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Cancer Associated Fibroblasts express pro-inflammatory factors in human breast and ovarian tumors

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ABSTRACT

Inflammation has been established in recent years as a hallmark of cancer. Cancer Associated Fibroblasts (CAFs) support tumorigenesis by stimulating angiogenesis, cancer cell proliferation and invasion. We previously demonstrated that CAFs also mediate tumor-enhancing inflammation in a mouse model of skin carcinoma. Breast and ovarian carcinomas are amongst the leading causes of cancer-related mortality in women and cancer-related inflammation is linked with both these tumor types. However, the role of CAFs in mediating inflammation in these malignancies remains obscure. Here we show that CAFs in human breast and ovarian tumors express high levels of the pro-inflammatory factors IL-6, COX-2 and CXCL1, previously identified to be part of a CAF pro-inflammatory gene signature. Moreover, we show that both pro-inflammatory signaling by CAFs and leukocyte infiltration of tumors are enhanced in invasive ductal carcinoma as compared with ductal carcinoma *in situ*. The pro-inflammatory genes expressed by CAFs are known NF-κB targets and we show that NF-κB is up-regulated in breast and ovarian CAFs. Our data imply that CAFs mediate tumor-promoting inflammation in human breast and ovarian tumors and thus may be an attractive target for stromal-directed therapeutics.

1. Introduction

Tumor-promoting inflammation was established as a hallmark of cancer [1]. An inflammatory microenvironment is associated with virtually all tumors, even those that have not been etiologically linked with inflammation. Hallmarks of inflammation, such as the infiltration of neoplastic tissue by innate and adaptive leukocytes, activated angiogenic vasculature, tissue remodeling and high levels of chemokines and cytokines, are found in most solid tumors [2].

Cancer Associated Fibroblasts (CAFs) are an activated subpopulation of stromal fibroblasts. CAFs have been shown to promote tumor growth by directly stimulating tumor cell proliferation and by enhancing angiogenesis [3–5]. We have demonstrated that CAFs are functionally required for mediating inflammation during squamous cell carcinogenesis, starting at the earliest pre-neoplastic stages [6,7]. CAFs isolated from pre-neoplastic skin lesions expressed a pro-inflammatory gene signature and promoted macrophage recruitment, neovascularization and tumor growth *in vivo*, in an NF-κB-dependent manner. Other recent studies support these observations implicating CAFs as novel mediators of tumor-promoting inflammation, thus enhancing tumor progression [8,9]. Importantly, we showed that this role is widespread as CAFs from end-stage mouse mammary and pancreatic tumors expressed components of the pro-inflammatory gene signature identified in skin CAFs, while normal tissue fibroblasts did not, suggesting that pro-inflammatory signaling by CAFs may be operative also in other cancer types [6].

Breast and ovarian cancers continue to be amongst the leading causes of cancer related mortality in women in the western world. Inflammation is correlated with bad prognosis in breast cancer, and is tightly connected with the development of ovarian cancer [10,11]. Multiple studies in recent years demonstrated the central role of immune cells in facilitating cancer progression and metastasis [12–16]. Tumor cell-intrinsic inflammatory signaling pathways were shown to contribute to mammary carcinogenesis [17,18] in particular, activation of the NF-κB pathway contributes to mammary tumor cell survival and self-renewal [19–21]. However, the role of CAFs in mediating tumor-promoting inflammation in breast and ovarian cancer is largely unknown. Breast tumors are characterized by an extensive desmoplastic stroma, abundantly populated by fibroblasts [22], and CAFs were shown to support the growth of mammary tumors [23]. In ovarian carcinoma, women with abundant tumor stroma have an overall decreased survival [24]. Moreover, CAFs in ovarian carcinoma were shown

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in several studies to impact tumor progression and metastasis, but the molecular mechanism of CAF-mediated tumor enhancement is poorly defined [25].

