Microenvironment and Immunology

Cancer Research

Fibroblast-Specific Protein 1/S100A4–Positive Cells Prevent Carcinoma through Collagen Production and Encapsulation of Carcinogens

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Abstract

Stromal restraints to cancer are critical determinants of disease but they remain incompletely understood. Here, we report a novel mechanism for host surveillance against cancer contributed by fibroblast-specific protein 1 (FSP1)$^+$/S100A4$^+$ fibroblasts. Mechanistic studies of fibrosarcoma formation caused by subcutaneous injection of the carcinogen methylcholanthrene (MCA) had suggested that IFN-γ receptor signaling may restrict MCA diffusion by inducing expression of collagen (foreign body reaction). We tested the hypothesis that this reaction encapsulated MCA and limited carcinogenesis by determining whether its ability to induce fibrosarcomas was impaired in the absence of proliferating fibroblasts. We found that FSP1$^+$/S100A4$^+$ fibroblasts accumulated around the carcinogen where they produced collagens, encapsulating MCA and protecting epithelial cells from DNA damage. Ablation of these cells at the site of MCA injection by local administration of ganciclovir in FSP-TK transgenic mice altered tumor morphology to an epithelial phenotype, indicating that, in the absence of encapsulating fibroblasts, MCA targeted epithelial cells. Notably, we showed that destruction of the fibrous capsule around the MCA by local injection of collagenase induced rapid tumor development in mice that were otherwise durably tumor free. Our findings demonstrate that the FSP1$^+$/S100A4$^+$ fibroblasts prevent epithelial malignancy and that collagen encapsulation of carcinogens protects against tumor development. Together, this study provides a novel mechanism for host surveillance against cancer. *Cancer Res; 73(9); 2770–81. ©2013 AACR.*

Introduction

The term "fibroblast" is used to describe a heterogeneous multifunctional group of cells of mesenchymal origin. Fibroblasts synthesize extracellular matrix (ECM), produce many immune modulators, and play an important regulatory role in inflammation, wound healing, and tissue fibrosis (1–5). Fibroblasts are also a major cell type in the tumor stroma (6, 7). The impact of fibroblasts on tumor growth and progression has been the subject of intensive investigation recently. Fibroblast infiltration, collagen deposition, and tissue fibrosis are commonly observed in human cancers (8–10), it is still not well understood how exactly fibroblasts and their ECM products influence the different stages of tumorigenesis and malignant transformation.

In many studies, fibroblasts have been shown to support tumor development (11–14). Cancer-associated fibroblasts (CAF) have been reported to stimulate cancer cell proliferation, angiogenesis (15), invasiveness, metastasis (16, 17), and cancer-related inflammation (18). We have previously reported that fibroblasts promote tumor development by enhancing monocyte chemoattractant protein-1–dependent macrophage infiltration and chronic inflammation (19).

There is also evidence that fibroblasts can inhibit tumor growth. For example, fibroblasts repress the early stages of tumor progression by facilitating the formation of gap junctions and exerting contact inhibition on cancer cells (20, 21). In addition, fibroblast-specific signaling pathways, such as TGF-β and Pten-Ets2 have been identified to suppress epithelial tumors (22, 23). The exclusive response of stroma fibroblasts to interleukin (IL)-4 is sufficient for the rejection of IL-4–secreting tumors (24). Therefore, the role of fibroblasts in tumor development warrants further investigation.

Polycyclic aromatic hydrocarbons (PAH) are a group of environmental pollutants, some of which (e.g., benzo(a)pyrene) occur naturally in coal, crude oil, and gasoline and have been shown to cause human cancers (25). Methylcholanthrene, another PAH molecule, has been widely used in mice to study chemical-induced carcinogenesis (26–28). For unknown reasons, methylcholanthrene often induces fibrosarcomas at the site of injection when injected in oil subcutaneously or intramuscularly. In a previous study, we showed that there is a good correlation between a protective response against

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methylcholanthrene and encapsulation of the carcinogen (27, 29), and we postulated that fibrosarcomas arise because fibroblasts acquire mutations during this protective foreign body response. A foreign body reaction is an evolutionary conserved strategy in which material that is foreign to the respective tissue is encapsulated. In mammals, this is accomplished first by encapsulating cells such as macrophages and fibroblasts and later by ECM deposition around the foreign body. In this study, we wanted to test if there is a causal relationship between methylcholanthrene-encapsulation and protection against tumor development.

Fibroblast-specific protein 1 (FSP1), also named as S100A4, belongs to the S100 superfamily of cytoplasmic calcium-binding proteins and can be expressed by different cell types of mesenchymal origin (30, 31). FSP1/S100A4$^+$ cells are mainly fibroblasts in skin (19), kidney (30), lung (32), and normal heart (33). In FSP-thymidine kinase (TK) transgenic mice, upon administration of ganciclovir, proliferating FSP1$^+$ cells can be depleted selectively (34, 35). Using the FSP-TK mice, we found that ablation of FSP1$^+$ cells altered tumor morphology from a fibroblastoid toward an epithelial phenotype in some tumors induced by methylcholanthrene inoculation subcutaneously. Furthermore, disruption of the capsule by collagen degradation resulted in rapid tumor development in long-term "tumor-free" mice. Our results strongly suggest that FSP1$^+$ cells (most likely fibroblasts because of their procollagen I expression) and collagens play an important protective role during chemical carcinogenesis and may have implications for cancer prevention.

Materials and Methods

Mice

BALB/c, C57BL/6, and 129/Sv/Ev mice were purchased from Vital River. FSP-TK transgenic mice were obtained from Dr. Eric G. Neilson (Northwestern University, Feinberg School of Medicine). Six- to 8-week-old sex- and age-matched mice were used for experiments. All animal studies were conducted with the approval of the corresponding Chinese and German authorities.

Animal experiments

Methylcholanthrene-induced tumorigenesis. BALB/c, C57BL/6, and 129/Sv/Ev mice were injected subcutaneously in the left abdomen or intramuscularly in the left hind leg with different amounts of methylcholanthrene (Sigma) suspended in 0.1 mL of sesame oil. Tumor development was observed 2 to 3 times weekly for 1.5 to 2 years. Mice with a tumor of 10 mm in diameter were counted as tumor positive.

Depletion of proliferating fibroblasts. FSP-TK mice and control littermates were first injected subcutaneously in the left abdomen with 25 μg methylcholanthrene suspended in 0.1 mL of sesame oil. To deplete fibroblasts *in vivo*, 1 mg ganciclovir was dissolved in water and mixed with 0.1 mL sesame oil and was injected next to the methylcholanthrene/oil injection. The time of methylcholanthrene/oil injection was defined as week 0. Ganciclovir was applied once a week from week 3 to 5. In control experiments (described in Supplementary Fig. S4), FSP-TK mice and control littermates ganciclovir treatment was applied in week 0 to 2 and week 8 to 10, respectively. Fibrosarcomas typically developed within 3 to 5 months.

