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CAPC negatively regulates NF-κB activation and suppresses tumor growth and metastasis

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Abstract

CAPC, also known as LRRC26, is expressed in normal prostate and salivary gland. We developed a monoclonal antibody to CAPC and used it to characterize the protein and study its function. CAPC protein was detected in normal prostate and salivary gland, in several human breast cancer cell lines and in the prostate cancer cell line LNCaP. Knock down of CAPC by siRNA in LNCaP cells enhanced anchorage-independent growth in soft agar. Conversely, over-expression of CAPC in MDA-231 breast cancer cells and the A431 epidermoid cancer cells inhibited growth in soft agar and tumorigenesis in nude mice, and suppressed metastasis of MDA-231 cells to the lung. Over-expression of CAPC down-regulated NF-κB activity and its target genes including GM-CSF (CSF2), CXCL1, IL8 and LTB1. It also suppressed genes encoding the serine protease mesotrypsin (PRSS3) and Cystatin SN (CST1). CAPC expressing tumors showed a decrease in the number of proliferating cells and a large increase in extracellular matrix. The role of CAPC in the suppression of tumor growth and metastasis may be through its alteration of the tumor microenvironment.

Keywords

CAPC; tumor growth; NF-κB; cytokines

Introduction

Breast and prostate cancers are the leading cause of cancer related death in women and men, respectively, in the United States (American Cancer Society, 2009). Many experimental approaches have been developed to identify genes and proteins that could be useful for

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Conflict of Interest

The authors declare no conflict of interest.

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diagnosis or treatment of cancers (Chatterjee and Zetter, 2005). To identify genes that encode membrane-associated or secreted proteins present in breast and prostate cancers, but with limited expression in normal organs, we prepared a cDNA library from membrane-associated polyribosomal RNA isolated from breast and prostate cancer cell lines enriched in RNAs encoding membrane or secreted proteins (Egland et al., 2003). From this library, we isolated a cDNA encoding CAPC, also known as LRRC26 (Gene ID: 389816). We observed that CAPC RNA is present in many breast, prostate, colon and pancreatic cancers; but in normal tissues expression is limited to the prostate and salivary gland (Egland et al., 2006). These findings suggest an important role of CAPC in cancer biology, and point to its potential value as a tumor marker or therapeutic target.

In this report, we describe the biological properties of the CAPC protein. To do this we prepared a mAb that specifically reacts with CAPC and used it to characterize the protein in tumor cell lines. We found that knock down of CAPC protein can stimulate anchorage-independent growth in the LNCaP cell line and that over-expression of CAPC suppressed tumor cell growth in vitro and in vivo. We also demonstrated that CAPC over-expression can suppress breast cell metastasis into lung. Our data indicates that CAPC inhibits tumor cell proliferation and tumor growth by regulating NF-κB and its target genes, as well as many secreted proteins.

Results

Characterization of CAPC protein by mAb

Figure 1A shows that the CAPC mAb recognizes a 37 kd doublet in a stably transfected line MDA-CAPC-18, but not in the untransfected cells. We then used the mAb to investigate CAPC expression in normal tissues and cancer cell lines. We found it detected very high CAPC protein expression in the prostate cancer cell line LNCaP, moderate expression in the breast cancer cell line HTB 20 (BT474) and low expression in MCF7. We did not detect the protein in MDA-435 cells (Fig. 1B). We also detected the CAPC protein in the prostate and salivary gland (Fig. 1C). No band was detected in pituitary gland where there is no RNA expression. Interestingly, there is no detectable protein expression in prostate tumor sample (Fig.1C, lane 2) which is from the same patient from which the normal prostate tissue was obtained (Fig. 1C, lane 1).

The predicated size of CAPC protein is 35 kd. In western blots we observed multiple bands above 35 kd, suggesting protein modification (Fig. 1A). To determine if the CAPC protein is glycosylated, a NP40 solubilized cell lysate was treated with PNGase F, which removes oligosaccharides from N-linked glycoproteins. As shown in Figure 1D, after treatment of PNGase the high molecular weight band disappeared, and the 35 kd band increased in intensity, indicating CAPC is a N-glycosylated protein. The CAPC protein may contain other modifications because after PNGase treatment we still observed double bands near 35 kd. To determine if the CAPC is a membrane protein, we separated the membrane fraction from the cytosolic fraction. CAPC was present in the membrane fraction (Fig. 1E) but moved to the soluble fraction when 1% NP40 was present. The CAPC protein was not solubilized by 2M urea or high salt (data not shown), indicating that CAPC is an integral membrane protein.

To clarify the organelle in which CAPC was located, we used a MCF7 cell line stably expressing CAPC-myc-tag protein. The expressing cells were double stained with both monoclonal anti-CAPC and polyclonal anti-myc antibody (Ab). The two Abs colocalized exactly in merged image (data not shown), indicating the CAPC Ab recognized the same protein as the anti-myc-tag antibody. CAPC-myc expression was then followed by staining the myc-tag with anti-myc Ab and either anti-PDI, an endoplastic reticulum (ER) protein marker, or anti-pan-cytokeratin. The green fluorescence (myc tag on CAPC) exactly merged with red PDI staining of the ER (Fig. 1F, red), but not with the pan-cytokeratin Ab (Fig. 1I, red), indicating CAPC localized to the ER. Also the specific CAPC staining did not co-localize with a trans-Golgi marker TGN46 (data not shown). Previously, CAPC was characterized by a rabbit polyclonal antibody, which recognized a 50 kd endogenous protein in addition to the over-expressed 37 kd CAPC by western blot (Egland et al., 2006). Using immunoprecipitation and GC-MS we have found that the 50 kd protein, recognized by the polyclonal anti-CAPC in previous studies, is not CAPC but cytokeratin 8 (data not shown). This explains why the CAPC protein previously reported to be associated with cytokeratin in microfilaments (Egland et al., 2006).