Here we show that CAFs found in the stroma of human breast and ovarian cancer contain high levels of NF-κB and express pro-inflammatory cytokines and chemokines from the NF-κB pathway including IL-6, Cox-2, and CXCL1, which we previously identified as part of a pro-inflammatory gene signature in skin CAFs [6]. Moreover, we show that pro-inflammatory signaling by CAFs in breast cancer is enhanced in correlation with tumor progression: expression of COX-2 and CXCL1 was significantly higher in Invasive Ductal Carcinoma (IDC) as compared with Ductal Carcinoma *In Situ* (DCIS). Thus, our data imply that the pro-tumorigenic activity of CAFs in breast and ovarian cancer is mediated, at least in part, by their inflammatory signaling.

2. Methods

2.1. Ethics statement

The research is non-genetic project of retrospective archival anonymous FFPE tissues. Samples were obtained from archives and the data were analyzed anonymously, therefore, informed consent was waved by the Institutional Helsinki Committee. Committee of the Sheba Medical Center, Israel. Approval number: 8153/10.

2.2. Human tissue specimens

Formalin-fixed paraffin embedded (FFPE) tumor samples were obtained from the following sources: Sheba Medical Center, Tel Hashomer, Israel, and Rabin Medical Center, Petah Tikva, Israel. An expert pathologist evaluated histological tumor type, tumor grade and tumor percentage using Hematoxylin and Eosin (HE)-stained samples derived from the first and/or last sections of each FFPE block. The tumor content was $\geqslant$50% in more than 90% of FFPE samples.

2.3. Immunohistochemistry

Paraffin sections of human breast and ovarian tumors and normal breast or ovary were deparaffinized in xylene and rehydrated through a series of graded ethanol to distilled water. Antigen retrieval was performed in microwave in EDTA solution. The sections were incubated with 15% hydrogen peroxide to inactivate endogenous peroxidase followed by washes with TBS. Incubation with normal goat serum was performed to inhibit nonspecific binding, followed by incubation with antibodies for NF-κB (Epitomics, dilution at 1:50), CXCL1 (Proteintech, dilution at 1:100), COX2 (Cell marque, dilution at 1:50), IL-6 (Abcam, dilution at 1:20) or α-SMA (sigma, dilution 1:100), overnight at 4 °C. Sections were then incubated with peroxidase conjugated secondary antibodies, followed by incubation with AEC solution, and counterstained with hematoxylin.

2.4. IHC scoring assessments

The intensity and percentage of IHC staining were recorded. Intensity was scored from 0 to 3+ and defined as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. An overview of the IHC for all tissue sections was performed by an expert pathologist (I Barshack). Two additional observers evaluated the staining results independently (N. Erez and S. Glanz).

2.5. Statistical analysis

Statistical analyses were done by Student's *t* test. Statistical significance was defined as $p < 0.01$.

3. Results

Inflammatory signaling by CXCL1 [26,27], COX-2 [28] and IL-6 [29,30] was shown to play a role in mammary tumorigenesis, progression and metastasis. However, previous studies demonstrating pro-tumorigenic signaling mediated by these factors had mostly described autocrine signaling by tumor cells. In order to test whether pro-inflammatory signaling is mediated by CAFs in breast carcinoma, we analyzed 31 cases of invasive ductal carcinoma and 14 cases of DCIS for the expression of COX-2, IL-6 and CXCL1, which we and others have previously identified as part of a CAF pro-inflammatory gene signature in mice [6,9]. We found that the expression of all three factors was significantly upregulated in mammary CAFs, as compared to fibroblasts found in the stroma of normal breast specimens. Normal breast stroma was negative for the expression of all three factors (Fig. 1 and Table 1), suggesting that CAFs mediate inflammation in breast tumors. Moreover, there was a significant increase in the number and intensity of fibroblasts expressing pro-inflammatory factors in invasive ductal carcinoma, as compared with DCIS (Fig. 1 and Table 1), indicating an enhancement in pro-inflammatory signaling with tumor progression. Since immune cells in the tumor microenvironment also express these pro-inflammatory factors, we wanted to verify that the cells expressing these factors are indeed fibroblasts. To that end, we performed a series of differential staining with markers for immune cells (CD-3, L-26), endothelial cells (Factor VIII) and myofibroblasts (α-SMA) and found that the spindle-shaped cells in the tumor stroma that expressed pro-inflammatory factors were not stained with immune or endothelial cell markers, but only with α-SMA and thus we concluded that they were indeed fibroblasts (Supp. Fig. 1).

