

Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF- κ B-Dependent Manner

Neta Erez,^{1,2,3} Morgan Truitt,^{1,2,3} Peter Olson,^{1,2,3} and Douglas Hanahan^{1,2,3,4,*}

¹Helen Diller Family Comprehensive Cancer Center

²Diabetes Center

³Department of Biochemistry and Biophysics

University of California San Francisco, San Francisco, CA 94143, USA

⁴Present address: Swiss Institute for Experimental Cancer Research, Swiss Federal Institute of Technology Lausanne, Lausanne CH-1015, Switzerland

*Correspondence: dh@ucsf.edu

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SUMMARY

Cancer-associated fibroblasts (CAFs) support tumorigenesis by stimulating angiogenesis, cancer cell proliferation, and invasion. We demonstrate that CAFs also mediate tumor-enhancing inflammation. Using a mouse model of squamous skin carcinogenesis, we found a proinflammatory gene signature in CAFs isolated from dysplastic skin. This signature was maintained in CAFs from subsequent skin carcinomas and was evident in mammary and pancreatic tumors in mice and in cognate human cancers. The inflammatory signature was already activated in CAFs isolated from the initial hyperplastic stage in multistep skin tumorigenesis. CAFs from this pathway promoted macrophage recruitment, neovascularization, and tumor growth, activities that are abolished when NF- κ B signaling was inhibited. Additionally, we show that normal dermal fibroblasts can be “educated” by carcinoma cells to express proinflammatory genes.

INTRODUCTION

Although tumorigenesis has classically been viewed as a largely cell-autonomous process involving genetically transformed cancer cells, the importance of stromal cell types populating the neoplastic microenvironment is now well accepted (reviewed in Bissell and Radisky, 2001; Hanahan and Weinberg, 2000; Tlsty and Coussens, 2006). Studies investigating the link between inflammation and cancer have been particularly revealing, demonstrating that inflammatory immune cells are recruited to neoplasias, where they in many cases markedly promote tumor progression by facilitating multiple hallmark capabilities (de Visser et al., 2006; Karin and Greten, 2005; Mantovani et al., 2008). Immune cells have been documented to supply soluble growth and survival factors, matrix remodeling enzymes, reactive oxygen species, and other bioactive molecules that

variously influence cancer cell proliferation, angiogenesis, invasion, and metastasis (De Marzo et al., 1999; Kuper et al., 2000; van Kempen et al., 2006).

A second stromal component of importance for tumorigenesis is cancer-associated fibroblasts (CAFs). CAFs are phenotypically and functionally distinguishable from their normal counterparts in their increased rate of proliferation and differential expression of extracellular matrix (ECM) components and growth factors (Bhowmick et al., 2004b; Kalluri and Zeisberg, 2006). Several studies have demonstrated that normal fibroblasts have a role in maintaining epithelial homeostasis by suppressing proliferation and oncogenic potential of adjacent epithelia (Begley et al., 2008; Bhowmick et al., 2004a; Trimboli et al., 2009). However, following neoplastic transformation of epithelia, CAFs have been shown to promote tumor growth by inducing angiogenesis, recruiting bone marrow-derived

Significance

Chronic inflammation is highly correlated with many types of human cancer. Here we show that cancer-associated fibroblasts (CAFs) express a proinflammatory gene signature in skin, breast, and pancreatic cancers. Proinflammatory CAFs from a transgenic mouse model of squamous cell carcinoma mediated immune cell recruitment, angiogenesis, and tumor growth. Inhibition of NF- κ B signaling abolished these tumor-promoting effects, demonstrating that NF- κ B is critical for tumor-enhancing inflammation mediated by CAFs. These results not only demonstrate a role for CAFs in promoting tumorigenesis, but also have important implications for therapeutic targeting of CAF regulatory or effector circuits, suggesting that inhibiting stromal NF- κ B signaling could be of value in treating certain forms of human cancer.

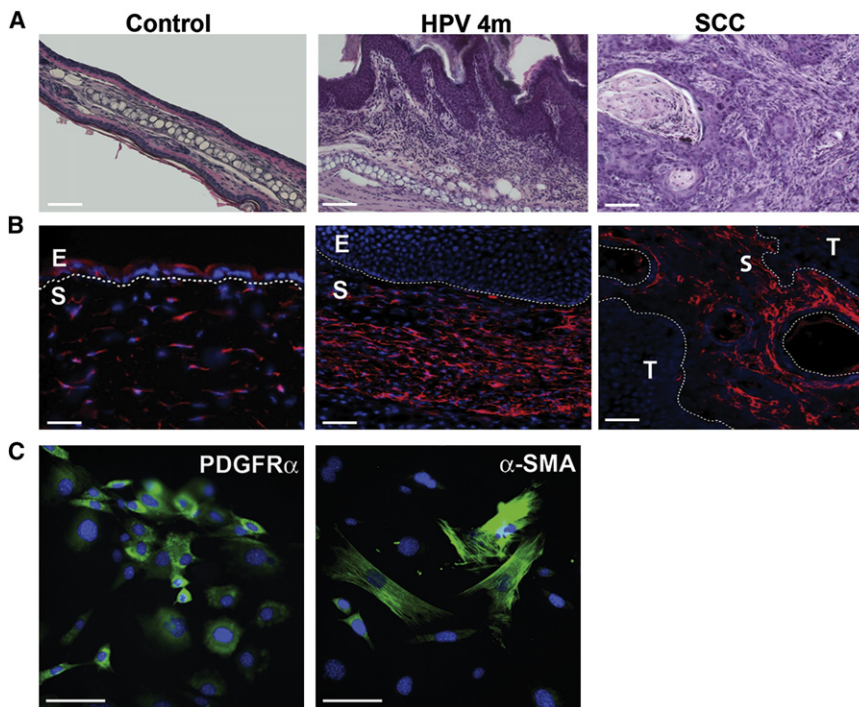


Figure 1. PDGFR α is Broadly Expressed in Normal and Neoplastic Skin Fibroblasts

(A) H&E staining of skin from nontransgenic control ears (left), a 4-month-old HPV16 mouse with dysplasia (middle), or a squamous cell carcinoma (SCC) (right).

(B) PDGFR α expression is restricted to the stromal compartment in skin and tumors. Immunohistochemical analysis of PDGFR α (Rhodamine-X, red) in the same tissue as in (A). E, epithelium; S, stroma. DAPI staining is shown in blue. The panels are representative of multiple fields of skin sections from two control and four HPV mice.

(C) Immunofluorescent staining of cultured fibroblasts sorted from dysplastic ear. All fibroblasts express PDGFR α (FITC). Only a subset of PDGFR α ⁺ fibroblasts expresses α -SMA (FITC). Scale bar, 100 μ M. See also Figure S1.

endothelial progenitor cells, and remodeling the ECM (Allinen et al., 2004; Olumi et al., 1999; Orimo et al., 2005; Pietras et al., 2008). Interestingly, CAFs can even mediate resistance to antiangiogenic therapy (Crawford et al., 2009). It is increasingly apparent that CAFs are a diverse cell population that can have different characteristics in different tumor types and tissue locales. Some CAFs are related to myofibroblasts, an activated form of fibroblast that plays an important role in wound healing and is characterized by expression of α -SMA. Not all CAFs, however, express α -SMA. Increasingly, fibroblasts in tumor tissues are being recognized as a diverse population of myofibroblastic cells intermixed with other fibroblastic cells that do not express α -SMA but may be tumor promoting nevertheless (Desmouliere et al., 2004; Micke and Ostman, 2004; Sugimoto et al., 2006).

