Supplemental Information

Dualism of FGF and TGF-β Signaling in Heterogeneous Cancer-Associated Fibroblast Activation with ETV1 as a Critical Determinant

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Figure S1. FGF2 and TGFβ1 have opposite effects on CAF gene expression in both human and mouse dermal fibroblasts, Related to Figure 1.

(A and B) Double immunofluorescence analysis of α-SMA (green) and MMP1 (red) in HDFs plus/minus FGF2 or TGFβ1. Shown are further representative images other than the ones shown in Figure 1G (A) and quantification of MMP1 and α-SMA average fluorescence signal intensity per cell per field (B). Scale bar 50 μm. n(fields per condition) = 8, mean ± SEM, one-way ANOVA/Dunnett’s test, * p<0.05, ** p<0.005, *** p<0.001.

(C and D) RT-qPCR analysis of the indicated CAF effector genes in a different HDF strain from the one analyzed in Figures 1D and 1E, plus/minus treatment with FGF2, TGFβ1 or both. Values are expressed as Log10 ratios of treated versus untreated HDFs. Genes are grouped in TGFβ1- (C) and FGF2- (D) induced genes. n(independent experiments) = 3, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05, ** p<0.005, *** p<0.001.

(E) RT-qPCR analysis of the indicated CAF effector genes in mouse dermal fibroblasts (from C57BL/6 wild-type mice) plus/minus treatment with FGF2, TGFβ1 or the combination of both. Values are expressed as Log10 ratios of treated versus untreated fibroblasts. Genes are divided in TGFβ1- (left) and FGF2- (right) induced genes. n(independent experiments) = 3, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05, ** p<0.005, *** p<0.001.

(F) RT-qPCR analysis of the indicated CAF effector genes in HDFs plus/minus treatment with FGF2, FGF5, FGF9 and FGF10. Values are expressed as Log10 ratios of treated versus untreated HDFs. Genes are grouped in TGFβ1- and FGF2-induced genes. n(independent experiments) = 3, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05, ** p<0.005, *** p<0.001.
Figure S2. The effects of FGF modulation on CAF effector gene expression are MEK/ERK-dependent and p53-independent, Related to Figure 2.

(A) RT-qPCR analysis of the indicated CAF effector genes in HDFs after 72 hours treatment with MEK1/2-inhibitor PD184352 versus vehicle (DMSO) control. n(independent experiments) = 3, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05, ** p<0.005, *** p<0.001.

(B) EdU labeling assays (red) of HDFs stably infected with either DNFGFR1- or DNTGFBR2-expressing lentiviruses versus empty-vector controls. Shown are representative images (left) and quantification of the results (right). Scale bar 50 μm. >6 fields per condition, n(independent experiments) = 4, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05, *** p<0.001.

(C) RT-qPCR analysis of indicated CAF effector genes and of p53-target gene CDKN1A in HDFs plus/minus CRISPR-mediated TP53 gene deletion and infection with a DNFGFR1-expressing lentivirus versus empty-vector control, 10 days after infection. Data shown are from one out of two experiments with similar results.
Figure S3. Global analysis of gene expression in HDFs upon modulation of FGF versus TGFβ signaling, Related to Figure 3.

(A and B) GSEA using RNA-seq expression profiles of HDFs plus/minus FGF2-treatment against an unbiased collection of curated gene sets comprising chemical and genetic perturbations and canonical pathways (Broad Institute). Shown are the lists of gene sets with most significant enrichment (FDR ≤ 0.1) in FGF2-up-regulated (A) and down-regulated (B) genes.
(C) GSEA as the one performed in panel A and B, run against two gene sets derived from published RNA-seq expression profiles of TGFβ1-treated HDFs (GEO accession GSE79621). Positively and negatively differentially expressed genes (Absolute log2FC ≥ 0.58, p-value ≤ 0.05) were used to build TGFβ1_UP (left) and TGFβ1_DOWN (right) gene sets respectively. The complete lists of genes that they comprise are shown in Table S2.

(D) GSEA using GeneChip array data of DNFGFR1- (left) or DNTGFBR2-expressing (right) HDFs versus controls, respectively against gene signatures of TGFβ1 or FGF2 down-regulated genes from RNA-seq profiles discussed in panels A-C.