Destruction of the fibrotic capsule by collagenase injection. First, to generate long-term tumor-free mice that harbor collagen-encapsulated methylcholanthrene as described previously (27, 29), 129/Sv/Ev mice were injected intramuscularly in the left hind leg with 100 μg methylcholanthrene/oil. The mice that remained tumor-free at day 300 were subsequently injected with 0.1 mg collagenase (Sigma) in 0.1 mL water-in-oil micro emulsion at the methylcholanthrene site. As control, only 0.1 mL water-in-oil micro emulsion was injected and tumor development was monitored twice a week.

Tumor transplantation experiments. A total of 1×10^5 cells of the cell lines Mc3-5a, Mc3-5b, and the control McO cell line were subcutaneously injected into nude mice and tumor development was monitored.

Immunohistochemistry

Tissues from the methylcholanthrene injection site and tumors from above animal experiments were prepared and paraffin and frozen sections were done as described previously (29). For E-cadherin (BD Pharmingen), cytokeratin (CK; Biolegend), FSP1 (a gift from Dr. Eric G. Neilson, Northwestern University, Feinberg School of Medicine), and thymidine kinase (a gift from Dr. Xianghui Yu, Jilin University, School of Life Sciences) staining, paraffin sections were incubated with above antibodies respectively, and then incubated with biotinylated secondary antibody followed by streptavidin-peroxidase. The peroxidase activity was detected with diaminobenzidine (Sigma). FSP1 and Gr-1 (BD Pharmingen), FSP1 and CD4 (BD Pharmingen), FSP1 and CD8 (BD Pharmingen), FSP1 and CD31 (BD Pharmingen), FSP1 and F4/80 (Biolegend), FSP1 and ER-TR7 (Abcam), FSP1 and laminin-1 (R&D), FSP1 and PCNA (Tianjin Sungene Biotech), FSP1 and α-smooth muscle actin (α-SMA; Abcam), FSP1 and procollagen I (Santa Cruz Biotechnology), FSP1 and fibronectin (Thermo Scientific), FSP1 and p65 (Santa Cruz Biotechnology), FSP1 and cyclin D1 (Santa Cruz Biotechnology), FSP1 and γ-H2AX (Millipore), CK and γ-H2AX, and F4/80 and Ki67 (BD Pharmingen) double staining was conducted as described previously (19, 36). Adjacent sections were used to double stain γ-H2AX and FSP1, γ-H2AX and CK, respectively. Sections were evaluated under the microscope (DP71; Olympus) for bright field and fluorescence microscopy. The results were expressed as the mean count of cells per high power fields (×200 or ×400).

Isolation of tumor cells and establishing of tumor cell lines

Tumors from mice treated with methylcholanthrene and ganciclovir at different time periods as described earlier were isolated. One part of the tumor was reserved for histologic analysis and the rest of the tumor material was minced, treated with the lysis buffer (Trypsin, EDTA, and collagenase) for 5 to 10 minutes at 37°C, and then cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The primary tumor cell cultures McO and McN were obtained from FSP-TK and control mice without ganciclovir treatment. Tumor cell cultures Mc3-5a and Mc3-5b were

obtained from FSP-TK mice treated with ganciclovir at week 3 to 5.

In vitro ganciclovir sensitivity assay

To determine FSP-TK gene expression in reisolated tumor cell lines, ganciclovir sensitivity in McO, McN, Mc3-5a and Mc3-5b cells was measured. A standard MTT assay was conducted with cells previously incubated with ganciclovir for 72 hours.

Statistical analysis

Data were analyzed using Fisher exact test and two-tailed unpaired Student *t* test. Mean ± SD were presented. *P* values less than 0.05 were considered statistically significant. For cell counting of immunohistochemistry staining, 4 fields were selected randomly in every section, 10 sections from each mouse, and the number of immunopositive cells per high-power field (HPF; ×400 or ×200) was counted.

Results

FSP1$^+$ cells actively participated in the host reaction to methylcholanthrene

Previously, we observed a foreign body reaction to methylcholanthrene in 129/Sv/Ev mice (29). To confirm that this result is not strain-dependent, groups of BALB/c mice were inoculated at first with a low dose (25 μg) of methylcholanthrene in sesame oil subcutaneously. A series of tissue sections of the inoculation site were stained for accumulating cells at week 1, 3, 5, and 7. As shown in Fig. 1A, the total number of infiltrating cells at the methylcholanthrene injection site increased with time. A large amount of FSP1$^+$ cells were detected at week 3 and these cells reached a peak in number at week 5 and dominated at week 7 (Fig. 1B). Double staining for PCNA and FSP1 showed that most of FSP1$^+$ cells were proliferating cells (Fig. 1C). This observation is in accordance with our previous studies with 129/Sv/Ev mice (29). Furthermore, the NF-κB signaling pathway in FSP1$^+$ cells was activated by methylcholanthrene (Supplementary Fig. S1). Notably, the active response of FSP1$^+$ cells to the carcinogen/oil emulsion is not specific to methylcholanthrene but also occurs after application of other carcinogens, such as 7,12-dimethylbenz(α)anthracene (DMBA) and diethylnitrosamine (DEN) when injected subcutaneously in oil (Supplementary Fig. S2).

FSP1$^+$ cells around the methylcholanthrene injection site were mainly fibroblasts

To characterize the FSP1$^+$ cell types that are induced by methylcholanthrene in the skin, tissue sections of the inoculation site were stained for different markers. As shown in Fig. 2A, the FSP1$^+$ cells rarely express Gr-1, CD4, CD8, or CD31. However, about 25% of the FSP1$^+$ cells were also F4/80$^+$, indicating that some of these cells were macrophages (Fig. 2B). Further staining with FSP1 and α-SMA or procollagen I showed that although approximately 18% of the FSP1$^+$ cells were α-SMA$^+$, approximately 72% of them were procollagen I$^+$. In addition, we found that the distribution of other ECM molecules, such as fibronectin, ER-TR7, and laminin-1 has a correlation with that of FSP1$^+$ cells (Supplementary Fig. S3). This result, together with that shown in Fig. 1, showed that most of the FSP1$^+$ cells around the methylcholanthrene were proliferating fibroblasts and the fibroblasts were activated by the reaction with methylcholanthrene. Accordingly, a Sirius red staining revealed that collagen deposits at the methylcholanthrene injection site colocalized with FSP1$^+$ cells (Fig. 2C). Similar results were obtained when C57BL/6 or 129/Sv/Ev mice were injected subcutaneously or intramuscularly with 300, 100, 25, or 5 μg methylcholanthrene in oil (data not shown), suggesting that neither mouse strain nor the concentrations of methylcholanthrene applied had a significant influence on the nature of the cellular response to methylcholanthrene, with regard to the accumulation of FSP1$^+$ cells and the foreign body response. These results confirm our previous finding that the primary host response to methylcholanthrene is a "foreign body reaction" (29) and further suggest that FSP1$^+$ cells, especially fibroblasts, play a major role during the chemical carcinogenesis.

