Original Research

Activated breast stromal fibroblasts exhibit myoepithelial and mammary stem cells features

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ARTICLE INFO

Keywords:
Breast cancer
Cancer-associated fibroblasts
Mammary stem cells
MET
Myoepithelial

ABSTRACT

Background: Active breast cancer-associated fibroblasts (CAFs) promote tumor growth and spread, and like tumor cells they are also heterogeneous with various molecular sub-types and different pro-tumorigenic capacities.

Methods: We have used immunoblotting as well as quantitative RT-PCR to assess the expression of various epithelial/mesenchymal as well as stemness markers in breast stromal fibroblasts. Immunofluorescence was utilized to assess the level of different myoepithelial and luminal markers at the cellular level. Flow cytometry allowed to determine the proportion of CD44- and ALDH1-positive breast fibroblasts, while sphere formation assay was used to test the ability of these cells to form mammospheres.

Results: We have shown here that IL-6-dependent activation of breast and skin fibroblasts promotes mesenchymal-to-epithelial transition and stemness in a STAT3- and p16-dependent manner. Interestingly, most primary CAFs isolated from breast cancer patients exhibited such transition and expressed lower levels of the mesenchymal markers N-cadherin and vimentin as compared to their adjacent normal fibroblasts (TCFs) isolated from the same patients. We have also shown that some CAFs and IL-6-activated fibroblasts express high levels of the myoepithelial markers cytokeratin 14 and CD10. Interestingly, 12 CAFs isolated from breast tumors showed higher proportions of CD24low/CD44high and ALDHhigh cells, compared to their corresponding TCF cells. These CD44high cells have higher abilities to form mammospheres and to enhance cell proliferation of breast cancer cells in a paracrine manner relative to their corresponding CD44low cells.

Conclusion: Together, the present findings show novel characteristics of active breast stromal fibroblasts, which exhibit additional myoepithelial/progenitor features.

Introduction

Breast cancer-associated fibroblasts (CAFs) have different origins, and while some remain quiescent the majority are active with cancer-promoting capacity, and are also referred to as myofibroblasts [1,2]. While fibroblasts exhibit a thin and small spindle morphology, active CAFs are large and flat cells with different shapes and prominent nucleoli [3]. Owing to lack of precise molecular definition of CAFs, these cells are most often defined by their morphological characteristics and the expression of some markers [4]. The pro-carcinogenic effects of myofibroblasts are related to the production of high levels of growth factors, cytokines and chemokines, which can promote epithelial-to-mesenchymal transition (EMT) as well as stemness [5–7]. Although most of these cells express high levels of α-SMA, active fibroblasts constitute a large and heterogeneous cell pool expressing different molecular markers, which are not specific for fibroblasts, such as fibroblast activation protein (FAP) and fibroblast-specific protein-1 (FSP-1) [2,8]. In a recent study, Su et al. have identified a subset of active CAF cells, which express CD10 and GPR77 and can promote stemness and chemoresistance within breast cancer cells [9]. Therefore, what are CAFs? In fact, these myofibroblasts share several molecular and cellular features with normal fibroblasts, their shape and secretome resemble those of senescent cells, express some myoepithelial markers and exhibit some cancer features [2,4,10]. These findings raised an important question on the identity of these cells.

Aside from promoting breast carcinogenesis, active fibroblasts can modulate cancer response, drug accessibility and immune regulation, which explains their association with poor patient survival [11].

Abbreviations: BSF, breast stromal fibroblast; CAF, cancer-associated fibroblast; IL-6, interleukin-6; SFM, serum-free media; SFCM, serum-free conditioned media; TCF, tumor counterpart fibroblast.

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https://doi.org/10.1016/j.tranon.2023.101721
Received 5 April 2023; Received in revised form 18 May 2023; Accepted 11 June 2023
Available online 15 June 2023
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Thereby, myofibroblasts have gained momentum in anticancer drug discovery, taking into account their genetic stability and their presence across different cancer types [11]. To achieve this, better characterization of these cells and their functional interplay with cancer cells is mandatory. We have recently shown using indirect co-culturing that triple-negative breast cancer cells can increase the expression of the α-SMA protein and activate breast stromal fibroblasts in an IL-6-dependent manner. This also enhanced their migratory/invasiveness as well as their proliferative capacities [12]. Therefore, we sought in the present study to further characterize these active CAFs at both the molecular and cellular levels. We have shown that the activation of breast stromal fibroblasts triggers mesenchymal-to-epithelial transition, and also promotes the expression of stemness markers.

