CXCL5 secreted from adipose tissue-derived stem cells promotes cancer cell proliferation

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Abstract. Accumulating data suggest that adipose tissue facilitates breast tumor initiation and progression through paracrine and endocrine pathways, and that adipose tissue-derived stem cell (ASC) is likely the major cell type responsible for tumorigenesis and tumor development. However, it remains unknown how ASCs exert their effects. In the present study, in cultured breast cancer cell lines, including estrogen receptor (ER)-positive MCF-7 cells and ER-negative MDA-MB-231 cells, the effects on tumor proliferation of isolated ASCs from human breasts were examined. The expression of 174 cytokines was additionally identified in this medium. With an anti-human C-X-C motif ligand 5 (CXCL5) monoclonal antibody, the effects of neutralization of CXCL5 on the actions of ASCs in a co-culture medium of ASCs and tumor cells were studied The results demonstrated that ASCs significantly increased the number of breast cancer cells compared with controls. Similarly, the co-culture medium of ASCs with breast cancer cells exhibited potent effects on tumor cell proliferation. In the co-culture medium of ASCs with breast cancer cells, CXCL5 levels were significantly increased. In addition, depletion of CXCL5 with its specific antibody in ASC-conditioned medium blocked the stimulatory effect of ASCs on the proliferation of breast cancer cells. To the best of our knowledge, these results indicate for the first time that ASC-secreted CXCL5 is a key factor promoting breast tumor cell proliferation.

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Abbreviations: ASC, adipose tissue-derived stem cell; bFGF, basic fibroblast growth factor; BM-MSC, bone marrow-derived mesenchymal stem cell; CXCL5, C-X-C motif chemokine ligand 5; ENA-78, epithelial cell-derived neutrophil-activating peptide-78; ER, estrogen receptor; FBS, fetal bovine serum; IL, interleukin

Key words: adipose tissue-derived stem cell, CXCL5, breast cancer, chemokine, proliferation

Introduction

Obesity is a major risk factor for a variety of diseases, including cancer, diabetes and cardiovascular diseases (1,2), and is particularly associated with an increased risk of developing breast cancer in post-menopausal females (3,4). Obese subjects exhibit more aggressive breast tumors and higher risk of recurrence. A high body mass index demonstrates a predictive value for poorer outcome in pre- and post-menopausal patients with breast cancer (4). In post-menopausal patients with breast cancer, >50% of mortalities are likely attributable to obesity (5). Evidence also suggests that exercise to reduce body weight during and following medical treatment decreases the risk of mortality by 30% in patients with breast cancer (6). Although the association between breast cancer and obesity is well documented epidemiologically, the molecular mechanisms underlying this correlation remain incompletely understood.

Normal adult female breast tissue is largely composed of adipocytes, which significantly out-number epithelial cells, thereby allowing adipocytes to exert a critical role in breast development (4,6). It has been demonstrated that adipose tissue contains specific cells that share characteristics of pluripotent mesenchymal stem cells isolated from other tissues (7). These adipose tissue-derived stem cells (ASCs) include a number of types of cells such as fibroblasts, pericytes, myofibroblasts, endothelial or hematopoietic cells and macrophages, which are programmed to produce different kinds of cytokines, including chemokines and growth factors (8), and form a microenvironment that tightly controls the proliferation of epithelial cells (9). During the initiation and progression of breast cancer, the cancer cells reorganize the microenvironment to support their proliferation and invasion into the surrounding tissue (10). Previous studies have suggested that cytokine expression in primary human breast cancers, including interleukin (IL)-6 (11,12), IL-8 (13,14) and C-C motif ligand 5 (CXCL5) (15) expression, is associated with reduced differentiation and poor clinical outcomes. However, it remains to be identified if specific factors that are involved in the interactions between ASCs and breast cancer cells may contribute to the tumorigenesis of breast cells.

To determine if and how ASCs affect the tumorigenesis and development of breast cancer, different human breast

cancer cell lines (MCF-7 and MDA-MB-231) were used in the present study to evaluate the effects of ASCs and their products on cell proliferation, and to identify ASC-secreted cytokines with cytokine array analysis. To the best of our knowledge, the present study demonstrated for the first time that CXCL5 secreted by ASCs in a co-culture medium with human breast cancer cell lines may mediate the effect of ASCs on breast tumor cell proliferation.

Materials and methods

Ethics. The protocol for the present study was approved by the Ethics Committee of Harbin Medical University (Harbin, China) and Heilongjiang Province Institution of Higher Education (Harbin, China), and it conforms to the provisions of the Declaration of Helsinki in 1995 (16). All participants (10 females, aged 25-35 years old) provided their written informed consent to participate in the study.