Tumors with epithelial phenotype appeared in the injection site in the absence of local proliferating FSP1$^+$ cells

To assess the role of fibroblasts in the host reaction to methylcholanthrene, FSP1$^+$ cells at the site of methylcholanthrene inoculation were selectively eliminated by local application of ganciclovir in FSP-TK mice. From 3 to 5 week after methylcholanthrene injection, when large amounts of fibroblasts had accumulated around methylcholanthrene and were proliferating vigorously as shown in Fig. 1B, mice were treated locally either with ganciclovir in oil or with oil alone as control. As shown in Fig. 3A and B, without ganciclovir treatment, all FSP-TK mice got tumors with a size of more than 10 mm in diameter at day 120 after methylcholanthrene injection, similar to the control mice with or without ganciclovir treatment. However, abatement of proliferating FSP1$^+$ cells in FSP-TK mice by ganciclovir treatment at week 3 to 5 led to a slight delay of tumor development (for about 3 weeks) in 4 of 8 mice. Nonetheless, in the long term, all tumors grew progressively.

Interestingly, the types of tumors that developed in ganciclovir-treated FSP-TK mice differed from tumors in other groups of mice. Three of 8 ganciclovir-treated FSP-TK mice developed a tumor with epithelial morphology after methylcholanthrene induction, whereas only fibrosarcomas formed in FSP-TK mice without ganciclovir treatment and in control mice with or without ganciclovir application. Figure 3C shows the morphology of epithelial tumors detected by Van Gieson staining. Tumors developing in FSP-TK mice without ganciclovir treatment contained massive spindle cells, a feature typical of conventional fibrosarcoma. However, in ganciclovir-treated FSP-TK mice, apoptotic fibroblasts were found in compartments enclosed by collagen fibers, between which epithelial malignancy developed with round or oval tumor cells. The expression of E-cadherin and cytokeratin in these tumor cells further confirmed their epithelial origin (Fig. 3D). These results suggest that ganciclovir treatment in FSP-TK mice led to malignant transformation of the surrounding epithelial cells and apoptotic fibroblasts within epithelial-like tumor.

To further study the morphology and origin of malignant cells and to test their tumorigenicity, tumor cell cultures

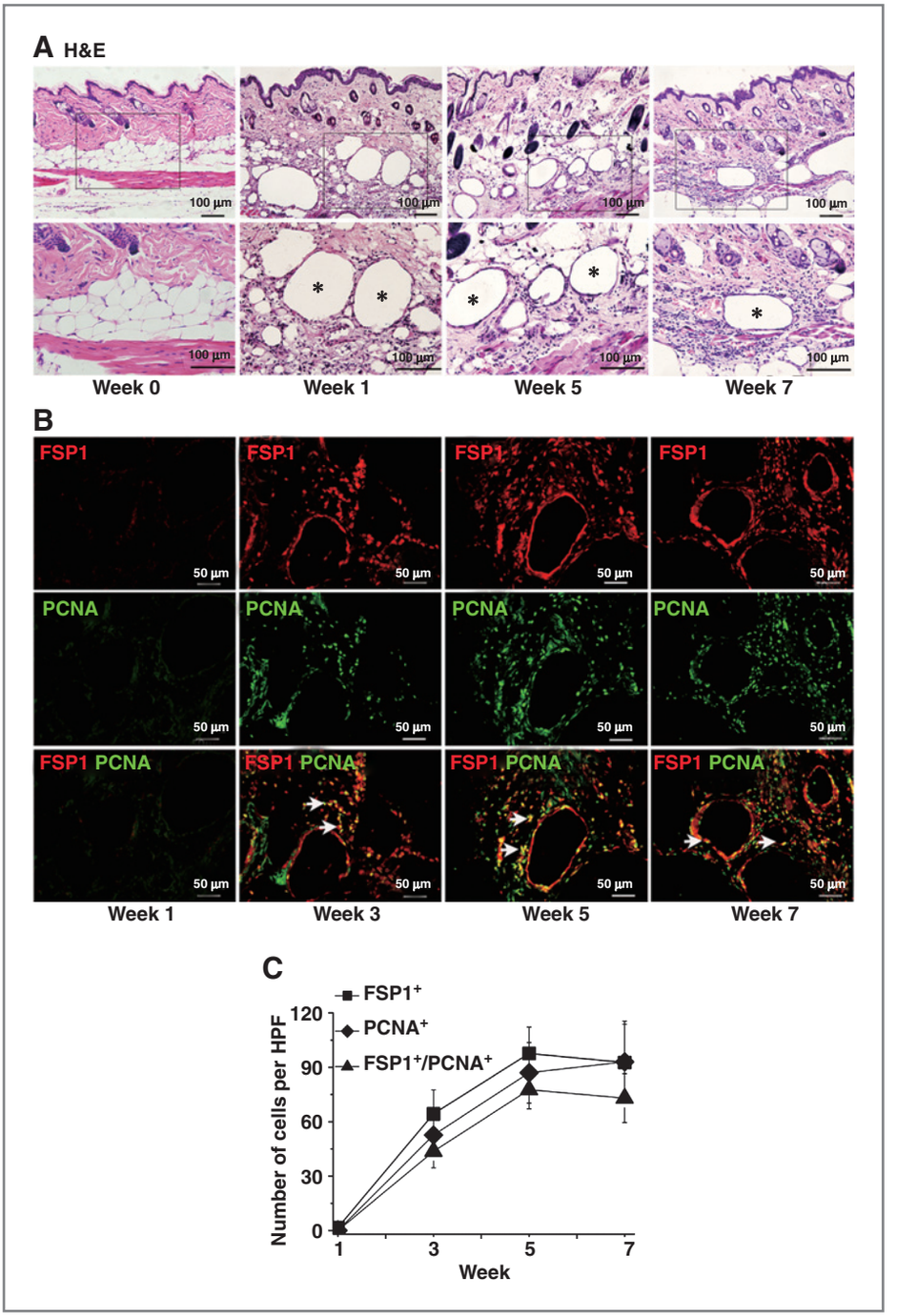

Figure 1. Proliferating FSP1$^+$ cells accumulate around the carcinogen. Groups of BALB/c mice (3–5/group) were injected subcutaneously with methylcholanthrene/oil. Skin sections were stained at different weeks after the methylcholanthrene injection. A, hematoxylin and eosin (H&E) staining shows the increasing amount of inflammatory cells that are recruited to the methylcholanthrene injection site. *, the representative injection site of the methylcholanthrene/oil suspension. B, double staining for FSP1 and PCNA identifies the proliferating FSP1$^+$ cells that accumulate around the injected methylcholanthrene. The FSP1$^+$/PCNA$^+$ cells are shown by arrows. C, the mean numbers of FSP1$^+$ cells, PCNA$^+$ cells, and FSP1$^+$/PCNA$^+$ cells in a HPF (×200) are shown.

were established. Mc3-5a and Mc3-5b are cells with epithelial phenotype established from ganciclovir-treated FSP-TK mice, whereas McO cells were established from FSP-TK mice without ganciclovir treatment and McN cell lines from FSP-TK–negative control mice. As shown in Fig. 3E, McO cells were killed dose-dependently by ganciclovir, but the malignant cells with epithelial phenotype were significantly resistant to ganciclovir *in vitro*, confirming that the FSP1 promoter was inactive in these cells. The control McN fibrosarcoma cells lacking the TK-gene, as expected, were resistant to ganciclovir.