Knock down of CAPC stimulated prostate tumor cell growth in soft agar

To study the role of CAPC in cancer, we first investigated the human prostate tumor cell line LNCaP, which expresses CAPC at very high levels (Fig. 1D, lane 1). CAPC protein was efficiently knocked down by transfecting a short siRNA oligo (Fig. 2A). Knock down of CAPC did not affect cell proliferation in standard tissue culture (Fig. 2B). To examine the possible role of CAPC in tumorigenicity, we assessed the anchorage-independent growth of cells treated with CAPC siRNA in comparison with a luciferase siRNA control. When LNCaP cells treated with a control siRNA were plated into soft agar, only a few small colonies developed in 2 weeks. In contrast, after knock down of CAPC with a specific siRNA, many colonies appeared (Fig. 2C and 2D).

Over-expression of CAPC suppressed tumor development of a breast cancer cell line

We established two CAPC expressing cell lines, MDA-CAPC-18 and MDA-CAPC-19, from the highly invasive human breast cancer cell line MDA-231. The CAPC protein expression in the parental cell line is undetectable (Fig. 3A, lane 1). We measured the growth rate of MDA-CAPC-18 and -19 lines in cell culture and found that over-expression of CAPC did not affect the growth rate of the cells over 4 days (Fig. 3B). We then measured the growth of the cells in soft agar and found that the MDA-231 cells formed 200–300 colonies per well, whereas the CAPC-18 and CAPC-19 lines did not form colonies (Fig. 3C).

To confirm that the growth inhibition in soft agar is due to CAPC expression, we knocked down CAPC expression by siRNA in the MDA-CAPC-18 line. We found that CAPC protein expression was decreased by 90–100% at 48 hours (Fig 3D), and remained low for up to 1 week (data not shown). After CAPC knock down, the MDA-CAPC-18 cells were capable of forming colonies in soft agar (Fig. 3E), indicating that the suppression of the growth of tumor cells in soft agar is caused by CAPC.

We then investigated the role of CAPC expression for xenograft tumor growth in immunodeficient mice. We compared MDA-CAPC-18 cells with the MDA-231 parental cells. The MDA-231 cells formed tumors that reached 120 mm^3 in size in 21 days, and 180 mm^3 in 52 days. In contrast, the MDA-CAPC-18 tumors only reached 30 mm^3 in 3 weeks, and then slightly regressed (Fig. 3F). These results indicate that CAPC expression reduces tumor cell growth in vivo.

Expression of CAPC suppressed tumor development of epidermoid carcinoma cell line

To determine if CAPC was capable of suppressing the growth of other tumors, we used a human A431 epidermoid cancer cell line, which does not have detectable CAPC mRNA when analyzed by RT-PCR (data not shown). We isolated two CAPC lines, A431-CAPC-20 and A431-CAPC-24; both express CAPC (Fig. 4A). The two A431-CAPC expressing cell lines grew at the same rate as a A431-vector control in cell culture (Fig. 4B). The A431 tumor cells rapidly formed colonies when grown in soft agar, but the A431-CAPC-20 and -24 cells did not (Fig. 4C). Furthermore, when cells were implanted into nude mice, the A431 cells rapidly grew into large tumors, which reached a volume of 1000 mm^3 in 2 weeks, whereas the two CAPC expressing cell lines developed tumors much more slowly (Fig. 4D). These data show that CAPC suppression of tumor cell growth is not limited to a breast cancer cell line.

Expression of CAPC suppresses breast tumor metastasis to lung

It has been previously shown that MDA-231 cells form lung tumors when injected intravenously into SCID mice and is a model of metastasis (Nam et al., 2006). To address the role of CAPC in metastasis, 1×10^6cells of MDA-231 or MDA-CAPC-18 cells were injected into the tail vein of SCID mice. After 6 weeks, 5/5 mice injected with MDA-231 cells had lung tumor metastasis (data not shown) and at 8 weeks, 4/4 of the MDA-231 group showed very extensive tumor cell infiltration of the lung (Fig. 5, top). In contrast, 4/4 mice receiving MDA-CAPC-18 cells were free of lung tumors (Fig. 5, bottom) in 8 weeks. In a separate experiment, we found that 4/5 mice in the MDA-231 group died with lung metastasis at week 9, but none of the MDA-CAPC-18 group died (data not shown). These results indicate that over-expression of CAPC suppressed metastatic growth.

Over-expression of CAPC down-regulate secreted proteins, NF-κB and a spectrum of cytokines and chemo-attractants in tumor cells

To investigate the mechanism of CAPC regulation of tumor cell growth, RNA samples from MDA-CAPC-18 and MDA-231 cells were analyzed by Illumina bead arrays containing 24,000 genes. The assembled microarray data was analyzed to identify the genes and pathways that were affected by CAPC over-expression. The data show that CAPC negatively regulates the NF-κB pathway, the local acute inflammatory response (lair) pathway and the inflammation pathway by more than 10-fold (Supplemental Fig. 1A). We identified 6 genes that are positively regulated by more than 2-fold when CAPC is over expressed and 48 genes that are negatively regulated by more than 2-fold (Supplemental Table 1). Of the 54 genes regulated by CAPC, 42 (78%) encode secreted or membrane anchored proteins, 10 encode cytoplasmic or nuclear proteins and 2 encode uncharacterized ORFs.

To confirm the microarray data, we chose a group of negatively regulated genes from the microarray list and performed real time PCR. As shown in Figure 6A, the mRNA levels for 7 genes were lowered by expression of CAPC. Cytokines SAA2 and CSF2 and LTB1 were down-regulated approximately 5-fold and IL8 and CXCL1 were decreased 1.5–2.5 fold. Two other secreted proteins, cysteine protease inhibitor Cystatin SN (CST1) and serine protease mesotrypsin (PRSS3), were decreased by 10–100 fold.

To determine if CAPC negatively regulated cytokine or chemokine genes in another cell line, we performed real time PCR after knock down of CAPC in LNCaP cells. When CAPC RNA levels were decreased by 60%, CXCL1, IL6, IL8, LTB1 and SAA2 were increased between 4–17 fold compared to control siRNA (Fig. 6B). We also found that CSF2, protease PRSS3 and CST1 were not affected (data not shown). It is possible that a 60% decrease in CAPC mRNA was not sufficient to induce a change in their RNA or that there are cell type effects of CAPC.