To better understand the characteristics and functions of CAFs, we set out to investigate CAFs in the K14-HPV16 mouse model of multistep squamous skin carcinogenesis. This model displays premalignant and malignant stages that are stereotypical of carcinogenesis in humans. K14-HPV16 transgenic mice have proven useful for investigating multiple aspects of carcinogenesis, including tumor-stroma interactions (Arbeit et al., 1994; Coussens et al., 1996). These mice express the HPV16 early-region that includes the E6/E7 oncogenes, under the control of the human keratin-14 promoter/enhancer. Animals develop hyperplastic and then dysplastic lesions that progress to invasive squamous cell carcinomas, typically within the epidermis of the ear or on the chest and truncal skin; by 4 months of age, 100% of transgenic animals have hyperplastic/dysplastic lesions on their ears. The preneoplastic stage is characterized by extensive remodeling of the underlying dermal stroma, which facilitates both angiogenesis and eventual tumor cell invasion. This extensive stromal remodeling develops early at the

dysplastic stage in all animals, well before malignant conversion, and is characterized by a chronic inflammatory response.

In this study, we performed expression profiling of fibroblasts from dysplastic skin of K14-HPV16 transgenic mice and

investigated the functional implications of tumor-promoting inflammation mediated by CAFs. Furthermore, we studied the molecular mechanism underlying activation of naive, stromal fibroblasts into proinflammatory, tumor-promoting CAFs beginning at the earliest stages of multistep skin tumorigenesis.

RESULTS

Fibroblasts from Skin Dysplasias are Proinflammatory

Stromal changes in neoplastic skin of K14-HPV16 (HPV) mice precede progression to invasive carcinomas. By 4 months of age, at sites where skin tissue is focally dysplastic, the dermis is transformed into a reactive stroma, composed of blood and lymphatic vessels, inflammatory cells, and fibroblasts, which persists during neoplastic progression to cancer (Coussens et al., 1999) (Figure 1A). We sought to identify genes whose expression changed in CAFs in early neoplastic skin lesions compared to their normal dermal counterparts. We chose to purify fibroblasts from normal and neoplastic tissues by FACS. Although the myofibroblast marker α -SMA is commonly used to identify CAFs, it is intracellular, and it is not certain that all CAFs are α -SMA positive (Sugimoto et al., 2006). We surveyed a number of surface markers for robust expression in skin fibroblasts and found PDGFR α to be the best (see Figure S1A available online). PDGFR α , which is reportedly expressed by up to 90% of stromal fibroblasts in solid tumors (Micke and Ostman, 2004), was found by immunostaining to be expressed exclusively in the stromal compartments of normal skin, dysplastic HPV tissue, and HPV skin tumors (Figure 1B). The purity of sorted PDGFR α ⁺ cells was verified by expression of fibroblast-specific genes and by a lack of expression of immune cell markers (Figure S1B). When α -SMA expression was assessed in sorted skin fibroblasts, we observed that only a subset of PDGFR α ⁺

Table 1. Summary of Microarray Results of Proinflammatory Genes

Gene Name	Gene Symbol	Affymetrix Probe ID ^a	Fold Change ^b	p
Chemokine (C-X-C motif) ligand 2	CXCL2	1449984_at	225.985	0.00085
Interleukin 6	IL-6	1450297_at	54.449	0.00085
Interleukin 1 beta	IL-1 β	1449399_a_at	47.236	0.03878
Chemokine (C-X-C motif) ligand 1	CXCL1	1457644_s_at	37.353	0.00085
Chemokine (C-X-C motif) ligand 5	CXCL5	1419728_at	37.709	0.02520
Cyclooxygenase 2	Cox-2	1417262_at	18.521	0.03465
Serglycine	SRGN	1417426_at	16.612	0.02520
Chemokine (C-C motif) ligand 3	CCL3	1419561_at	15.162	0.03878
Matrix metalloproteinase 12	MMP12	1449153_at	11.502	0.04352
Interleukin 1 family, member 9	IL-1F9	1425958_at	6.867	0.04200
Matrix metalloproteinase 3	MMP3	1418945_at	3.663	0.04515
Fibrinogen, α polypeptide	FGA	1424279_at	6.942	0.03864
Nuclear receptor subfamily 4, member 1	Nur77/NR4A	1416505_at	7.920	0.04673
Secretory leukocyte peptidase inhibitor	Slpi	1448377_at	8.996	0.04869
Osteopontin (secreted Phosphoprotein 1)	OPN/Spp1	1449254_at	9.364	0.19182

^a Affymetrix probe ID corresponds to the Mouse Genome 430 2.0 Array.

^b Fold change represents HPV-16 CAFs from dysplastic skin tissue as compared to age-matched nontransgenic controls.

fibroblasts expressed α -SMA (Figure 1C), indicating that we were indeed including a larger and more heterogeneous population of CAFs in our survey than just myofibroblasts.

We expression profiled fibroblasts sorted from dysplastic skin tissue of K14-HPV16 mice and from age-matched controls, using PDGFR α as a cell surface marker. Comparison of the transcriptome of CAFs from dysplastic tissue to normal dermal fibroblasts (NDFs) using the genMAPP pathway profiling software implicated “inflammatory response” and “immune cell chemotaxis” pathways. We chose to focus on the most highly upregulated genes from these pathways (Table 1). Because we have previously shown that CAFs are proangiogenic in the related HPV16 mouse model of cervical cancer (Pietras et al., 2008), we also analyzed the microarray results for proangiogenic genes. The expression of a number of known proangiogenic genes (e.g., VEGF, FGF-2, and FGF-7) was not significantly changed in dermal CAFs, compared with controls (data not shown). Interestingly, however, one proangiogenic gene, *CYR61* (Brigstock, 2002; Monnier et al., 2008), was upregulated in dermal CAFs.

For further analysis, we selected a set of highly upregulated “signature” genes (Figure 2F). This predominantly proinflammatory gene signature consists of two chemokines that are chemoattractants for neutrophils and macrophages (CXCL1, and CXCL2); the proinflammatory cytokines IL-1 β and IL-6, the latter of which recently was implicated in the link between inflammation and cancer (Bollrath et al., 2009; Grivennikov et al., 2009); the proangiogenic gene *CYR61*; COX-2, which is widely upregulated in cancer and has been functionally implicated in mediating inflammatory responses via prostaglandin production (Harris, 2009; Sahin et al., 2009); and osteopontin (OPN), which is known to affect inflammation, angiogenesis, and metastasis (Cook et al., 2005). Interestingly, OPN was recently implicated in tumor enhancement by fibroblasts (Anderberg et al., 2009; Pazolli et al., 2009). Expression of the signature genes identified from the microarray data was validated by quantitative RT-PCR analysis of fibroblasts sorted from dysplastic skin tissue, compared

with NDFs (Figure 2A). Notably, CAFs sorted from end-stage squamous cell carcinomas in this multistep pathway continue to express the proinflammatory gene signature identified in premalignant dysplasias (data not shown).

CAFs in Mouse Pancreatic Ductal Adenocarcinoma and Mammary Tumors, but Not Cervical Tumors Are Proinflammatory

We next sought to assess the generality of the proinflammatory gene signature in CAFs associated with other tumor types. We therefore analyzed expression of the inflammatory signature genes in CAFs sorted from mouse cervical, mammary, and pancreatic tumors. The HPV16 transgenic mouse model gives rise to cervical cancer in female mice treated with estrogen (Arbeit et al., 1996; Elson et al., 2000). Cervical carcinogenesis in this model is also characterized by recruitment of proangiogenic macrophages and the induction and persistence of angiogenesis (Giraudo et al., 2004). Despite their similarities in tumor type and initiating oncogenes, fibroblasts sorted from cervical dysplasias (Figure 2B) and cervical tumors (data not shown), unlike dermal HPV CAFs, did not express the inflammatory gene signature, suggesting that other cell types orchestrate the inflammatory response in this pathway. The proangiogenic gene *CYR61* was, however, highly expressed by cervical CAFs and thus may participate in CAF-mediated angiogenic stimulation in this model.