To determine the tumorigenicity of the malignant cells, BALB/c nu/nu mice were injected with 1×10^5 of Mc3-5a, Mc3-5b cells, or as control, McO cells. As shown in Fig. 3F, all groups of cultured malignant cells developed into a tumor in nude mice, albeit with different kinetics. The tumor of McO cells was only fibroblastoid and negative for E-cadherin. However, Mc3-5a- and Mc3-5b-derived tumors were heterogeneous with

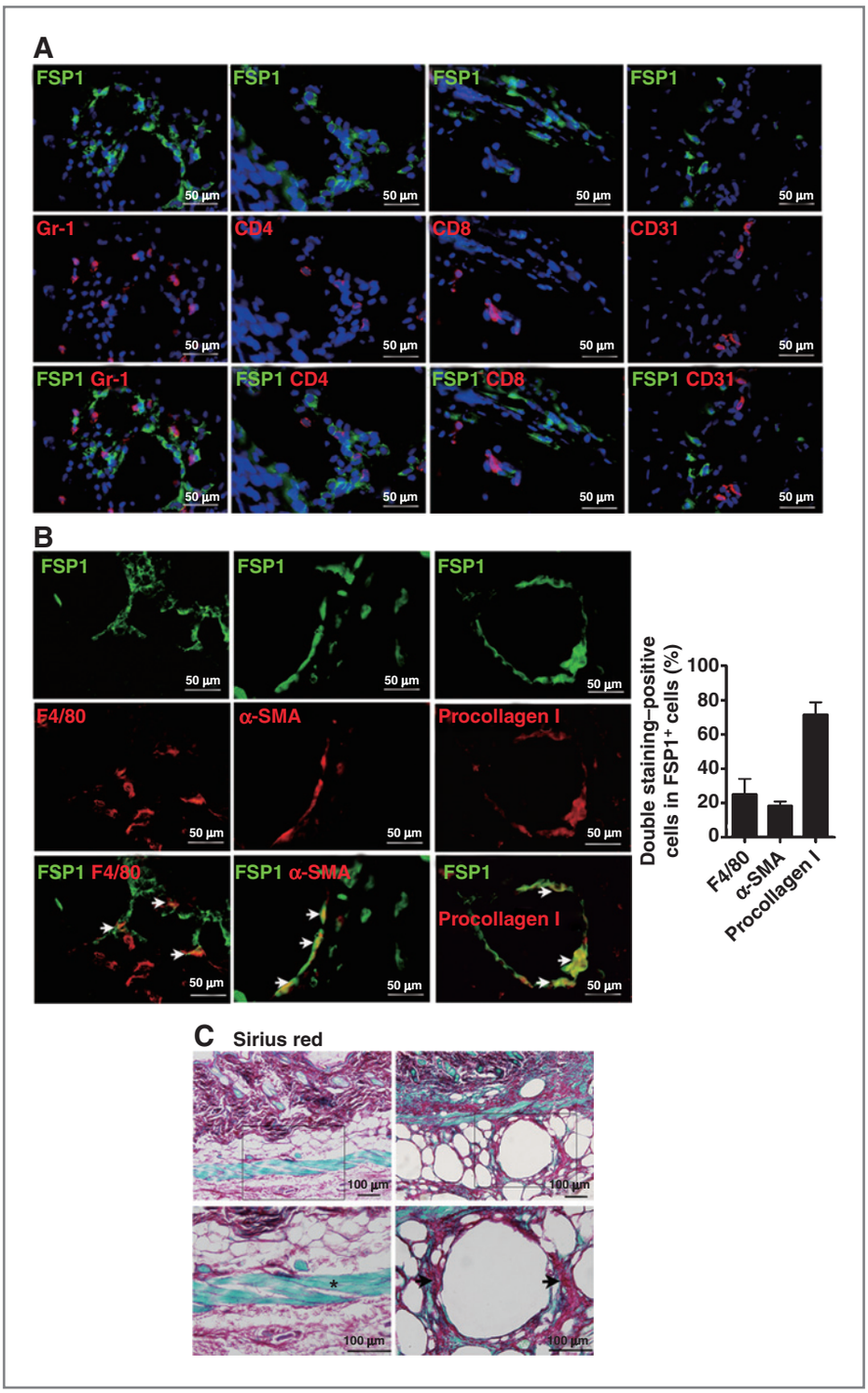

Figure 2. The majority of FSP1$^+$ cells that accumulate around the methylcholanthrene injection site are stained positive for procollagen I. BALB/c mice were injected subcutaneously with methylcholanthrene ($n = 5$). Five weeks later, skin sections were stained for markers as indicated. A, double staining of FSP1 and Gr-1, CD4, CD8, or CD31. An overlay of the staining shows that FSP1$^+$ cells are rarely granulocytes, lymphocytes, or endothelial cells (bottom). B, double staining of FSP1 and F4/80, α-SMA or procollagen I and the percentages of double positive cells are shown. Double positive cells are shown by arrows. C, Sirius red staining showing the deposition of collagens around the methylcholanthrene injection site. *, the injection site of the methylcholanthrene/oil suspension.

the morphology of epithelial tumor nests, and many E-cadherin–positive cells were found in these tumors (Fig. 3G). Furthermore, thymidine kinase expression was almost absent in these tumors, confirming that the FSP1 promoter was inactive in Mc3-5a and Mc3-5b cells. Only a small number of stromal cells probably derived from host expressed FSP1 in these epithelial tumors (Fig. 3H). The fact that ablation of proliferating FSP1$^+$ cells around methylcholanthrene results in epithelial tumors strongly suggests that FSP1$^+$ cells play a crucial role in protecting epithelial cells from malignant transformation.

