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# Mutations in Bone Marrow-Derived Stromal Stem Cells Unmask Latent Malignancy

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Neoplastic epithelia may remain dormant and clinically unapparent in human patients for decades. Multiple risk factors including mutations in tumor cells or the stromal cells may affect the switch from dormancy to malignancy. Gene mutations, including p53 mutations, within the stroma of tumors are associated with a worse clinical prognosis; however, it is not known if these stromal mutations can promote tumors in genetically at-risk tissue. To address this question, *Apc<sup>Min/+</sup>* and *Apc<sup>Min/+</sup>Rag2<sup>-/-</sup>* mice, which have a predilection to mammary carcinoma (as well as wild-type (wt) mice), received mesenchymal stem cells (MSC) with mutant p53 (p53MSC) transferred via tail vein injection. In the wt mouse, p53MSC circulated in the periphery and homed to the marrow cavity where they could be recovered up to a year later without apparent effect on the health of the mouse. No mammary tumors were found. However, in mice carrying the *Apc<sup>Min/+</sup>* mutation, p53MSC homed to mammary tissue and significantly increased the incidence of mammary carcinoma. Tumor necrosis factor (TNF)- $\alpha$ -dependent factors elaborated from mesenchymal cells converted quiescent epithelia into clinically apparent disease. The increased cancer phenotype was completely preventable with neutralization of TNF- $\alpha$  or by transfer of CD4<sup>+</sup> regulatory T cells from immune competent donors, demonstrating that immune competency to regulate inflammation was sufficient to maintain neoplastic dormancy even in the presence of oncogenic epithelial and stromal mutations. The significant synergy between host immunity and mesenchymal cells identified here may restructure treatments to restore an anticancer microenvironment.

## Introduction

**B**Y MIDLIFE, THE MAJORITY of people have microscopic foci of malignant cells throughout their body, yet the incidence of clinically apparent tumors is strikingly lower [1]. Autopsy studies reveal a profound discrepancy between the presence of subclinical malignancy and clinically apparent cancer [2–4]. Multiple factors may affect this switch from dormant to overt disease, including additional gene mutations within tumor cells, changes in the systemic immune response, and/or alterations in the microenvironment and stromal cells proximate to the cancer cells.

Bone marrow-derived cells (BMDC) invade and reside within inflamed tissues. Within these tissues, cells

derived from the marrow can differentiate as myofibroblasts and fibroblasts and contribute substantially to the signaling environment surrounding regenerating tissue [5], and they are instrumental in directing epithelial cell regeneration and wound repair. Within established tumors, BMDC are recruited as cancer-associated fibroblasts (CAF), which have a phenotype similar to that of activated fibroblasts thus sharing a “wound-healing” repertoire of chemokine and cytokine [6] production. Mesenchymal stem cells (MSC), which can be found in the marrow, also localize to areas of established carcinoma and infiltrate into the tumor as tumor stroma [7]. MSC promote breast cancer metastasis through effects on local tumor growth and migration, and

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via priming of distant sites to become conducive docking sites for tumor cells [7].

Cancer-associated fibroblasts have been shown to carry genetic and epigenetic mutations, which are thought to impact their clinical behavior [8–10]. Recently, it has been suggested that CAF may play as much a role in cancer formation as do the epithelial-derived tumor cells themselves. Mutations in stromal p53 have been demonstrated in CAF associated with multiple tumor types. Mammary tumors containing p53-deficient stromal fibroblasts developed faster and are more aggressive than tumors containing wild-type (wt) stromal fibroblasts [11]. Though it is clear that stromal cells have dramatic impact on established tumors, it is less clear what effect these cells have on the earliest stages of malignancy or what role, if any, they may have in converting microscopic malignant foci within at-risk tissue to frank malignant disease. Interestingly, it has been suggested that mammary stroma may be a critical target for chemical-induced transformation. Studies using NMU as a transforming agent demonstrate that the mammary stroma can be a direct target of chemical transformation and precedes the transformation of rat mammary epithelial cells in a rat model of chemical-induced mammary carcinoma [8].

Here we show that mutant p53MSC home to mammary tissue under the influence of dysregulated inflammation. Once within at-risk tissue, p53MSC accelerated neoplastic cell growth. Homing and proliferation of p53MSC within the mammary epithelium resulted in a dramatic increase in disease incidence and tumor size, and a decrease in the time to clinically apparent disease in mice at-risk for mammary carcinoma. The p53MSC themselves did not promote mammary tumors in wt mice that were not at risk for the disease. Further, the increased cancer phenotype was dependent upon tumor necrosis factor (TNF)- $\alpha$ , and accelerated tumor formation was completely reversed with neutralization of TNF- $\alpha$  via neutralizing antibody or by transfer of competent T-regulatory cells ( $T_{REG}$ ). Altered MSC signaling converted quiescent in situ carcinoma to clinically apparent disease in a tissue-specific fashion. The significant contribution of immune dysregulation to pro-cancer MSC activity provides additional mechanisms for the clinical stratification of breast cancer and may re-structure treatment targets toward restoring an anticancer microenvironment.

## Materials and Methods

Please see Supplemental Methods for additional details.

### Human tissue

Human breast cancer and normal breast tissue samples from de-identified individuals were obtained from the UMass Cancer Center Tissue and Tumor Bank <http://www.umassmed.edu/cancercenter/tissuebank/index.aspx> with IRB approval and evaluated for p53 expression (see Supplemental Methods).

### Experimental animals

Animal work was performed at Massachusetts Institute of Technology or at the University of Massachusetts Medical

School under IACUC approval. Female C57BL/6*Apc*<sup>Min/+</sup> (B6Min), *Apc*<sup>Min/+</sup>*Rag2*<sup>-/-</sup> (B6RagMin), and *wt* (B6) littermates were genotyped prior to experiments, and at euthanasia. Experiments were conducted using separate trials with 5–10 mice each and results combined for statistical analyses. C57BL/6*Apc*<sup>Min/+</sup> mice are at risk for mammary tumors and 1% of female mice develop tumors by 26 weeks of age. *Apc*<sup>Min/+</sup>*Rag2*<sup>-/-</sup> mice are at a higher risk of mammary carcinoma and 10% of female mice develop mammary tumors by 26 weeks of age.

### Wild-type bone marrow transplantation and MSC infusion

Bone marrow transplantation was performed as previously described [12] using enhanced green fluorescent protein (eGFP)-labeled total bone marrow from C57BL/6J- $\beta$ -actin-EGFP mice (Jackson laboratories, Bar Harbor, ME) or eGFP-MSC plus 3–5 million unmarked support marrow cells transplanted into irradiated (900 rads) recipients via tail vein injection. Recipient mice were 6–8 weeks of age. Donor mice were 8–12 weeks of age. Freshly cultured cells were used at first passage. Cultured wtMSC or p53MSC were age-matched for experiments and used at Passages 12–15.

### Isolation, culture, and infusion of p53MSC and wtMSC

Wild-type bone marrow was isolated and cultured as previously described [13]. p53wt (wt) and MSC with a CAF phenotype and carrying p53 mutation (p53MSC) were identified, characterized, and transfected with a monomeric DS-red containing plasmid as described in Supplemental Methods.

### Immunomodulation

$T_{REG}$  transfer and neutralization of TNF- $\alpha$  by anti-TNF- $\alpha$  antibody is described in Supplemental Methods.

### Primary tumor, tumor transplantation, and histological evaluation of tissues

p53MSC-induced B6Min mammary tumors were excised. One half was processed for histology and Ki-67 expression, and ½ was minced, digested, and cell suspensions injected into the mammary fat pad of recipient female B6Min mice. All mammary tissue and tumors were evaluated for histology and epithelial cell proliferation as described in Supplemental Methods. For ex vivo proliferation studies, cells were placed in tissue culture with Dulbecco's modified Eagle's medium (DMEM) 10% fetal calf serum (FCS) and grown as a monolayer.

