## Supplementary Material

# p53 status in stromal fibroblasts modulates tumor growth in SDF-1-dependent manner

Yoseph Addadi, Neta Moskovits, Dorit Granot, Guillermina Lozano, Yaron Carmi, Ron N. Apte, Michal Neeman and Moshe Oren

## Supplementary Materials and Methods

### Retroviruses

The procedure was as described by Milyavsky et al [1]. The pRS-shSDF-1 puro, targeting mouse SDF-1, was generated based on Dharmacon design guidelines. The mouse SDF-1 sequence targeted for knock-down was: 5'-GAGCCAACGTCAAGCATCT-3'. Oligonucleotides were cloned into BglII-HindIII digested pRetro-SUPER vector [2].

### Real-time reverse transcription-PCR analysis

Total RNA was extracted using NucleoSpin kit (Macherey Nagel, Germany). One microgram of each RNA sample was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random hexamer primers. QRT-PCR was performed using SYBR PCR Master Mix (Applied Biosystems, UK) on an ABI 7300 instrument (Applied Biosystems, Singapore). Expression levels of *SDF-1* and *CXCL1* mRNA were normalized to those of the *GAPDH* or *HPRT*. The following primers pairs were used (F= forward, R= reverse):

| | | |
|---|---|---|
| *m-HPRT* | F: GCAGTACAGCCCCAAAATGG | R: GGTCCTTTTCACCAGCAAGCT |
| *m-beta actin* | F: GCTTCTTTGCAGCTCCTTCGT | R: CGTCATCCATGGCGAACTG |
| *m-SDF-1* | F: GAAAGGAAGGAGGGTGGCAG | R: TCCCCGTCTTTCTCGAGTGT |
| *m-CXCL-1* | F: GCTTGAAGGTGTTGCCCTCA | R: CTATGACTTCGGTTTGGGTGC |

**Immunoblotting**

Cells were lysed in Tris-Triton lysis buffer and cell extract aliquots containing equal amounts of total protein (Bradford assay) were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Kent, United Kingdom), which were probed with the following primary antibodies: a mixture of the p53-specific monoclonal antibodies PAb1801 and DO-1 for human p53; rabbit polyclonal anti-p53 (CM5; Novocastra, Newcastle upon Tyne, United Kingdom) for mouse p53 and mouse monoclonal anti-GAPDH (MAB374; Chemicon International, Chandlers Ford, United Kingdom). Western blots were developed with the enhanced chemiluminescence reagent (Amersham Pharmacia, Piscataway, NJ).

**Histological analysis**

Tumors were fixed in buffered formaldehyde (4%). Fixed paraffin-embedded tumor blocks were sectioned and stained with haematoxylin and eosin (H&E), Representative slides were taken for immunohistochemical staining, Sections were deparaffinized with xylene for 5 min, followed by sequential ethanol hydration and double-distilled water. Sections were then washed with PBS for 5 minutes and blocked by incubation with 1% BSA in PBS at room temp. To visualize blood endothelial cells, sections were stained with anti CD34 (Cedarlane laboratories) for young blood vessel or anti SMA for mature blood vessels. Slides were counter-stained with Hoechst (Molecular Probes) for visualization of nuclei.

To visualize GFP-expressing fibroblasts, tumors were frozen in liquid nitrogen immediately following excision from the animal, 10μ sections were fixed with 2.5% paraformaldehyde for 10 minutes, washed with PBS for 5 minutes and blocked by incubation with 20% normal horse serum and 0.2% for 1 hour, followed by incubation with anti-GFP (Abcam) overnight. Slides were counter-stained with Hoechst (Molecular Probes) for visualization of nuclei.