(E) Lists of relevant FGF and TGFβ1 ligands and receptors differentially expressed in GeneChip array data of HDFs expressing DNFGFR1 (top) or DNTGFBR2 (bottom) versus control HDFs, with indicated Log2(Fold Change) values.

(F) GSEA using the same gene expression profiles as in panel D, run against the Hallmark gene sets collection (Broad Institute). Shown are the lists of gene sets with most significant enrichment (FDR ≤ 0.1) in DNFGFR1-up-regulated (top) and DNTGFBR2-up-regulated (bottom) genes.

(G) Similar GSEA as in F against an Inflammatory response gene set derived from the Molecular Signature Database (Broad Institute).

(H) RT-qPCR analysis of ETV1 in HDFs treated with indicated recombinant growth factors/cytokines described in Figure 1A. Values are expressed as Log10 of the ratio of treated versus untreated HDFs. n(HDF strains) = 3, mean ± SEM, repeated-ANOVA with Dunnett’s multiple comparison test, *** p<0.001.

(I) RT-qPCR analysis of ETV1 in HDFs plus/minus CRISPR-mediated TP53 gene deletion and infection with a DNFGFR1-expressing lentivirus versus empty-vector control as in Figure S2C. Data shown are from one out of two experiments with similar results.
Figure S4. FGF- TGFβ dualism in skin CAF heterogeneity in vivo, Related to Figure 4.

(A – C) Triple immunofluorescence analysis of 3 AK lesions for vimentin (cyan), ETV1 (red) and α-SMA (green), followed by quantification of single cell fluorescent signals. Shown are full scans of lesions and squared areas used for high magnification image analysis (A), together with representative high magnification confocal images (Image1 and Image2).
Image2, from AK1 and AK2, respectively) of stromal areas enriched for α-SMA versus ETV1 positive fibroblasts (arrow-heads) (B). (C) Quantification of the results plotted as individual cell values in arbitrary units (dots). For each lesion, >250 cells were counted, in n(fields per lesion) >8, mean± SEM, ANOVA with Bonferroni’s multiple comparison test, **** p<0.0001. Scale bars 200 μm (A) and 10 μm (B).

(D and E) Full scans (D) and representative high magnification images used for quantification (E) of two other SCCs besides those of Figures 4D-4H, analyzed by triple immunofluorescence with antibodies against vimentin (cyan), ETV1 (red) and α-SMA (green). Squares indicate areas used for high magnification image analysis (D). Arrow-heads point to examples of ETV1high/α-SMA-low versus ETV1-low/α-SMA-high vimentin-positive cells (E). Scale bars 500 μm (D) and 10 μm (E).

(F and G) Double immunofluorescence analysis of sequential tissue sections of SCC1 and SCC3 (shown in Figure 4D and 4E) with antibodies against vimentin (cyan) and pan-keratin (red). Shown are full scans of the lesions (F) and representative high magnification images (G). Scale bars 500 μm (F) and 10 μm (G).

(H) Immunofluorescence analysis with an antibody against pan-keratin (red) of two desmoplastic versus two non-desmoplastic SCC lesions, followed by selection of pan-keratin-positive islands (left, white selections) and calculation of their perimeter/area average ratio per field to evaluate the invasion pattern (right). n(fields per lesion) = 8, mean ± SEM, two-tailed unpaired t-test of combined desmoplastic versus non-desmoplastic SCCs, *** p<0.001. Scale bars 100 μm.

(I) Immunohistochemistry analysis for CD68 on the same SCC lesions described in the previous panel. Shown are representative images (left) and quantifications of CD68-positive cells per field (right). n(fields per lesion) = 8, mean ± SEM, two-tailed unpaired t-test of combined desmoplastic versus non-desmoplastic SCCs, *** p<0.001. Scale bars 100 μm.
Figure S5. HDFs with loss of either FGF or TGFβ signaling show tumor-promoting properties in vitro, Related to Figure 5.

(A) 2D co-cultures of CAL27 cells in 1:1 ratio with DNFGFR1- or DNTGFBR2-expressing HDFs versus controls. Cancer cell proliferation was assessed by EdU incorporation (cyan) and double immunofluorescence analysis for pan-keratin (red) and vimentin (green) for CAL27 and HDF cell identification respectively. Shown are representative images, while the quantification of EdU-positive CAL27 cells is reported in Figure 5B. Scale bar 100 μm.