Materials and methods

Cells and cell culture

Breast fibroblast cells were obtained and used as previously described [10]. Fibroblast cells were purified through 2 sorting steps as previously described [13]. Briefly, breast tissues were cut into small pieces (1–2 mm diameter), placed in digestion solution (400 IU collagenase, 100 IU hyaluridinase and 10% Serum (Stem Cell Technologies), containing antibiotics and antymiotics (Gibco), and agitated at 37 °C overnight in an Adams Nutator Mixer (Becton Dickinson, Franklin Lakes, NJ). Cells were separated by differential centrifugation at 110 g for 2 min at 4 °C. The supernatant (mesenchymal-enriched fraction) containing fibroblasts was centrifuged at 485 g for 8 min, resuspended in fibroblast growth medium and cultured at 37 °C, 5% CO2. Normal breast fibroblasts (NBF-6) cells that were obtained from plastic surgery were further purified by sorting by the antibody cocktail Ep-CAM APC, CD90 in addition to CD49F-PE, MUC-1 FITC, and CD10 PE-Cy7 utilizing BD FACSARIA cell sorter (BD Biosciences, USA). These cells were maintained as primary and were used at relatively low passages (8–12). HFSN-1 are primary skin fibroblasts. MCF-7 and MDA-MB-231 cells were purchased from ATCC and were authenticated using short tandem repeat profiling by ATCC, propagated, expanded, and frozen immediately into numerous aliquots after arrival. Cells were regularly screened for mycoplasma contamination using MycoAlert Mycoplasma Detection Kits (Lonza). All supplements were obtained from Sigma (Saint Louis, MO, USA) except for antibiotic and antymycotics solutions, which were obtained from Gibco (Grand Island, NY, USA). Cells were maintained at 37 °C in humidified incubator with 5% CO2.

All methods were performed in accordance with relevant guidelines and regulations. Written informed consent was not required and a waiver was granted by institutional review board approval (Research Ethics Committee, under RAC#2,180,018) of King Faisal Specialist Hospital and Research Center, since the study was retrospective and samples were anonymized to the research team. This was granted by the institutional review board approval (Research Ethics Committee, under RAC#2,180,018) of King Faisal Specialist Hospital and Research Center.

Human IL-6 recombinant protein (hBA-184) (Santa Cruz, CA).

Cellular lysate preparation and immunoblotting

This has been performed as previously described (5). Antibodies directed against, α-SMA, IL-6 and Vimentin (RV202) were purchased from Abcam (Cambridge, MA). ALDH1/2 (H-85), CD24 (C-20), and GAPDH (FL-335) were purchased from Santa Cruz Biotech (Santa Cruz, CA). p16 was purchased from BD. E-cadherin (24E10), N-cadherin, AKT (C73H10), p-AKT (Thr308), Sox2 (D6D9) and EpCAM (D1B3) Cell Signaling Technology (Danvers, MA). CD44 was purchased from (Sigma-Aldrich).

RNA purification and qRT-PCR

Total RNA was purified using the miRNeasy mini kit (Qiagen, USA) according to the manufacturer’s instructions and was treated with RNase-free DNase. RNA (1 μg) was used to synthesize complementary deoxyribonucleic acid (cDNA) utilizing the Advantage RT-PCR kit. qRT-PCR was performed in triplicate using cDNA (4 μl) mixed with 2x FastStart Essential DNA Green qPCR master mix (5 μl) and primers mix (1 μl). Amplifications were performed utilizing the LightCycler 96 Real-time PCR detection system. GAPDH expression levels were used for normalization, and gene expression differences were calculated using the threshold cycle (Ct). The respective primers are:

- **GAPDH**: 5’-GAGGTCACAGGGCTTCTC-3’ and 5’-GGGGTGCTAAGCAGTTGTG-3’
- **CDH2**: 5’-CTTCCA GAG TTT ACT GCC ATG AC-3’ and 5’-GTA GGA TCT CGG CCA ATG ATT C-3’
- **CDH1**: 5’-CCC GCC TTA TGA TTC TGG CTG T-3’ and 5’-GCC GTC CAT GTC AGC GAG TCT G-3’
- **ALDH1A1**: 5’-TCC TTA TTT TTT TCC CCC TCC T-3’ and 5’-ACC ATC TTT GAA GGG TTG GC-3’

siRNA transfection

STAT3 siRNA and control siRNA were obtained from Cell Signaling technology. The transfections were carried out using the High Perfect reagent (Qiagen, USA) as recommended by the manufacturer. Exponentially growing cells were treated with 30 nM siRNA or a scrambled sequence for 3 days, and then the medium was changed to complete medium.