An inflammatory environment is thought to be tightly linked with ovarian carcinoma development and progression [11]. However, most of the known signaling is tumor cell-derived. Thus, we next assessed the expression of the above-described pro-inflammatory factors by CAFs found in the stroma of high-grade ovarian cyst adenocarcinomas. Similarly to breast carcinoma, we found that CAFs in ovariantumors significantly upregulated IL-6, COX-2 and CXCL1, suggesting that CAFs in ovarian tumors mediate tumor-promoting inflammation (Fig. 2 and Table 2). The fibroblastic identity of the stained cells was verified as detailed above.

IL-6, CXCL1 and COX-2 are known targets of the NF-κB transcription factor. We next wanted to assess NF-κB activity in breast and ovarian CAFs. To that end, we performed immunostaining for the NF-κB subunit p65 (RelA). When active, the NF-κB heterodimer (RelA-p50) translocates to the nucleus, where it activates the transcription of its target genes [31]. Indeed, we found that in addition to its accumulation in the tumor cells, NF-κB was upregulated in CAFs in the stroma of breast and ovarian tumors (Figs. 3 and 4). We found a mixture of CAFs with either nuclear or cytoplasmic p65 staining (Figs. 3A and 4A), in both breast and ovarian tumors stroma. p65 was not upregulated in normal breast or in normal ovarian fibroblasts (Figs. 3B and 4B). Furthermore, in agreement with the increase in pro-inflammatory factors expression with tumor progression, there was an enhancement in p65 staining in invasive ductal carcinoma as compared with fibroblasts in DCIS (Fig. 3 and Table 1). Thus, NF-κB pro-inflammatory signaling is activated in tumor cells as well as in CAFs in the microenvironment of breast and ovarian carcinomas, leading to upregulation of its pro-inflammatory downstream genes CXCL1, IL-6 and COX-2.