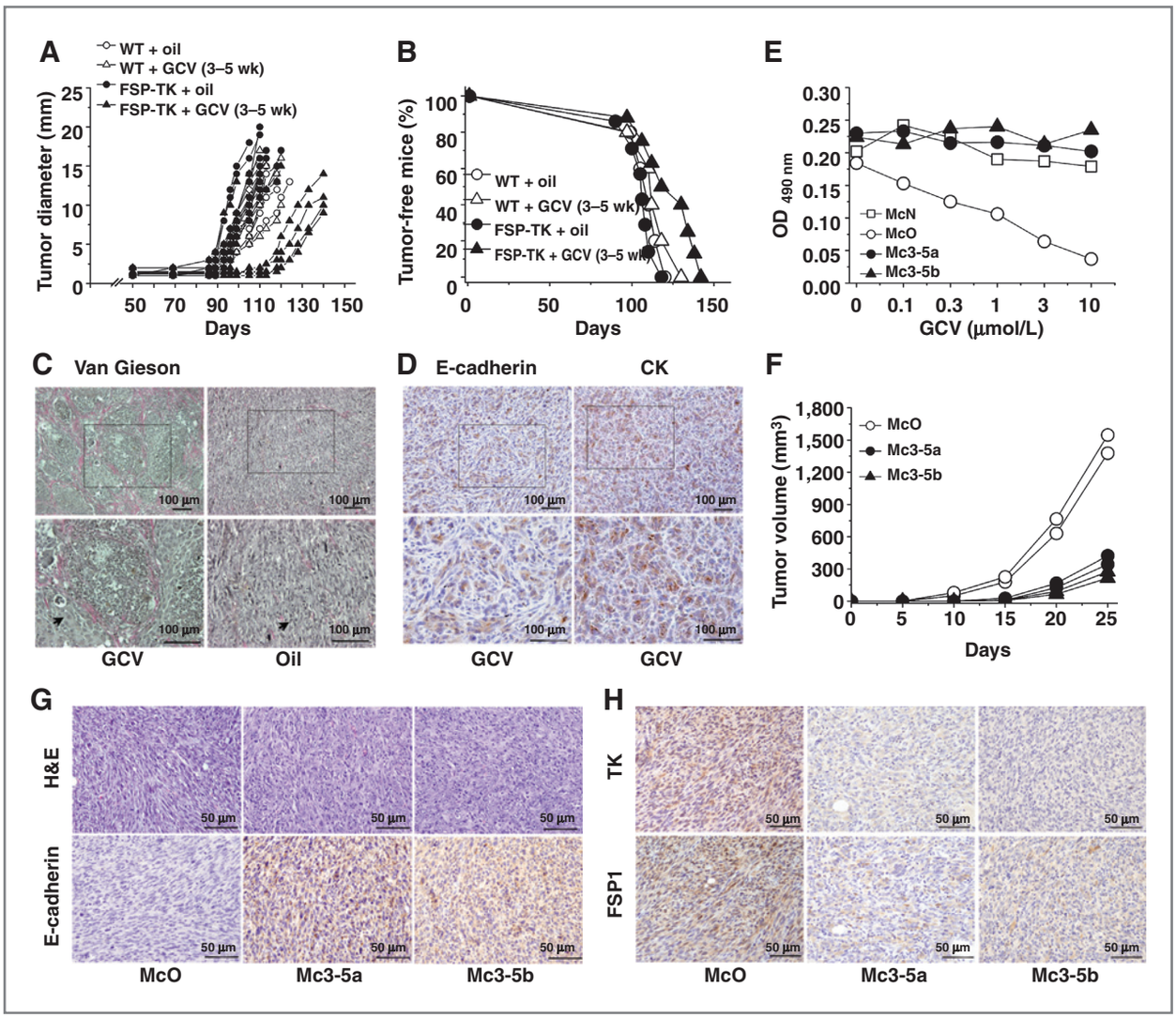

Figure 3. Tumors with an epithelial morphology occurred after local ablation of proliferating FSP1$^+$ cells. FSP-TK mice and control littermates (8–10/group) were injected subcutaneously with methylcholanthrene/oil and treated either with ganciclovir (GCV) in oil at weeks 3 to 5 or with oil alone as control. A, tumor development in the presence or absence of FSP1$^+$ cells. Each line represents the tumor growth of a single mouse. B, tumor incidence at different time points. C, Van Gieson staining shows collagens in tumor tissue. The arrow shows tumor cells with an epithelial morphology induced by ganciclovir treatment in FSP-TK mice (left) and fibrosarcoma cells arising without ganciclovir treatment (right). D, tumors from ganciclovir-treated FSP-TK mice are stained positive for the epithelial markers E-cadherin and cytokeratin. E, the epithelial tumor cells are resistant to ganciclovir treatment *in vitro*. Cells were cultured in the presence of increasing amounts of ganciclovir and viability of different tumor cells was assessed using an MTT assay. F, the epithelial tumor cell lines grew slower than fibrosarcoma cell lines *in vivo*. A total of 1×10^5 McO (○), Mc3-5a (●), and Mc3-5b (▲) cells were injected subcutaneously into nude mice. Each line represents the tumor growth kinetic of a single mouse. G, expression of E-cadherin on transplanted tumors of ganciclovir-treated mice. Tumors from nude mice in F were stained for hematoxylin and eosin (H&E) and E-cadherin. H, the FSP1 promoter is silent in Mc3-5a and Mc3-5b epithelial tumors. Two adjacent tumor sections from nude mice were stained with thymidine kinase (TK) and FSP1.

Protection of epithelial malignancy by proliferating FSP1$^+$ cells was time dependent

To exclude the possibility that ganciclovir alone or ganciclovir in combination with methylcholanthrene preferentially induces tumors with epithelial morphology, we chose 2 different time points of ganciclovir administration.

FSP-TK mice were treated with ganciclovir/oil from week 0 to 2 after methylcholanthrene injection, when the fibroblasts were still very scarce at the methylcholanthrene site (Fig. 1B) and from week 8 to 10, when methylcholanthrene/oil had already been encapsulated. As shown in Supplementary Fig. S4, there was no significant difference in tumor incidence between the ganciclovir-treated and control groups. As a control, both FSP-TK and control mice were also injected subcutaneously only with ganciclovir/oil, and none of the mice developed a tumor during the observation period of 200 days. Furthermore, all tumors developing in mice that were treated with ganciclovir were fibrosarcomas. These results indicate

that the elimination of proliferating FSP1$^+$ cells are responsible for the epithelial morphology of the tumors in FSP-TK mice treated with ganciclovir 3 to 5 weeks after methylcholanthrene inoculation.

The encapsulation of methylcholanthrene was impaired after the ablation of proliferating FSP1$^+$ fibroblasts in mice

It has recently been described that the FSP1 protein is not only expressed in fibroblasts, but also by some hematopoietic cells (31). To further investigate the mechanism with which FSP1$^+$ cells protect hosts from epithelial tumors, groups of FSP-TK mice and control littermates were subcutaneously injected with methylcholanthrene. Three weeks later, the mice were treated locally with ganciclovir/oil to eliminate proliferating FSP1$^+$ cells. As shown in Fig. 4A, most of the FSP1$^+$ cells at the methylcholanthrene inoculation site were depleted 4 weeks upon ganciclovir treatment in FSP-TK mice but not in wild-type (WT) littermates. Interestingly, approximately 94% of the remaining FSP1$^+$ cells were F4/80$^+$ in the ganciclovir-treated FSP-TK mice (Fig. 4B) and most of these cells do not proliferate (Fig. 4C), indicating that FSP1$^+$ fibroblasts, rather than FSP1$^+$ macrophages, were depleted by ganciclovir treatment in the skin.

Sirius red staining of the skin tissue prepared from FSP-TK or control mice, treated with or without ganciclovir as described earlier, showed that collagen encapsulation of methylcholanthrene/oil was diminished only in FSP-TK mice treated with ganciclovir but not in other groups of mice (Fig. 4D). Methylcholanthrene is a polycyclic aromate that can become visible on tissue sections through auto-fluorescence (29). Methylcholanthrene in ganciclovir- and vehicle-treated FSP-TK mice detected under fluorescence and light microscope is shown in Fig. 4E. Methylcholanthrene crystals were found near the epithelium in ganciclovir-treated FSP-TK mice. Ablation of proliferating fibroblasts led to a broad diffusion of methylcholanthrene into the neighboring tissue of the skin. These results suggest that it is FSP1$^+$ fibroblasts that protected epithelial cells from methylcholanthrene-induced mutation and malignancy.