Breast tumors and pancreatic ductal adenocarcinomas (PDACs) are both characterized by an extensive desmoplastic stroma, abundantly populated by fibroblasts (Korc, 2007; Mahadevan and Von Hoff, 2007; Walker, 2001). We assessed the CAFs from mammary adenocarcinomas in the MMTV-PyMT transgenic mouse model (Guy et al., 1992; Lin et al., 2003). Fibroblasts sorted from mammary tumors of 3-month-old mice expressed components of the inflammatory signature, whereas normal mammary fibroblasts did not (Figure 2C). CAFs sorted from PDACs of p48-Cre, LSL-Kras^{G12D}, p53^{fl/+} mice (Bardeesy

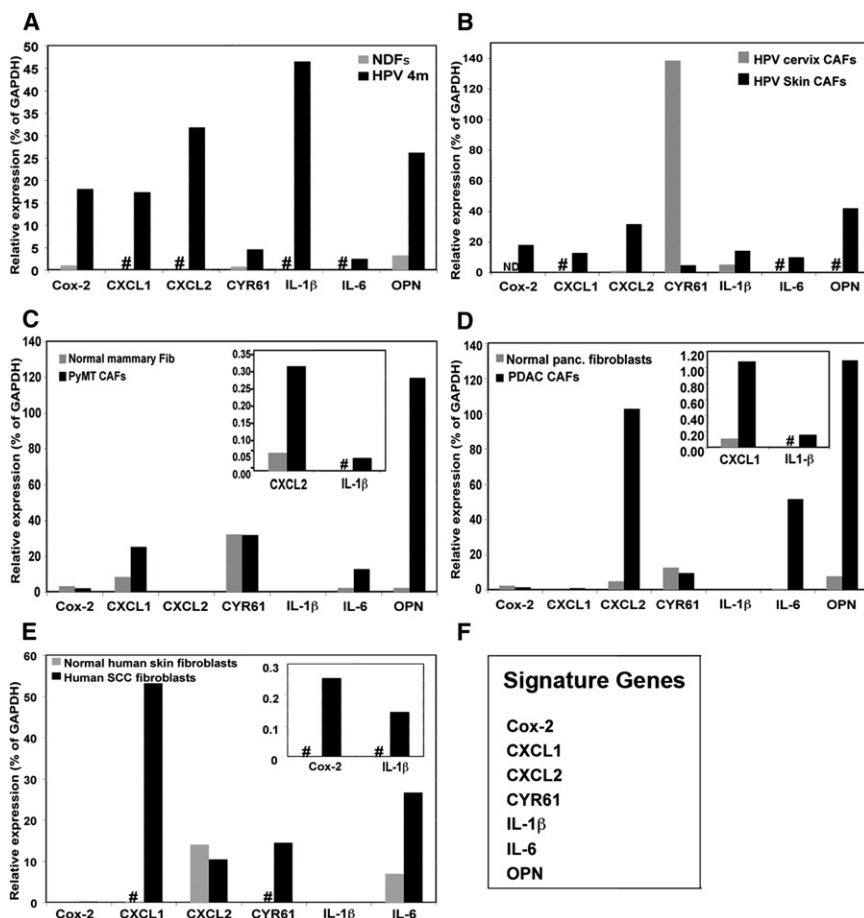


Figure 2. Expression of a Proinflammatory Gene Signature by CAFs in Skin, Cervical, Mammary, and Pancreatic Tumors

(A) qRT-PCR of PDGFR α ⁺ fibroblasts FACS sorted from ear tissue of 4-month-old HPV mice or normal dermal fibroblasts (NDFs) sorted from nontransgenic control mice. Fibroblasts were sorted from a pool of tissue from 15 age-matched mice. Results are representative of at least three independent experiments. Pound sign (#) indicates undetectable/very low expression.

(B) CAFs were FACS sorted from 3-month-old estrogen-treated female HPV mouse cervixes or from dysplasias on the ears of 4-month-old HPV mice. Results shown are from pools of tissue from 10 age-matched mice. Normal cervical fibroblasts were unavailable for comparison because of the difficulty of obtaining a sufficient amount of sorted fibroblasts from normal mouse cervixes. ND, not done.

(C) CAFs were sorted from two MMTV-PyMT tumors compared to normal fibroblasts from the mammary glands of six age-matched nontransgenic control mice.

(D) CAFs were sorted from two pooled PDAC tumors compared to pooled normal fibroblasts sorted from the pancreas of eight age-matched normal controls. All experiments were repeated at least twice.

(E) CAFs were sorted from freshly resected human SCC tumors or normal human skin. Results shown are representative of two different tumors and two normal skin samples.

(F) List of genes included in the inflammatory gene signature. See also Figure S2.

et al., 2006) were also found to express proinflammatory genes, whereas normal mouse pancreatic fibroblasts did not (Figure 2D). These results suggest that proinflammatory signaling by CAFs is operative in certain other tumor types.

CAFs in Human Squamous Cell Carcinoma and PDAC Are Proinflammatory

To investigate whether this proinflammatory signature is expressed in human cancer CAFs, we analyzed the expression of the signature genes in fibroblasts sorted from freshly resected human SCC or PDAC tumors. Notably, CAFs sorted from human SCC tumors expressed genes of the inflammatory signature, whereas normal human skin fibroblasts did not (Figure 2E). Similarly, although normal pancreatic fibroblasts were unavailable for comparison, the CAFs sorted from human PDAC tumors expressed genes of the inflammatory signature (Figure S2). To further assess the relevance of the inflammatory signature to human cancer, we surveyed expression data from multiple human SCC, breast, and pancreatic tumors available via the Oncomine database (Rhodes et al., 2004). The available data are of total tumor tissue, and not specifically of CAFs, but may be predictive nevertheless, because the stromal component is prominent in these tumor types. Strikingly, we found that all the signature genes are strongly upregulated across multiple SCC studies. Most of the genes are also upregulated in human breast and pancreatic cancer (Figure S3).

Buoyed by these associations with other mouse and human tumor types, we sought to functionally investigate the inflammatory signature in skin CAFs of the mouse squamous carcinogenesis pathway.

Inflammatory Fibroblasts Enhance Tumor Growth, Vascularization, and Macrophage Recruitment

To assess CAF functions, we adopted a well-established bioassay, involving coinoculation with transformed epithelial cells into an orthotopic site, where the effects of the fibroblasts on tumor growth could be assessed (Olumi et al., 1999; Orimo et al., 2005). Importantly, fibroblasts derived from HPV dysplastic skin maintained expression of the inflammatory gene signature in culture (Figure S4). PDSC5 cells, an HPV16-derived skin carcinoma cell line, were injected intradermally into FVB/n mice either alone, admixed with HPV dysplastic skin fibroblasts (i.e., CAFs), or admixed with fibroblasts derived from control mice (i.e., NDFs). Tumors coinjected with CAFs grew significantly faster and were larger than tumors in mice injected with PDSC5 cells alone (Figure 3A). Interestingly, the tumors in mice coinjected with NDFs had an intermediate phenotype.

We hypothesized that HPV CAFs might support tumor growth in part through the stimulation of angiogenesis, either directly via such genes as *CYR61* and *OPN*, or indirectly through the recruitment of inflammatory cells. Therefore we assessed the vascularity and necrosis of PDSC5 tumors injected with or without

