Cancer-associated fibroblasts enact field cancerization by promoting extratumoral oxidative stress

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Histological inspection of visually normal tissue adjacent to neoplastic lesions often reveals multiple foci of cellular abnormalities. This suggests the presence of a regional carcinogenic signal that spreads oncogenic transformation and field cancerization. We observed an abundance of mutagenic reactive oxygen species in the stroma of cryosectioned patient tumor biopsies, indicative of extratumoral oxidative stress. Diffusible hydrogen peroxide (H₂O₂) was elevated in the conditioned medium of cultured skin epithelia at various stages of oncogenic transformation, and H₂O₂ production increased with greater tumor-forming and metastatic capacity of the studied cell lines. Explanted cancer-associated fibroblasts (CAFs) also had higher levels of H₂O₂ secretion compared with normal fibroblasts (FIBs). These results suggest that extracellular H₂O₂ acts as a field effect carcinogen. Indeed, H₂O₂-treated keratinocytes displayed decreased phosphatase and tensin homolog (PTEN) and increased Src activities because of oxidative modification. Furthermore, treating FIBs with CAF-conditioned medium or exogenous H₂O₂ resulted in the acquisition of an oxidative, CAF-like state. In vivo, the proliferative potential and invasiveness of composite tumor xenografts comprising cancerous or non-tumor-forming epithelia with CAFs and FIBs could be attenuated by the presence of catalase. Importantly, we showed that oxidatively transformed FIBs isolated from composite tumor xenografts retained their ability to promote tumor growth and aggressiveness when adoptively transferred into new xenografts. Higher H₂O₂ production by CAFs was contingent on impaired TGFβ signaling leading to the suppression of the antioxidant enzyme glutathione peroxidase 1 (GPX1). Finally, we detected a reduction in Smad3, TAK1 and TGFβRII expression in a cohort of 197 clinical squamous cell carcinoma (SCC) CAFs, suggesting that impaired stromal TGFβ signaling may be a clinical feature of SCC. Our study indicated that CAFs and cancer cells engage redox signaling circuities and mitogenic signaling to reinforce their reciprocal relationship, suggesting that future anticancer approaches should simultaneously target ligand receptor and redox-mediated pathways.

Cell Death and Disease (2017) 8, e2562; doi:10.1038/cddis.2016.492; published online 19 January 2017

Tumor initiation, growth and propagation are frequently accompanied by desmoplasia and the acquisition of a reactive stroma mainly comprising cancer-associated fibroblasts (CAFs).1–3 The dynamic and reciprocal relationship between the epithelial and mesenchymal compartments of the tumor dictates almost every aspect of cancer progression, even governing the efficacy of therapy and influencing the risk of disease relapse.4,5 Importantly, a population of epithelial and stromal cells adjacent to the primary neoplastic lesion acquires early genetic changes but lacks histopathology, indicating that tumors prime the surrounding tissue for malignancy, an effect known as field cancerization.6,7 Field cancerization results in multifocal primary tumors in close proximity with a higher chance of recurrence after surgical resection of malignant tumors.8 Although genetic and epigenetic anomalies have been well described as the driving force behind field cancerization of premalignant epithelia,6 mediators of field cancerization by the stroma remain poorly understood.

The expansion of the cancer field requires soluble factors that promote oncogenic transformation in a non-cell-autonomous manner and prime the adjacent naïve stroma to support tumor growth. Although these functions are usually ascribed to growth factors and cytokines, reactive oxygen species (ROS), which are persistently elevated in almost all cancers, have been recently identified as critical intermediates of cellular signaling.9–11 The oxidative modification of proteins by ROS modulates intracellular signaling to influence cellular behavior and contribute to the pathophysiology of many human diseases.12,13 ROS are by-products of aberrant metabolism in the tumor epithelia, and their cell-autonomous mutagenic effects have been studied extensively. Much less is known about the regulation of ROS in CAFs and how ROS impact field cancerization. Notably, not all ROS are well suited for paracrine cell signaling. Superoxide anions (O₂−) and hydroxyl radicals (OH•) have a very short diffusion distance because they are highly reactive, resulting in a very short half-life in the aqueous phase.14 They also have a very low permeability coefficient for lipid bilayers.15 In contrast,
hydrogen peroxide (H$_2$O$_2$) has a longer half-life, is lipid soluble and can diffuse across the cellular plasma membrane, making it an ideal field effect carcinogen. In this study, we investigated the regulation of ROS, and in particular, H$_2$O$_2$ production, in CAFs and how this tumor stroma-mediated oxidative stress causes a premalignant field defect.