(B) Sphere forming assays of CAL27 cells admixed in 1:1 ratio with DNFGFR1- or DNTGFBR2-expressing HDFs versus controls. Cancer cell proliferation was assessed by EdU incorporation (cyan) and double immunofluorescence analysis for pan-keratin (red) and vimentin (green) for CAL27 and HDF cell identification respectively. Shown are representative images, while the quantification of EdU-positive CAL27 cells is reported in Figure 5B. Scale bar 100 μm.

(C) Conditioned Medium (CM) on HDF-derived ECM

(D) Conditioned Medium (CM) on HDF-derived ECM

(E) Conditioned Medium (CM) on HDF-derived ECM

(F) Conditioned Medium on other HDFs

(G) Pro-Mitogenic Factors

(H) SCC13 - Conditioned Medium

(I) Conditioned Medium on other HDFs

Related to Figure 5.
controls and grown on Matrigel. Shown are representative images, while the quantification of number of spheres per field for each condition is reported in Figure 5D. Scale bar 500 μm.

(C) Diagram representing the setup of the experiments with SCC13 cells cultured with fibroblast-derived conditioned medium (CM) and extracellular matrix (ECM).

(D and E) EdU labeling assays (red) of SCC13 cells cultured with conditioned medium (D) or on top of ECM (E) derived from DNFGFR1- or DNTGFR2-expressing HDFs versus controls. Shown are representative images (left) and quantifications of the results (right). The pictures in panel E have been assessed for vimentin expression in Figure 5E. Scale bar 200 μm. >500 cells assessed from >10 fields per condition. n(independent experiments) = 3, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05.

(F) Normal HDFs were cultured for 72 hours with conditioned medium derived from either DNFGFR1- or DNTGFR2-expressing HDFs versus controls, followed by RT-qPCR analysis of indicated CAF effector genes. n(independent experiments) = 3, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05.

(G) List of CAF effector genes up-regulated in either DNFGFR1- or DNTGFR2-expressing HDFs versus controls with reported pro-mitogenic properties, derived from GeneChip array data (deposited under GEO accession number GSE122372) discussed in Figures 3B-3D (FC>1.5, p-value<0.05) and from the genes validated by RT-qPCR (in bold) shown in Figures 2G-2H.

(H) SCC13 cells were cultured with conditioned medium derived from different HDFs as in panel D, followed by RT-qPCR analysis of indicated EMT genes. n(independent experiments) = 3, mean ± SEM, repeated-ANOVA/Dunnett’s test, * p<0.05, ** p<0.005, **** p<0.0001.

(I) Representative images of hematoxylin and eosin staining of organotypic assays performed with CAL27 cells and the indicated fibroblasts, described in Figure 5G.
Figure S6. HDFs with loss of either FGF or TGFβ signaling or with ETV1 modulation boost tumorigenicity of SCC cells, Related to Figure 6.

(A and B) RFP-expressing SCC13 cells were admixed with HDFs infected with either DNFGFR1-(A) or DNTGFBR2-expressing lentiviruses (B) versus empty-vector controls, followed by parallel co-injection into mouse ears. Shown are representative images of injected ear pairs at day 16 after injection (left) and quantifications of relative red fluorescence intensity values normalized to day 1 values (right). Scale bar 2 mm. (A) n(injected ear pairs) = 11. (B) n(injected ear pairs) = 17. Mean ± SEM, two-tailed paired t-test, * p<0.05.

(C) Hematoxylin and eosin staining of parallel ear lesions formed by CAL27 cells co-injected with HDFs infected with DNTGFBR2-expressing lentiviruses versus empty-vector controls. Scale bar 500 μm.

(D) Representative images of immunofluorescence analysis with anti-pan-keratin antibodies (red) of lesions formed by CAL27 cells admixed with DNTGFBR2-expressing HDFs. Quantifications reported in Figure 6D.

(E and F) Hematoxylin and eosin staining of parallel ear lesions formed by SCC13 cells co-injected with HDFs infected with either DNFGFR1-(E) or DNTGFBR2-expressing lentiviruses (F) versus empty-vector controls. Scale bar 500 μm.

(G and H) Hematoxylin and eosin staining of parallel ear lesions formed by CAL27 cells co-injected with HDFs infected with either shETV1 (G) or ETV1-expressing lentiviruses (H) versus empty-vector controls. Scale bar 500 μm.
Figure S7. Western blot full scans, Related to Figures 1, 2 and 3.