**CDKN2A-ORF transfection**

Lentivirus-based vectors bearing **CDKN2A-ORF** and the corresponding control were used to carry out transfections using human dermal fibroblast nucleofector 2000 transfection kit (Invitrogen, USA) following the manufacturer’s recommendations. Exponentially growing cells were transfected with 1 μg of DNA, and after 5 days, transfected cells were selected with puromycin (1 μg/mL).

Cell sorting, flow cytometry and aldeflour assay

CD44- and CD44- cells were selected from different sets of CAFs by flow cytometric cell sorting (FACS). The FACS was performed on single cell suspensions using the flow cytometer (BD FACSaria). Before FACS sorting, exponentially growing fibroblasts were detached by trypsin, centrifuged and then washed with PBS. DAPI staining confirmed that more than 95% cells were live. Cells were then resuspended in PBS containing 1% PBS and incubated with antibody against CD44 for 30 min at 4 °C. For CD44/CD24 flow cytometry analysis, single cells were resuspended in PBS containing 1% PBS and were stained with fluorescent-conjugated antibody against CD44 and CD24. Aldeflour assay was performed according to manufacturer’s instruction (StemCell Technology). Flow cytometry data were acquired using NovoCyte Flow Cytometer (ACEA Bioscience).

Immunofluorescence

Cells for immunofluorescence were blocked with 4% paraformaldehyde for 15 min at room temperature, washed with PBS and permeabilized with 0.25% Triton X-100 in PBS for 10 min. After washing with PBS, cells were incubated with 4% BSA for 30 min, incubated with indicated primary antibodies overnight at 4 °C. Washed with PBS and incubated with Alexa Fluor conjugated secondary antibodies for 30 min. Next, cells were counter stained with Alexa Flour 488 Phallolidin for cytoskeleton and DAPI for nuclei. Images were captured by Olympus microscope. Primary antibodies used: Cytokeratin 14
(LL002), Cytokeratin 19 (EP1580Y) were purchased from Abcam.

**Sphere formation assay**

FACS-sorted CD44\(^{\text{high}}\)/CD44\(^{\text{low}}\) CAF cells (5000 cells/ml) were cultured in ultra-low attachment plates (Corning) in serum-free DMEM-F12 (Life Technologies) containing stem cell culture supplements. After 10 days in culture, spheres >50 μm were counted under phase-contrast microscopy (Olympus), and the size and number of spheroids were calculated from 5 random fields using Image J (images.nih.gov). The number of CD44\(^{\text{high}}\) spheres >50 μm formed under the sphere culture condition was 8–12 per field, while the number of CD44\(^{\text{low}}\) spheres formed >50 μm was 0–2 per field. The spheres less than 50 μm were not counted. Three independent experiments were conducted.

**Co-culture experiments**

Co-culture experiments were performed by seeding MCF-7 breast cancer cells (5.10\(^3\)) in the lower chamber and FACS sorted CD44\(^{+}\)/CD44\(^{-}\) CAF cells (5.10\(^4\)) in the upper chamber of 6-well transwell apparatus with 0.4 μm pore (Corning NY, USA), and were incubated for 48 h. MCF-7 cells were trypsinized and counted. Three independent experiments were conducted.

**Cell migration, invasion and proliferation assays**

This has been performed as previously described [14]. These assays were performed in a label-free real-time setting using the xCELLigence RTCA technology (Roche, Germany) that measures impedance changes in a meshwork of interdigitated gold microelectrodes located at the well bottom (E-plate) or at the bottom side of a micro-porous membrane (CIM plate 16). Cell migration and invasion were assessed as per the manufacturer’s instructions. In brief, 2 × 10\(^4\) cells in serum-free medium were added to the upper wells of the CIM-plate coated with a thin layer of Matrigel (BD Biosciences) basement membrane matrix diluted 1:20 in serum-free medium (invasion) or non-coated (migration). Complete medium was used as a chemo-attractant in the lower chambers. Subsequently, the plates were incubated in the RTCA for 24 h and the impedance value of each well was automatically monitored by the xCELLigence system and expressed as Cell Index (CI) value, which represents cell status based on the measured electrical impedance change divided by a background value. Each assay was biologically performed in triplicate.

For the proliferation assay, exponentially growing cells (2 × 10\(^4\)) were seeded in E-plate with complete medium as per the manufacturer’s instruction. Cell proliferation was assessed for 72 h. All data were recorded and analyzed by the RTCA software. Cell Index was used to measure the change in the electrical impedance divided by a background value, which represents cell status. Each assay was biologically performed in triplicate.