Results

Elevated ROS in the microenvironment of numerous tumor types. We first examined the oxidative status of tumor sections from patients with late stage (stage 3 to metastatic) skin squamous cell carcinoma (SCC), gastric, breast and colon cancers using the ROS-sensitive fluorescent dye CM-H$_2$DCFDA. Pan-cytokeratin staining distinguished between tumor epithelia and stromal elements. All tumor sections stained positive for ROS (Figure 1a). SCC sections exhibited the highest intensity of ROS staining. Interestingly, ROS were not restricted to the tumor epithelia but were frequently observed in the stromal compartments of tumors. To identify the cell types responsible for elevated microenvironmental ROS, we measured extracellular H$_2$O$_2$ in conditioned medium from various SCC lines of different aggressiveness (Figure 1b). HaCaT is a non-tumorigenic human keratinocyte cell line, whereas benign A-5, malignant II-4 and highly aggressive A-5RT3 are sublines of different tumorigenic potential derived from HaCaT cells. HaCaT cells secreted the lowest amount of H$_2$O$_2$, whereas conditioned medium from A-5RT3 cells had the highest level of H$_2$O$_2$ (Figure 1b). As CAFs dominate the tumor stroma and diffusible H$_2$O$_2$ is well suited for a role in tumor stroma crosstalk, we measured the amount of H$_2$O$_2$ in conditioned medium from CAFs explanted from four patient skin SCC biopsies alongside their paired normal fibroblasts (FIBs) (Figure 1c). We consistently detected higher extracellular H$_2$O$_2$ in the conditioned medium from SCC CAFs compared with FIBs.

Microenvironment oxidative stress causes a premalignant field defect. Next, we determined whether microenvironmental oxidative stress promotes premalignant development, that is, field cancerization. As the tumor promotion protocol involves a mutagen, N-methyl-N’-nitro-N-nitrosoguanidine (MNNG), we first determined the cytotoxic dose of MNNG in combination with various concentrations of H$_2$O$_2$. There were no significant differences in the percentages of apoptotic (Annexin V$_{\text{high}}$/PI$_{\text{low}}$ and Annexin V$_{\text{high}}$/PI$_{\text{high}}$) and

![Figure 1](image)

Figure 1  Elevated ROS in the microenvironment of numerous tumor types. (a) Dichlorofluorescein diacetate (DCF) staining to detect ROS on sections of patient tumor biopsies from the skin, gastric, breast and colon. Pan-cytokeratin (CK) antibody staining was used to delineate epithelial-stromal boundaries. Sections were counterstained with DAPI. Scale bar: 100 μm. (b and c) Extracellular concentrations of H$_2$O$_2$ in the conditioned medium from b, various human SCC lines, and (c) paired FIBs and CAFs from human SCC explants, as measured by Amplex Red H$_2$O$_2$ assay. *P<0.05, **P<0.01
Figure 2. H$_2$O$_2$ has a transforming effect on epithelia and fibroblasts in vitro. (a) Quantification of epithelial colonies in soft agar at 1 month after the indicated treatments with MNNG and H$_2$O$_2$. (b) Percentages of proliferating (S-phase) cells after the indicated treatments with H$_2$O$_2$ as determined by BrdU labeling and FACS analysis. (c) Relative fluorescence (SYTO90 dye) readings of invaded cells after the indicated treatments in the transwell invasion assay. (d) PEG-switch assay followed by immunoblot analysis of oxidatively modified PTEN and Src in keratinocyte lysates subjected to the indicated treatments. Bars represent the percentage of protein targets that have undergone oxidative modification. (e-g) Immunoblot analysis of key signaling mediators of the PI3K and ERK pathways in keratinocytes subjected to the indicated treatments. Bars represent normalized densitometry measurements of phosphorylated protein targets normalized against the cognate unphosphorylated forms. (h–k) Median fluorescence intensities of human fibroblasts subjected to the indicated treatments, followed by staining with DCF and PE-conjugated anti-FAP antibody and FACS analysis. (l) Measurements of GSH/GSSG ratio in CAF and FIBs subjected to the indicated treatments. A low GSH/GSSG ratio is an indicator of oxidative stress in cells. The data are presented as the mean ± S.D. of three independent experiments. Statistical tests were performed against cognate untreated cells for each cell type. NS, not significant, *P < 0.05, **P < 0.01, ***P < 0.001.
necrotic (Annexin Vlow/PIhigh) keratinocytes treated with 1–100 nM H2O2 alone or in combination with 10 ng/ml MNNG (Supplementary Tables S2 and S3). Thus, this range of H2O2 and MNNG was used to examine the effects of H2O2 on colony formation in soft agar. A marked increase in colony number was observed with cells pretreated with MNNG and exposed to H2O2 for 4 weeks (Figure 2a). Keratinocytes treated with a higher concentration of H2O2 alone formed
colonies in soft agar, suggesting that chronic exposure to elevated extracellular H$_2$O$_2$ facilitates the transformation of epithelial cells. It is conceivable that normal and neoplastic epithelial cells respond differently to H$_2$O$_2$. Indeed, we observed differential growth response and invasiveness to exogenous H$_2$O$_2$. The proliferation of HaCaT and A-5RT3 cells peaked at 10 nM H$_2$O$_2$, whereas the proliferation of A-5 and II-4 cells peaked at 100 nM H$_2$O$_2$. The proliferation of HaCaT cells began to decrease as the concentration of H$_2$O$_2$ increased to 10 μM. In contrast, the growth of the three tumorigenic sublines remained significantly elevated compared with untreated cognate controls (Figure 2b). Exogenous H$_2$O$_2$ also increased the invasiveness of the malignant II-4 and A-5RT3 cells, whereas that of normal HaCaT and benign A-5 cells was reduced as determined by a transwell invasion assay (Figure 2c). The differential growth response and invasiveness was not due to H$_2$O$_2$-induced apoptosis, as apoptosis of the cell lines was only observed at H$_2$O$_2$ concentrations >100-fold higher than those used for the proliferation and invasion assays as determined by Annexin V/PI FACS analysis. (Supplementary Figure S1A). These observations indicate that sub-cytotoxic levels of H$_2$O$_2$ differentially modulate the behavior of normal and neoplastic epithelial cells.