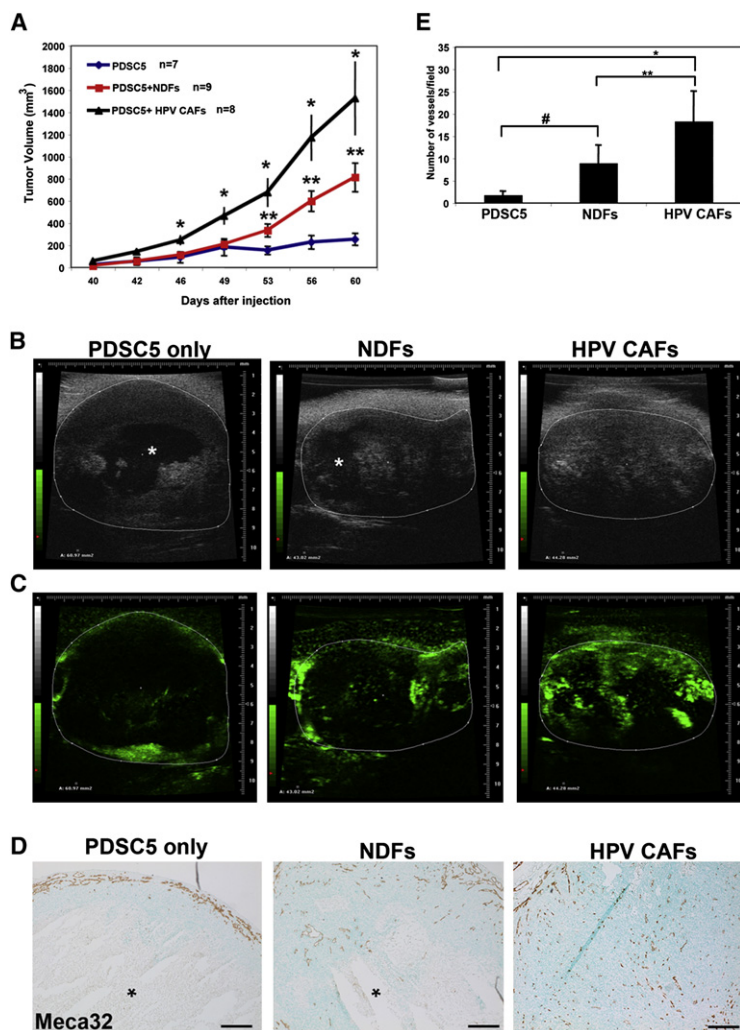


Figure 3. Skin CAFs Enhance Tumor Growth and Angiogenesis

(A) HPV CAFs enhance the growth of PDSC5 cells when coinjected intradermally (* $p < 0.05$). Tumors resulting from coinjection of PDSC5 cells and NDFs had an intermediate phenotype but were significantly different from tumors resultant from injecting PDSC5 cells alone (** $p < 0.05$). Results are representative of three independent experiments with two separate HPV-derived tumor cell lines, PDSC5.2 and PDSC5.6. Each orthotopic experiment was performed with a different batch of fibroblasts.

(B and C) In vivo ultrasound imaging of orthotopic tumors. Error bars show mean \pm SEM.

(B) Representative ultrasound images of tumors. Three mice from each group were analyzed. Asterisk indicates necrotic region.

(C) Ultrasound imaging of tumor vasculature following intravenous injection of nontargeted microbubbles. See also Movies S1–S3. (D) Immunostaining of blood vessels with anti-Meca32 antibody. The panels are representative of multiple fields of tumor sections from at least seven tumors per group. Scale bar, 100 μ M.

(E) Quantification of blood vessel density in PDSC5 tumors. Blood vessels in five different fields of three different tumors were quantified. Error bars show mean \pm SD. * $p = 0.0002$; ** $p = 0.021$; # $p = 0.0057$. See also Figure S3.

(Figure 4A). Furthermore, in vivo Matrigel plug bioassays for angiogenesis revealed that plugs injected with CAFs were significantly more vascularized and contained more macrophages than did plugs injected with NDFs or without fibroblasts (Figures 4B and 4C). Importantly, these results demonstrate that CAFs are not only critical for the recruitment of inflammatory cells into tumors and for enhancement of angiogenesis, but also that they can directly mediate these effects in the absence of tumor cells.

NF- κ B is Activated in HPV Skin Fibroblasts

Several of the inflammatory genes upregulated in HPV CAFs are known targets of the NF- κ B transcription factor (COX-2, IL-6, CXCL1, CXCL2, and IL-1 β). NF- κ B is upregulated in several inflammation-linked cancers (Karin and Greten, 2005; Pikarsky et al., 2004); however, its activity has been previously implicated in the cancer cells (Meylan et al., 2009; Wu et al., 2009) or in tissue macrophages (Greten et al., 2004). To assess NF- κ B activity in HPV CAFs, we performed immunostaining for the NF- κ B subunit p65 (RelA). When active, the NF- κ B heterodimer (RelA-p50) translocates to the nucleus, where it activates the transcription of its target genes (Karin and Ben-Neriah, 2000; Pereira and Oakley, 2008). Nuclear p65 staining was detected in the stroma of HPV skin sections but not in the skin of nontransgenic control mice (Figure 5A). To confirm that p65 is indeed active in fibroblasts, we performed immunostaining on cultured low-passage primary fibroblasts and found nuclear p65 staining in HPV dysplasia-derived fibroblasts but not in dermal fibroblasts derived from control mice, demonstrating that NF- κ B signaling is activated in CAFs (Figure 5B).

IKK β is the catalytic subunit of IKK and is essential for NF- κ B activation (Chen et al., 2003). To determine whether the tumor-promoting activity of the fibroblasts was dependent on NF- κ B, we knocked down Ikk β in CAFs using shRNA (Figure 5C).

fibroblasts. Ultrasound live imaging showed that tumors injected with CAFs were less necrotic than tumors injected without fibroblasts or with NDFs (Figure 3B). Furthermore, tumors injected with CAFs were significantly more vascularized, as assessed by both ultrasound imaging using contrast enhanced micro-bubbles to visualize tumor blood flow (Figure 3C and Movies S1–S3), and by immunostaining for the endothelial cell marker Meca-32 (Figure 3D). When the number of blood vessels was quantified, the blood vessel density in tumors injected with CAFs was found to be significantly higher than in controls (Figure 3E), supporting the idea that HPV CAFs are protumorigenic, at least in part because of their ability to promote angiogenesis.

We next examined possible mechanisms by which fibroblasts might facilitate tumor vascularization. Several of the proinflammatory genes upregulated in CAFs are known chemoattractants for macrophages and neutrophils (e.g., CXCL1, CXCL2, and CXCL5). Therefore, we suspected that macrophages, which are known to support tumor angiogenesis (Condeelis and Pollard, 2006), might be differentially recruited to PDSC5 tumors injected with CAFs. Indeed, immunostaining for the macrophage marker CD68 showed that tumors injected with CAFs were more heavily infiltrated by macrophages than were controls

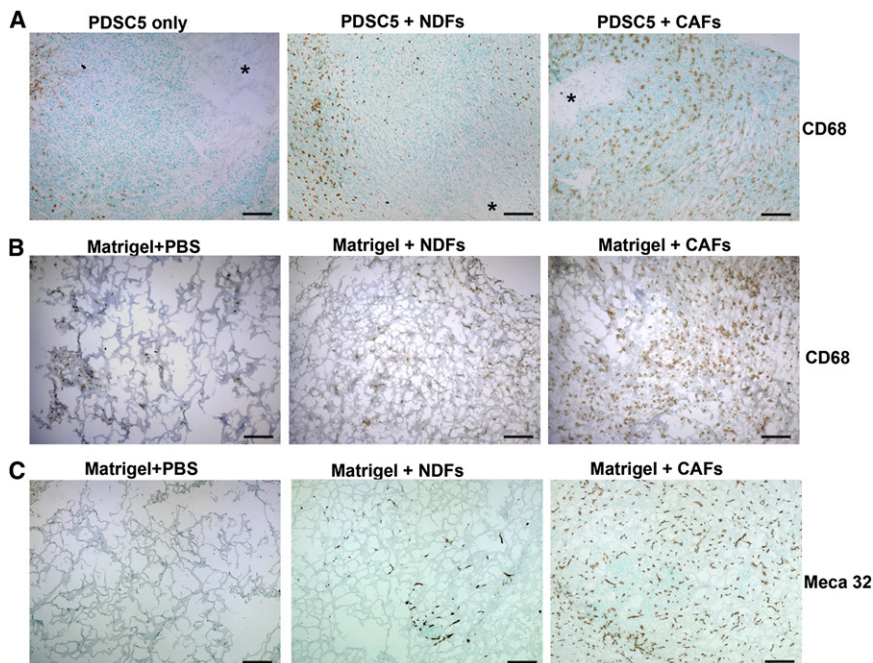


Figure 4. CAFs Recruit Macrophages and Stimulate Angiogenesis

(A) Immunostaining of macrophages in PDSC5 orthotopic tumors with an antibody to CD68. Asterisk indicates necrosis. The panels are representative of multiple fields of tumor sections from seven to nine tumors per group.