**Conditioned media**

Cells were cultured in medium without serum for 24 h, and then media were collected and briefly centrifuged. The resulting supernatants were used either immediately or were frozen at -80 °C until needed.

**Quantification of protein expression level**

The protein signal intensity of each band was determined using ImageQuant TL software (GE Healthcare). Next, dividing the obtained value of each band by the value of the corresponding internal control allowed a correction of the loading differences. The fold change in the protein levels was determined by dividing the corrected values by that of the control.

**Statistical analysis**

Statistical analysis was performed using Microsoft Excel and Prism softwares. The results are presented as mean ± SED. of independent experiments. Statistical significance was determined using a two-tailed Students t-test and P values of 0.05 and less were considered as statistically significant.

**Results**

**Activation of human fibroblasts promotes mesenchymal-to-epithelial transition**

We have previously shown that serum-free medium (SFM) conditioned with MDA-MB-231 cells (MDA-SFCM) and the interleukin-6 activates breast stromal fibroblasts [12]. To confirm this, whole cell lysates were prepared from normal breast fibroblast (NBF-6) cells exposed either to IL-6 (3.5 ng/mL) or SFCM from MDA-MB-231 cells treated either with IL-6 specific siRNA or a scrambled sequence used as control [12]. SFM was used as a negative control. Fig. 1A shows IL-6- and MDA-SFCM-dependent up-regulation of the myofibroblast marker α-SMA, and this effect was abolished in cells wherein IL-6 was knocked-down as compared to controls. Next, we investigated the possible changes in the expression of the epithelial and/or mesenchymal markers. Fig. 1A shows IL-6-dependent increase in the epithelial markers (E-cadherin and EpCAM), and a decrease in the mesenchymal markers (N-cadherin and vimentin). Similarly, MDA-SFCM and IL-6 up-regulated α-SMA as well as the epithelial markers (E-cadherin and EpCAM), while reduced the level of the mesenchymal markers (N-cadherin and vimentin) in the human skin fibroblasts HFSN1 (Fig. 1B). In addition, the migration/invasion as well as proliferation of HFSN1 cells were higher in cells treated with IL-6 or MDA-SFCM as compared to SFM (Fig. 1C). This was confirmed at the molecular level by showing the activation of AKT, an important pro-migratory/invasive protein kinase [15], upon treatment with IL-6 or MDA-SFCM (Fig. 1D). This indicates the induction of the mesenchymal-to-epithelial transition (MET) upon activation of fibroblast cells.

**IL-6 induces MET features in breast stromal fibroblasts in a STAT3- and p16-dependent manner**

We have previously shown that myofibroblasts express low levels of p16, and IL-6-dependent activation of breast stromal fibroblasts is inhibited by p16 up-regulation or STAT3 knock-down [12,16]. Therefore, we sought to investigate the effect of these genes in IL-6-dependent induction of the MET features in breast stromal fibroblasts (NBF-6). Fig. 1E shows that ectopic expression of p16 inhibits IL-6-related up-regulation of α-SMA and also the MET-related features as compared to control cells. Indeed, p16 up-regulation abolished IL-6-dependent increase in the level of the epithelial markers (E-cadherin and EpCAM) as well as the decrease in the level of the mesenchymal markers (N-cadherin and vimentin) compared to controls (Fig. 1E). Furthermore, p16 abolished IL-6-dependent activation of AKT (Fig. 1E). Interestingly, similar results were obtained when the IL-6 downstream target STAT3 was knocked-down by specific siRNA (Fig. 1F). This indicates that p16 down-regulation and STAT3 activation are important for IL-6-dependent induction of the MET process in breast fibroblasts.

**Breast cancer-associated fibroblasts display mesenchymal-to-epithelial transition**

To confirm that active breast stromal fibroblasts express high levels of the epithelial markers and low levels of the mesenchymal ones, we assessed the expression of some key markers of the MET process in 11 pairs CAF/TCF (TCF: tumor counterpart fibroblasts, isolated from
IL-6 induces MET features in human fibroblast cells