Next, we performed a polyethylene glycol (PEG)-switch assay on H$_2$O$_2$- and MNNG-treated keratinocytes to identify oxidatively modified proteins. As an oxidant, H$_2$O$_2$ oxidizes cysteine residues in proteins and modifies their activities. The PEG-switch assay results in the conjugation of PEG to the oxidized cysteine residue, thereby increasing the molecular weight of the oxidized protein and slowing its migration within an SDS-PAGE gel. Proteins bordering the cell membrane are particularly susceptible to modification by extracellular H$_2$O$_2$. Phosphatase and tensin homolog (PTEN) and Src are well-established mediators of tumorigenesis that are membrane associated. The oxidation of PTEN forms a disulfide bridge that inactivates its tumor-suppressive catalytic activity. Src oxidation increases its autophosphorylation and concomitant activation. Indeed, an elevated level of oxidized PTEN and Src in keratinocytes treated with H$_2$O$_2$ and MNNG was detected by PEG-switch assay, further confirmed by Oxyblot (Figure 2d, Supplementary Figures S1B and C). MNNG alone only contributed to ≤ 4% of oxidized PTEN and Src, whereas H$_2$O$_2$ contributed to ~20% oxidized PTEN and ~10% oxidized Src (Figure 2d). Notably, oxidized JNK and EGFR were also detected by PEG-switch, reflecting the promiscuous nature of H$_2$O$_2$ interactions (Supplementary Figure S1D). PTEN and Src participate in the activation of the PI3K/PKBα and MAPK/ERK signaling pathways, which have pivotal roles in tumor development and progression. Immunoblot analysis confirmed increased activation of downstream mediators of the PI3K/PKBα and MAPK/ERK pathways (Figures 2e-g, Supplementary Figure S1E). A focused qPCR array comparing H$_2$O$_2$-treated epithelia and those cocultured with CAFs revealed similar altered expression profiles of many pro-oncogenic and pro-tumor genes. Similarly, the expression of tumor suppressors and growth inhibitory mediators was reduced (Supplementary Figure S1F).

In addition to epithelial cells, H$_2$O$_2$ may promote field cancerization by affecting adjacent FIBs. We first investigated whether CAF-conditioned medium (CCM) alters the behavior of FIBs. FIBs cultured in CCM had increased ROS levels and elevated expression of fibroblast-activated protein (FAP), a biomarker of CAFs (Figures 2h-i, Supplementary Figures S2A and B). This effect can be attributed, partially, to extracellular ROS because H$_2$O$_2$ was detected in CCM (Supplementary Figure S2C), and the increase in CAF marker expression was attenuated when CCM was pretreated with the antioxidant NAC. Similar experiments were also performed using H$_2$O$_2$ alone. Normal FIBs exposed to exogenous H$_2$O$_2$ also displayed elevated ROS and increased FAP levels after 1 day, and αSMA expression was increased after 3 days (Figures 2j-k, Supplementary Figure S2D-F). Consistently, analysis of the GSH:GSSG ratio revealed that CAFs and H$_2$O$_2$- or CCM-treated FIBs experienced oxidative stress (reflected by a low GSH:GSSG ratio) compared with non-treated FIBs. NAC increased the GSH:GSSG ratio of CCM-treated FIBs (Figure 2l). These collective observations suggest that extratumoral H$_2$O$_2$ is a transformative agent in stromal field cancerization, stimulating genetic and phenotypic changes in the epithelial cells and normal stromal fibroblasts to generate a field effect.

Oxidative transformation of FIBs to CAF-like cells was attenuated by catalase in vivo. To determine whether extratumoral H$_2$O$_2$ generates a field effect in vivo, we generated an admixed tumor xenograft containing three different cell types, namely, epithelial cells, CAFs and VybrantDiO-labeled FIBs, in the presence or absence of 500 U/ml extracellular catalase (Figure 3a). Cancerous A-5RT3 cells with CAFs:FIBs formed large tumors, whereas non-tumorigenic HaCaT cells with CAFs:FIBs formed small palpable neoplasms (Figure 3b). Notably, tumor growth was attenuated by catalase in the microenvironment (Figure 3b). Consistent with the in vitro observations, the tumor xenografts showed reduced activation of key mediators of the