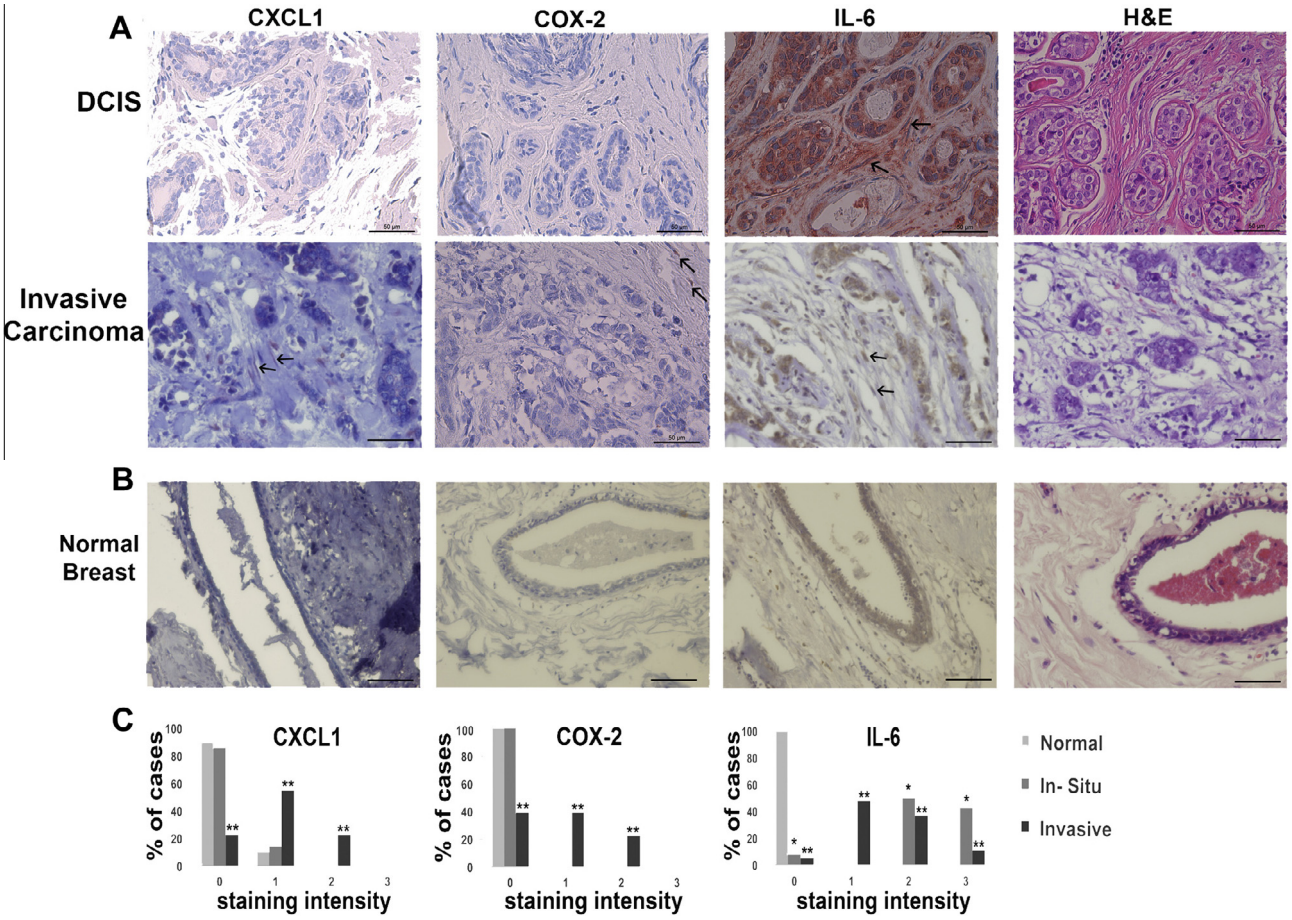

Fig. 1. CAFs in human breast tumors express pro-inflammatory factors in correlation with tumor invasiveness.(A) Tumor tissue sections from breast cancer patients were immunostained with antibodies for CXCL1, COX-2, IL-6 or with Hematoxylin and Eosin, as indicated. Upper and lower panel show representative sections from two different patients, out of 16 patients tested. (B) Normal breast tissue sections were stained with antibodies as in (A). Arrows indicate stained CAFs. Scale bar = 50 μM. (C) *Expression of pro-inflammatory factors by mammary fibroblasts is enhanced in invasive breast tumors*. Staining Intensity in sections from all patients analyzed was scored from 0 to 3 as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. $^{*}p < 0.01$ *in situ* vs. normal. $^{**}p < 0.01$ invasive vs. normal.

Table 1
Summary of positive cases for expression of pro-inflammatory factors by CAFs, in the tested samples of breast ductal carcinoma *in situ*, invasive ductal carcinoma, and normal control tissues, as indicated.

	Normal breast ($N = 10$) (%)	Carcinoma *in situ* ($N = 14$) (%)	Invasive carcinoma ([a]) (%)	*p* Value *in situ* vs. normal	*p* Value invasive vs. *In situ*	*P* Value invasive vs. normal
CXCL1	10	14	77	0.766	$3.47 * 10^{-5}$	$9.18 * 10^{-5}$
COX2	0	0	59	N/A[b]	$3.35 * 10^{-5}$	$3.64 * 10^{-4}$
IL-6	0	93	95	$2.39 * 10^{-10}$	0.89	$5.29 * 10^{-12}$
NF-κB	10	64	97	0.006	0.02	$4.68 * 10^{-9}$

[a] For CXCL1, COX-2, IL-6 and NF-κB – $N = 30, 17, 19, 31$, respectively.
[b] All cases of normal breast and carcinoma *in situ* were negative for COX-2.