### Migration by monolayer wound-healing assay

MCF-7 cells were grown to 90% confluence and the cell monolayer was then wounded by a plastic tip (1 mm). Monolayers were washed, photographed, and media replaced with control medium or p53MSC-conditioned medium  $\pm$  chemokines and healing measured as described in Supplemental Methods.

### Statistical analyses

Mammary tumor incidence was compared using Fisher exact *t*-test. Ki-67 index and assessment of stroma was compared between groups using 2-tailed *t*-test. In vitro data were compared using Student's *t*-test using a minimum of 3 replicates per experimental point, and experiments repeated 2–3 times for reproducibility.

### Results

#### Cancer-associated fibroblasts from human breast cancer contain a population of p53-mutated stromal cells

Stromal cells with p53 mutations have been identified associated with breast [14,15], prostate [16], and other epithelial cancer cells and are predictive of aggressive tumor behavior and metastasis. While there is controversy with regard to the specific mutation and frequency of stromal p53 mutations in human breast cancer specimens [15,17–19], there is a growing literature that demonstrates a role for nonautonomous p53 effects between stroma and mammary epithelial cells [11]. We first assessed p53 expression in human breast cancer stroma. Specific p53 immunohistochemistry (IHC) of 15 consecutive breast cancer specimens archived by the UMass tumor bank was done and our data support the notion that overexpression of p53, which can be associated with clinically relevant mutations, occurs commonly within a population of CAF (Table 1). p53<sup>+</sup> stromal cells were found in 67% of breast cancer specimens (Fig. 1A–1C) and expressed  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) indicative of an activated fibroblast phenotype (Fig. 1D–1G). p53<sup>+</sup> cells were found as isolated cells within activated stroma. We evaluated

non-neoplastic mammary glands and associated connective tissue for p53 and  $\alpha$ -SMA expression. Small glands were devoid of  $\alpha$ -SMA expression. Myoepithelial cells along lobular ductules stain strongly positive for  $\alpha$ -SMA. Fibroblasts within the stroma were devoid of  $\alpha$ -SMA signal. We did not detect any p53 staining within any cell of benign mammary tissue (Fig. 1H and 1I). These findings confirm the published observation of rare (1%–5%) p53<sup>+</sup> CAF cells within breast cancer stroma [11,15,17–19].

#### Wild-type MSC are not associated with an increase risk of mammary carcinoma

We hypothesized that p53 mutations in stromal cells could drive the transition of occult malignant cells to frank mammary carcinoma in at-risk tissue. As a first step in evaluating the p53MSC promotion of mammary carcinoma, we characterized the in vivo activity of wtMSC in wtC57BL/6 mice (B6) not prone to mammary carcinoma compared with wtMSC given to C57BL/6 *Apc*<sup>Min/+</sup> (B6Min) mice that have a low but significant spontaneous rate of developing mammary carcinoma, and found that wtMSC did not contribute to a malignant phenotype in either mouse model (see Supplemental Table 1; Supplementary materials are available online at <http://www.liebertonline.com/scd>).

#### Infused p53MSC-CAF are not associated with mammary carcinoma in B6 mice

We next investigated the fate of MSC hemizygous for p53 307 Thr–Ile mutation [13] (p53MSC) (which coincides with position 312 in the DNA-binding region of human p53, a mutation found in human cancers [20]) in the B6 mice. This

TABLE 1. HUMAN BREAST CANCER—CLINICAL INFORMATION

Sample number	Age	Cancer type	Grade	pT	pN	pM	ER/PR/Her2	p53	
								Epithelial	Stromal
1	65	Ductal	2	1b	0	–	+/-/-	–	+
2	83	Ductal	2	1b	0	–	+/+/-	–	+
3	66	Ductal	2	1b	3a	–	+/-/-	–	–
4	69	Ductal/lobular	2	2	0	–	+/+/-	–	–
5	59	Ductal	3	2	0	–	+/-/-	+	+
6	79	Ductal	2	1c	0	–	+/+/-	–	+
7	72	Ductal	2	1b	0	–	+/+/-	+	+
8	61	Ductal	2	1b	0	–	+/+/-	–	+
9	44	Ductal	2	2	0	–	+/+/-	+	+
10	61	Ductal	2	1b	0	–	+/+/-	–	+
11	82	Ductal	2	4	1b	–	+/+/-	–	+
12	73	Ductal	2	1c	0	–	+/+/-	–	–
13	45	Ductal	1	1b	1mi	–	+/+/-	–	–
14	47	Ductal	3	1b	0	–	+/-/-	+	+
15	59	Ductal	3	1c	0	–	-/-/+	+	–

Grade: 1, well-differentiated; 2, moderately differentiated; and 3, poorly differentiated.

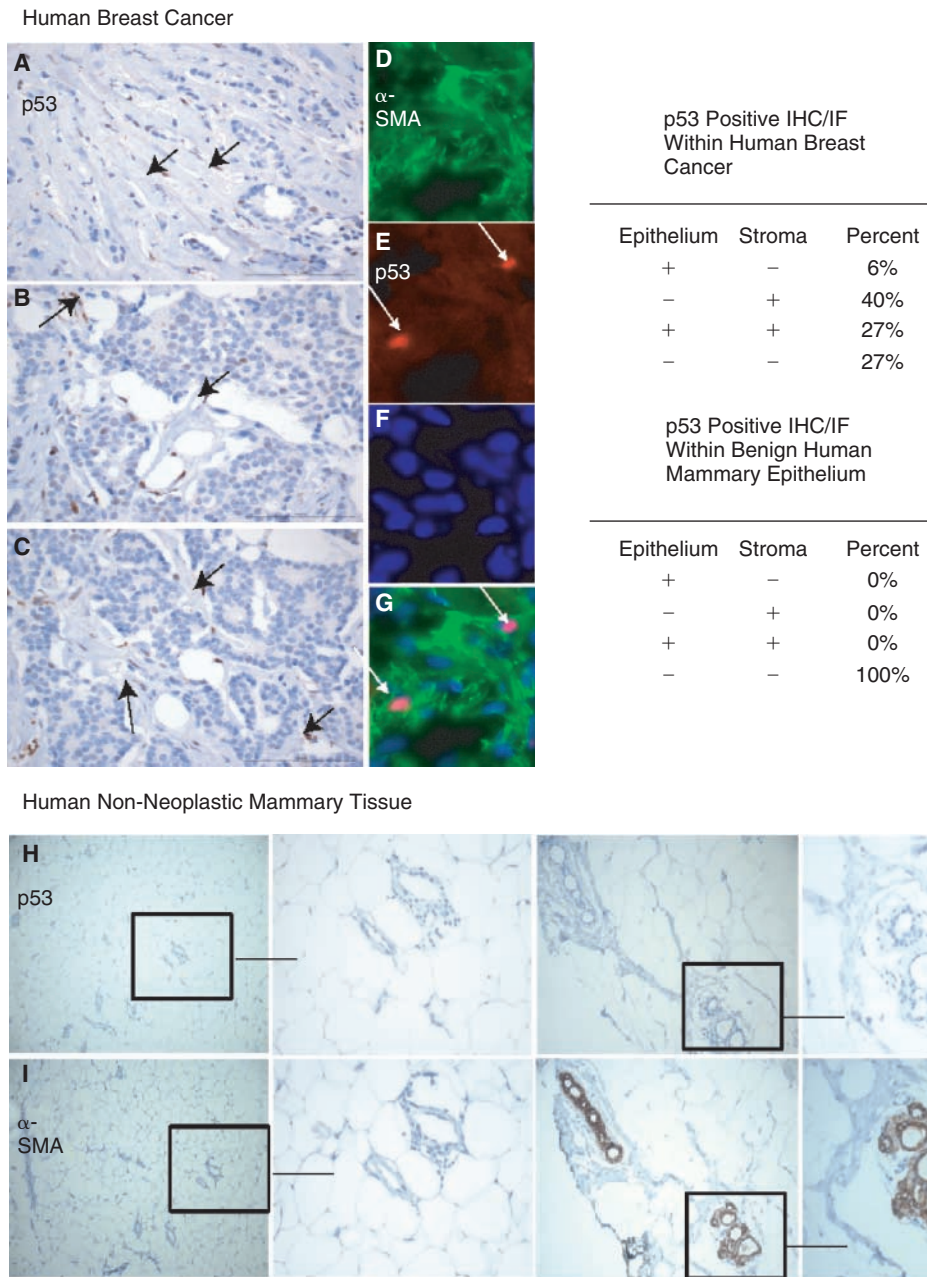
pT: tumor size and extent.

pN nodes: *a* ≤ 0.2 cm; *b* ≥ 0.2 cm; mi, micrometastasis <0.2 cm.

pM metastasis: +/-.