![Figure 3](http://example.com/figure3.png)
PI3K/PKBα and ERK pathways when catalase was present (Figure 3c, Supplementary Figure S3A). The number of Ki67-positive proliferating epithelial cells was also reduced in the presence of catalase compared with the saline control (Figure 3d). Depletion of microenvironmental H2O2 by catalase partially restored basement membrane integrity between the epithelial layer and the adjacent stroma, as shown by a clear laminin 332 staining pattern, suggesting a reduced risk of malignancy (Figure 3d). To determine whether extratumoral H2O2 transforms normal FIBs into a CAF-like phenotype in vivo, we examined intracellular ROS levels and FAP expression in VybrantDIO-labeled FIBs isolated from tumor xenografts by FACS analysis (Supplementary Figure S3B). We observed that 10% of the cells were ROShigh/FAPhighFIBs, herein referred to as transformed FIBs (tFIBs), compared with only 3% tFIBs in the presence of catalase (Figure 3e). Next, we asked whether the tFIBs had tumor-promoting capabilities compared with untransformed FIBs (ROS Orn/FAP OrnFIBs). To this end, we performed an adoptive transfer of tFIBs and untransformed FIBs into new tumor xenografts (Figures 3f and g). We observed rapid xenograft growth when cancerous A-5RT3 cells were co-injected with tFIBs compared with FIBs. Non-tumorigenic HaCaT cells also grew at an accelerated rate when co-injected with tFIBs compared with FIBs (Figure 3f). Immunofluorescence staining further confirmed a more proliferative and aggressive epithelial cell phenotype in the presence of tFIBs (Figure 3g). Moreover, adoptively transferred tumors comprising A-5RT3 cells and tFIBs exhibited slower growth and less invasion in the presence of extratumoral catalase compared with controls (Supplementary Figure S3C). Taken together, our in vitro and in vivo observations suggest that ROS, specifically H2O2, induce an oxidative CAF-like state in FIBs for stromal-mediated field carcinogenesis.

Impaired TGFβ signaling reduces GPX1 expression in fibroblasts to cause a defect in ROS detoxification. Next, we investigated how extratumoral H2O2 activated FIBs to participate in field carcinogenesis. TGFβ is a potent mediator of FIB activation, which signals through the canonical Smad and non-canonical TGFβ-activated kinase-1 (TAK1) pathways. We showed that exogenous H2O2 had similar activating effects on FIBs, such as elevated ROS levels, increased FAP and αSMA expression and reduced GSH:GSSG ratio (Figures 2j-l, Supplementary Figure S2F). Next, we probed the effect of H2O2 on fibroblast TGFβ signaling. A luciferase assay for TGFβ response revealed that CAFs have impaired TGFβ signaling, and FIBs became desensitized to TGFβ after 2 and 3 days of H2O2 treatment (Figure 4a). Correspondingly, Smad3 and TGFβRII mRNA expression in FIBs decreased after 2 and 3 days of H2O2 treatment, and TAK1 mRNA expression decreased after 3 days of H2O2 treatment (Figure 4b). FIBs treated with CCM showed similar impairment of TGFβ signaling, which was rescued by treatment with NAC (Figures 4a and b).

Oxidative stress also triggers NFκB activity in fibroblasts, leading to a CAF-like phenotype.21,22 Indeed, FIBs treated with exogenous H2O2 displayed a significant increase in p65-NFκB phosphorylation after 2 and 3 days of H2O2 treatment (Supplementary Figure S3D). siRNA silencing of p65-NFκB expression in FIBs partially abrogated the H2O2-mediated suppression of Smad3, TAK1 and TGFβ mRNA and protein expression (Supplementary Figures S3E-G). These findings suggest that FIBs exposed to H2O2 initially remain responsive to TGFβ and this facilitates their activation and acquisition of FAP expression at an early time point, after just 1 day of H2O2 treatment. Simultaneously, H2O2 triggers NFκB activation that negatively regulates TGFβ signaling while potentiating FIB activation. NFκB activation is elevated after 2 days of H2O2 exposure, thus NFκB maintains FAP expression (and therefore, the CAF-like state) in H2O2-treated FIBs at later time points when TGFβ signaling becomes impaired.

To determine whether FIBs with impaired TGFβ signaling promote the growth of adjacent epithelia, we performed siRNA knockdown of Smad3 (FSmad3), TAK1 (FTAK1) and TGFβRII (FTGFβRII) in FIBs and measured mitogenic factor secretion and ROS levels. The efficiency of siRNA knockdown was validated by qPCR and immunoblotting (Figures 4c and d). Compared with F ctrl, no differences in proliferation or apoptosis of knockdown fibroblasts were observed by FACS analysis (Supplementary Figure S4A). We examined the secreted growth factor protein profile in CCM from these knockdown fibroblasts by ELISA. Out of a total of 76 distinct proteins screened, the expression levels of 7 secreted factors were elevated (Supplementary Figure S4B). A comparative analysis revealed that these factors were differentially modulated by Smad3 and TAK1 in FIBs (Supplementary Figure S4B). To further strengthen our observation, focused real-time PCR arrays comparing epithelia cocultured with CAFs and the various knockdown FIBs were performed. Notably, the expression profiles of many pro-oncogenic or tumor-associated genes in the epithelia cocultured with CAFs were also observed in the epithelia cocultured with F Smad3, F TAK1, F TGFβRII or treated with H2O2 (Supplementary Figure S1F).