Tumor-related inflammation is characterized by infiltration of leukocytes into the tumor microenvironment [2]. In order to test whether pro-inflammatory signaling by CAFs is correlated with immune cell recruitment into breast tumors, we assessed leukocyte infiltration in all breast tissue sections (normal, DCIS and IDC). We found a significant increase in leukocyte infiltration in correlation with tumor progression: While in normal breast there was minimal leukocyte infiltration (all samples scored 0–1), in DCIS and IDC, 28% and 44% of the tumor samples presented with strong (scored 2–3) leukocyte infiltration, respectively ($p = 0.009$, IDC vs. normal). This is in agreement with the progression in pro-inflammatory signaling by breast fibroblasts, presented herein.

4. Discussion

In this study we have shown that cancer-associated fibroblasts express pro-inflammatory factors in breast and ovarian carcinoma. We found that IL-6, COX-2 and CXCL1, known to have pro-tumorigenic activity, are significantly upregulated in fibroblasts found in the stroma of human breast and ovarian tumors, as compared with fibroblasts in normal breast and ovarian tissues. These cytokines were previously shown to be part of a pro-inflammatory gene signature expressed by CAFs in skin and gastric cancers [6,9]. Furthermore, we show that the expression of pro-inflammatory factors by CAFs is enhanced in invasive ductal carcinoma of the breast as

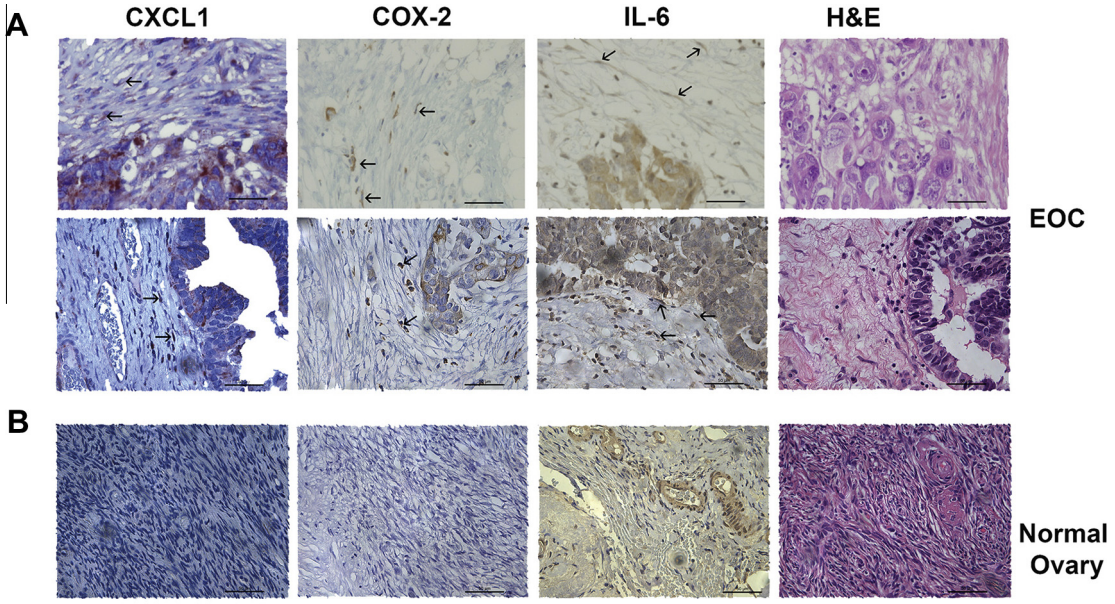

Fig. 2. CAFs in human ovarian carcinoma express pro-inflammatory factors. (A) Tumor tissue sections from patients with ovarian tumors were immunostained with antibodies for CXCL1, COX-2, IL-6 or with Hematoxylin and Eosin, as indicated. Upper and lower panel show representative sections from two different patients, out of 11 patients analyzed. EOC = epithelial ovarian cancer. (B) Normal ovary tissue sections were stained with antibodies as in (A). Arrows indicate stained CAFs. Scale bar = 50 μM.