ER/PR/Her2: estrogen receptor, progesterone receptor, and Her2 status of tumor sample.

p53 status for epithelium and stroma: Negative (–) if there is no staining, (+) if any cell in the appropriate compartment stains for nuclear p53.



**FIG. 1.** Human breast cancer stroma contains nuclear p53. Specific anti-p53 IHC of 3 different representative human breast cancer specimens containing (A) abundant stroma (B) moderate amount of stroma, and (C) little stroma. Arrows point out p53<sup>+</sup> nuclei of stromal cells (brown nuclear staining). Frozen tissue was used to colocalize (D) anti- $\alpha$ -smooth muscle actin (SMA) (green), (E) p53 (red, arrows), (F) nuclei (blue). (G) Merged image shows p53 colocalizing with dapi (purple, arrows) within  $\alpha$ -SMA<sup>+</sup> cells. (H) Anti-p53 (brown staining) and (I) anti- $\alpha$ -SMA (brown staining) immunohistochemistry of consecutive sections of non-neoplastic mammary tissue containing mammary epithelial and stromal structures. Boxed areas shown in higher power as indicated. The chart summarizes the number of p53<sup>+</sup> cells within the epithelial and stromal compartments of the human tumors and within benign mammary tissue.

mutation is a gain-of-function mutation (see Supplemental Fig. 1; Supplementary materials are available online at <http://www.liebertonline.com/scd>). wtMSC cultured under the same conditions, and at the same age, do not have demonstrable p53 protein by immunofluorescence (IF) or by western blot analysis, while p53MSC stain heavily by p53 IF, and have abundant protein by western blot analysis [13]. Freshly isolated MSC and cultured MSC populations

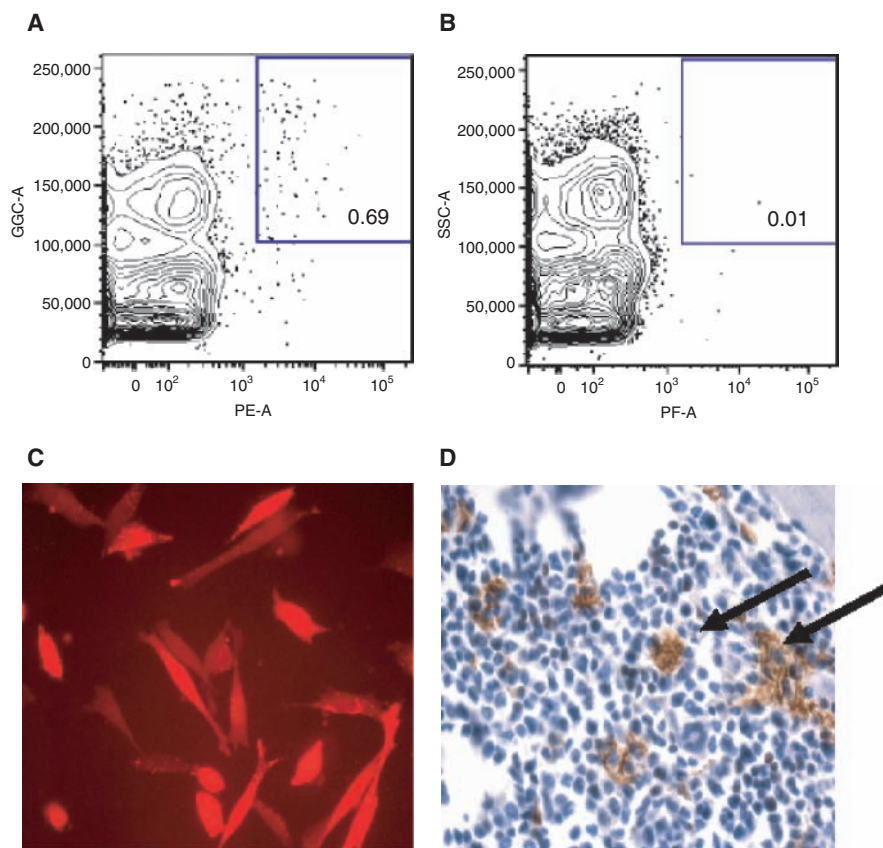
were verified by fluorescence-activated cell sorting (FACS) to lack hematopoietic stem cell markers and lineage-specific markers (negative for CD45, CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, Gr1, F4/80 NK1.1, and CD31) and were positive for CD105, CD73, and CD44. All MSC populations used maintained trilineage differentiation (fat, cartilage, and bone) capacity in culture. wtMSC and p53MSC maintained similar phenotypes and growth characteristics

with doubling times of 26 and 28 h, respectively. B6 mice were infused with 1 million p53MSC or age-matched cultured wtMSC labeled with red fluorescent protein (RFP) as control. Infused cultured wtMSC were not maintained at detectable levels long term in host tissue, and were not found after 3, 6, 9, or 12 months postinfusion (Supplemental Fig. 2; Supplementary materials are available online at <http://www.liebertonline.com/scd>). In contrast to wtMSC, p53MSC remained detectable in the blood for many months postinfusion and were recovered at all time points tested (Fig. 2A and 2B and Supplemental Fig. 2). There were no gross or microscopic tumors in any tissue from B6 mice. The spleen and the marrow cavity contained a few scattered RFP<sup>+</sup> cells (Fig. 2D). To confirm the presence of p53MSC in the bone marrow, we cultured the bone marrow and recovered  $35 \pm 5$  RFP-p53MSC colony-forming units per mouse (Fig. 2C), which we calculated was found to be ~3% of the stroma. These MSC lacked specific hematopoietic or lineage markers (CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, Gr1, F4/80, and NK1.1), and retained CD44 positivity and the ability to differentiate into bone fat and cartilage, confirming they were MSC. Further, these data demonstrate that p53-mutated MSC have a survival advantage in vivo over

wtMSC but, in the absence of inflammation or a predisposition to tumors, do not themselves initiate epithelial malignancy (see Supplemental Methods for further details).

#### *p53MSC and mammary carcinoma in the *Apc*<sup>Min/+</sup> cancer prone mouse*

To address the effects of stroma on the growth of dormant or premalignant mammary cells, we chose a mouse model that develops mammary carcinoma at a low but reproducible rate. Less than 1% of B6Min mice housed at MIT or UMass develop mammary carcinoma as they age (personal observation). Tumor incidence is directly linked to inflammatory triggers [21–23] as tumor formation occurs with chronic infection [23]. Congenital or acquired immunodeficiency is strongly associated with an increased incidence of tumors in both human and mice. Immune cells are thought to prevent tumor outgrowth by either direct elimination of tumor cells or by maintaining an equilibrium state where occult tumors are kept in check. These processes are termed immunosurveillance and immunoediting [24–26]. Rag2-deficient mice lacking T, B, and NK-T immune cells are at an increased risk for chemically induced tumors attributed to genetic



**FIG. 2.** Analysis of red fluorescent protein (RFP)-p53MSC recovered in the mature wild-type mouse after IV injection. RFP-labeled p53MSC injected IV into wild-type C57BL/6 mice are identified within the circulation and bone marrow cavity. (A) Fluorescence-activated cell sorting (FACS) analysis of peripheral blood 9 months after injection of  $1 \times 10^6$  RFP p53MSC. (B) FACS analysis of peripheral blood 9 months after injection of PBS. Boxed area indicates the number of RFP<sup>+</sup> cells detected. (C) Bone marrow flushed from the right femur of mouse shown in Panel A was grown in culture and RFP-p53MSC recovered. RFP<sup>+</sup> cells in culture are seen under the fluorescent inverted tissue culture microscope. (D) RFP-specific IHC of the marrow cavity of the left femur of the same mouse. Arrows highlight brown-colored positive signal in RFP<sup>+</sup> cells (DAB chromogen and hematoxylin counterstain 10 $\times$ ).