Importantly, immunoblot analysis of the epithelial layers of organotypic cocultures of keratinocytes with various knockdown fibroblasts showed the activation of the PI3K/PKB and ERK pathways associated with elevated epithelial proliferation, as indicated by PCNA (Supplementary Figure S4C). Next, we measured ROS levels in knockdown fibroblasts. Compared with F ctrl, ROS levels were elevated in F Smad3 (8.9-fold), F TAK1 (8.4-fold) and F TGFβRII (8.2-fold) (Figures 4e and f). We also detected a significant increase in extracellular H2O2 from the knockdown fibroblasts compared with F ctrl. The concentration of extracellular H2O2 was derived using a calibration curve created using known H2O2 concentrations (Figure 4g). F TGFβRII exhibited the highest amount of extracellular H2O2 (Figure 4g).

To investigate how impaired TGFβ signaling increases H2O2 levels in FIBs, we examined the expression of key enzymes affecting H2O2 detoxification in FIBs, namely, catalase, superoxide dismutase (SOD) and glutathione peroxidase (Gpx), using real-time PCR. No significant changes were observed in the mRNA levels of SOD1, SOD2 or catalase (Figure 4h). Interestingly, the mRNA and protein expression levels of Gpx1 were reduced in F Smad3, F TAK1 and F TGFβRII compared with F ctrl. H2O2 treatment of FIBs also resulted in an initial spike followed by a gradual loss of Gpx1 expression, corresponding to impaired TGFβ signaling (Figure 4b). Consistent with the qPCR data, Gpx protein expression and activity were reduced in CAFs.
and fibroblasts with impaired TGF\(\beta\) signaling, suggesting that the GPx1 gene is regulated by Smad3- and TAK1-mediated pathways (Figures 4h and i). These observations indicate that epithelial cells are bathed in an oxidative microenvironment supplemented with mitogenic factors secreted by stromal fibroblasts with impaired TGF\(\beta\) signaling.

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**Figure 4** Impaired TGF\(\beta\) signaling reduces GPX1 expression in FIBs to cause a defect in ROS detoxification. (a) Relative TGF\(\beta\)-responsive reporter (luciferase) readings of FIBs and CAFs subjected to the indicated treatments followed by assay for TGF\(\beta\) response. (b and c) Relative Smad3, TAK1, TGF\(\beta\)RII and GPX1 mRNA levels in FIBs and CAFs after the indicated treatments. The ribosomal RNA gene Rpl27 served as the housekeeping gene. (d) Immunoblot for Smad3, TAK1 and TGF\(\beta\)RII protein expression in control (scrambled) FIBs and siRNA knockdown FIBs. \(\beta\)-Tubulin from the same samples was used as a loading and transfer control. (e and f) Levels of ROS in FIBs with siRNA knockdown of Smad3, TAK1 and TGF\(\beta\)RII as determined by DCF staining and FACS. (e) The values in brackets indicate the mean fluorescence signal intensities. (f) Median DCF fluorescence intensity. (g) Extracellular H\(2\)O\(2\) in conditioned medium from various knockdown FIBs as measured by Amplex Red assay. The figure insert shows the calibration curve for determining H\(2\)O\(2\) concentration. (h) Relative SOD1, SOD2, CAT and GPx1 mRNA levels in knockdown FIBs. Rpl27 served as the housekeeping gene. Immunoblot of the GPx1 protein from the indicated cell lysates. \(\beta\)-Tubulin from the same samples was used as a loading and transfer control. (i) GPx1 activity measurements for the indicated cells normalized against total protein in the cell lysates. The data are presented as the mean \(\pm\) S.D. of three independent experiments. *\(P<0.05, **P<0.01, ***P<0.001\)
Regulation of Gpx1 by cJUN and Smad3 in CAFs from SCCs. In silico analysis of the human Gpx1 proximal promoter revealed two putative AP1-binding sites and one SMAD3-binding site (Figure 5a). TAK1 is also a strong activator of cJun N-terminal kinase, which consequently activates cJUN. To determine whether Gpx1 is a target gene of cJUN and Smad3, chromatin immunoprecipitation (ChIP) experiments for cJUN and Smad3 followed by re-ChIP for p300, a cJUN and Smad3 coactivator, were performed. No binding of phospho-cJUN was detected at AP1(−12), indicating that this binding site is non-functional (Supplementary Figure S5A). There was amplification of the
AP1(-262)-binding site in the human Gpx1 promoter after ChIP for phospho-cJUN (Supplementary Figure S5A) and re-ChIP for p300 in Fctrl and FSmad3, but not in FTAK1 and FTGFβRII (Figure 5a). There was also amplification of the Smad3 (-172)-binding site after ChIP for phospho-Smad3 (Supplementary Figure S5A) and re-ChIP for p300 in Fctrl and FTAK1, but not in FSmad3 and FTGFβRII (Figure 5a). No binding was detected with preimmune serum, and there was no binding to a control sequence ∼2-kb upstream of the transcriptional start site. These observations indicate that the expression of the human Gpx1 gene in fibroblasts is regulated by canonical and non-canonical TGFβ-mediated signaling.