Fig. 1. IL-6 induces MET features in human fibroblast cells. (A) NBF-6 cells were cultured either in SFM in absence (−) or presence of IL-6 (+IL-6) or in MDA-SFCM post-transfection with specific IL-6 siRNA (IL-6) or a scrambled sequence (Ctl) for 24 h. Whole cell lysates were prepared, and immunoblotting analysis was performed using antibodies against the indicated proteins. GAPDH was used as internal control. (B) and (D) HFSN1 cells were incubated for 24 h in either SFM, SFM containing IL-6 or MDA-SFCM. Whole cell lysates were prepared, and immunoblotting analysis was performed using antibodies against the indicated proteins. (C) Exponentially growing HFSN1 cells were treated as indicated, and then the proliferation, migration and invasion capabilities were assessed using the RTCA-DP xCELLigence system. Data are representative of different GAPDH was used as internal control. (B) and (D) HFSN1 cells were incubated for 24 h in either SFM, SFM containing IL-6 or MDA-SFCM. Whole cell lysates were prepared, and immunoblotting analysis was performed using antibodies against the indicated proteins. (C) Exponentially growing HFSN1 cells were treated as indicated, and then the proliferation, migration and invasion capabilities were assessed using the RTCA-DP xCELLigence system. Data are representative of different experiments performed in triplicate. (E) NBF-6 cells were transfected with a vector bearing the CDKN2A gene, while an empty vector was used as control (Ctl). Cells were then exposed for 24 h to SFM or SFM containing IL-6 as indicated. Subsequently, whole cell lysates were prepared, and then immunoblotting analysis was performed using specific antibodies against the indicated proteins. (F) STAT3 was knocked-down in NBF-6 cells using specific siRNA (a scrambled sequence was used as control, Scr). Whole cell lysates were prepared, and then immunoblotting analysis was performed using specific antibodies against the indicated proteins. The numbers below the bands show protein level fold change relative to the corresponding control after normalization against the internal control GAPDH, while the phospho-protein was further normalized to the total protein. The immunoblotting experiments were repeated at least twice. The recombinant human IL-6 was used at 3.5 ng/mL.

The fact that active CAFs express high levels of α-SMA and also exhibit epithelial characteristics, prompted us to investigate whether they gained some myoepithelial features. Therefore, we assessed the expression of the main myoepithelial marker cytokeratin 14 [17] in 2 CAF/TCF pairs (chosen randomly) as well as in normal breast fibroblast cells (NBF-6) by immunofluorescence. Fig. 3A shows that NBF-6 cells express very low level of cytokeratin 14, which was up-regulated in the active IL-6 treated cells. Similarly, the active CAF-64 and CAF-180 cells expressed higher levels of cytokeratin 14 relative to their correspondent adjacent counterparts TCF-64 and TCF-180, respectively (Fig. 3A). A triple immunofluorescence staining using F-actin shows the shape of cells and higher level of cytokeratin 14 in CAF-64 relative to the corresponding TCF-64 cells (Fig. 3B). In addition, immunoblotting analysis showed upregulation of the myoepithelial marker CD10 [18] in 3 out of 4 CAFs as compared to their corresponding TCFs (Fig. 3B). For the CAF/TCF-87 pair, the level of CD10 was very high even in TCF-87 cells (Fig. 3B). These results indicate that most active breast fibroblasts acquired myoepithelial features.

Breast myofibroblasts exhibit myoepithelial features

The fact that active CAFs express high levels of α-SMA and also exhibit epithelial characteristics, prompted us to investigate whether they gained some myoepithelial features. Therefore, we assessed the expression of the main myoepithelial marker cytokeratin 14 [17] in 2 CAF/TCF pairs (chosen randomly) as well as in normal breast fibroblast cells (NBF-6) by immunofluorescence. Fig. 3A shows that NBF-6 cells express very low level of cytokeratin 14, which was up-regulated in the active IL-6 treated cells. Similarly, the active CAF-64 and CAF-180 cells expressed higher levels of cytokeratin 14 relative to their correspondent adjacent counterparts TCF-64 and TCF-180, respectively (Fig. 3A). A triple immunofluorescence staining using F-actin shows the shape of cells and higher level of cytokeratin 14 in CAF-64 relative to the corresponding TCF-64 cells (Fig. 3B). In addition, immunoblotting analysis showed upregulation of the myoepithelial marker CD10 [18] in 3 out of 4 CAFs as compared to their corresponding TCFs (Fig. 3B). For the CAF/TCF-87 pair, the level of CD10 was very high even in TCF-87 cells (Fig. 3B). These results indicate that most active breast fibroblasts acquired myoepithelial features.