TABLE 2. MAMMARY TUMOR FREQUENCY IN MOUSE MODELS

Group	Mammary tumor incidence
B6	0 ( <i>n</i> = 10)
B6+wtMSC (infused)	0 ( <i>n</i> = 10)
B6+BM+marked wtMSC (transplant)	0 ( <i>n</i> = 10)
B6+p53MSC	0 ( <i>n</i> = 10)
B6Min	0 ( <i>n</i> = 10)
B6Min+T <sub>REG</sub>	0 ( <i>n</i> = 10)
B6Min+wtMSC (infused)	0 ( <i>n</i> = 10)
B6+BM+marked wtMSC (transplant)	0 ( <i>n</i> = 10)
B6Min+p53MSC	50% ( <i>n</i> = 10)*
B6Min+p53MSC+T <sub>REG</sub>	0 ( <i>n</i> = 10)
B6Rag	0 ( <i>n</i> = 10)
B6MinRag	0 ( <i>n</i> = 10)
B6MinRag+T <sub>REG</sub>	0 ( <i>n</i> = 10)
B6MinRag+p53MSC	80% ( <i>n</i> = 10)**
B6MinRag+p53MSC+T <sub>REG</sub>	0 ( <i>n</i> = 10)
B6MinRag+p53MSC+anti-TNF- $\alpha$	0 ( <i>n</i> = 10)
B6MinRag+p53MSC+isotype control IgG	80% ( <i>n</i> = 10)**

Mice 6–8 weeks of age at the start. Tumor frequency determined up to 6 weeks. Several mice removed from study at 4 weeks due to large tumor size. All groups with 0 tumor frequency observed for at least 6 weeks. \**P* = 0.0325, \*\**P* = 0.0007.

Tumor incidence between the C57BL/6Apc<sup>Min/+</sup> (B6Min)+MSC and C57BL/6Apc<sup>Min/+</sup>/Rag2<sup>-/-</sup> (B6MinRag)+MSC groups was not statistically significant; *P* = 0.35.

mutations and escape of immune-mediated surveillance [21] and for microbially induced tumors attributed to inability to down-regulate host inflammatory response [27]. Placing the Apc<sup>Min/+</sup> mutation on the Rag2 background increases the incidence of spontaneous mammary carcinoma to ~10% by 5 months of age. Tumors are usually solitary.

#### *p53MSC are recruited to mammary tissue of the B6Min and B6MinRag mouse, surround ducts, and dramatically increase tumor incidence*

We tested our hypothesis that circulating p53MSC contribute to carcinoma progression. One million p53MSC, wtMSC, or vehicles were injected via the tail vein into female B6, B6Min, or B6MinRag mice at 8 weeks of age. Mice were followed for an additional 4–6 weeks. Fifty percent of the B6Min mice and 80% of the B6MinRag mice that received p53MSC developed single mammary tumors ranging from 0.3 to 2.0 cm in size by 4–6 weeks postinjection (Table 2). In marked contrast, B6, B6Min, or B6MinRag mice that received either vehicle or wtMSC developed no tumors over 6 weeks of observation.

p53MSC-associated mammary tumors in the B6Min and the B6MinRag mouse had histological features similar to spontaneous tumors that are found in the aged (>26-week-old) B6Min and B6MinRag mice (Fig. 3A–3D, higher-power images of B6MinRag p53MSC tumor in Supplemental Fig. 3; Supplementary materials are available online at <http://www.liebertonline.com/scd>). In p53MSC recipients, mammary adenosquamous carcinoma was characterized by highly irregular infiltrative growth pattern with both keratinized and nonkeratinized gland units forming small nests, cords, and trabeculae. Cellular atypia and increased proliferation

were evident. Tumors in B6Min mice had a larger proportion of keratinized glands compared with the tumors in the B6MinRag mice. The amount of supporting stroma varied from small to moderate amounts within a group, and did not differ between groups as a whole; p53MSC did not alter the amount of stroma in tumors (B6MinRag spontaneous tumor 8.39% stroma vs. B6MinRag+p53MSC 8.41% stroma). Neutrophilic infiltrate was noted in all tumors and terminal ductular (alveolar) differentiation was an occasional finding.

#### *p53MSC-induced mammary tumors are transplantable into a second host*

We next assessed p53MSC-induced tumors for malignant characteristics. Cell suspensions were prepared from B6Min p53MSC tumors and injected into the mammary fat pad of secondary B6Min hosts. Tumors formed by 8 days after injection in 1/3 B6Min mice (1.6 × 0.9 × 0.8 cm). Hematoxylin and eosin (H&E) staining revealed adenosquamous carcinoma with interspersed fat and stroma, similar in characteristics to the original parent tumor as well as to the histology of spontaneous mammary tumors arising in aged B6Min mice (Fig. 3E and 3F [transplanted tumor] compared with Figure 3A and 3B [parent tumor]). Marked nuclear atypia was noted within disorganized glands (Fig. 3F), and a large number of cells were proliferative by Ki-67 staining (Fig. 3G). Transplanted tumors contained 1% p53MSC within the stroma, which were Ki-67<sup>-</sup>. Recovered tumor from the secondary host contained ~1% RFP-p53MSC within a predominantly p53<sup>-</sup> (by IHC) stroma. Cells from the secondary tumor grew readily in tissue culture, and maintained an epithelial morphology in monolayer (Fig. 3H). We did not isolate any p53MSC in



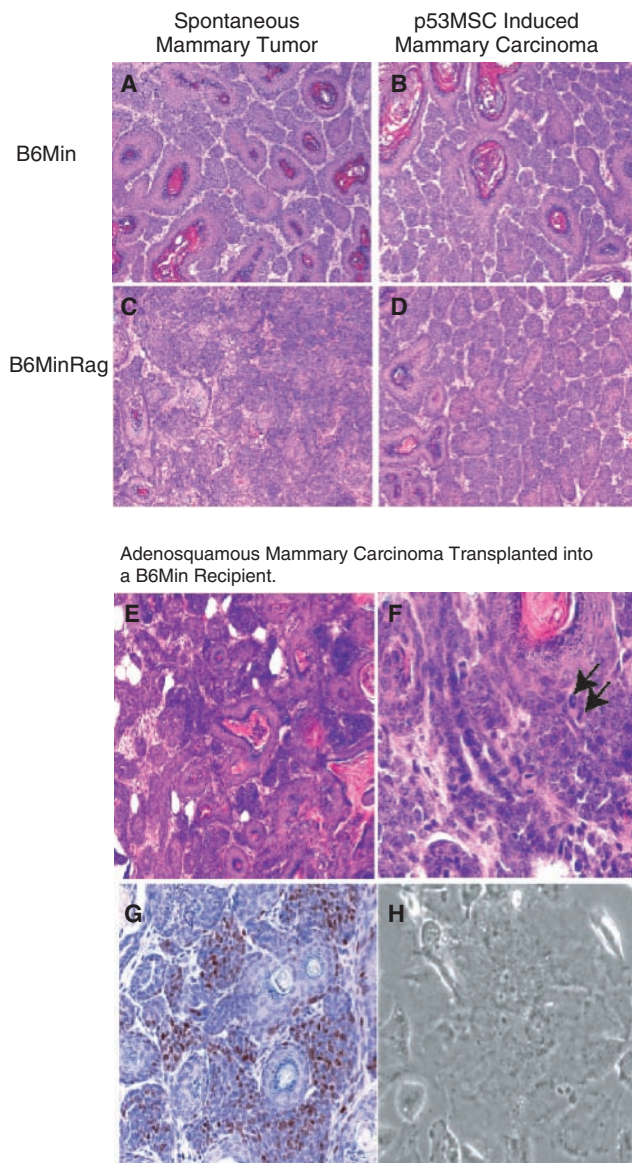
culture; however, the quantity of tissue for this purpose was limited.