FSP1$^+$ fibroblasts protected epithelial cells from DNA damage

DNA damage is recognized as the initial step in chemical carcinogenesis and is also responsible for its progression (37). To test the hypothesis that FSP1$^+$ fibroblasts protect epithelial cells from DNA-damage, a monoclonal antibody against the phosphorylated histone H2AX (γ-H2AX) was used to detect DNA double-strand breaks in methylcholanthrene-treated skin biopsies. In accordance with the theory that fibroblasts protect epithelial cells from DNA damage by encapsulating methylcholanthrene, and during this process, acquire mutational events themselves, the DNA damage was often found in fibroblastoid cells adjacent to methylcholanthrene and could be detected as early as week 7 after methylcholanthrene injection (Fig. 5). Most of γ-H2AX–positive cells were FSP1$^+$ fibroblasts, whereas epithelial cells were stained negatively with γ-H2AX. However, the abatement of proliferating fibroblasts around methylcholanthrene led to a striking increase in DNA damage in many epithelial cells 4 weeks after the ganciclovir treatment. There was no obvious DNA damage in untreated and only ganciclovir/oil–treated control mice. These results show that fibroblasts prevent epithelial cells from methylcholanthrene-induced DNA damage.

Degradation of collagen at the methylcholanthrene injection site led to a rapid tumor development in long-term "tumor-free" mice

Previously, we and others observed that at lower doses of methylcholanthrene, a number of mice remained tumor-free for more than 40 weeks after methylcholanthrene injection (29, 38). For example, about 40% of 129/Sv/Ev mice injected with 100 μg methylcholanthrene, 50% of BALB/c mice injected with 5 μg methylcholanthrene, and 15% of C57BL/6 mice injected with 25 μg methylcholanthrene were tumor-free for more than 300 days (Fig. 6A). Depending on the time point of investigation, pools of methylcholanthrene/oil or dispersed pieces of methylcholanthrene crystals were observed and were mostly encapsulated by collagen capsules (Fig. 6B).

To test if these collagen capsules around methylcholanthrene are protective, collagenase was injected at the methylcholanthrene site in the long-term "tumor-free" 129/Sv/Ev mice to disrupt the collagen capsule. As shown in Fig. 6C, the collagen capsules can be efficiently broken down by injecting collagenase. Three of 6 collagenase-injected tumor-free mice developed tumors in about 15 weeks after the treatment, but all 6 of 6 control mice treated with only solvent remained tumor-free (Fig. 6D). In another experiment, similar results were obtained with 3 of 10 collagenase-injected tumor-free mice developing tumors. Together, the results indicate that FSP1$^+$ fibroblasts produced collagen, encapsulated methylcholanthrene, and thereby protected surrounding epithelial cells from methylcholanthrene-induced malignancy.

Discussion

In this study, we asked if depletion of replicating (FSP1$^+$/S100A4$^+$) fibroblasts after methylcholanthrene injection would result in impaired encapsulation of the carcinogen. Furthermore, we hypothesized that elimination of proliferating fibroblasts from the skin area around the methylcholanthrene would result in the proliferation of other cells in the skin (e.g., epithelial cells), which have become a target of the unencapsulated carcinogen. Indeed, local depletion of FSP1/S100A4$^+$ fibroblasts resulted in impaired collagen encapsulation of methylcholanthrene and malignant transformation of the surrounding epithelial cells.

FSP1 can be expressed by different cell types of mesenchymal origin. We found FSP1 was mainly expressed by procollagen I$^+$ fibroblasts in skin tissue treated with methylcholanthrene/oil. Also, approximately 25% of the FSP1$^+$ cells were F4/80$^+$ (Fig. 2B). However, we also found that after ganciclovir treatment in FSP-TK mice, approximately 94% of the remaining FSP1$^+$ cells were F4/80$^+$ (Fig. 4B). This suggests that most of FSP1$^+$ macrophages were not depleted. In our opinion, this is because the suicide gene-*TK* is