The genes most affected, as identified by the microarray analysis (Supplemental Table 1), are cytokines or chemokines. To determine if the RNAs were translated into protein we used a chemokine protein array. Cell culture medium collected from MDA-231 or MDA-CAPC-18 cells was analyzed. As shown in Figure 6C, the most significantly affected protein is CSF2 (GM-CSF, spot 1). CXCL1 (Groα, spot 2), IL6 (spot 3) and IL8 (spot 4) are also suppressed by CAPC with 1.5–3 fold changes (see also lower exposure in Supplemental Fig. 1B). The down-regulation of these four proteins is consistent with real time PCR analysis. Additionally, cytokine CCL5 (spot 7) was down-regulated. Interestingly, CAPC up-regulated cytokine IL1ra (IL1F3, spot 8), which is an inhibitor of IL1 action (Apte et al., 2006). In summary, CAPC over-expression down regulated pro-inflammatory cytokines (IL6, IL8, CSF2, CXCL1, CCL5) and up-regulated an anti-inflammatory cytokine (IL1ra).

Since most of the genes affected by CAPC are NF-κB targets, we investigated whether NF-κB activation is affected by over-expression of CAPC. NF-κB exists as an inactive complex in the cytoplasm in unstimulated cells. However, in some transformed tumor cells, NF-κB is activated and is found in the nucleus (Pacifico and Leonardi, 2006; Lee et al., 2007). We examined whether the amount of NF-κB in the nuclear fraction in MDA-231 cells was changed by CAPC over-expression. Figure 6D and 6E showed that NF-κB is reduced 2–3 fold in the nuclei of MDA-CAPC-18 cells compared to the parental MDA-231 cells. To confirm the negative regulation of NF-κB activity by CAPC, we performed NF-κB luciferase assays. As shown in Figure 6F, over-expression of CAPC significantly down-regulated transcription from the NF-κB promoter-reporter constructs in both MDA-231 and MCF7 cells. These results indicate that over-expression of CAPC suppresses the constitutive NF-κB activation in these tumor cells.

Recently, CAPC was identified as an auxiliary protein allowing BK potassium channel activation without calcium (Yan and Aldrich, 2010). We investigated whether CAPC regulation of NF-κB activation is dependent on BK channel. Since there is no detectable BKα protein in MDA-231 cells (data not shown), we used MCF7 and LNCaP. As seen in Supplemental Figure 2, CAPC over-expression suppressed NF-κB activity in the MCF7 cell line as expected; however, knock down of BKα protein by siRNA did not affect NF-κB

activity as measured in the luciferase assay in either the MCF7 or the MCF7-CAPC cell lines. Additionally, knock down of BKα protein did not affect the NF-κB activity in LNcaP cells. These results indicated that CAPC regulation of NF-κB activity is independent of the BK channel.

CAPC effect on tumor xenografts

Since CAPC expression down-regulated a set of genes encoding secreted proteins in vitro, we studied the same set of genes in tumor xenografts. As shown in Figure 7A, chemokine/cytokines CXCL1, IL8, LTB1, protease PRSS3 and Cystatin SN (CST1) were dramatically decreased in MDA-231 tumors expressing CAPC compared with the parental MDA-231 tumors.

We also examined the histology of the tumor xenografts. As shown in Figure 7B, Hematoxylin and Eosin (H&E) staining of sections from CAPC expressing tumors show there were substantially fewer tumor cells and much more extracellular matrix. Treating the sections with Masson stain indicated the presence of substantial amounts of collagen type I in the extra cellular matrix (ECM). Furthermore, the number of proliferating cells as determined by Ki67 staining was reduced, whereas the number of apoptotic cells as determined by staining for cleaved-caspase-3 was unchanged. These results indicate that CAPC expression inhibited tumor cell proliferation and greatly modified the tumor microenvironment.

Discussion

CAPC was discovered in a search for protein targets for antibody-based therapies. The goal was to find proteins located on the plasma membrane of cancer cells that were not expressed on essential normal organs such as liver, kidney, colon, brain, so that elimination of the cancer cells would not damage essential organs. In this approach expression in non-essential organs is not a serious limitation, because removal of these organs is often carried out as part of cancer treatment. The only normal tissues that express CAPC are the prostate and salivary gland, two tissues that have a major secretary function. When we examined three prostate cancer lines for CAPC expression, we found CAPC protein expression in LNCaP, but not in poor differentiated PC3 and DU145 cells. These data indicate that CAPC is a differentiation antigen that has an important function in normal prostate and continues to be expressed in cancers arising in the prostate.

Because CAPC is highly expressed in normal prostate, we examined its function in LNCaP cells and found that lowering CAPC expression using RNAi led to a striking increase in the ability of these cells to form colonies in soft agar consistent with an increase in tumorigenicity. Next, because breast cancers frequently express CAPC, we investigated its function in breast cancer by introducing CAPC into the MDA-231 line that has undetectable CAPC protein and found that these cells had a decreased ability to grow in soft agar and to form tumors in nude mice. In addition, we examined the ability of the cells to grow in the lungs of mice after i.v. injection and found that increased CAPC also decreased tumor growth in this assay, which is used to measure metastatic capacity. Furthermore, lowering CAPC levels in the CAPC-expressing MDA-231 cells restored ability to grow in soft agar.

To determine if this inhibitory effect of CAPC was more general, we used the A431 cancer line and again observed that CAPC over-expressing cells were less tumorigenic in animals and in the soft agar assay, even though the growth rate in standard cell culture were unaffected. Furthermore, CAPC expression in breast cancer cell line demonstrated by western blots (Fig. 1B) correlate with the invasiveness of the cells: the highly invasive cell lines MDA-231 and MDA-435 have no or weak expression, the weakly invasive cells MCF7 and HTB20 showed moderate levels of CAPC protein. These data suggest that CAPC expression was related to cancer cell behavior. Although we previously reported that CAPC RNA expressed in breast tumor samples by RT-PCR (Egland et al., 2006), we could not detect any CAPC protein by either immunohistochemistry or by western blot (data not shown). Similarly, CAPC RNA was detected in human lung sample by RT-PCR (Egland et al., 2006), but the protein is undetectable by western blot (data not shown). In tumor cells, CAPC protein expression is probably very low, so they can evade the suppressing effects of CAPC protein on tumor growth and metastasis.