(B) Staining of Matrigel plug sections with anti-CD68 to reveal macrophages following inoculation of Matrigel with or without admixed NDFs or CAFs. Results shown are representative of three independent experiments.

(C) Staining of blood vessels with anti-Meca32 antibody in adjacent sections of the same Matrigel plugs admixed or not with NDFs or CAFs. Panels are representative of multiple fields from seven or eight plugs per group. Scale bar, 100 μ M.

Knockdown of IKK β did not have an effect in vitro on CAF survival (data not shown). IKK β -shRNA CAFs were then coinjected orthotopically with PDSC5 cells. Tumors coinjected with shRNA IKK β fibroblasts grew significantly slower than did tumors coinjected with control fibroblasts (Figure 5D). Furthermore, the tumors contained fewer infiltrating macrophages and were significantly less vascularized than controls (Figures 5E and 5F). These findings provide evidence that the inflammation and tumor enhancement mediated by HPV CAFs is NF- κ B dependent.

Education of Normal Fibroblasts by Carcinoma Cells

Intriguingly, admixing normal skin fibroblasts with carcinoma cells resulted in tumor growth that, although slower than that of tumors mixed with CAFs, was significantly greater than that of tumor cells alone. This observation suggested in vivo “education” of NDFs by the tumor cells. To test this hypothesis, primary skin fibroblasts from nontransgenic control mice were incubated with PDSC5-conditioned medium. Indeed, the inflammatory signature was largely induced (Figure 6A). Interestingly, *CYR61* was not induced, suggesting that it is under distinct regulation. Furthermore, FACS-purified fibroblasts from end stage orthotopic tumors coinjected with NDFs expressed proinflammatory genes, even though these fibroblasts were originally negative for the inflammatory gene signature (Figure 6B). These findings provide evidence for the in vivo “education” of dermal fibroblasts by tumor cells, consistent with the ex vivo education of dermal fibroblasts in culture.

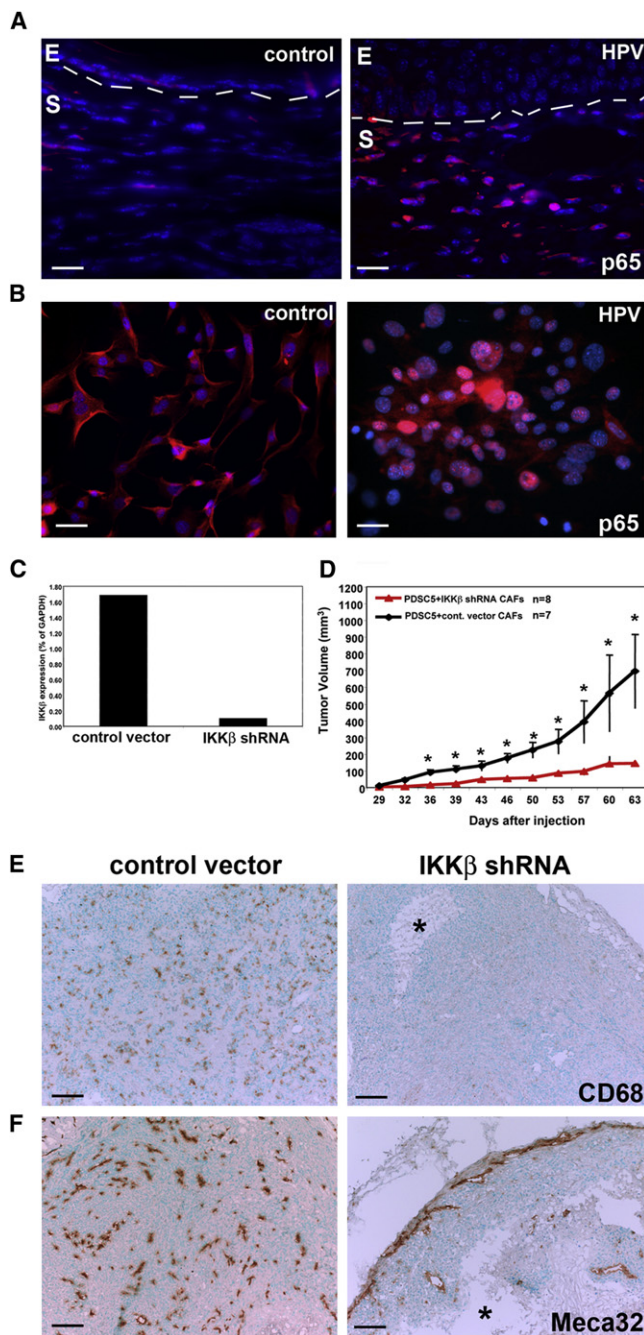
Because the genes in our proinflammatory signature were largely known to be regulated by NF- κ B in other contexts, we next asked whether IL-1 β , a potent inducer of the NF- κ B signaling pathway, could elicit expression of the signature genes in fibroblasts. Indeed, when NDFs were incubated with IL-1 β , there was a strong induction of the signature genes known to be NF- κ B targets (Figure 6C). To ascertain whether IL-1 β could

be inducing expression of the inflammatory signature in NDFs, we assessed the effects of neutralizing IL-1 β on NF- κ B target genes in the inflammatory signature. Indeed, induction of the known NF- κ B target genes in NDFs was inhibited when a neutralizing antibody to IL-1 β was added to PDSC5 conditioned medium, suggesting that education of NDFs by tumor cells is mediated by IL-1 β (Figures 6D–6G).

Proinflammatory CAFs First Appear in Incipient Neoplasia

We next asked whether the proinflammatory gene signature identified in CAFs from dysplastic tissue was already induced in stromal fibroblasts isolated from the earlier hyperplastic stage of tumor progression. Fibroblasts were sorted from hyperplastic skin lesions of 1-month-old HPV mice and were assessed for expression of the proinflammatory gene signature. With the exception of IL-1 β , the proinflammatory gene signature was evident in fibroblasts sorted from these hyperplastic lesions (Figure 7A), suggesting that such activated fibroblasts may play a prominent role in both initiating and driving tumor-promoting inflammation in skin carcinogenesis. Thus, CAFs with a proinflammatory signature are first induced in the earliest incipient neoplasias and persist as neoplastic progression leads to invasive cancer.

Our data implicate IL-1 β as the factor inducing the inflammatory gene signature in NDFs. We therefore investigated possible cellular sources of IL-1 β in the preneoplastic stages. FACS-sorted cell populations from dysplastic skin tissue of 4- or 6-month-old HPV mice were analyzed by real-time PCR, with similar results. We found that fibroblasts, macrophages, and dysplastic epithelial cells all expressed *IL-1 β* mRNA in dysplastic tissue. The expression of *IL-1 β* in dysplastic epithelial cells was further upregulated at 6 months, compared with 4 months (Figure 7B). Interestingly, only CD45⁺ immune cells expressed *IL-1 β* at the earlier hyperplastic stage, suggesting that an immune cell type is inducing the initial expression of the inflammatory signature in NDFs (Figure 7C). Notably, a previous study had implicated signaling by B-lymphocytes in the recruitment of the innate inflammatory cells that stimulate neoplastic