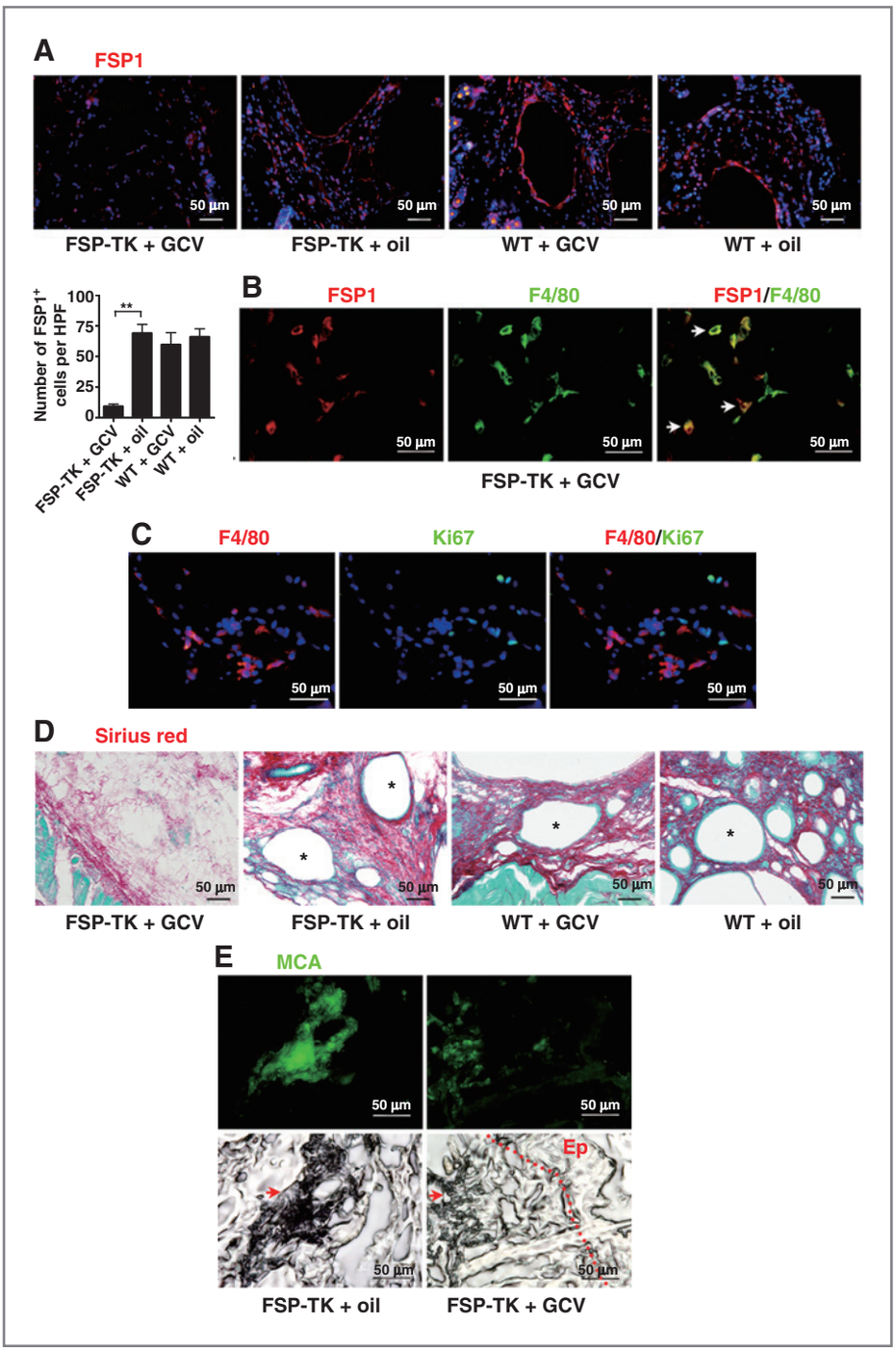

Figure 4. Ablation of proliferating FSP1$^+$ cells in methylcholanthrene-treated mice interferes with collagen encapsulation of methylcholanthrene. FSP-TK mice and control littermates (3–5/group) were injected subcutaneously with methylcholanthrene. Three weeks later, mice were treated either with ganciclovir in oil or with oil alone as control once a week, for 3 weeks. Skin sections were stained at week 7. A, the number of FSP1$^+$ cells decreased obviously after ganciclovir treatment in FSP-TK mice, but not in control groups (**, $P < 0.01$). B, the majority of FSP1$^+$ cells after ganciclovir treatment were macrophages, as shown by staining of skin sections from ganciclovir-treated mice for FSP1 and F4/80. C, most of skin F4/80$^+$ macrophages did not proliferate. Double staining of F4/80 (red) and Ki67 (green) in the skin sections from methylcholanthrene-treated BALB/c mice as described earlier. D, Sirius staining of the skin tissue prepared from the mice as described earlier. Local ablation of proliferating FSP1$^+$ cells was followed by disruption of the collagen capsule around methylcholanthrene. *, the injection sites of the methylcholanthrene/oil suspension. E, methylcholanthrene injected subcutaneously was detected under fluorescence and light microscope from undewaxed paraffin skin sections. Methylcholanthrene diffused close to epithelial cells after ganciclovir treatment of FSP-TK mice. Methylcholanthrene is indicated by arrows and epidermis (Ep) and dermis boundary is marked by dotted line.

effective only during cell proliferation (39). van Furth and colleagues have reported that at the site of skin inflammation more than 99% of skin macrophages were monocyte derived and less than 1% originated by local division of macrophages (40). In our experiments, ganciclovir was applied in water–oil emulsion locally in skin tissue. Meanwhile, skin F4/80$^+$ macrophages mostly do not proliferate (Fig. 4C), which instead derived from continuously recruited monocytes, therefore cannot be efficiently killed by ganciclovir. Whether and how macrophages act during fibrosarcoma development still need further investigation.

We show that FSP1$^+$/S100A4$^+$ fibroblasts accumulate around the carcinogen and produce collagens, leading to the encapsulation of methylcholanthrene and protection of epithelial cells from DNA damage and play a role in preventing epithelial malignancy. DNA damage and defects in the

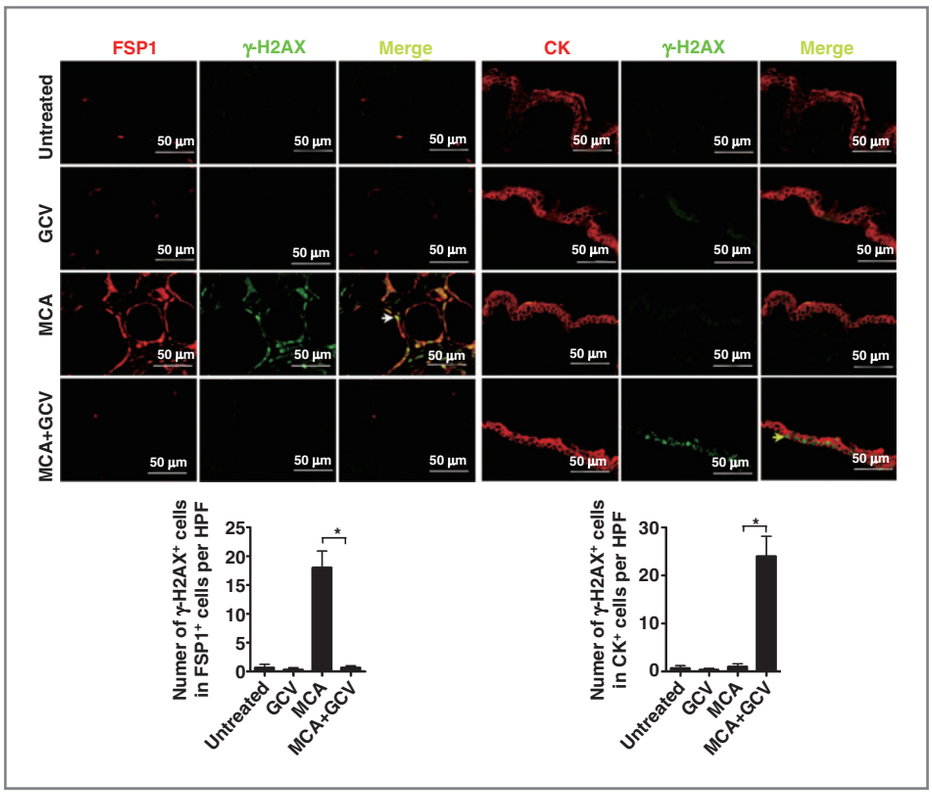

Figure 5. FSP1$^+$ cells protect epithelial cells from DNA damage. Groups of FSP-TK mice were left untreated ($n = 3$; first row) or injected subcutaneously with ganciclovir (GCV; $n = 3$; second row), or methylcholanthrene (MCA; $n = 6$; third row). Three weeks later, half of the mice in methylcholanthrene group were treated with ganciclovir in oil once a week, for 3 weeks ($n = 3$; fourth row). Skin sections were stained 7 weeks after methylcholanthrene injection for γ-H2AX, FSP1, or cytokeratin. Arrows show the DNA damage in FSP1$^+$ cells around methylcholanthrene (white) or in CK$^+$ epithelial cells (yellow) after a local ablation of proliferating FSP1$^+$ cells with ganciclovir. The mean numbers of γ-H2AX$^+$ cells in FSP1$^+$ cells and CK$^+$ cells in a HPF (×400) are shown (*, $P < 0.05$).

related cell-cycle regulation network can contribute to the development of mutations and promote tumorigenesis (37, 41). In our study, fibroblasts around the methylcholanthrene injection site acquire most of the DNA-damage, as shown by positive staining of H2AX (Fig. 5), highly expressing cell-cycle regulator cyclin D1 (Supplementary Fig. S5) and proliferating heavily (Fig. 1B). The results may provide an explanation why subcutaneous or intramuscular injection of methylcholanthrene/oil usually results in the formation of fibrosarcoma. Rapid tumor development in long-term tumor-free mice after destruction of the fibrous capsule around the methylcholanthrene by local injection of collagenase further confirmed that the collagen encapsulation of the carcinogen is protective against tumor development. However, besides collagens, FSP1$^+$ fibroblasts were also associated with the production of fibronectin, ER-TR7, and laminin-1 (Supplementary Fig. S3). These ECM molecules might also play roles in capsulation of the carcinogen. At the same time, methylcholanthrene injection also induced secreted proteases from activated fibroblasts, macrophages and neutrophils (data not shown). The role of these matrix metalloproteinases (MMP) during methylcholanthrene-induced ECM remodeling and carcinogenesis still needs further investigation.