IL-6 induces stem/progenitor markers in fibroblast cells in a STAT3- and p16-dependent manner

In addition to cytokeratin 14, CAFs as well as IL-6-activated fibroblasts also expressed high levels of cytokeratin 19 (Fig. 3A and B). In addition, qRT-PCR analysis confirmed cytokeratin 19 (KRT19) up-regulation in 4 CAFs as compared to their adjacent counterpart TCFs (Fig. 3C). While CAF-144 showed lower level of the KRT19 mRNA as compared to control cells (Fig. 3C). The fact that this cytokeratin has been proposed to be a stem/progenitor marker, suggested that these myofibroblasts may also possess progenitor/stem features [19,20]. Therefore, we sought to assess the expression of other important stemness markers. We have found that the treatment with IL-6 or MDA-SFCM induced mammary stem cells features (CD44high/CD24low/ALDHpositive) in both NBF-6 as well as HFSN1 fibroblasts (Fig. 4B and C). This suggests that myofibroblasts or CAFs exhibit epithelial as well as stemness features. Fig. 4D shows that ectopic expression of p16 inhibits IL-6-related
up-regulation of ALDH/CD44 and down-regulation of CD24. Similar effect was obtained with STAT3 specific siRNA (Fig. 4E). This shows that, like MET, IL-6-dependent induction of stemness features in breast stromal fibroblasts requires STAT3 induction and p16 down-regulation.

Cancer-associated fibroblasts express low levels of the mesenchymal markers

Fig. 2. Cancer-associated fibroblasts express low levels of the mesenchymal markers. (A) Whole cell lysates were prepared from the indicated TCF/CAF pairs and the expression levels of the indicated proteins were assessed by immunoblotting. The numbers below the bands show protein level fold change relative to the corresponding control after normalization against the internal control GAPDH. These experiments were repeated at least twice. (B) and (C) Total RNA was extracted from the indicated TCF/CAF pairs, and then the mRNA levels of the indicated genes were determined by qRT-PCR. Error bars indicate mean ± SD, (n = 3). *: p ≤ 0.05; **: p ≤ 0.01.

Active fibroblasts express high levels of myoepithelial markers

Fig. 3. Active fibroblasts express high levels of myoepithelial markers. (A) and (B) Representative images of immunofluorescence staining in the indicated breast fibroblasts using the antibodies specific for the indicated cytokines, in addition to DAPI and F-actin. Scale bar = 100 µm. (C) Whole cell lysates were prepared from the indicated TCF/CAF pairs, and then immunoblotting analysis was performed using antibodies against the indicated proteins. The numbers below the bands show protein level fold change relative to the corresponding control after normalization against the internal control GAPDH. These experiments were repeated at least twice. (D) Total RNA was extracted from the indicated cells, and then the mRNA level of the cytokeratin 19 was determined by qRT-PCR. Error bars indicate mean ± SD, (n = 3). *: p ≤ 0.05; **: p ≤ 0.01.
CAFs from breast cancer tissues exhibit stemness features

Next, we have checked the status of the stemness markers in CAF/TCF pairs isolated from breast cancer tissues. Flow cytometry analysis shows clear increase in the proportion of CD24$^{\text{low}}$/CD44$^{\text{high}}$ subpopulation in CAF cells (CAF-64, CAF-87 and CAF-900 with 6.17%, 7.21% and 4.87%, respectively) as compared to their corresponding counterparts (TCF-64, TCF-87 and TCF-900 with 1.21%, 2.01% and 1.16%, respectively) (Fig. 5A). Additionally, we examined the ALDH (aldehyde dehydrogenase) activity in paired CAFs and TCFs cells. We used gating strategy for the exclusion by incubating cells with DEAB and were used as negative control (Fig. 5B). Remarkably, all CAF cells (CAF-64, CAF-87 and CAF-900) exhibited higher ALDH activity (57.21%, 21.31% and 5.3%) as compared to their corresponding TCF cells (TCF-64, TCF-87 and TCF-900) with ALDH activity as low as 37.89%, 14.27% and 1.52%, respectively (Fig. 5B).

Furthermore, Fig. 5C shows that most CAFs express the markers of mammary stem cells (CD44$^{\text{high}}$/CD24$^{\text{low}}$/ALDH$^{\text{high}}$) as compared to their corresponding TCFs. Indeed, relative to their corresponding TCFs, 9 out of 12 CAFs expressed higher level of ALDH1, and CD44,
while 10 out of 12 showed lower level of CD24 (Fig. 5C). Interestingly, 8 out of 12 CAFs showed the CD44\textsuperscript{high}/CD24\textsuperscript{low} characteristic, and 6 out of 12 CAFs exhibited the (CD44\textsuperscript{high}/CD24\textsuperscript{low}/ALDH\textsuperscript{high}) feature of mammary stem cells (Fig. 5C). The increase in the expression level of ALDH1 was confirmed at the mRNA level, by showing that 6 CAFs expressed higher levels of the ALDH1 mRNA compared to their corresponding TCFs (Fig. 5D). On the other hand, the pluripotency marker Sox-2 was very lowly expressed in the majority of the CAF/TCF pairs, and showed only a slight increase in CAF-64, CAF-153 and CAF-69 cells relative to their corresponding TCFs (Fig. 5C). These results indicate that active fibroblasts show MET as well as stemness features but they did not reach pluripotency.