### *p53MSC home to at-risk mammary epithelium*

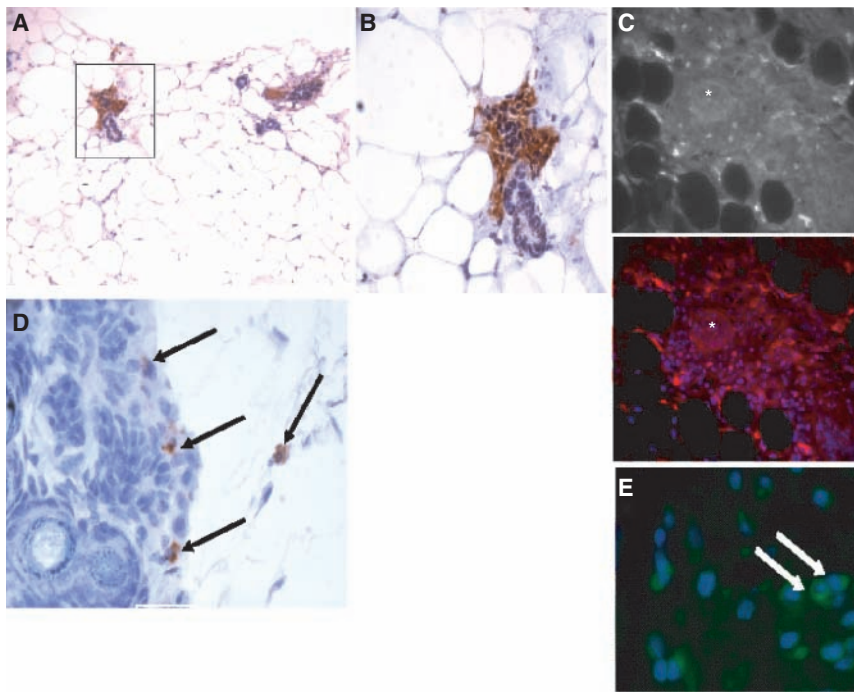
p53MSC were detected in the mammary tissue of mice following injection with RFP-labeled p53MSC, but the level of engraftment differed substantially between recipient mouse strains. B6 mice had rare single MSC juxtaposed to mammary ducts (Supplemental Table 1). B6Min (data not shown) and B6MinRag mice had foci of abundant p53MSC-derived cells surrounding several ducts (Fig. 4A and 4B), while other ducts within the same mouse had few to no detectable RFP-p53MSC (summarized in Supplemental Table 1) even in the absence of tumors. This patchy engraftment was consistent between B6Min and B6MinRag mice. In areas of tumor, p53MSC were identified both at the periphery of tumors, and within the infiltrating stroma, and accounted for 1%–5% of cells within tumors (Fig. 4C and 4D and Supplemental Table 1). Carcinomas were consistently infiltrated with, and

surrounded by a small population of RFP-p53MSC. We confirmed p53 nuclear expression (indicative of mutant protein expression) within a subset of the stromal cells by anti-p53 IF staining, consistent with continued *in vivo* expression of the mutant protein (Fig. 4E). The majority of stromal cells within the tumor were RFP<sup>-</sup>, and therefore of host origin (Fig. 4C and 4D and Supplemental Table 1). This pattern closely resembled the findings in our human samples, where p53<sup>+</sup> stromal cells comprised 1%–5% of the tumor stroma, while 95%–99% of the cells failed to stain for p53.

Peripheral blood of the B6Min and B6MinRag mice that had developed mammary carcinoma contained <1% circulating RFP-expressing p53MSC-derived cells, similar to wt mice. The spleen contained between 1% and 5% RFP-expressing cells. The p53MSC in the spleen maintained CD44 expression, while lacking surface markers of differentiation: CD45, CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, Gr1, F4/80, CD31, and NK1.1 by FACS analysis. Bone marrow contained few RFP-p53MSC. The p53MSC recovered from B6min or B6MinRag mice with tumors had the same distribution and

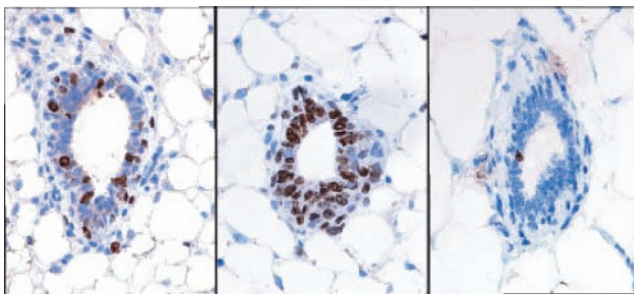


**FIG. 3.** p53MSC induce mammary adenosquamous carcinoma in the B6Min and B6MinRag mouse. Comparison of histology of spontaneous mammary carcinoma and p53MSC-induced mammary tumor in the mouse model. Tumor tissue from aged (>26-week-old) untreated B6Min and B6MinRag mice from previously published studies was used for comparison purposes, as no spontaneous tumors arose during our study period. Tumors from (A) aged B6Min, (B) 8-week-old B6Min injected with p53MSC and evaluated 4–6 weeks later, (C) aged B6MinRag, (D) 8-week-old B6MinRag injected with p53MSC and evaluated 4–6 weeks later demonstrate striking similarities when compared with each other including highly irregular infiltrative growth pattern with nonkeratinized and keratinized glands arranged in variable sized nests and cords, cellular atypia, and nuclear pleomorphism of neoplastic cells. p53MSC-induced mammary tumor (such as the one shown in Panel B) was transplanted as a cell suspension into the mammary fat pad of secondary recipient B6Min mice. (E) Low-power and (F) high-power images of transplanted tumor reveal suspended tumor cells have been organized into abnormal neoplastic glands. Increased mitotic figures, including 2 abnormal ones (arrows) typify active neoplastic cell proliferation. (G) Ki-67-specific immunostaining of transplanted mammary carcinoma highlights increased proliferative activity consistent with neoplasia. (H) Cells from the tumor shown in E and F grow readily in tissue culture as an epithelial monolayer. 40× inverted microscope image. Images (A–F), hematoxylin and eosin staining. (G), Ki-67 IHC, hematoxylin counter stain. Original magnification (A–E, G) 10×, (E) 20×, (F, H) 40×.