Methylcholanthrene injection is one of the most widely used chemical carcinogenesis protocols in mouse experiments. As environmental pollution progresses, the amount of chemical carcinogen chronically inhaled by the exposed human population will also increase. In particular, PAHs (such as methylcholanthrene) are globally distributed environmental pollutants known for their carcinogenic and mutagenic effects on humans (42–45). Fibroblasts are also present in human tissues exposed to carcinogen as, for example, asbestosis, making it important to study if encapsulation of carcinogen can be found in humans, especially in the light of the report from the International Agency for Research on Cancer (Lyon, France) that diesel engine exhaust is definitely carcinogenic to humans (a group 1 carcinogen, which is actually the same category as methylcholanthrene; refs. 46). Despite differences in dose and route of administration between methylcholanthrene and the mixture of PAH carcinogens likely present in diesel exhaust, it is reasonable to assume that the evolutionary conserved mechanism of the foreign body response may also contribute to protection in humans especially, as our results have shown that the encapsulation is not specific for the carcinogen methylcholanthrene but also takes place when other mutagens (DMBA and DEN) dissolved in oil are injected (Supplementary Fig. S2).

Fibroblasts may play different roles on tumorigenesis at different stages of tumor development. Stromal fibroblasts participate in host defense against carcinogenesis via capsulation of carcinogen, which very likely protects the surrounding cells from acquiring initial mutations and therefore occurs before tumor cells arise. On the other hand, fibroblasts may also promote tumor growth and progression by enhancing angiogenesis, stemness (47), invasiveness, and metastasis (11, 12). Using the same FSP-TK mice, we have shown that fibroblasts promote DMBA/TPA–induced skin tumor development

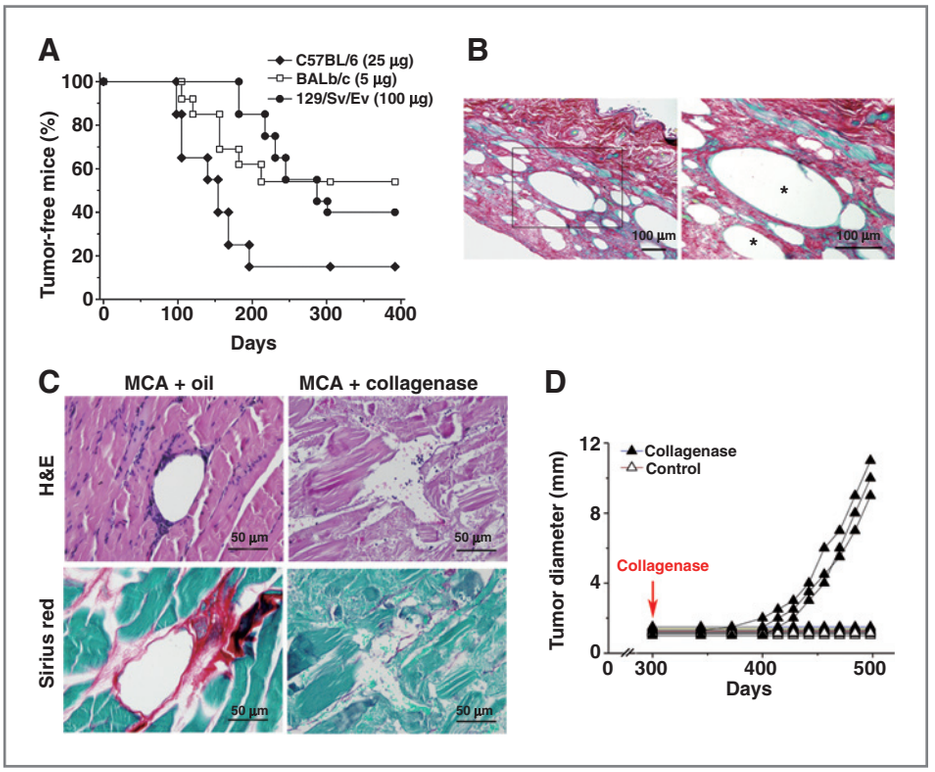

Figure 6. Collagenase injection next to the methylcholanthrene (MCA) injection site induces tumor development in long-term tumor-free mice. A, tumor incidence in BALB/c, C57BL/6, and 129/Sv/Ev mice. Groups of mice ($n = 20$) were injected subcutaneously with methylcholanthrene/oil as described in the Materials and Methods. The percentages of tumor-free mice at different time points after methylcholanthrene injection are shown. B, shown is encapsulated methylcholanthrene/oil. Six months after methylcholanthrene injection, tissue sections of C57BL/6 mice ($n = 5$) that remained tumor-free were stained with Sirius red. *, the injection site of the methylcholanthrene/oil suspension. C, the local injection of collagenase induced the destruction of the fibrous capsule around methylcholanthrene. Groups of 129/Sv/Ev mice were injected intramuscularly with methylcholanthrene/oil as described in the Materials and Methods. Three weeks after the injection of methylcholanthrene, the mice were treated with collagenase in water-in-oil emulsion ($n = 3$) or with the solvent alone as control ($n = 3$). Tissue sections from the injection site in collagenase-treated and control mice were stained with hematoxylin and eosin (H&E) and Sirius red. D, groups of 129/Sv/Ev mice that remained tumor-free at day 300 after an injection of methylcholanthrene were treated with collagenase ($n = 6$) or with the solvent alone as control ($n = 6$). Tumor growth kinetics in each single mouse of tumor-free mice in collagenase treated (▲) and control mice (△) are shown. The arrow indicates the time point of collagenase application.

by promoting chronic inflammation (19) and O'Connell and colleagues also found that depletion of FSP1$^+$ fibroblasts significantly reduced metastatic colonization with 4T1 transplant tumor model (17). In these tumor models, FSP1$^+$ fibroblasts cannot form encapsulation. Therefore, these opposite effects of fibroblasts on tumorigenesis may result from different mechanisms occurring at different stages of tumor development. How fibroblasts differentiate into effectors of tumor-inhibition, instead of tumor-promotion is certainly an important issue for tumor biology and therapy and further investigation will be necessary to elucidate the exact cellular and molecular mechanisms underlying these tumor-promoting and the tumor-protective effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Zhang, T. Kammertoens, T. Blankenstein, Z. Qin
Development of methodology: J. Zhang, L. Chen, X. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zhang, X. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zhang, L. Chen, T. Blankenstein
Writing, review, and/or revision of the manuscript: J. Zhang, L. Chen, T. Kammertoens, T. Blankenstein, Z. Qin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Liu
Study supervision: Z. Qin

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