Table 2
Summary of the number of positive cases for expression of pro-inflammatory factors by CAFs, in the tested samples of epithelial ovarian carcinoma and normal control tissue.

	Normal ovary ($N = 3$) (%)	Invasive carcinoma ($N = 11$) (%)	p Value
CXCL1	0	90	0.0002
COX2	0	54	0.104
IL-6	0	81	0.0052
NFκB	0	100	N/A

compared with ductal carcinoma *in situ*, indicating an enhancement in pro-inflammatory signaling with tumor progression. This enhancement is also evident by an increase in leukocyte recruitment into invasive tumors, as compared with DCIS and normal breast. Finally, we show that the transcription factor NF-κB is accumulated and activated in breast and ovarian CAFs, suggesting that pro-inflammatory signaling by CAFs is mediated through the NF-κB pathway.

CAFs have been implicated in facilitating the growth of mammary tumors by directly stimulating tumor cell proliferation and by enhancing angiogenesis [3–5]. However, while several recent studies linked inflammation with breast cancer [10,15], the role of CAFs in mediating cancer-related inflammation in breast tumors remained unresolved. We previously showed that CAFs isolated from *de novo* mammary tumors in transgenic mice express a pro-inflammatory gene signature [6]. Here we expand our previous findings and show that CAFs in human breast tumors express the pro-inflammatory factors IL-6, CXCL1 and COX-2, suggesting that in addition to their known up-regulation in tumor cells, signaling by these factors is also mediated by mammary CAFs. Moreover, we demonstrate an increase in the expression of pro-inflammatory factors as well as in leukocyte recruitment in correlation with progression to invasive tumors. Further studies are required in order to elucidate the functional sequence of events and to examine whether the increase in pro-inflammatory signaling by CAFs contributes to the enhanced recruitment of leukocyte into invasive tumors.

Interestingly, previous studies indicated that COX-2, expressed by breast carcinoma cells, might be a predictive marker of DCIS progression to invasive carcinoma [32]. Thus, stromal expression of COX-2 may also be a biomarker for progression in breast cancer.

Inflammation is a key driving force in the development and progression of ovarian carcinoma. Many chemokines and cytokines, including IL-6, COX-2 and CXCL1 were shown to be involved in tumor-related inflammation in Epithelial Ovarian Cancer (EOC) [25]. In particular, IL-6 is secreted by macrophages in ascites of advanced tumors, as well as by the tumor cells [33,34]. Tumor cell-derived CXCL1 signaling was shown to be pro-angiogenic [35], and anti-inflammatory drugs that block COX-2 were reported to reduce the risk for ovarian cancer [36]. However, although CAFs were suggested to be linked with tumor progression and invasion in ovarian cancer [25], their role in mediating inflammation through secretion of these factors is unknown. Our data suggest that tumor-enhancing inflammation in ovarian cancer stroma is, at least in part, mediated by CAFs. Interestingly, CXCR2, the receptor for CXCL1, was recently shown to be upregulated in ovarian cancer cells, and to promote proliferation and angiogenesis [37]. Our results suggest that ovarian CAFs may be the source for its ligand, CXCL1, thus facilitating tumor growth.