**Quantification of secreted chemokine levels**

WT, p53-KO and p53-mutant MEFs were grown to 50% confluence, and then the medium was replaced with fresh medium containing 10% FCS. After 3 days, the conditioned medium was collected and subjected to ELISA for SDF-1 and CXCL1. Values were corrected for the cell number at the time of collection,

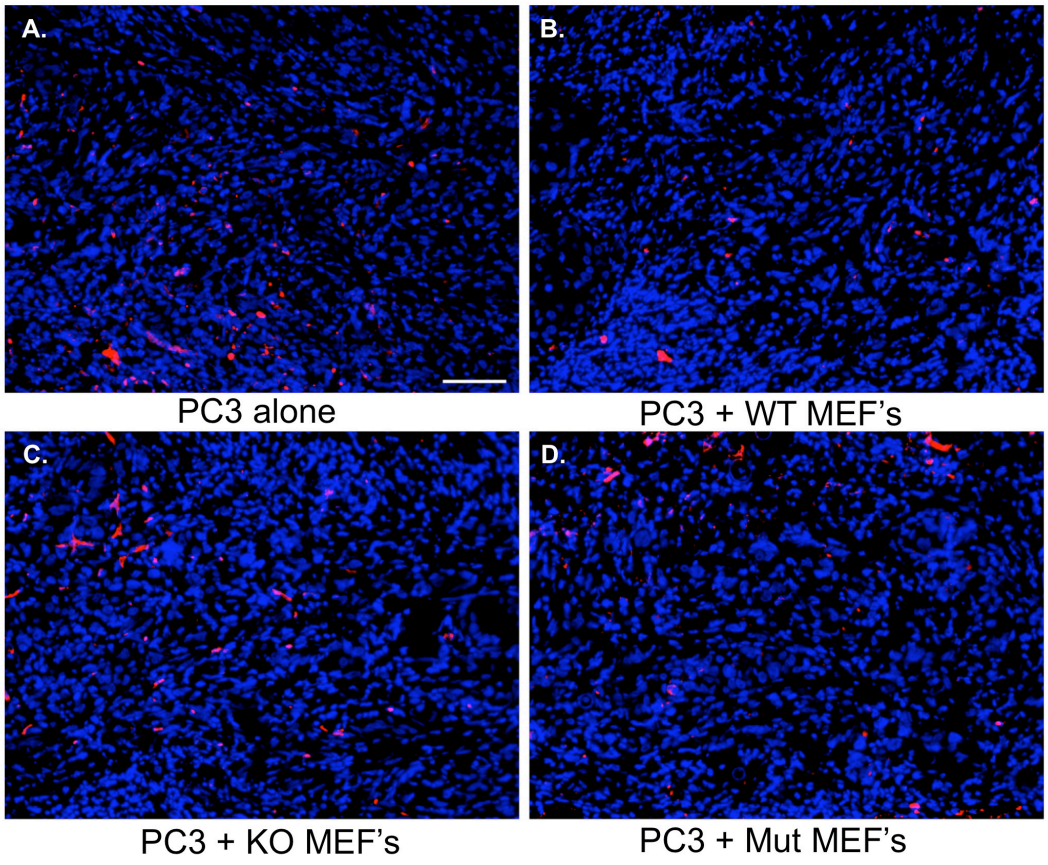
Levels of SDF-1α were measured using specific ELISA kits (DuoSet, R&D Systems, Minneapolis, MN). CXCL1 was measured by using the Qplex ™ Mouse Cytokine array (Quansys Biosciences,

Logan, UT) according to the manufacturer's instructions. The images were captured by a cooled CCD camera (MicroMAX-1300B, Roper Scientific, Tucson, AZ).

References


1. Milyavsky, M., et al., *Prolonged culture of telomerase-immortalized human fibroblasts leads to a premalignant phenotype.* Cancer Res, 2003. **63**(21): p. 7147-57.
2. Brummelkamp, T.R., R. Bernards, and R. Agami, *A system for stable expression of short interfering RNAs in mammalian cells.* Science, 2002. **296**(5567): p. 550-3.