To underscore the clinical relevance of TGFβ signaling in stromal fibroblasts, we examined the mRNA expression of Smad3, TAK1 and TGFβRII in laser-microdissected CAFs from human SCC and adjacent peri-normal fibroblasts (NFs) from 197 patients. During microdissection, we collected the stroma (vimentin-positive) immediately associated with tumor epithelial cells rather than stromal tissue distal to the tumor, and excluded lymphocytes and obvious vascular structures. We observed reduced expression of Smad3, TAK1 and TGFβRII, alone or in combination, in CAFs compared with NFs (Figure 5b). Immunoblot analysis of these TGFβ signaling mediators in CAFs and cognate NFs isolated from five resected SCC samples selected at random from the available patient sample pool showed corresponding changes (Figure 5c). ChIP and re-ChIP experiments were also performed using NFs and CAFs (patient #5; reduced TGFβRII expression). Consistent with our earlier observations, the Smad3(-172) and AP1(-262)-binding sites of the human Gpx1 promoter were occupied by Smad3:p300 and cJUN:p300 in NFs, whereas their occupancies were significantly reduced in CAFs (Figure 5d, Supplementary Figure S4B). These results collectively indicate that impaired TGFβ signaling is a clinical feature of CAFs and is responsible for their loss of Gpx1 expression and activity, which consequently leads to micro-environmental oxidative stress and field cancerization.

Discussion

CAFs are one of the most abundant stromal cells in the tumor microenvironment, facilitating the development, propagation, and invasiveness of tumors.25 Our study showed that CAF-derived ROS, specifically H2O2, induced oxidative stress in normal FIBs and trigger de novo CAF transformation in vitro and in vivo. Adoptive transfer of oxidatively IFIBs into malignant and non-tumorigenic epithelia xenografts resulted in faster-growing and more aggressive tumors, indicating exacerbated oncogenic transformation. This self-amplifying, field cancerization effect was due to impaired TGFβ signaling, leading to deficient Gpx1 expression and activity in CAFs. We showed that the human Gpx1 gene was regulated by TGFβ1-mediated Smad3 and TAK1/cJUN pathways in fibroblasts. Reduced expression of Smad3, TAK1 or TGFβRII was also detected in CAFs from a cohort of 197 SCC patients.

 Fibroblast-specific deletion of TGFβRII in mice led to spontaneous tumors in the prostate and foreestomach, which was partially attributed to the activation of paracrine HGF signaling.26,27 However, TGFβ is required for fibroblast activation and transition to a CAF-like state. To reconcile these paradoxical observations, we noted that TGFβ signaling operates in a negative feedback loop mediated by the inhibitory Smad, Smad7,28,29 which is upregulated by TGFβ via the pro-inflammatory transcription factor NFκB.30,31 Oxidative stress is also strongly associated with NFκB activation.32,33 Hence, FIBs exposed to H2O2 are initially responsive to TGFβ, which facilitates their activation by tumor epithelia- and CAF-derived TGFβ. Simultaneously, H2O2 triggers NFκB activation to suppress TGFβ signaling and Gpx1 expression while potentiating FIB activation. In mature tumors, TGFβ is abundant and the tumor microenvironment is chronically subjected to low-grade inflammation and redox imbalance. These combined characteristics synergistically create an ecosystem that encourages the conversion of FIBs to CAFs, then renders CAFs refractory to TGFβ signaling and highly oxidative because of Gpx1 deficiency. The expression of Gpx1 is also regulated by p53,34 and the ablation or mutation of p53 in stromal fibroblasts increased growth and metastatic spread of PC3 prostate cancer cells because of increased production of SDF-1.35 Although the redox status of p53-deficient fibroblasts is unknown, it is conceivable that elevated H2O2 may contribute to their pro-tumorigenic effect. Together with our own observation that silencing NFκB expression could prevent the H2O2-mediated impairment of TGFβ signaling in fibroblasts, these findings suggest that transcriptional control has a critical role in modulating the pro-tumorigenic effects of CAFs.