We found that three pathways were down-regulated over 10-fold by CAPC over-expression. These are the NF-κB-induced pathway (Dixit and Mak, 2002; Naugler and Karin, 2008) and the local acute inflammation response and inflammatory pathways (Karin and Greten, 2005; Loza et al., 2007). All three pathways have been implicated in cancer progression and metastases (Lee et al., 2007; Park et al., 2007; Raman et al., 2007; Porta *et al*., 2009; Siebenlist et al., 1994; Wu and Zhou, 2009; Li and Sethi, 2010). Our microarray analysis showed that CXCL1, IL8, LTB1, CSF2 and SAA2 are down-regulated 2–5 fold and we confirmed that these were decreased using a protein array (Fig. 6). These cytokines or chemokines were also decreased in mouse tumor xenografts (Fig. 7). Measurements of NF-κB activity showed it was decreased in cells over-expressing CAPC. The NF-κB pathway is a crucial mediator of inflammation-induced tumor growth and progression. Other studies have shown that NF- κB inhibition can reduce the incidence of cancers of colon, lung and Hodgkins lymphoma (Karin and Greten, 2005; Wang et al., 2009). CAPC suppression of tumor growth may, in part, due to the suppression of NF- κB activity and its downstream targets. An alternate explanation is that knock down or over-expression of CAPC affected the tumor initiating cells (or cancer stem cells), which would affect tumor development.

Cell surface proteases degrade and remodel the ECM to help dissemination of cancer cells into normal adjacent tissues. Cancer cells use secreted metalloproteinases, serine proteases and cathepsins to cleave and remove different types of ECM substrates, including collagen (Basbaum and Werb, 1996; Lee et al., 2004; Friedi and Wolk, 2008; Joyce and Pollard, 2009). We found tumors and cells over-expressing CAPC had decreased expression of genes PRSS3 and CST1. PRSS3 has been found to promote the growth of breast cancer cells, and knock down of PRSS3 attenuated cancer cell growth (Hockla et al., 2010). The function of CST1 is still unclear, but it contributes to tumor cell proliferation and its expression was linked positively to many types of cancer, including gastric cancer and colon cancer in humans (Choi et al., 2009; Yoneda et al., 2009). We also found that CAPC expression promoted accumulation of collagen presumably by inhibiting its degradation since CAPC expression did not increase collagen mRNA levels in tumors (unpublished data). Our findings support the role of PRSS3 and CST1 in promoting tumor development. Thus

expression of CAPC can suppress pro-tumorigenesis factors and change the tumor microenvironment to decrease tumor cell proliferation, malignant progression and metastasis.

Materials and Methods

Cell culture

Human breast cancer cell lines MCF7, MDA-MB-435, MDA-MB-231 (MDA-231), HTB20 and prostate cancer cell line LNCaP were cultured in RPMI and A431 epidermoid carcinoma cell lines in DMEM, supplemented with 10% fetal calf serum and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2. To establish CAPC stable lines, 2 μg of plasmid pcDNA3-CAPC was transfected into cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 6 well dishes. CAPC stably transfected cells were selected with either 500 μg/ml (MDA-231) or 750μg/ml (A431 and MCF7) G418 for 2 wk. Individual clones were selected and grown in the presence of G418. Control lines (transfected vector only) were selected using the same concentration of G418.

Generation of mAb

Monoclonal anti-CAPC antibody was generated using the C-terminal 14 amino acids (ARRRRLRTAALRPP) to immunize mice in collaboration with BD Pharmingen (San Diego, CA).

Cell proliferation assay

Cells wereplated in 96-well plates at 2000 cells perwell. Cells were allowed to adhere overnight andgrowth measured by the CCK-8 colorimetric growth assay (Dojindo, Rockville, MD).

SiRNA

All cells were transfected with short oligo siRNA using the Amaxa system (Lonza, Germany) following the manufacture's instructions. The target sequence for CAPC is 5′-CGGGCUGCACUCGGUGC-3′ (Dharmacon, Lafayette, CO).

Western blot analysis and membrane fractionation

Cells were washed in phosphate buffered saline (PBS) and lysed in lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) with 1% NP40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 uM PMSF. Cytoplasmic and nuclear fractions were isolated using a kit from Pierce (Rockford, IL). For membrane fractionation, cell pellets were washed with PBS and suspended in lysis buffer without NP40 and divided into 3 aliquots. One served as a no detergent, no salt control. NP40 was added to another at a final concentration of 1% NP40. The other was exposed to 2M urea. After high-speed centrifugation, supernatant and pellets were subjected to SDS-PAGE and western blot analysis.

Luciferase assay

Cells were transfected with NF-κB-RE and Renilla (pGL4.32 and pGL4.74, respectively) (Promega, Madison, WI) for 48 hr using nucleofector (Lonza). Dual-Glo luciferase assays were performed following manufacture's instruction.

Cytokine array

Cells (8×10^5) of both MDA-231 and MDA-CAPC-18 cells were cultured in 60 mm dishes in 2 ml medium for 2 days. One ml of media were centrifuged to remove cell debris and applied to a human cytokine array (R&D System, Panel A array kit, Minneapolis, MN) following the manufacture's instructions.

Immunofluorescence microscopy

Cells growing on cover slips were fixed in 4% paraformaldehyde at room temperature for 20 min. The samples were blocked with buffer containing 3% BSA, 0.05% Tween 20 in PBS and incubated with primary antibody with anti-myc, 9E10 (Cell Signaling, Danvers, MA), anti-PDI or anti-pan-cytokeratin (Sigma, St. Louis, MO) in blocking buffer for 1–2 hr, followed by Alex Flour-conjugated secondary antibodies (Invitrogen). Nuclei were counter stained with DAPI. The images were taken with Zeiss 510 LSM confocal microscopy.

Anchorage-independent soft agar assays

For soft agar assays, 4000 cells were plated in 1.5 ml of DMEM containing 0.3% soft agarose, overlaid on 1.5 ml of 0.7% agarose in 6 well dishes. Cultures were maintained for 15–21 days. Colonies from triplicate wells were stained by 0.05% crystal-violet and visible colonies were counted by eye.