progression (de Visser et al., 2005). Thus, in HPV16/RAG1^{-/-} mice, incipient neoplasias do not progress beyond the hyperplastic stage, as a result of the failure to recruit innate inflammatory cells. To test the hypothesis that adaptive immune cells might be required for activation of NF-κB signaling in the induction of NDF to CAF, we performed immunostaining for p65 in skin tissue from HPV16/RAG1^{-/-} mice, compared with wild-type HPV mice. Indeed, there was no nuclear p65 staining in HPV16/RAG1^{-/-} skin tissue (Figure 7D). Furthermore, when we audited expression of the inflammatory gene signature in HPV16/RAG1^{-/-} fibroblasts, compared with that in HPV CAFs, the NF-κB-regulated subset proved to be very low (Figure 7E). Thus, hyperplasia-associated fibroblasts in HPV16/RAG1^{-/-} mice do not become proinflammatory, which likely contributes to the lack of proangiogenic immune cell recruitment and malignant progression in these mice. To further decipher the mechanism by which fibroblasts become activated, we asked whether Ig deposition by B cells, previously implicated as a regulator of the tumor-promoting inflammatory response (de Visser et al., 2005), is required for CAF activation. To that end, we analyzed the expression of the inflammatory signature in CAFs derived from HPV16/Fcγ^{-/-} mice. These mice exhibit the same phenotype as the HPV16/RAG1^{-/-} mice, in that they do not progress beyond benign hyperplasias (Andreu et al., 2010). Fibroblasts isolated from hyperplastic lesions in HPV16/Fcγ^{-/-} mice proved to express low levels of the NF-κB-regulated subset of the proinflammatory gene signature (Figure 7F), much like in Rag null mice, and in contradistinction to fibroblasts from hyperplasias (and later stages) in wild-type HPV transgenic mice. Because Fcγ is not expressed by skin fibroblasts (data not shown), these results suggest that B cells signal to Fcγ-expressing innate immune cells that in turn activate NDFs to become proinflammatory CAFs. Thus, the NF-κB-dependent proinflammatory program in CAFs is initially induced within incipient neoplasias by resident immune cells that had been stimulated by adaptive immune cells to express IL-1β.

DISCUSSION

In this study, we identify a proinflammatory gene signature responsible for the tumor-promoting behavior of CAFs. We show that CAFs mediate inflammation and angiogenesis by recruiting macrophages and stimulating angiogenesis, thus enhancing tumor growth in squamous cell carcinoma. Fibroblasts from hyperplastic, dysplastic, and neoplastic skin of K14-HPV16 mice express a proinflammatory gene signature that, in significant part, depends on NF-κB signaling. The induction of this inflammatory gene signature in fibroblasts at the earliest discernable stage (hyperplasia) of multistep squamous carcinogenesis suggests that fibroblasts play a crucial role in generating the inflammatory microenvironment that promotes malignant progression in this model. Furthermore, fibroblasts isolated from mouse models of mammary and pancreatic adenocarcinoma and from human skin and pancreatic tumors expressed many of the genes present in the proinflammatory

(E and F) CD68 and Meca32 staining in tumors from (D). Panels are representative of multiple fields of tumor sections from seven tumors. Scale bar, 100 μm.

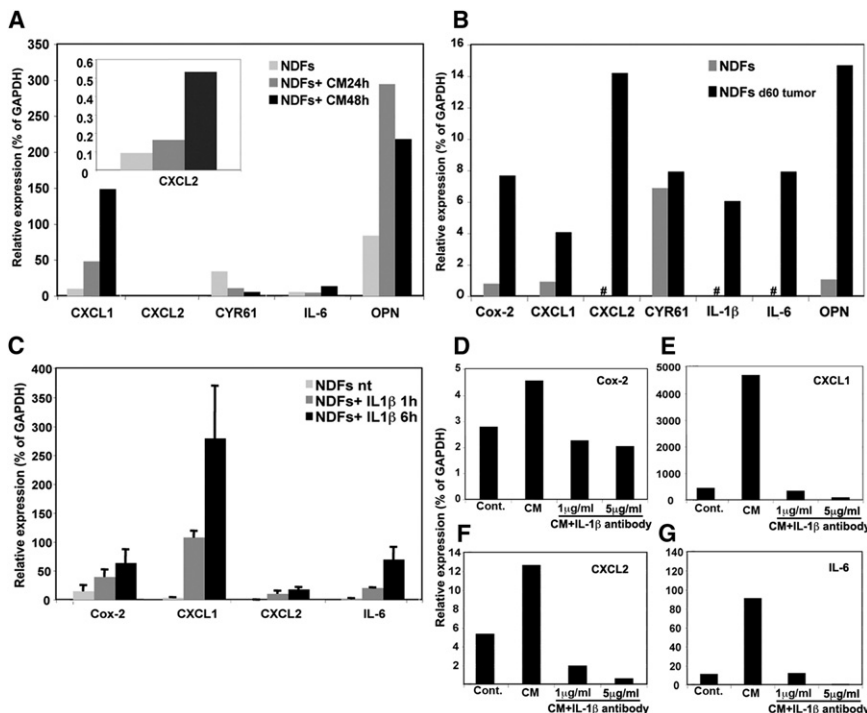


Figure 6. Education of Fibroblasts by Carcinoma Cells

(A) Induction of the inflammatory signature in NDFs incubated overnight with PDSC5-conditioned medium. Control fibroblasts were incubated in SFM. A representative experiment out of three performed is shown.

(B) Gene expression analysis in fibroblasts isolated from end-stage orthotopic tumors injected with NDFs. Fibroblasts were sorted from a pool of three tumors. Results are representative of at least two experiments. Pound sign (#) indicates undetectable/very low expression.

(C) Cultured NDFs were treated with 10 ng/ml IL-1 β for 1 or 6 hr. Data shown are mean \pm SD of two experiments performed in duplicate.

(D–G) NDFs were incubated overnight with PDSC5 CM with or without anti-IL-1 β neutralizing antibody. Control fibroblasts were incubated in SFM. Results are representative of two experiments performed in duplicate.

gene signature, implicating a general role for fibroblast-mediated inflammation in multiple cancers and in human disease. Collectively, our results demonstrate that CAFs can promote tumor growth by orchestrating the recruitment of inflammatory cells and stimulating tumor angiogenesis throughout carcinogenesis, beginning with incipient neoplasias.

Although the involvement of innate and adaptive immune cells in cancer-promoting inflammation is well established (Condeelis and Pollard, 2006; de Visser et al., 2006; Whiteside, 2008), few studies have implicated fibroblasts in this process. CAFs have been shown to promote tumor growth by directly stimulating tumor cell proliferation and by enhancing angiogenesis (Allinen et al., 2004; Bhowmick et al., 2004a; Orimo et al., 2005). Fibroblasts are also key players in wound healing (Hinz et al., 2007; Kisseleva and Brenner, 2008), mediating different aspects of this complex process, including extracellular matrix remodeling and generation of contractile forces. Furthermore, fibroblasts have been implicated in the pathogenesis of chronic inflammatory diseases, such as rheumatoid arthritis and chronic obstructive pulmonary disease (Baglioni et al., 2008; Flavell et al., 2008; Martey et al., 2004). It has also been suggested that fibroblasts mediate the transition from acute to chronic inflammation by inappropriately providing recruitment, survival, and retention signals to infiltrating leukocytes, thus inhibiting the normal resolution of inflammation (Buckley et al., 2001; Smith et al., 1997). Previous studies have reported that fibroblasts are a source of cytokines or chemokines or both in tumors (Gallagher et al., 2005; Mueller et al., 2007; Silzle et al., 2003). These reports, however, involved *in vitro* assays and were potentially susceptible to alterations in gene expression caused by cell culture conditions. Recently, laser capture microdissection revealed CXCL12 and CXCL14 to be upregulated in stroma of prostate and basal cell carcinomas (Augsten et al., 2009; Micke et al.,

2007). Notably, the microdissected stroma also contained immune cells, which were inseparable from the fibroblasts. Extending on these results, our experimental methodology involving

purification by flow cytometry enabled profiling of *in vivo*-derived fibroblasts, unequivocally demonstrating that CAFs functionally orchestrate the proangiogenic inflammatory response during squamous carcinogenesis.