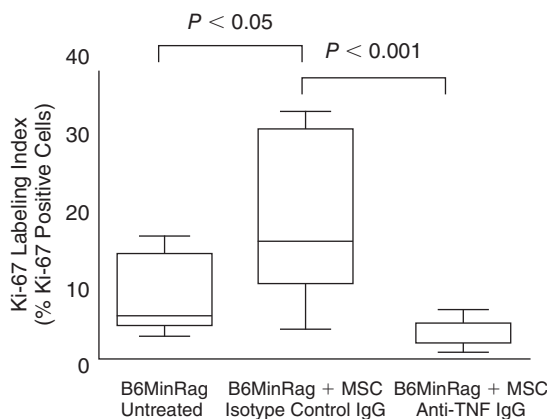


**FIG. 4.** p53MSC in mammary epithelium of the B6MinRag increase mammary duct epithelium proliferation via tumor necrosis factor (TNF) signaling. (A) 10× and (B) 40× (boxed image from (A)) red fluorescent protein (RFP)-immunohistochemistry (IHC) (brown cytoplasmic staining) showing clusters of p53MSC closely opposed to mammary duct epithelium. (C) Direct fluorescence imaging of tissue surrounding mammary carcinoma. Abundant RFP<sup>+</sup> (top image black and white fluorescent image, below; pseudocolored image). Asterisk denotes a duct embedded within RFP<sup>+</sup> stroma. (D) RFP-IHC reveals RFP<sup>+</sup> MSC at the edge of mammary tumor and within stroma (arrows). (E) p53 IF staining (green) of stroma surrounding mammary carcinoma. Green nuclei of p53<sup>+</sup> stromal cells are highlighted by arrows. (F) Ki-67-specific IHC of mammary epithelium of B6MinRag mouse receiving no intervention (no treatment—first image), p53MSC and isotype control IgG (second image), or p53MSC plus anti-TNF-α IgG (third image). Ki-67 staining is confined to epithelial cells. The stromal cells were predominantly nonproliferative. We did not detect any Ki-67 staining in p53MSC. Tumor incidence and Ki-67 proliferation index is shown below histology as a chart form and as a bow-and-whisker plot for Ki-67 staining.

**F** Ki67 Staining of Mammary Ductal Epithelium of B6MinRag Mice



Group	No Treatment	MSC + Isotype Control IgG	MSC + Anti-TNF IgG
Tumor Incidence	0/10	8/10	0/10
Ki67 Staining Index	7.78 ± 1.46	17.3 ± 3.24	3.00 ± 0.55



behavior (when placed in culture or injected back into the wt mouse) as those isolated from wt nontumor-bearing mice. Specifically, reinjected p53MSC into B6 mice did not form

tumors, and behaved as previously described for primary p53MSC. These cells lacked the surface markers: CD45, CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, Gr1, F4/80, CD31, and

NK1.1, retained CD44 positivity, and retained the ability to differentiate into bone fat and cartilage, confirming they were MSC. Further, these data suggest that our results do not reflect phagocytosis of p53MSC by macrophages. These data also suggest that the p53MSC did not gain *in vivo* growth characteristics as a result of passage through the mouse. The phenotype of p53MSC recovered from B6, B6Min, and B6MinRag was similar with regard to the number in circulation, the number recovered from the marrow cavity (~3% of the stromal cells of the marrow), and surface marker expression. Thus these results support our hypothesis that tissue environment of at-risk tissue itself orchestrates engraftment, proliferation, and proneoplastic effects of p53MSC in the cancer prone model, but that outside of the local environment of the tumor, p53MSC behave normally.

#### *Wild-type CD25<sup>+</sup> regulatory T cells abolish p53MSC augmentation of tumor formation in B6Min and B6MinRag mice*

CD4<sup>+</sup>CD25<sup>+</sup> regulatory (T<sub>REG</sub>) cells significantly contribute to immune homeostasis in autoimmune diseases [27], chronic inflammatory diseases [29], and cancer [27,30,31] through down-regulation of destructive inflammatory responses arising from CD4<sup>+</sup> T cells as well as cells of innate immunity. In our present system, infusion of wt T<sub>REG</sub> cells completely inhibited the cancer phenotype. While 50% of the B6Min and 80% of the B6MinRag mice injected with p53MSC developed clinically relevant mammary carcinoma, none of the B6Min or B6MinRag mice injected with T<sub>REG</sub> cells along with the p53MSC developed mammary tumors (Table 2). This finding was not the result of T<sub>REG</sub> elimination of p53MSC, as we were able to identify p53MSC in the peripheral blood and bone marrow of these mice. The pattern, number, and distribution of p53MSC in the blood and marrow were similar between mice receiving T<sub>REG</sub> and vehicle. Examination of the mammary tissue of T<sub>REG</sub> recipients revealed several important differences compared with controls. Few, single p53MSC were found in the mammary epithelium of B6MinRag mice receiving p53MSC and T<sub>REG</sub> (Supplemental Table 1). These cells were juxtaposed to mammary epithelium suggesting that, in the presence of T<sub>REG</sub> cells, p53MSC remain viable and retain their ability to home to the mammary tissue at risk. However, despite their localization to mammary tissue, no tumor formed with T<sub>REG</sub> cells present. Thus, immune dysregulation appears to be required for p53MSC-orchestrated tumor initiation and growth in the mammary epithelium.

#### *p53MSC-induced mammary tumor formation in B6Min and B6MinRag mice was TNF- $\alpha$ -dependent*

Previous studies from our group show that intestinal and mammary tumor development in *Apc<sup>Min/+</sup>* mice depends upon overexpression of TNF- $\alpha$  [22,23,32]. In these models, T<sub>REG</sub> cells modulate the immune response, in part by restoring elevated TNF- $\alpha$  levels to normal. In our present study, anti TNF- $\alpha$ , but not isotype control Ig, effectively prevented tumor formation in both the B6Min and B6MinRag mice receiving p53MSC, indicating that p53MSC modulated cancer via a TNF- $\alpha$ -dependent signaling pathway (Table 2). Anti-TNF- $\alpha$  therapy did not alter peripheral circulating levels of p53MSC and p53MSC were present in mammary

tissue (Supplemental Table 1) verifying that the anti-TNF- $\alpha$  treatment did not affect p53MSC viability or engraftment ability, but dramatically impacted the ability of p53MSC to induce tumor in at-risk tissue similar to our findings with T<sub>REG</sub> infusion.

#### *Proliferation of malignant cells in at-risk tissue*

p53MSC are detected juxtaposed to normal duct epithelium in the B6Min and B6MinRag mice, and rarely in mammary tissue of B6 mice that do not develop mammary carcinoma. We examined Ki-67 expression in these nonmalignant mammary tissues to gauge the level of cell proliferation within the ductal epithelium prior to onset of overt neoplasia. Baseline proliferation of mammary ductal cells in both the B6Min (data not shown) and B6MinRag (Fig. 4E and 4F) mice were higher than control B6 mice (data not shown). Proliferation within the B6MinRag tissue increased dramatically with the addition of p53MSC, and was unaffected by the addition of control Ig Ab treatment (Fig. 4E and 4F). Addition of T<sub>REG</sub> cells or anti-TNF- $\alpha$  antibody to B6Min (data not shown) and B6MinRag mice (Fig. 4E and 4F) with p53MSC decreased the proliferation rate below baseline levels for these strains to proliferative levels that were indistinguishable from levels found in wt non-Min mice. These data indicated that at baseline, at-risk mammary epithelium in Min mice had a higher proliferation rate, which was dramatically augmented by p53MSC. Anti-TNF- $\alpha$  therapy prevented the p53MSC-mediated increase in proliferation, and restored epithelial proliferation rates to levels found in normal risk non-Min individuals.