CD44\textsuperscript{high} CAF cells have higher ability to form spheres and enhance breast cancer cell growth in vitro

After showing that a proportion of CD44\textsuperscript{high} CAF cells express mammary stem cells markers and exhibit higher ALDH activity, we sought to investigate the potential of these cells in forming spheres. To this end, we have first sorted CD44\textsuperscript{high} cells from various CAF cells, and then equal numbers of the sorted cells were used for sphere formation in ultralow attachment plates. Interestingly, CD44\textsuperscript{high} cells generated significantly more spheres than CD44\textsuperscript{low} cells from all three CAF cultures (Fig. 6A). Next, we investigated the paracrine pro-carcinogenic effect of these CAF-CD44\textsuperscript{high} cells, by investigating their effect on the proliferation of MCF-7 cells in a coculture setting. Therefore, we isolated CD44\textsuperscript{high} and CD44\textsuperscript{low} cells from 3 different CAF cells as described above and cocultured them separately with the slowly proliferating MCF-7 cells. Fig. 6B shows clear differences in MCF-7 cell growth between CD44\textsuperscript{low} and their corresponding CD44\textsuperscript{high} cells. Indeed, MCF-7 growth was significantly enhanced in the presence of CD44\textsuperscript{high} cells as compared to their corresponding CD44\textsuperscript{low} cells (Fig. 6B). These results suggest that CAF cells contain CD44\textsuperscript{high} subpopulation with stem cell features and higher paracrine pro-carcinogenic effects.

Discussion

Cancer-associated fibroblasts are very active promoters of breast carcinogenesis through functional cross-talks with cancer cells via secretions that contain metabolites, cytokines and other types of molecules. CAFs constitute a large heterogeneous population of cells that express various types of markers, which are not unique for these cells. This vast heterogeneity could be owing to various origins of CAFs or their mode/condition of activation [2,4]. We have shown in the present study that MDA-MB-231- and IL-6-dependent activation of normal fibroblasts upregulates the epithelial markers (E-cadherin and EpCAM) but downregulates the mesenchymal markers (N-cadherin and vimentin). This suggests that the activation of fibroblasts is accompanied by a transition from the mesenchymal to the epithelial state (MET). Intriguingly, despite this MET, these cells showed higher migration, invasion and proliferation capacities as compared to controls (Fig. 1C and [12]). This could be explained by the fact that these cells express high levels of the active form of the pro-invasive/migratory and -proliferative protein kinase AKT [15] (Fig. 1D). Interestingly, ectopic expression of p16 or knock-down of STAT3 in breast stromal fibroblasts suppressed the IL-6-related activation of these cells and their transition to epithelial status. This indicates that while p16 inhibits the IL-6 induced MET, STAT3 activation was required for the acquisition of the epithelial traits.

The development of epithelial features and down-regulation of mesenchymal markers were confirmed in 12 patient-derived CAFs and their adjacent counterpart TCFs. The active status of CAFs was confirmed by the higher expression level of α-SMA, which was accompanied in most CAFs with increased level of E-cadherin and reduced level of N-cadherin and vimentin as compared to their corresponding

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**CD44\textsuperscript{high} CAF cells form mammospheres and promote breast cancer cells growth**

Fig. 6. CD44\textsuperscript{high} CAF cells form mammospheres and promote breast cancer cells growth. (A) Representative images of spheres formed from CAF sorted CD44\textsuperscript{high}/CD44\textsuperscript{low} cells and growth in ultralow attachment stem cells culture conditions. The number of spheres was determined and was plotted as bar graphs. (B) Breast cancer MCF-7 cells were co-cultured CD44\textsuperscript{high}/CD44\textsuperscript{low} CAF cells for 24 h, and then cells were counted and plotted as bar graphs. Three independent experiments were conducted for each CAF cells.
TCFs. This indicates that, like MDA-MB-231 cells and IL-6, breast cancer cells activate fibroblasts and trigger MET within tumors. It has been previously shown that the complete reprogramming of fibroblasts into pluripotent stem cells entails also up-regulation of the epithelial markers and down-regulation of the mesenchymal markers [21–23]. This confirms the importance of the MET process during fibroblast reprogramming.