Tumor xenografts

Animals were maintained according to the NIH Animal Care and Use Committee guidelines under approved animal study protocols. Cells (5×10^6) were suspended in 200 μl of PBS and injected subcutaneously into either the right thoracic mammary fat pads of female nude mice (MDA-231 and MDA-231-CAPC) or into the rear flanks of nude mice (A431 and A431-CAPC) with 5 mice in each group. Tumor dimensions were determined every 2–3 days using calipers. Tumor volume (mm^3) was calculated by the following formula: $(a) \times (b^2) \times 0.4$, where a is tumor length and b is tumor width in centimeters. 2 week-old tumor xenografts were removed for real time PCR analysis and histology.

In vivo metastasis assays and histology

One million cells from MDA-231 or MDA-CAPC-18 were injected into the tail vain of SCID mice. After sacrifice, the lungs were removed and fixed with formalin. A representative lung from each group was sent to Histoserve (Gaithersburg, MD) for sectioning and staining with H&E, with Masson stain and for immunohistochemical detection of anti-ki67 or anti-cleaved caspase-3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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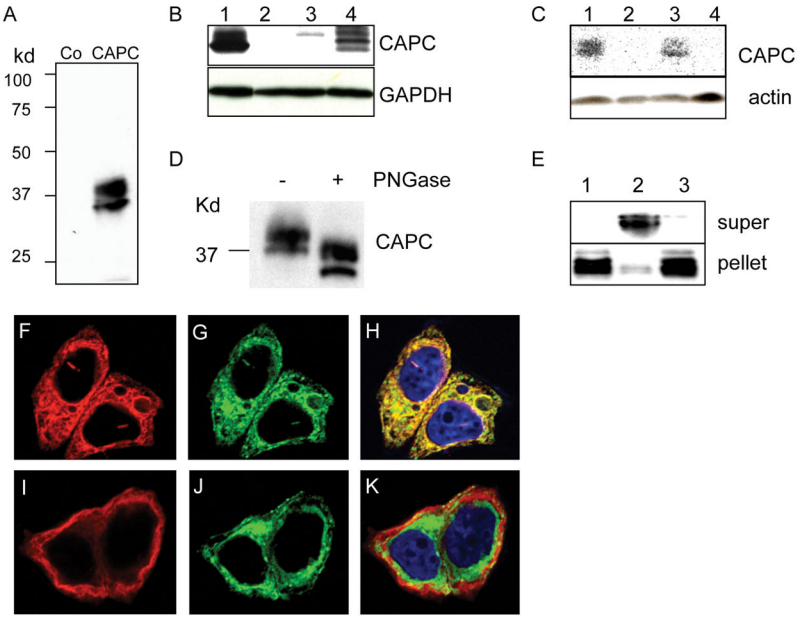

Figure 1.

Characterization of CAPC protein. **A**. Cell lysates of MDA-231 (Co) or MDA-CAPC-18 (CAPC) analyzed by western blot with anti-CAPC antibody. **B**. Western blot analysis of cell lines, LNCaP [1], MDA-435 [2], MCF7 [3] and HTB20 [4] with anti-CAPC or anti-GADPH. **C**. Human tissue lysate from prostate [1], prostate tumor [2], salivary gland [3] and pituitary gland [4] analyzed by western blot with anti-CAPC or anti-actin. **D**. Cell lysate of MDA-CAPC-18 treated with PNGase (+) or buffer (−) and analyzed with anti-CAPC. **E**. MDA-CAPC-18 cell lysates [1] was treated with NP-40 [2] or 2M urea [3] and separated into membrane and soluble fractions and blotted with anti-CAPC for western blot. **F-K**. Confocal images of MCF7-CAPC-myc stable line double stained with anti-myc (green, **G** and **J**), anti-PDI (red, **F**) or anti-pan-cytokeratin (red, **I**). **H** and **K** are merged images of **F** and **G** or **I** and **J**, respectively.

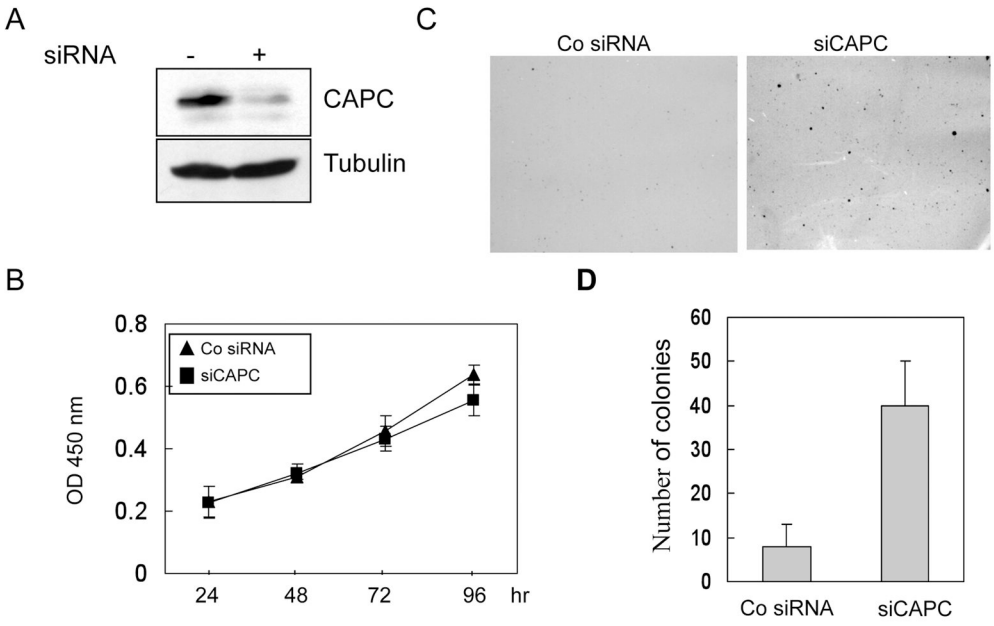

Figure 2.