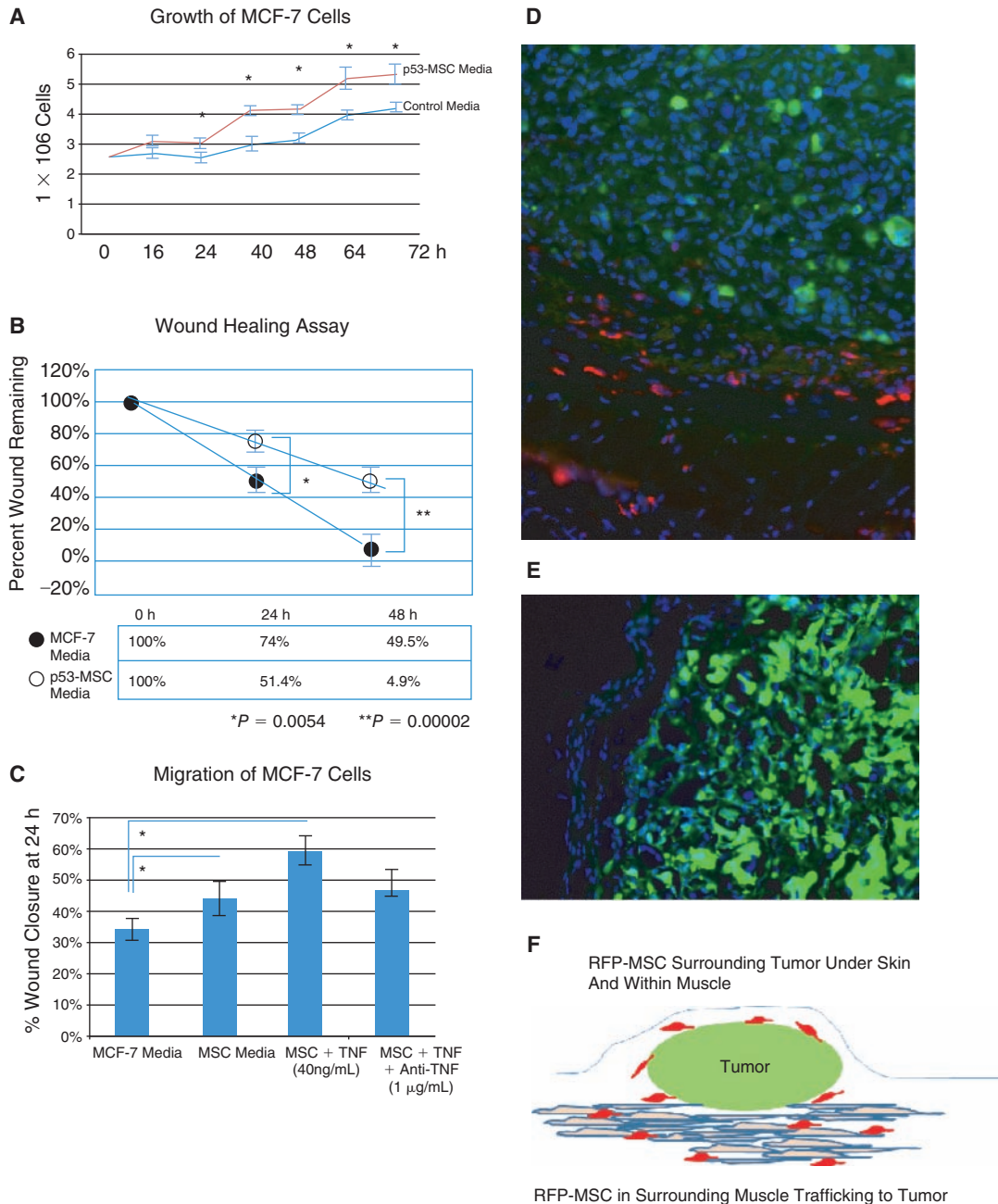
#### *p53MSC increased proliferation and migration of human breast carcinoma cells in vitro and in vivo*

To tease out the mechanisms involved in immune regulation of mammary carcinoma, and establish relevance to human disease, we utilized a combination of *in vitro* cell culture systems and human breast cancer xenograph models. We first evaluated the effects of soluble factors produced by p53MSC on the MCF-7 breast cancer cell line. p53MSC had a phenotype consistent with the one that is reported for CAF. p53MSC expressed higher levels of MMP3, MMP9, fgf1, and VEGF [13] and secreted high levels of MCP-1 and RANTES when compared with wtMSC and had low but detectable levels of IL-6, TNF- $\alpha$ , and IFN- $\gamma$  (Supplemental Table 2; Supplementary materials are available online at <http://www.liebertonline.com/scd>). Conditioned cell-free media from 24-h p53MSC cultures (but not wtMSC cultures) increased proliferation of the MCF-7 human breast cancer cell line in a consistent and sustained fashion (Fig. 5A) and increased the mobility of MCF-7 cells in a wound-healing assay (Fig. 5B). Direct addition of TNF- $\alpha$  to MCF-7 wounded monolayers did not significantly increase the motility of the breast cancer cells (data not shown). In contrast, p53MSC cell-free conditioned media by itself (which contain on average 153 pg/mL of secreted TNF- $\alpha$ ) stimulated motility and migration of MCF-7 cells significantly over control conditions (Fig. 5C). The addition of exogenous TNF- $\alpha$  to p53MSC cultures prior to harvesting media increased motility further, allowing 60% healing by 24 h, and complete healing by 48 h. Adding neutralizing anti-TNF- $\alpha$  during p53MSC culture resulted in conditioned media that did not support

MCF-7 cell wound healing (Fig. 5C). However, neutralizing the TNF- $\alpha$  in the conditioned media just prior to adding it to the MCF-7 monolayers did not have a significant effect on healing kinetics. These data suggest that TNF- $\alpha$  stimulates the p53MSC in an autocrine manner to secrete factors

responsible for increased motility of breast cancer cells. The effect of TNF- $\alpha$  is on MSC themselves rather than a direct effect of TNF- $\alpha$  on the breast cancer cells.

Next we assessed the interaction between p53MSC and MCF-7 cells in vivo. NOD-SCID mice received 1 million



**FIG. 5.** Factors elaborated by p53-mutated mesenchymal stem cells (MSC) increase growth and motility of human breast cancer in vitro and allow homing to breast cancer in vivo. (A) Human breast cancer cell line MCF-7 was grown in control media or cell-free p53MSC-conditioned media. Cell counts were determined at the indicated times. (B) Confluent MCF-7 wounded monolayers were grown in MCF-7 media (open circle) or p53MSC-conditioned media (black circle) for 48 h and the wound remaining measured and recorded as a percent of the original wound. (C) MCF-7-wounded monolayers were grown in media as indicated and percent wound closure recorded at 24 h. Cell counts and wound-healing experiments were performed using triplicate plates, repeated a minimum of one additional time, and reported as the mean  $\pm$  1 SD. For A–C; \* $P$  < 0.05 unless otherwise noted. (D) Red fluorescent protein (RFP)-p53MSC surround GFP-labeled MCF-7 cells in the NOD-SCID mouse. (E) wtMSC labeled with RFP do not migrate toward GFP-labeled MCF-7 cells. (F) Cartoon depicting the distribution of p53MSC within human breast cancer stroma and within surrounding muscle.

p53MSC, or wtMSC as a single intracapsular injection, followed 1 week later by  $1 \times 10^6$  GFP-labeled MCF-7 cells or mouse embryonic fibroblast (MEF) cells injected onto the flank. At 4 weeks, p53MSC were detected in the surrounding skin and muscle and encasing small well-demarcated GFP-MCF-7 tumors (Fig. 5D, and cartoon in Fig. 5F). p53MSC did not migrate to normal flank nor to normal mammary fat pads, or in response to the primary GFP-MEF cell culture (data not shown). In contrast to p53MSC, wtMSC could not be recovered in MCF-7 tumors or peripheral tissues including mammary fat at times up to 4 weeks (Fig. 5E). These findings confirm that p53MSC migrate toward human breast cancer cells, providing relevance to human disease.

## Discussion

Adults have in situ malignancy in multiple organs by midlife [1–4], but remarkably few people manifest clinical disease. We sought to understand factors involved in allowing subclinical cancers to progress to overt clinical disease. We chose our *Apc*<sup>Min/+</sup> mouse model because of the low incidence of mammary tumors in a controlled environment, and the association of clinically apparent tumor formation with increasing systemic inflammation. This pattern follows the known association of chronic inflammation with many types of human tumors. *Apc* mutations can also be found in human breast cancer [33] and breast cancer cell lines [34]. Thus the *Apc*<sup>Min/+</sup> mouse represents a model whereby the mammary tissue is at-risk for malignancy, but depending upon the local environment, cancer potential may or may not be realized.