Importantly, active CAFs expressed high levels of the myoepithelial markers cytokeratin 14 in addition to α-SMA, which is highly expressed in both myofibroblasts and myoepithelial cells. This indicates that some active CAFs acquired some myoepithelial cell characteristics. These findings confirm what has been recently shown by Su et al. who have shown the expression of the myoepithelial cell marker CD10 on a subset of active CAF cells [9]. This was confirmed here by showing that CAFs express high level of the CD10 protein. This suggests that active fibroblasts develop myo-fibroblast as well as myo-epithelial traits, with a novel shape different from that of fibroblast and myoepithelial cells. Indeed, while myoepithelial cells are stellate in shape, and fibroblasts have spindle-shape, myofibroblasts are characterized by branching and flat morphology [2,4]. In addition, active fibroblasts expressed higher levels of cytokeratin 19, which is considered as a neutral switch cytokeratin whose expression is compatible with cell type flexibility and acquisition of progenitor/stem features [20,24]. Moreover, Villadsen et al. have detected cytokeratin 14/keratin 19 double positive cells in adult human mammary glands, and have shown that these cells exhibit progenitor cell characteristics [19]. This suggests that a proportion of active fibroblasts have developed some progenitor features. This was confirmed by showing that CAF cells express higher level of the progenitor/stem marker c-Kit than their corresponding counterparts TCF cells.

In addition, we have shown that IL-6-activated breast and skin fibroblasts also exhibited the (CD44high/CD24low/ALDHhigh) feature of mammary stem cells, in a p16- and STAT3-dependent manner. Furthermore, we have shown that the proportions of CD24low/CD44high as well as ALDHhigh sub-populations were higher in CAF cells compared to their counterpart TCF cells isolated from the same patient. This showed that activated fibroblasts acquired stem/progenitor cell features. This was confirmed in several pairs CAF/TCF cells isolated from 12 different patients. Indeed, most of these cells exhibited the (CD44high/CD24low/ALDHhigh) feature of mammary stem cells (Fig. 5C). The high level of ALDH1 in CAFs was also confirmed at the mRNA level in 6 CAF/TCF pairs (Fig. 5D). Together, these findings indicate that CAFs are myofibroblasts that express myoepithelial as well as stem cell markers, with high migratory/invasive and proliferative capacities. This confirms that they do not represent a cell type but rather a cell state, as has been previously proposed by Madar et al. [25].

Interestingly, we have also shown that this CD44high sub-population of CAF cells has much higher capacity to grow in suspension and form mammospheres compared to CD44low sub-population. Furthermore, these cells have higher capacity to promote cell growth of breast cancer cells in a co-culture setting (Fig. 5). Similarly, it has been previously shown that CD44 was abundantly expressed on immortalized CAF cells isolated from melanoma bearing mice, and that CD44-positive cells supported the stemness and drug resistance of cancer cells [26]. This indicates that a proportion of CAFs exhibit stemness features and have higher paracrine pro-carcinogenic effects than the corresponding CD44-negative cells. The fact that the pluripotency marker Sox2 was only low expressed in most CAF/TCF pairs, indicates that these CAF-stem cells did not reach pluripotency. Therefore, which type of stem cells are formed in active breast fibroblasts? and what are their origin? Ishikawa et al. have shown simultaneous expression of cancer stem cell-like and CAF-like properties in a primary culture of breast cancer cells [27]. In another study, it has been reported the presence of multipotent CAF cells in hepatocellular carcinoma tissues [28]. Furthermore, Herrera et al. showed that most pro-migratory CAFs from human colon tumors expressed stem cell markers [29]. Together, these findings indicate that most active CAFs contain a sub-population of cells with stem features, and that this population possesses the higher pro-carcinogenic ability. In a recent publication, Nair et al. have proposed a cancer stem cell model as the point of origin of CAFs [30]. This suggests that CAF cells in a tumor may have different origins with different gene expression signature and consequently different effects on cancer cells. It’s also possible that CAF heterogeneity arises from different paracrine signaling effects from heterogeneous cancer cells as well as other active stromal cells such as adipocytes and macrophages. Indeed, we have shown here that TNBC cells (MDA-MB-231) as well as IL-6 can promote stemness in breast stromal fibroblasts.

In conclusion, the present findings indicate that great proportion of active breast stromal fibroblasts develop progenitor/stem cells features. This allows a better understanding of these cells, which represent a permanent cell state rather than a cell type, and opens new avenues towards their specific therapeutic targeting.

Consent for publication
Not applicable.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRedit authorship contribution statement
Abdelilah Aboussekhra: Conceptualization, Writing – review & editing. Syed S. Islam: Data curation, Writing – original draft. Noura N. Alraouji: Data curation, Writing – original draft.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments
We are thankful to Mrs. Fauziah Hendrayani for technical support. This work was performed under the RAC proposal # 2180018.

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