Recent studies have revealed an important relationship between the extracellular redox state and cancer aggressiveness.9 The activation of oncogenes in epithelial cells results in the loss of caveolin-1 and the elevated expression of lactate transporters in stromal fibroblasts. These CAF-like fibroblasts exhibited increased ROS levels and elevated glucose uptake, which complement cancer cell metabolism.26,36 We showed that extratumoral H2O2, either
from epithelial tumor cells or CAFs, reinforced the already dysregulated epithelial–mesenchymal communication to promote field cancerization. Indeed, the prolonged exposure of primary human keratinocytes to low levels of \( \text{H}_2\text{O}_2 \) elevated the levels of oxidatively modified PTEN and Src, which triggered oncogenic signaling to promote epithelial transformation and growth; oxidation of the tumor suppressor PTEN and the oncogene Src results in protein inactivation and activation, respectively. \( \text{H}_2\text{O}_2 \) can also differentially influence the proliferation and invasiveness of cancer cells at various stages of progression. Lending support to a pro-mitogenic role of \( \text{H}_2\text{O}_2 \), \( \text{H}_2\text{O}_2 \) antagonized the cytostatic functions of TGF\( \beta \)1 by the activation of Akt-ERK1/2-linked signaling.\(^{37} \) Although the precise target of \( \text{H}_2\text{O}_2 \) was not identified, many studies have shown that Akt-ERK1/2 are downstream mediators of Src and PTEN.\(^{34,36} \) Similarly, the migration of MCF-7 breast cancer cells was enhanced by elevated Nox4 expression in a human mammary fibroblast cell line.\(^{36} \) Nox4 is known to predominantly produce \( \text{H}_2\text{O}_2 \) rather than \( \text{O}_2^- \).\(^{39} \)

Finally, CAFs exert their pro-epithelial activity by secreting mitogenic factors. Many of these factors are known to be druggable targets, and it has been proposed that the inhibition of these ligand/receptor-activated signaling networks could limit neoplastic growth. We detected an increase in pro-mitogenic factors such as HGF and FGF7 in the conditioned media harvested from keratinocytes cocultured with fibroblasts with impaired TGF\( \beta \) signaling. These elevated mitogenic factors, together with extracellular \( \text{H}_2\text{O}_2 \) promoted cancer cell proliferation, as supported by the activation of pro-oncogenic PKB\( \alpha \), ERK, and JNK pathways in the epithelia. Our findings that the oxidative action of \( \text{H}_2\text{O}_2 \) also enables CAF-mediated field cancerization suggest that anticancer strategies that singularly target the ligand-receptor pathways may not be effective. For example, a neutralizing antibody to HGF exhibited potent antitumor effect in the treatment of glioblastoma multiforme.\(^{40} \) However, tumor growth became HGF-independent after repeated treatments.\(^{41} \) Conceivably, the promiscuous nature of \( \text{H}_2\text{O}_2 \) interactions with cellular signaling components, especially membrane-associated molecules such as EGFR and Met, renders the cells less dependent on ligand-activated pathways to promote transformation and growth. Indeed, initial efforts focused on the inhibition of PDGF with a single agent, imatinib, generated poor outcomes.\(^{42,43} \) In contrast, combination treatment with hydroxyurea and imatinib has provided very encouraging findings.\(^{44,45} \) Interestingly, hydroxyurea is a potent antioxidant that has been shown to increase the expression of Gpx1 in a p53-dependent manner, among other effects.\(^{46} \) Future exploration of new multi-modal approaches that simultaneously target ligand-receptor signaling and redox-mediated pathways will offer new promising anticancer strategies.

**Materials and Methods**

**Human tumor samples.** Human SCC biopsies along with their paired normal tissues were provided by the National Skin Centre, Singapore. CAFs and FIBs were isolated by laser capture microdissection and subjected to protein and RNA extraction for immunoblotting and real-time PCR analyses, respectively.\(^{47,48} \) The study was approved by the National Healthcare Group Domain-Specific Review Boards (NHG-DSRB). All tumor samples were de-identified before the analyses.

### In vitro tumor colony promotion protocol.

Keratinocytes were seeded at a density of \( 1 \times 10^5 \) per 35-mm dish containing complete OTC medium (Denova Sciences, Singapore) at day 0. On day 1, cultures were treated for 1 h with the initiating agent, MNNG, or solvent control (DMSO). The cells were washed, and the medium was replaced with complete medium for 24 h before exposure to different concentrations of \( \text{H}_2\text{O}_2 \). After 1 week, cells were harvested from individual dishes and plated at a density of \( 1 \times 10^7 \) per 35-mm dish in \( \text{H}_2\text{O}_2 \)-containing medium. Concurrently, \( 2 \times 10^5 \) harvested cells were embedded in 0.35% noble agar (Sigma Aldrich, St. Louis, MO, USA) and layered onto 0.7% noble agar-coated 35-mm dishes during each passage of the cells. These soft agar cultures were consistently replenished with fresh \( \text{H}_2\text{O}_2 \)-containing medium every other day. After 1 month, viable transformed colonies were stained with 1 mg/ml thiazolyl blue tetrazolium in PBS and counted.

### Cell proliferation, apoptosis assays and FACS analysis.

Bromodeoxyuridine (BrDU) incorporation and total DNA content in proliferating cells were analyzed by BrdU and PI staining using the BrdU Flow kit (BD Biosciences, San Jose, CA, USA). Epithelial cells were treated for 24 h with the indicated concentrations of \( \text{H}_2\text{O}_2 \) in serum-free medium. The cells were incubated with BrdU (40 \( \mu \)M) for 30 min. Apoptotic and necrotic cells were quantified using an Annexin V/PI staining kit (BioLegend, San Diego, CA, USA) and subsequent FACS analysis.