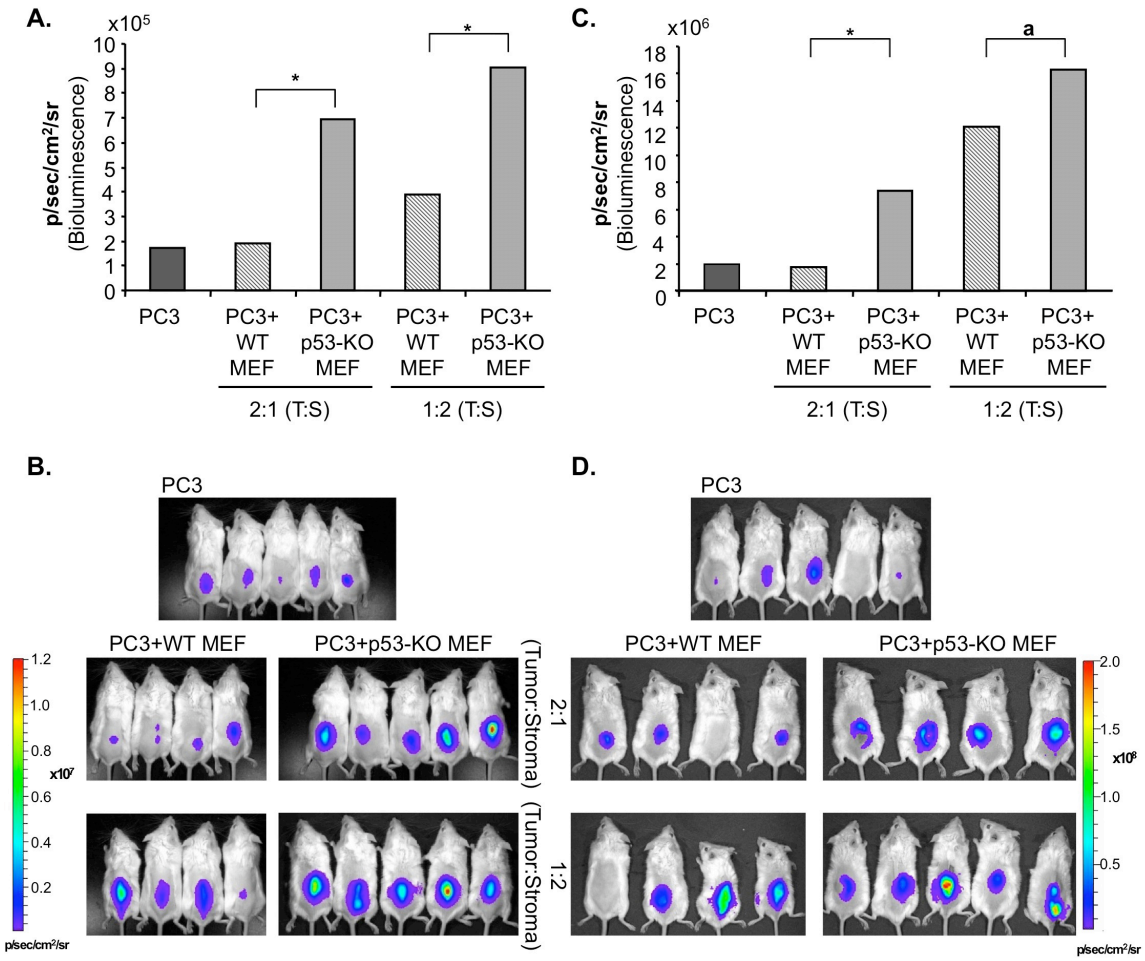
**Supplementary Figures**




**Supplementary Figure 1**

**Similar vascular involvement in tumors containing WT and p53 KO MEFs**

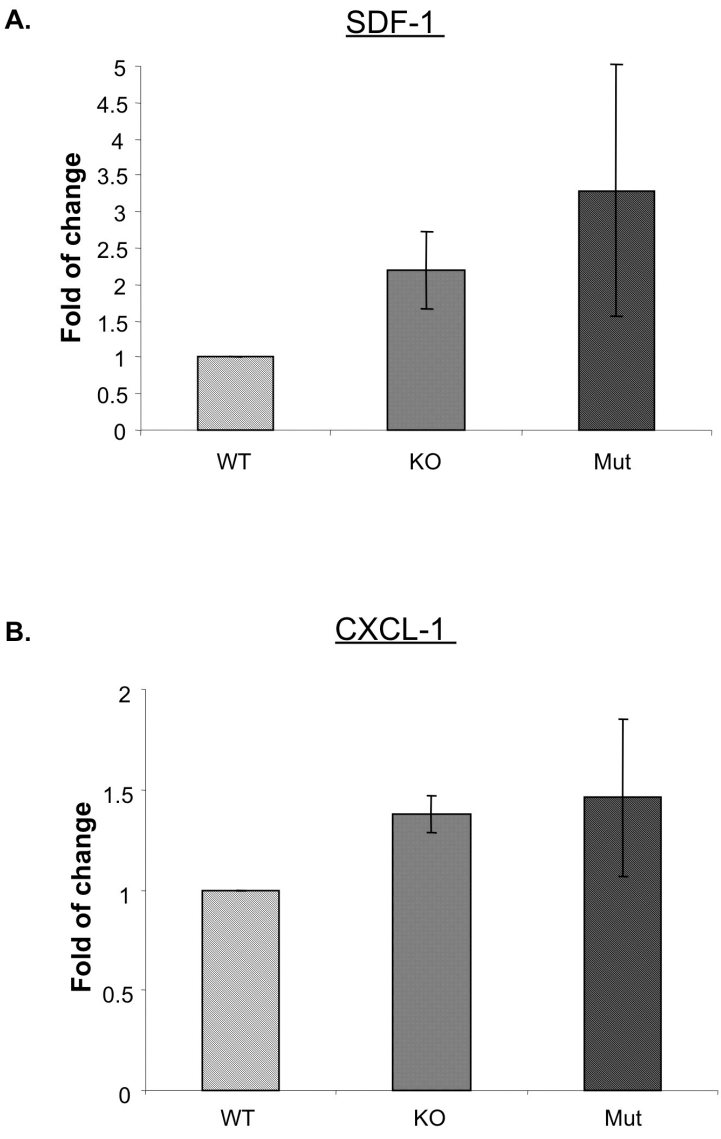
Representative images of sections of tumors derived by subcutaneous injection of PC3 cells alone (A) or in combination with WT MEFs (B), p53 KO MEFs (C) or p53-mutant MEFs (D), stained for CD34 as a marker for angiogenic vessels. No significant differences in intesity and pattern of staining are observable between the different groups. Bar = 50 μ.




**Supplementary Figure 2**

**Excess of exogenous fibroblasts abolishes the differential impact of their p53 status on tumor growth**

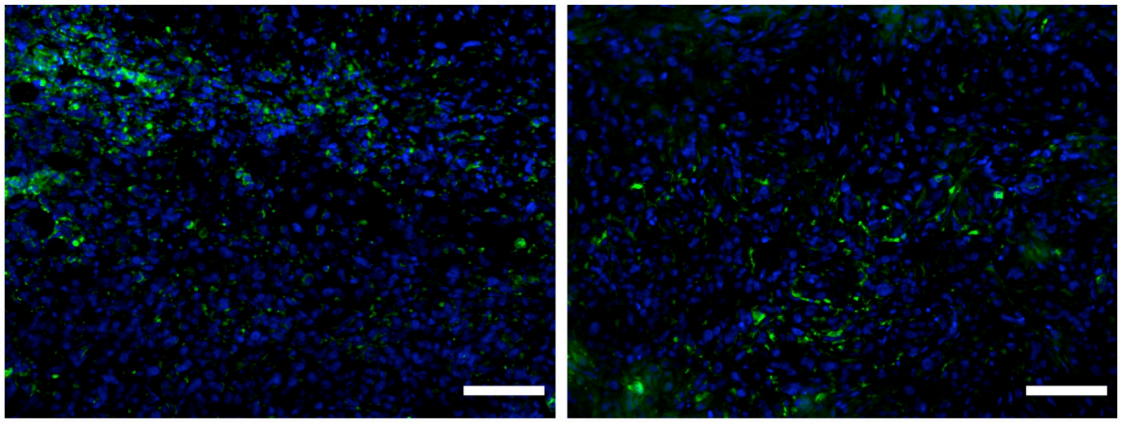
PC3 cells expressing luciferase were inoculated subcutaneously, either alone or together with WT MEFs or p53-KO MEFs, in the back of male SCID mice. Cells were mixed at two different ratios, either 2:1 ($2x10^6$ PC3:$1x10^6$ MEFs) or 1:2 ($2x10^6$ PC3:$4x10^6$ MEFs). Three (A,B) or twelve weeks (C,D) post inoculation tumor growth was measured by bioluminescence. **A,C.** Average bioluminescence calculated for each group of mice (n=4-5 mice per group). * denotes p-value<0.05; a p-value=0.32. **B,D** Color-coded bioluminescence images acquired with the IVIS system. **B**. Min Value- $8x10^4$, Max value-$1.2x10^7$ **D.** Min Value- $2.2x10^6$, Max value-$2x10^8$.




