulated the dynamics of macrophage-lineage cells, revealing the *de novo* generation of CAFs (green cluster) from the TAMs (brown and blue clusters) in the LLC-tumor at transcriptome level with single cell resolution.
Supplementary Figure S7. Expression of LysM is undetectably low in fibroblast. The mouse fibroblasts (NIH/3T3, ATCC CRL-1658) and BMDMs were stimulated with LLC-CM for 1, 3, and 5 days. By real-time PCR analysis, we found that the expression level of macrophage markers (A) LysM and (B) F4/80 were undetectable in the LLC-CM stimulated mouse NIH/3T3 fibroblasts (ATCC CRL-1658) compared to BMDM (NS p>0.05 vs control groups without LLC-CM stimulation, ***p<0.001 vs 3T3 fibroblast groups, one-way ANOVA, n=3). (C) In addition, expression of LYZ (human gene homologue of the mouse LysM) was extremely low in the α-SMA+ CAF cluster of the human NSCLC dataset used in Figure 1A.
Supplementary Figure S8. Transcriptome analysis revealed the protumoral phenotypes that are specifically showed in the in vivo generated MMTs. (A) The up- and down-regulated DEGs of the MMTs compared to their controls, BMDMs without TGF-β1 stimulation in vitro\textsuperscript{[28]} and α-SMA\textsuperscript{-ve} F4/80\textsuperscript{+} TAMs (Figure 4A) in vivo, were extracted from their scRNA-seq datasets. (B) The numbers of common and MMT-specific DEGs were shown as a hierarchical clustering diagram. GO analysis of the up-regulated DEGs found specifically in the (C) in vivo and (D) in vitro generated MMTs, where results associated with protumoral functions were highlighted in red.
Supplementary Figure S9. Protein expression of α-SMA in the in vitro generated MMTs. Immunofluorescence detected a strong co-expression of both CAF (α-SMA) and macrophage (CD68) markers in the MMTs generated from BMDMs under 5 days of TGF-β1 stimulation in vitro.
Supplementary Figure S10. TGF-β1 signaling in TAMs is important for inducing CAF phenotypes. (A) Unbiased bioinformatic platform MetaCore (Clarivate Analytics) highlighted the importance of TGFβ-induced myofibroblast activity in the MMTs of NSCLC by submitting the up-regulated DEGs of α-SMA+CD68+ cells from the dataset of Figure 1A to a KEGG pathway analysis. (B) Furthermore, our results showed that TGF-β1 (5 ng/ml) markedly induced the expression of myofibroblast markers (α-SMA and FAP) and effector (VEGF) in the BMDMs on day 5 in vitro (**p<0.01, *p<0.05 vs control, t-test, n=3).
Supplementary Figure S11. Protumoral effect of MMTs in the macrophage-depleted mice. Diphtheria toxin mediated macrophage depletion largely suppressed (A) MMT as well as (B, C) tumor growth of the LLC-bearing LysM-DTR mice (DT) compared to their control group (PBS), which was effectively restored in the depleted mice by adoptive transferring the in vitro generated MMTs (DT+MMT) (**p<0.01 vs PBS control, #p<0.05 vs DT, one-way ANOVA, n=3-4). Scale bar, (A) 50 µm.
Supplementary Figure S12. Level of overall α-SMA⁺ CAFs showed a weak association with the mortality of lung adenocarcinoma. (A) Representative images of specimens with high and low levels of MMT (α-SMA⁺ CD68⁺) in the NSCLC cohort of Figures 2 and 5. (B) No significant difference of the overall α-SMA⁺ CAFs between subtypes was detected in the same NSCLC cohort (NS, one-way ANOVA, n=222). (C) Where high level of overall CAFs (α-SMA⁺ area > 10%) was weakly associated with a poorer survival of the patients with lung adenocarcinoma (*p=0.045, Log-rank test, n=161).