### PEG-switch assay and OxyBlot protein oxidation detection.

PEG-switch assay was performed as previously described.\(^{49} \) Cells treated with 100 nM \( \text{H}_2\text{O}_2 \) or 10 ng/ml MNNG for 24 h were lysed using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA). Oxidized thiol levels were reduced using DTT and alkylated using 2 mM PEG-maleimide with 0.5% SDS for 2 h at room temperature. The alkylation reaction was quenched using 100 mM Tris–HCl buffer pH 6.8, 4% SDS, 20% glycerol, and 0.01% bromophenol blue and 100 mM maleimide and the samples were resolved by SDS-PAGE and subjected to immunoblot. Oxidatively modified PTEN was analyzed as previously described with some modifications.\(^{50} \) Cells treated with 100 nM \( \text{H}_2\text{O}_2 \) for 30 min were washed with oxygen-free ice-cold PBS containing 1 mM N-ethylmaleimide (NEM). Cells were lysed in buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 30 mM NEM). Lysates were further clarified using a QiAShredder (Qiagen, Valencia, CA, USA). Equal amounts of proteins were mixed with NEM-loading dye (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 20 mM NEM and 0.02% bromophenol blue) and resolved by non-reducing SDS-PAGE. As a control, proteins were completely reduced by heating in SDS-loading dye containing DTT. Oxidized Src was detected as previously described.\(^{19} \) The separated proteins were subjected to immunoblot analysis using antibodies against PTEN and Src.

### \( \text{H}_2\text{O}_2 \) and CCM treatment of FIBs in vitro.

FIBs and CAFs were seeded at a density of \( 1 \times 10^4 \) per 35-mm dish in FibroGRO-LS (Millipore, Billerica, MA, USA). The 48-h CCM was collected from CAFs, sterile filtered and used immediately. CCM, with or without 100 \( \mu \)M N-acetylcysteine (Sigma Aldrich) pretreatment, was added to wells containing FIBs and refreshed daily over 3 days, after which FIBs were harvested for FACS analysis. Separately, FIBs were treated with 100 nM \( \text{H}_2\text{O}_2 \) in serum-free FibroGRO-LS for 30 min twice daily with an 8-h interval between treatments for a maximum of 3 days. FIBs were harvested for FACS analysis 24 h after 1 day, 2 days and 3 days of \( \text{H}_2\text{O}_2 \) treatment.

### In vivo tumor growth and adoptive transfer of 1FIBs.

A total of \( 1 \times 10^6 \) HaCaT or A-5RT3 cells were admixed with \( 2 \times 10^5 \) CAFs and 1 \times 10^6VybrantDiO (Life Technologies, Waltham, MA, USA)-labeled FIBs to a ratio of 1:1 and implanted subcutaneously into NOD-SCID mice (Jackson Laboratory, Bar Harbor, ME, USA) (Figure 3a). Three weeks post-implantation, tumors were excised and dissociated using the MACS tumor dissociation kit and a gentleMACS Octo Dissociator (Miltenyi Biotec, Germany). Then, the tumors were homogenized using a Dounce homogenizer and cell lysates were subjected to immunoblot analysis. Tumors were analyzed in the National Healthcare Group Domain-Specific Review Boards (NHG-DSRB). All tumor samples were de-identified before the analyses.
ChIP and re-ChIP. ChIP was performed using phospho-cJun and phospho-Smad3 antibodies (Cell Signaling, Danvers, MA, USA), and re-ChIP was performed using a p300 antibody (Upstate Biotechnology, Lake Placid, NY, USA) as previously described.50 The primer sequences are listed in Supplementary Table S1.

Immunoblot assay. Far-infrared immunoblotting was performed as previously described.51

Measurement of extracellular H2O2, intracellular ROS and GSSH/GSSG ratio. Extracellular H2O2 and intracellular ROS were measured as previously described.47,52 The specificity of the assay for H2O2 was verified with catalase, and the degradation of H2O2 or inhibition of the assay system by the sample was analyzed by determining the recovery of exogenously added H2O2. GSSH/GSSG ratio was measured using the GSSH/GSSG ratio detection assay kit green (Abcam, Cambridge, UK) according to the manufacturer’s recommendations.

Mitogenic factor array. The expression profiles of the secreted factors present in the conditioned medium (48 h) were identified using the RayBio Human Growth Factor Array 1 and Human Inflammation Antibody Array 3 (RayBiotech, Inc., Norcross, GA, USA). These membranes were incubated with conditioned media from the different cocultures and then processed.

Statistical analysis. Statistical differences were determined using a two-tailed Mann-Whitney U-test. A P-value of < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. The work was supported by grants from the Singapore Ministry of Education (MOE2010-T2-2-009 and AcRF Tier 1 RG134/15) to NST. JSKC is a recipient of the Nanyang President Graduate Scholarship; TP is an NTU-KI PhD scholar.

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