NF-κB was shown to be upregulated in several inflammation-linked cancers [38], and we previously showed that pro-inflammatory signaling by CAFs in squamous cell carcinoma is NF-κB dependent [6]. In this study, we observed strong up-regulation of p65 in CAFs. However, only a fraction of the p65 accumulation in CAFs was nuclear. Interestingly, the same phenomenon of mixed nuclear and cytoplasmic staining of p65 was previously reported in breast tumor cells, where nuclear p65 staining was found to be in correlation with higher tumor grade [39]. Indeed, NF-κB was suggested to be a link between inflammation and cancer [38,40]. In summary, our previous and current data strongly suggest that pro-tumorigenic signaling in the microenvironment of breast and ovarian tumors via the NF-κB pathway is mediated, at least in part, by CAFs. Furthermore, there is an enhancement in pro-inflammatory signaling mediated by CAFs, as well as in leukocyte infiltration into tumors, in correlation with tumor progression. These results have important implications for therapeutic targeting of CAF-mediated inflammation, suggesting that pro-inflammatory signaling by CAFs may be an attractive target for stromal-directed therapeutics in breast and ovarian cancers.

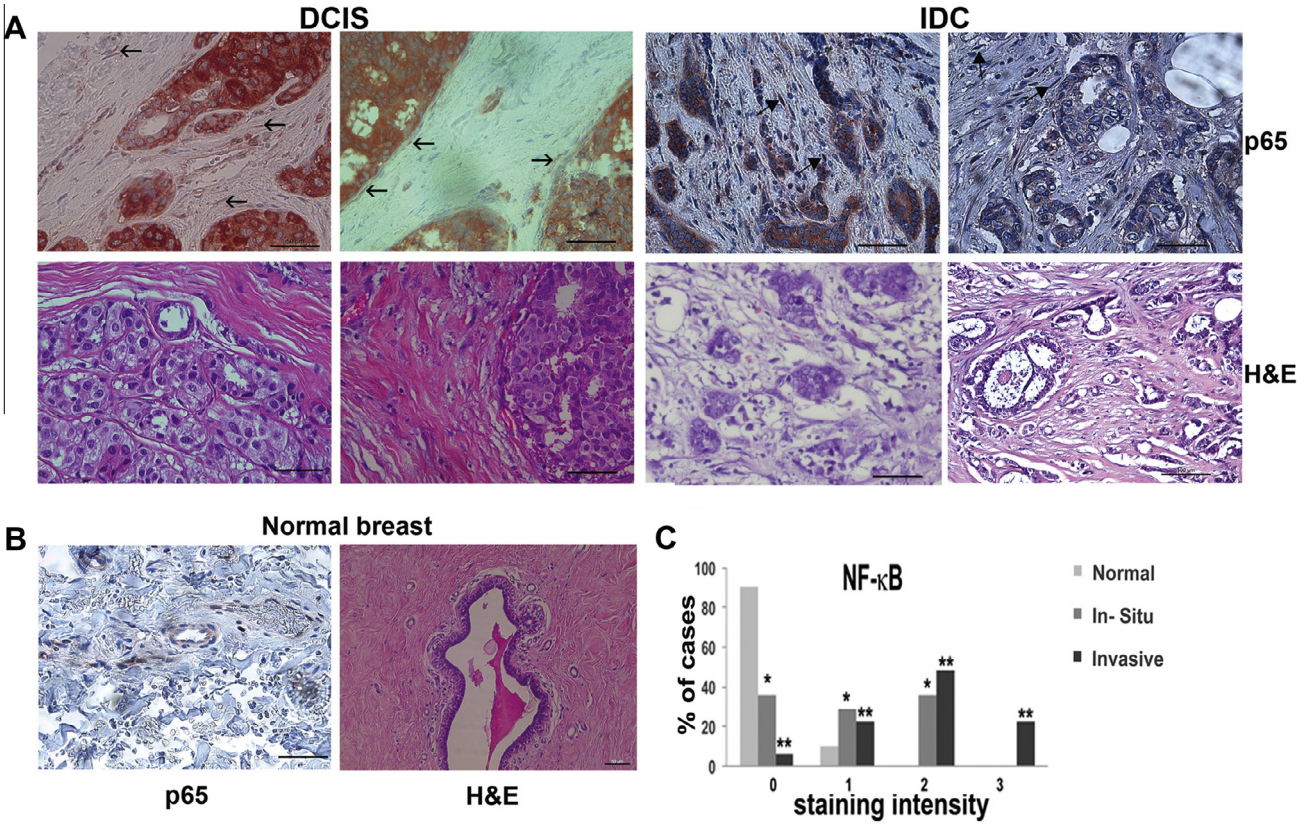