CAF from other organ sites have been shown to directly enhance tumor angiogenesis by either recruiting endothelial progenitor cells via their secretion of SDF-1/CXCL12 (Orimo et al., 2005) or secreting proangiogenic factors (Nyberg et al., 2008; Pietras et al., 2008). Here, we show that skin CAFs enhanced angiogenesis both in orthotopic squamous carcinoma tumors and in an *in vivo* Matrigel plug bioassay lacking cancer cells. The upregulation of the proangiogenic gene *CYR61* in HPV CAFs suggests that angiogenesis may be directly mediated, at least in part, by these CAFs. However, recruited macrophages are demonstrably proangiogenic in the HPV16-driven mouse models of skin and cervical cancers, through their secretion of MMP-9 and its mobilization of VEGF (Coussens et al., 2000; Giraudo et al., 2004). Thus, tumor angiogenesis may be mediated directly by CAFs or indirectly by macrophages recruited by the proinflammatory CAFs.

When other mouse models of cancer were examined, we found that CAFs sorted from cervical tumors did not express the inflammatory signature. This finding could be explained by tissue-specific differences in fibroblast populations. Indeed, fibroblasts derived from different anatomical locations have been shown in genomewide expression studies to be quite heterogeneous (Kalluri and Zeisberg, 2006; Rinn et al., 2006). In contradistinction, CAFs from two other tumor types known to have a strong desmoplastic response, mammary and PDAC tumors, similarly express proinflammatory genes, as did CAFs isolated from human squamous skin carcinoma and human PDAC, suggesting that fibroblast-mediated inflammation may be a common phenomenon, operative in multiple tumor types in human and mouse.

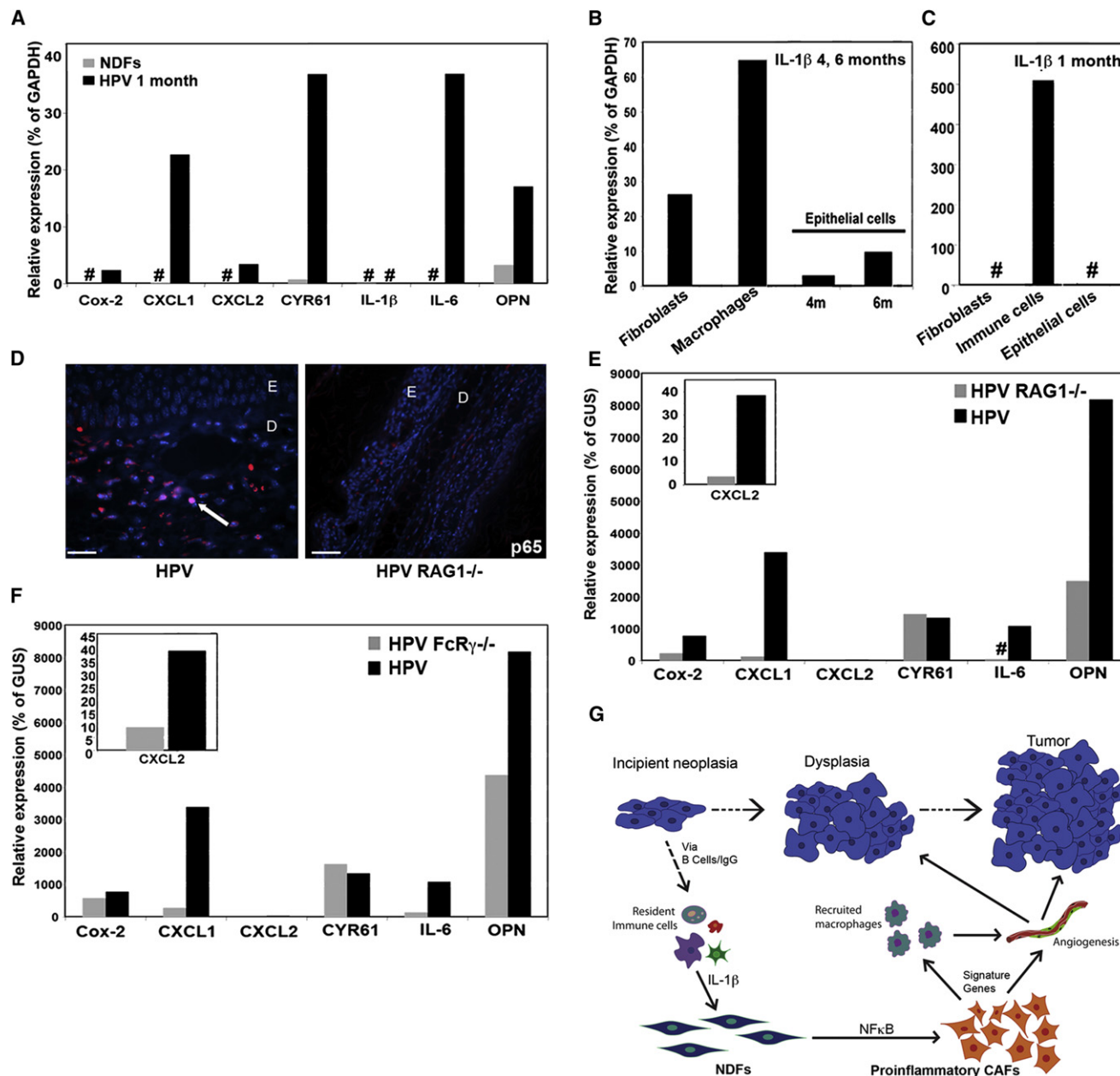


Figure 7. Early Activation of NDFs in HPV, but Not in HPV16/RAG1 $^{-/-}$ or HPV16/FcR $\gamma^{-/-}$ Skin

(A) Fibroblasts were sorted from hyperplastic or normal ear tissue of 1-month-old HPV or control mice, respectively. Fibroblasts were sorted from a pool of tissue from 15 age-matched mice. Results are representative of at least three independent experiments. Pound sign (#) indicates undetectable/very low expression.

(B) Fibroblasts, macrophages (CD68 $^{+}$, F4/80 $^{+}$) and epithelial cells (E-Cadherin $^{+}$) were FACS-purified from 4- or 6-month-old HPV mice. Results shown are from pools of tissue sorted from ten age-matched mice.

(C) Fibroblasts, immune cells (CD45 $^{+}$), and epithelial cells were similarly FACS-purified from skin tissue of 1-month-old HPV mice. Results shown are from pools of tissue sorted from 14 age-matched mice.

(D) Ear tissue sections from 3-month-old HPV mice or from age-matched HPV16/RAG1 $^{-/-}$ mice were immunostained with an anti-p65 antibody (Rhodamine-X) for the NF- κ B subunit p65. Arrow indicates positive nuclei. Nuclei are shown in blue with DAPI. Panels are representative of multiple fields of skin sections from three HPV16/RAG1 $^{-/-}$ mice and four HPV mice. Scale bar, 100 μ m.

(E and F) qRT-PCR for the inflammatory signature in fibroblasts derived from 4-month-old HPV and age-matched HPV16/RAG1 $^{-/-}$ mice (E) or from HPV16/FcR $\gamma^{-/-}$ mice (F).

(G) A model for the role of CAFs in mediating tumor-promoting inflammation. Signaling interactions among cell types in the neoplastic microenvironments of the stages in squamous skin carcinogenesis are shown, illustrating, in particular, the induction of CAFs and their functional role in amplifying the proangiogenic and tumor-promoting inflammatory response.

Importantly, expression of the proinflammatory signature in HPV CAFs is maintained in culture, implying a stable alteration. Indeed, permanent changes in expression patterns have been shown for synovial fibroblasts derived from patients with rheumatoid arthritis, compared with fibroblasts from noninflamed joints (Buckley et al., 2001), and also in stromal fibroblasts and stromal tissue associated with breast cancers (Finak et al., 2008; Hu et al., 2005). We have shown that both induction and maintenance of this proinflammatory signature are NF- κ B dependent. Thus, knock-down of NF- κ B activity in HPV CAFs by expression of an shRNA targeting IKK β abolished the tumor-enhancing effect of HPV fibroblasts in orthotopic skin tumors. NF- κ B is known to play a central role in mediating protumorigenic inflammatory signaling, but its activity has previously been ascribed to macrophages or cancer cells (Bollrath et al., 2009; Grivennikov et al., 2009; Wu et al., 2009). Notably, the NF- κ B target genes COX-2, which is also in the gene signature we identified, and *IL-8* were both shown to be expressed in human myofibroblasts in colorectal carcinoma tissue sections (Adegboyega et al., 2004; Vandroos et al., 2006) and in cultured CAFs from colorectal liver metastasis (Mueller et al., 2007), suggesting that NF- κ B signaling may also be operative in CAFs from colorectal cancer. Our data expand and compliment these observations and present functional evidence that activation of NF- κ B-dependent proinflammatory signaling is critical for CAF-mediated tumor enhancement in vivo.