Knock down of CAPC stimulated LNCaP cell growth in soft agar. **A**. LNCaP cells were transfected by CAPC siRNA or control GL2 siRNA, after 2 days, the cell lyastes were analyzed by western blot with anti-CAPC and anti-tubulin. **B**. Cell growth comparing CAPC knock down and control siRNA by cell proliferation assay. **C**. LNCaP cells were plated into soft agar for 14 days after siRNA treatment. The cells were stained and scanned (**C**) and the colonies counted (**D**). The experiment was performed in triplicate on 3 separate occasions. A summary graph of the results is shown.

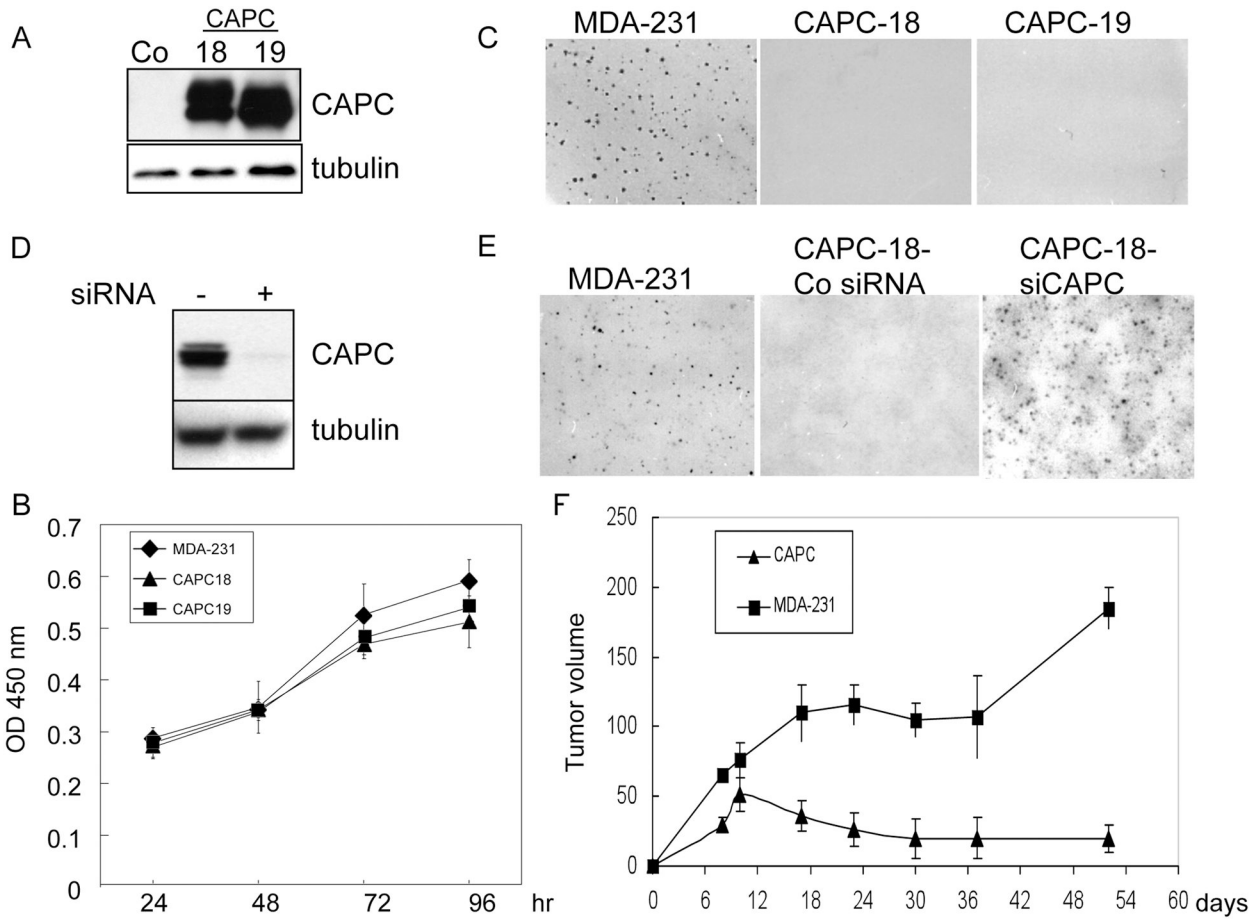

Figure 3.

Over-expression of CAPC suppressed anchorage-independent growth and tumor xenograft growth of MDA-231. **A**. CAPC expression on MDA-231 and CAPC stable lines MDA-CAPC-18 and -19 by anti-CAPC. Anti-tubulin is a loading control. **B**. Cell growth curve of MDA-231, MDA-CAPC-18 and -19 by cell proliferation assay. **C**. Soft agar assay of MDA-231, CAPC-18 and 19 as described in **Materials and Methods. D**. Western blot analysis with anti-CAPC or anti-tubulin after MDA-CAPC-18 cells were transfected with CAPC siRNA (+) or control siRNA GL2 (−) for 48 hr. **E**. Soft agar assay after MDA-CAPC-18 transfected with control siRNA or CAPC siRNA. MDA-231 is the positive control. **F**. Xenograft tumor growth in nude mice: MDA-231 or MDA-CAPC-18 were injected into mice and the tumor volume was measured at indicated time. The mean volume of 5 mice in each group is presented as a function of time after injection.

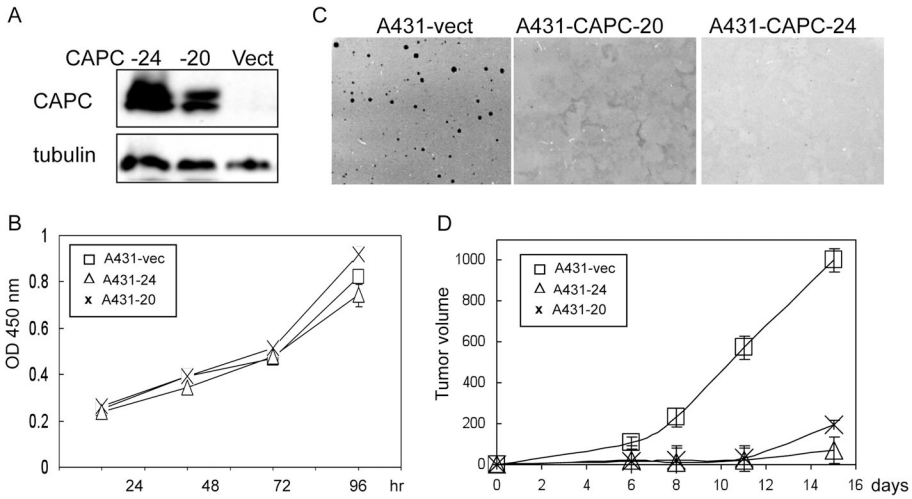

Figure 4.