Fig. 3. NF-κB is upregulated in breast carcinoma CAFs. Tissue sections from patients with DCIS, Invasive ductal carcinoma (A) or from normal breasts (B) were immunostained with anti-p65 antibody for the NF-κB subunit p65. Panels are representative of multiple fields from 16 patients and healthy controls. Arrows indicate cells with cytoplasmic staining of p65 in CAFs, Filled arrows indicate nuclear staining. Scale bar = 50 μM. (C) *Expression of NF-κB by mammary fibroblasts is enhanced in invasive breast tumors.* Staining Intensity in sections from all patients analyzed was scored from 0 to 3 as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. $^{*}p < 0.01$ *in situ* vs. normal. $^{**}p < 0.01$ invasive vs. normal.

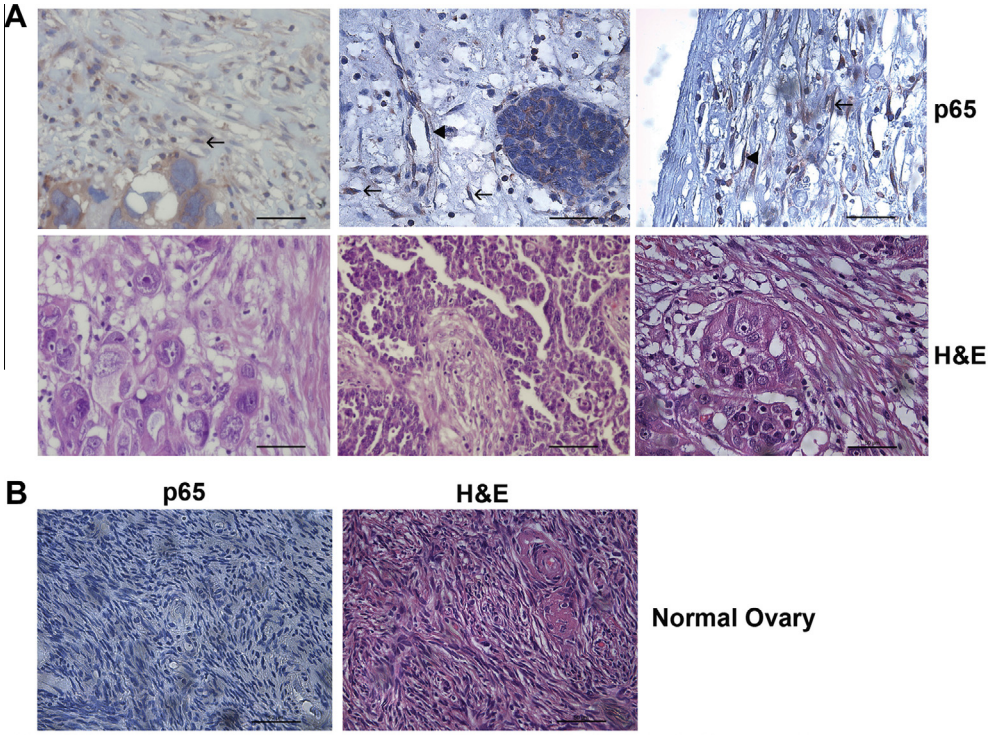

Fig. 4. NF-κB is upregulated in ovarian carcinoma CAFs. (A and B) Tissue sections from patients with ovarian carcinoma (A) or from normal ovaries (B) were immunostained with anti-p65 antibody for the NF-κB subunit p65. Panels are representative of multiple fields from 11 patients and healthy controls. Arrows indicate cells with cytoplasmic staining of p65 in CAFs, filled arrows indicate nuclear staining. Scale bar = 50 μM.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.089.

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