Our experiments show that genetic changes including p53 mutations in MSC are well-tolerated in genetically normal tissues, and are not associated with an increase in cancer incidence and that p53MSC have a homing advantage over wtMSC to “at-risk” peripheral tissue by an as of yet unclear mechanism. Alone, p53MSC are unable to induce disease; however, in combination with systemic immune dysregulation, they allow neoplastic growth in at-risk mammary tissue. Restoration of immune homeostasis prevents disease despite unchanged genetic mutations within both the stromal and epithelial compartments strongly supporting the notion that immune modulation may be an effective means to prevent cancer in high-risk individuals.

Epigenetic and genetic changes within tumors, which drive proliferation and aggressive clinical behavior, have historically been assessed at the level of the cancer cell itself, with less attention given to gene alterations within the supporting stroma. Animal models of cancer typically focus on gene mutations within the epithelial cell compartment, and in many cases multiple gene alterations are required to initiate tumors. The behavior of resulting tumors differs from those found in human and rarely is the spectrum of disease caused by epithelial mutations alone as diverse as the diseases seen in humans. One way to view these discrepancies is to recognize that genes targeted to specific cell populations alter the behavior of “tumor” cells, but do not take into account the contribution of stromal cells. Several recent reports demonstrate the importance of mutations within tumor stroma and the dramatic influence these cells have on the behavior of the tumor as a whole [8,14,15]. It is not clear, however, if the neoplastic epithelial cells influence and dictate behavior

of the stroma, or vice versa, that is, if the stroma forces a neoplastic phenotype on the epithelial cell, thus creating the classical chicken and egg dilemma. Our studies using p53MSC and wtMSC in the *Apc*<sup>Min/+</sup> mouse demonstrate that stromal cell mutations accelerate tumor progression in at-risk tissue.

For the most part, it has been assumed that transformed cells within an organ proliferate and orchestrate stroma recruitment and organization via influence on local fibroblasts [35–37], or through epithelial–mesenchymal transition of normal or transformed epithelial cells [38] or by recruiting bone marrow-derived mesenchymal [39–41] and other progenitor cells [42]. Factors elaborated by stromal cells create a unique milieu that has profound effects on cancer cell proliferation, invasion, and migration [43–45] similar to the contribution of activated stromal cells to wound healing. This viewpoint of stromal cell–cancer cell interaction presupposes that the tumor cells orchestrate the complex interaction with the stromal cells, and these stromal cells likely undergo gene mutations as a result of the hyperproliferative environment of the tumor. This view maintains the stromal cells are under the influence and direction of the cancer cell. Our data support an alternate view that stromal cells may initiate epithelial transformation via cytokine-mediated signaling of at-risk tissue and cause the transition of preneoplastic tumor foci to overt malignant tumors by promoting tumor cell proliferation.

Though controversial, there are data suggesting that p53 mutations can be found selectively within the stromal component of breast cancer and may be associated with a more aggressive phenotype [14,15,17–19]. The vast majority (95%–99%) of stromal cells within the B6Min+p53MSC and B6MinRag+p53MSC mammary tumors were host-derived, and thus wt for p53. Although rare, p53MSC were found both within tumors, and at the very edge of the epithelial cancer cells interface. Thus, sampling at any distance from the tumor itself would lead to a negative result for mutant p53, and sampling within the tumor could yield negative results due to sampling error based on the p53MSC being a small minority of cells within the stroma. Immunohistochemistry and IF analysis of our archived human breast cancer samples verifies that in 67% of cases examined, overexpression of p53 was found suggesting a mutant protein, but p53<sup>+</sup> stromal CAF were only 1%–5% of the total stromal cells. Thus, p53CAF are <5% of the total stroma, but may have a major impact on the biology of the tumor.

Despite this controversy, our human data and compelling data from other systems warrants investigation of the association between p53MSC and breast cancer. MCF-7 mammary carcinoma cells grow faster and demonstrate a more aggressive phenotype when mixed with p53-deficient fibroblasts compared with wt fibroblasts suggesting a nonautonomous mechanism of p53-mutated stroma and transformed mammary epithelial cells [11]. In these studies, p53-deficient fibroblasts are derived from p53 null mice. Signaling differs between p53-deleted animal models and clinically relevant p53 point mutations that occur in human disease. Our model addresses the function of clinically relevant p53 mutations [20] in mesenchymal cells and their ability to initiate cancer in a tissue-specific fashion in an at-risk mammary carcinoma model. Our results in both

the  $Apc^{Min+/-}$  and the MCF-7 models implicate p53 mutation in the stroma as a significant contributor to tumor cell proliferation.

MSC may acquire significant gene mutations after extended replication [13,46,47]. Significantly, MSC acquire p53 point mutations within the DNA-binding region, consistent with point mutations commonly found in human tumors [13], making this a highly relevant model for human cancer. MSC originating in the bone marrow circulate and engraft in peripheral tissues in low numbers as fibroblasts. During wound repair or with chronic inflammation, MSC enter solid organs in higher numbers [12,48] and maintain tissue integrity through direct transdifferentiation to epithelial cells [49,50] or as stromal cells [39]. MSC may not be permanent residents within organs, but rather may reenter the circulation and travel back to the bone marrow cavity to remain quiescent, or travel to other organs. It is conceivable then that mutations acquired by MSC at one site can affect distant tissues by MSC migration.

This sets up a compelling scenario where the interplay between at-risk epithelial cells, the stromal cells that may have acquired mutations at other sites, and the local immune environment each dictates tumor outcome. In our model, mice that harbor mesenchymal p53 mutations alone remained healthy with a normal life span. Similarly, mice with an epithelial cell  $Apc$  mutation rarely developed mammary carcinoma in the absence of systemic inflammatory triggers. Combining both epithelial cell mutations and stromal mutations resulted in accelerated tumor formation. Combining stromal and epithelial mutations with dysregulated immune response as with Rag-deficient mice receiving p53MSC had profound consequences, and resulted in dramatic increase in mammary tumor incidence. This tumor phenotype could be reversed by restoring immune regulation with  $T_{REG}$  or anti-TNF- $\alpha$  treatment.

p53MSC are recruited to mammary tissue by a yet unknown mechanism. p53MSC secrete many factors including MCP-1 and IL-6, which are potent inducers of inflammation. MMP-2 and -9 may further act to process epithelial-, stromal-, and inflammatory-derived growth factors and activate receptors [45] that contribute to tumor growth and neovascularization. Under the influence of TNF- $\alpha$ , MSC increased ductal proliferation. Tumor formation was directly linked to TNF- $\alpha$  activity in vivo. Our data strongly link the synergistic requirement of epithelial mutations, stromal mutations, and dysregulated persistent inflammation with the conversion of dormant disease to active malignancy. While we do not yet have direct evidence for a similar mechanism in human disease, the indirect evidence is striking. It was recently shown that the production of induced pluripotent stem cells (iPS) cells could be dramatically simplified by disabling the p53 pathway, suggesting that the pathways used for pluripotency and cancer formation overlap [51–53]. These findings suggest that cancer formation may involve a reprogramming-like mechanism of the stromal cells or the cancer cells themselves.

Cancer-associated stroma, especially stromal cells with dysregulated p53, may orchestrate local behavior of tumor cells and coordinate metastatic and invasive behavior of malignant cells [14,11]. A role for TNF- $\alpha$  in promotion and progression of cancer is well-documented and an area of intense interest [54]. Our findings that stromal cells assist in the conversion of dormant disease to active disease suggest

that new targets for preventative therapy for at-risk human populations can be developed. One can also envision the possibility that quiescent metastatic foci, which may become active many years after seemingly curative cancer therapy, could be kept in check by therapy targeted at disrupting inflammation–stroma–cancer cell interactions.

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