Expression of CAPC suppressed A431 tumor cell growth. **A**. Western blot analysis of CAPC expression in A431-CAPC-20 and -24 compared with vector control by anti-CAPC and anti-tubulin. **B**. A431-vector and A431-CAPC-20 or -24 cell growth at indicated time by cell proliferation assay. **C**. A431 and A431-CAPC-20 and -24 growth by soft agar assay. The images are representative of two separate triplicate experiments. **D**. Tumor xenograft growth of A431-vector and A431-CAPC-20 and -24. Tumor volume was measured by caliper and the mean represent 5 mice in each group.

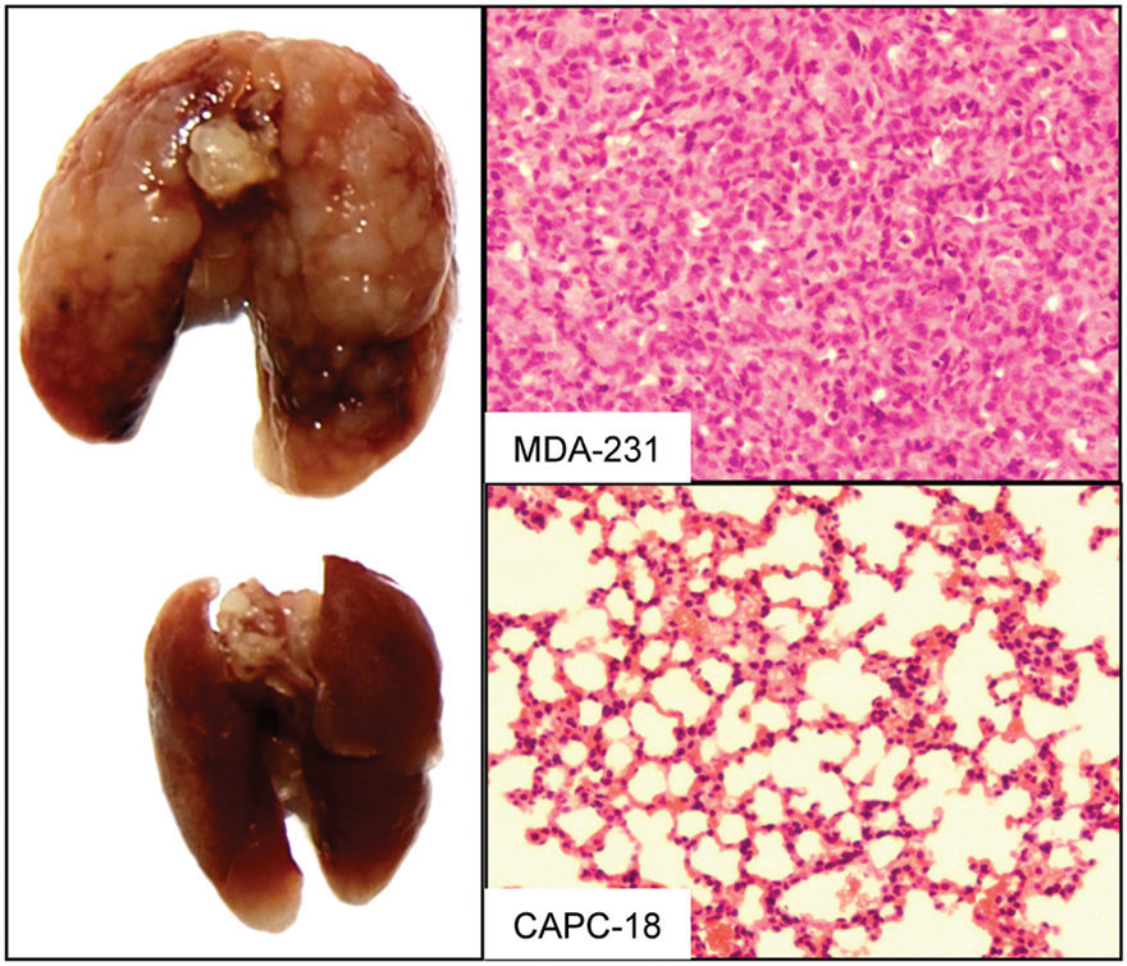

Figure 5.

Lung metastasis of breast tumor line MDA-231 and MDA-CAPC-18. The MDA-231 or MDA-CAPC-18 cells were injected into athymic mice through the tail vein. After 8 wk, the lungs were fixed and photographs taken (top left, lung injected with MDA-231; bottom, MDA-CAPC-18). The right two panels are representative lungs sectioned and stained with H&E to show the morphology of lung and lung tumors (100X).