**Supplementary Figure 3**

**Quantification of secreted SDF-1 and CXCL1**

The conditioned medium of cells grown in 10% FCS was subjected to SDF-1 and CXCL1 protein determination by ELISA as described under Supplementary Materials and Methods. Relative cytokine concentrations are shown; the concentration present in the conditioned medium of the WT MEFs in each individual experiment was taken as 1.




**Supplementary Figure 4**

**Similar proliferation of wild type and p53 KO MEFs *in vivo***

Representative images of sections of tumors from each group stained for GFP (green) expressed by exogenously injected WT and KO MEFs. The image suggests similar abundance and distribution of MEFs of both genotypes. Nuclei were visualized by staining with Hoechst (blue). Bar = 50 μ .

Supplementary Table 1

| Group | Mouse number | Positive lymph node | Metastasis signal p/sec/cm$^2$/sr | Percentage of group | Average group signal p/sec/cm$^2$/sr |
|---|---|---|---|---|---|
| PC3 alone | 1 | None | 0 | 0% | 0 |
| | 2 | None | 0 | | |
| | 3 | None | 0 | | |
| | 4 | None | 0 | | |
| | 5 | None | 0 | | |
| | 6 | None | 0 | | |
| | 7 | None | 0 | | |
| | 8 | None | 0 | | |
| | 9 | None | 0 | | |
| | 10 | None | 0 | | |
| | 11 | None | 0 | | |
| | 12 | None | 0 | | |
| PC3 + WT Mef's | 1 | None | 0 | 8.33% | 4.68E+02 |
| | 2 | None | 0 | | |
| | 3 | None | 0 | | |
| | 4 | None | 0 | | |
| | 5 | None | 0 | | |
| | 6 | None | 0 | | |
| | 7 | None | 0 | | |
| | 8 | None | 0 | | |
| | 9 | None | 0 | | |
| | 10 | None | 0 | | |
| | 11 | Inguinal left | 5.62E+03 | | |
| | 12 | None | 0 | | |
| PC3 + KO Mef's | 1 | None | 0 | 41.67% | 2.23E+03 |
| | 2 | None | 0 | | |
| | 3 | Inguinal right | 5.99E+03 | | |
| | 4 | Inguinal right | 9.28E+03 | | |
| | 5 | None | 0 | | |
| | 6 | Inguinal right | 1.26E+03 | | |

| | | | | | |
|---|---|---|---|---|---|
| | 7 | None | 0 | | |
| | 8 | Inguinal right | 4.23E+03 | | |
| | 9 | None | 0 | | |
| | 10 | None | 0 | | |
| | 11 | Inguinal left | 6.05E+03 | | |
| | 12 | None | 0 | | |
| PC3 + Mutant Mef's | 1 | None | 2.28E+04 | 54.55% | 9.77E+03 |
| | 2 | None | 0 | | |
| | 3 | None | 0 | | |
| | 4 | None | 0 | | |
| | 5 | None | 0 | | |
| | 6 | Inguinal left | 4.17E+03 | | |
| | 7 | Inguinal left | 2.43E+04 | | |
| | 8 | Inguinal left | 2.70E+04 | | |
| | 9 | Inguinal left | 2.35E+04 | | |
| | 10 | Inguinal left | 1.55E+04 | | |
| | 11 | None | 0 | | |
| | 12 | None | 0 | | |

Metastasis rate data

Metastasis was imaged by bioluminescence following coverage of main tumor signal, in order to protect the camera from saturation. Lymph metastasis was counted and signal was quantified per animal and averaged per group.