The in vivo “education” or “reprogramming” of normal fibroblasts by tumor cells demonstrated herein is relevant to the origins of CAFs (Orimo and Weinberg, 2006). Epithelial to mesenchymal transition (EMT) is thought to be one of the sources of CAFs in tumors (Kalluri and Weinberg, 2009). Although we cannot formally exclude the possibility of EMT concomitant with suppression of transgene expression, the possibility seems unlikely, because we show that nontransgenic normal fibroblasts can be educated to become inflammatory CAFs both in vitro and in vivo. Indeed, we found that when normal fibroblasts were co-injected with cancer cells and then isolated by flow cytometry from the resultant orthotopic tumors, fibroblasts expressed the characteristic proinflammatory CAF signature. It was previously shown that coculturing of pancreatic or lung cancer cells with stromal fibroblasts induced the expression of COX-2 and *IL-8* (Anderson et al., 2000; Sato et al., 2004). The concept of in vivo education of normal cells into tumor-promoting stromal cells was previously ascribed to the recruitment of monocyte progenitors and their differentiation into tumor-associated macrophages, which are functionally distinct from resident tissue macrophages (Pollard, 2004). Thus, cancer cells take advantage of the plastic nature of reactive cell populations, such as fibroblasts and macrophages, to generate a tumor-enhancing microenvironment.

Although the cancer cells can demonstrably educate CAFs, our findings suggest that dermal fibroblasts are first educated by immune cells at the initial stages of tumorigenesis. CAFs expressed the proinflammatory gene signature in the earliest detectable incipient neoplasias, the hyperplastic stage in this multistep pathway to squamous cell carcinomas. A variety of stimuli could, in principle, elicit the early, premalignant expression of cytokines and chemokines in fibroblasts. We focused on IL-1 β , a known NF- κ B activator (Ghosh and Karin, 2002),

which is expressed in skin CAFs but not NDFs, suspecting that it might be the inducer of the NF- κ B pathway. We found that IL-1 β is expressed exclusively by immune cells in early hyperplastic lesions, suggesting that activated resident immune cells or sentry inflammatory cells initially induce normal fibroblasts to become proinflammatory CAFs.

Thus, a complex heterotypic signaling network evidently controls the induction, persistence, and dynamic variation of the tumor microenvironment during squamous carcinogenesis (Figure 7G). First, hyperplasia of the HPV16-expressing squamous epithelia elicits B lymphocyte-dependent signaling to innate immune cells to produce IL-1 β , which then activate normal fibroblasts to become CAFs. In turn, CAFs serve to induce or amplify a tumor-promoting inflammatory response by secreting proinflammatory cytokines, thereby recruiting proangiogenic macrophages. The involvement of B cells as initial triggers of fibroblast activation was previously established by the analysis HPV16/RAG1 $^{-/-}$ mice (which lack B cells) and HPV16/FcR $\gamma^{-/-}$ mice (which are defective in B cell signaling via IgG), both of which fail to progress beyond hyperplasias because of the lack of innate immune cell inflammation (de Visser et al., 2005; Andreu et al., 2010). We found that fibroblasts isolated from hyperplasias in HPV16/RAG1 $^{-/-}$ and HPV16/FcR $\gamma^{-/-}$ mice did not express the NF- κ B-regulated subset of the inflammatory gene signature, consistent with the postulate that the impaired neoplastic progression of these mice results from the inability to induce inflammatory signaling in CAFs, which in turn fail to recruit proangiogenic macrophages that are instrumental for tumor progression in this model.

Importantly, the signaling interactions evolve with neoplastic progression. Thus, in contrast to the hyperplastic stage, where only immune cells express IL-1 β , other cell types in advanced dysplasias and squamous carcinomas express IL-1 β in addition to immune cells, including CAFs and epithelial cells. As such, IL-1 β is both an inducer and a member of the CAF signature. Notably, once NDFs are educated to become CAFs, the CAF phenotype becomes meta-stable (evidenced by maintenance in cell culture), likely facilitating malignant progression and expansion. A recent study showed that hyperactivating PI3K signaling in mammary CAFs results in tumor enhancement (Trimboli et al., 2009). Our results suggest that paracrine signaling by IL-1 β could be the physiological instigator of the PI3K-dependent activation of stromal fibroblasts observed in this study, because IL-1 β has been shown in other contexts to activate PI3K (Cahill and Rogers, 2008) as well as NF- κ B. Interestingly, IL-1 β is evidently not only an initial inducer but also a downstream effector of the CAF phenotype: IL-1 β is upregulated in the various mouse and human CAFs we have studied, as well as in the mouse mammary CAFs studied by Trimboli et al. (2009). Thus, both PI3K and NF- κ B may be components of an intracellular regulatory circuit that causes CAFs to become amplifiers of the tumor-promoting inflammatory response.

In summary, we have demonstrated that dermal fibroblasts are activated during early preneoplastic stages of tumorigenesis to express and maintain a proinflammatory gene signature. These proinflammatory CAFs mediate innate immune cell recruitment and augment tumor angiogenesis, thereby enhancing tumor growth, in an NF- κ B-dependent manner. These results further highlight the functional importance of CAFs and suggest

that therapeutic targeting of CAF regulatory or effector circuits (e.g., by inhibiting NF κ B signaling) could be of value in treating certain forms of human cancer.

EXPERIMENTAL PROCEDURES

Transgenic Mice and Human Samples

Generation and characterization of HPV16 and HPV16/RAG1^{-/-} mice have been described elsewhere (Arbeit et al., 1994; Coussens et al., 1996; de Visser et al., 2005). HPV16/FcR γ ^{-/-} mice were provided by Lisa Coussens's laboratory at University of California, San Francisco (UCSF) (Andreu et al., 2010). All mice were maintained within the UCSF Laboratory for Animal Care barrier facility, and all experiments involving animals were approved by the Institutional Animal Care and Use Committee of UCSF. Human tissue was obtained from the Surgery Department and Dermatologic Surgery and Laser Center at UCSF. Written informed consent was obtained from each patient prior to enrollment. The protocol and informed consent document was approved by the UCSF Committee on Human Research (Institutional Review Board).

Orthotopic Tumors

PDSC5 cells (5×10^5) were injected intradermally into FVB/n mice alone or admixed with 1.5×10^6 early passage NDFs or HPV CAFs. To prepare IKK β knockout fibroblasts, IKK β shRNA and control vector were purchased from OpenBiosystems. Virus was prepared by the UCSF Lentiviral Core. HPV CAFs were infected overnight with fresh, unconcentrated virus followed by 48 hr selection with Puromycin. Cells were resuspended in PBS and mixed 1:1 in volume with Growth Factor Reduced Matrigel (BD Biosciences) immediately prior to injection. Tumors were measured every 3–4 days with calipers, and tumor volumes were calculated using the formula $X^2 \times Y \times 0.52$, where X = smaller diameter and Y = larger diameter. Tumor growth was measured until tumors reached a size of 20 mm in any diameter. Mice were then killed, and tumors were embedded in OCT for histological analysis.

Statistical Analysis

Statistical analyses were done by Student's t test. Statistical significance was defined as $p < 0.05$.

ACCESSION NUMBERS

The data discussed in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) and are accessible at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi> (GEO Series accession: GSE17817).

SUPPLEMENTAL INFORMATION

Supplemental information includes four figures, three movies, and Supplemental Experimental Procedures and may be found with this article online at doi:10.1016/j.ccr.2009.12.041.

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