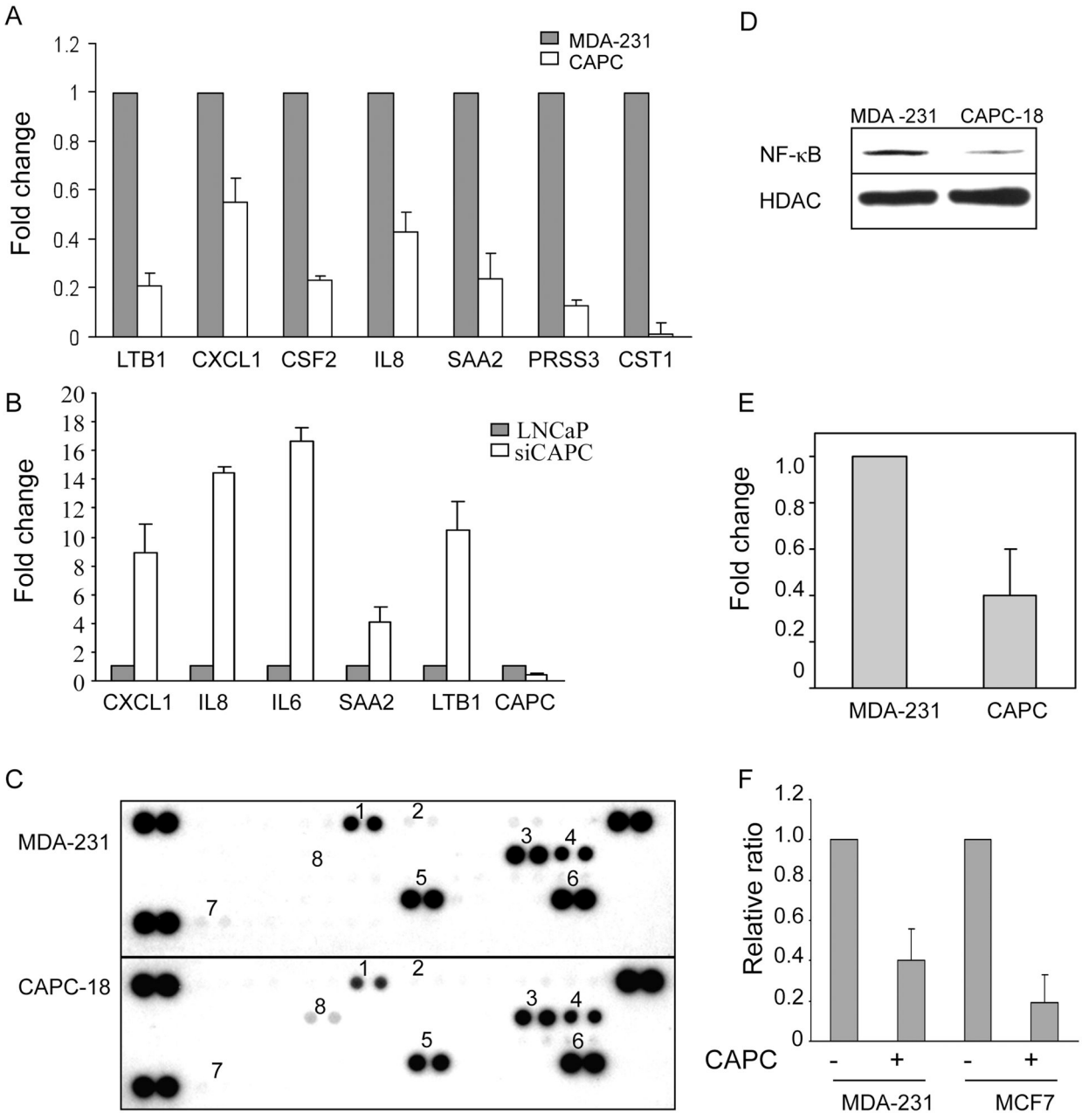

Figure 6.

CAPC over-expression modulated secreted genes and proteins and NF-κB activation. **A** and **B**. Real time PCR analysis of gene expression comparing MDA-231 and MDA-CAPC-18 (**A**) or LNCaP and siCAPC (**B**). The relative expression level was adjusted with endogenous actin as control by R.Q. manager software. **C**. Human cytokine array. 1, CSF2; 2, CXCL1; 3, IL6; 4, IL8; 5, MIF (GIF); 6, Serpin; 7, CCL5; and 8, IL1ra. **D**. Nuclear fractions of MDA-231 and MDA-CAPC-18 were isolated and nuclear extracts were blotted with anti-NF-κB p65. HDAC is a loading control. **E**. Quantitative analysis of NF-κB expression in nucleus using NIH Image J software. The data is the average of three separate experiments. **F**. Cells were transfected with NF-κB-Luc and Renilla plasmids. Luciferase activity was

measured after 48 hr transfection. The Luciferase activity was normalized based on Renalla activity. The relative ratios were an average of three independent experiments.

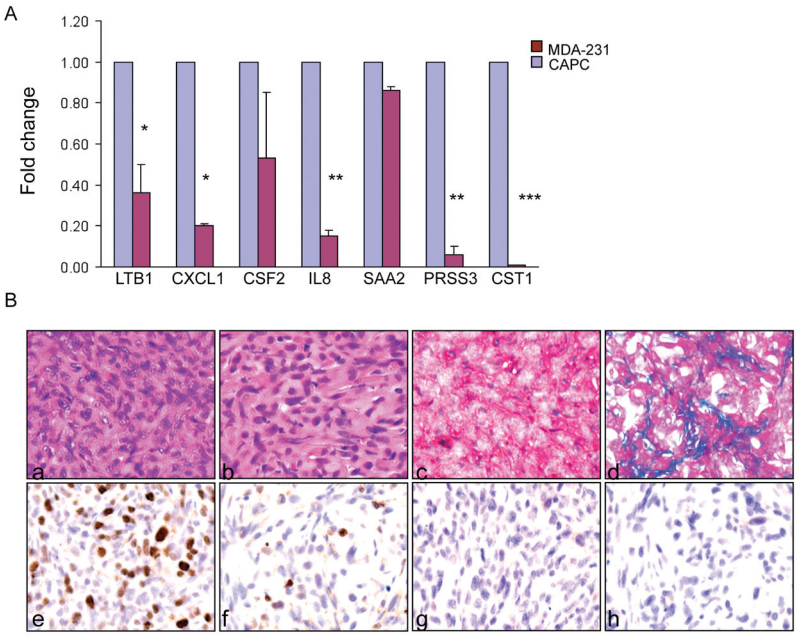

Figure 7.

CAPC expression changed tumor microenvironment and cell proliferation in mouse tumor xenografts. **A**. Real time PCR analysis of genes encoding cytokines and proteases in mouse tumor xenografts (three separate tumor from three mice). **B**. Morphology of mouse tumor xenograft from MDA-231 (a, c, e, g) and MDA-CAPC-18 (b, d, f, h) stained by H&E (a, b) and Masson (c, d) or stained with anti-Ki67 (e, f) and anti-cleaved caspase-3 (g, h). Original magnification ×200. Tumor samples were 2-wk-old tumor xenografts.