Cell culture and reagents. All the culture cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. The human MCF-7 and MDA-MB-231 breast cancer cell lines and the human WI-38 fibroblast cell line were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), where they were characterized for isozyme, mycoplasma and cell viability detection. If the cells did not pass these examinations, no further investigation would be conducted. WI-38 cells were used as a control for breast cancer cells based on a previous study (17). Human mammary epithelial cells (HMECs; catalog no., CC-2551), also used as a control, and their culture medium (catalog no., CC-3150) were purchased from Lonza Group AG (Basel, Switzerland). MCF-7, MDA-MB-231 and WI-38 cells were grown in α minimal essential medium (αMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, catalog no., F9665; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 U/ml penicillin-streptomycin. Cell number was counted using a hemocytometer, and the number of viable cells present in the culture was assessed with the Trypan Blue exclusion method according to the manufacturer's protocol (Trypan Blue Solution was obtained from Thermo Fisher Scientific, Inc., Waltham, MA, USA; catalog no., 15250061).

The co-culture of ASCs and cancer cells was performed using a 24-mm (diameter) chamber with filter inserts (pore size, 0.4 μm) in 24-well dishes (Corning Costar; Sigma-Aldrich; Merck KGaA). A total of $5x10^4$ MCF-7 and MDA-MB-231 tumor cells in 500 μl culture medium were placed in the upper chamber, while $1x10^5$ ASCs or WI-38 cells were placed in the lower chamber. Since the pore size of the filter was smaller than the diameter of either ASCs or cancer cells, these cells could not pass to the other side of the filter. Accordingly, cancer cells and ASCs can be separated physically. Cells were grown in αMEM supplemented with 10% FBS, 10 ng/ml basic fibroblast growth factor (bFGF, catalog no., 8910; Cell Signaling Technology, Inc., Danvers, MA, USA) and 100 U/ml penicillin/streptomycin.

The co-culture medium from the upper chamber was harvested, centrifuged at 1,842 x g for 5-6 min at room temperature, and passed through an sterile filter (catalog no., SLHV033RS; EMD Millipore, Billerica, MA, USA). The supernatant was stored at -80˚C in aliquots for subsequent use.

Preparation of human ASCs. Using previously described protocols (18), human ASCs were isolated from breast tissue obtained from reduction mammoplasty procedures, while abdominal adipose tissue was obtained from abdominal liposuction procedures in cancer-free donors at the Fourth Affiliated Hospital of Harbin Medical University (Harbin, China) between March 2011 and August 2011.

These subjects exhibited no family history of diabetes or other chronic diseases, had normal glucose tolerance and body mass index, were free from any major organ diseases, and demonstrated a stable body weight for at least 1 year. During the surgical procedures, a hollow blunt-tipped cannula was inserted into the subcutaneous space through small (~1 cm) incisions. The cannula was attached to a vacuum device for gentle suction and moved through the adipose region, while a mixed solution containing saline and the vasoconstrictor epinephrine (catalog no., E4375; Sigma-Aldrich; Merck KGaA) was infused into the adipose region to minimize blood loss and tissue contamination.

The harvested lipoaspirate (~1 g/patient) was washed extensively with PBS and digested at 37˚C for 45-60 min with 0.075% collagenase (catalog no., 17454; Bio-Sun Sci&Tech Co., Ltd., Shanghai, China). Then, the dissociated tissue was filtered by a 100-μm nylon mesh (catalog no., F613463; Sangon Biothech Co., Ltd., Shanghai, China) to remove the debris, and the adipocytes were separated from the stromal vascular fraction by centrifugation at room temperature at 7,371 x g for 10 min. The pelleted cells were resuspended and washed with PBS three times. The ASCs were plated at a density of $2x10^3/cm^2$ on 10-cm tissue culture petri dishes, and incubated for 24 h at 37˚C and 5% CO_2 in αMEM supplemented with 10% FBS, 2 mM L-glutamine (catalog no., ST083; Beyotime Institute of Biotechnology, Haimen, China) and 100 U/ml penicillin-streptomycin. Following incubation at 37˚C for 30 min, cultures were washed three times with PBS, provided with fresh medium, and maintained at 37˚C and 5% CO_2. Daily washing with PBS was performed to remove non-attached and red blood cells, and the medium was changed every 3 days. The ASCs were sub-cultured every 5-7 days, and cells between passages 2 and 6 were used for all experiments. In preliminary observations, it was revealed that ASCs from liposuction and breast reduction exhibited the same effect on the proliferation of breast tumor cell lines. Thus, these cells were mixed in a 1:1 ratio and used in all the experiments.

Flow cytometric analysis of the phenotype of ASCs. The ASCs of the third or fourth passage were harvested by treatment with trypsin-EDTA (catalog no., 25200072; Thermo Fisher Scientific, Inc.) and then fixed in 1% paraformaldehyde-PBS. Following fixation, cells were washed three times with PBS. Cell aliquots (1,200 cells/ml) were stained with primary antibodies for 30 min at room temperature in the dark. The primary antibodies were fluorescein isothiocyanate-conjugated anti-human CD44 (dilution, 1:50; catalog no., MABF1556; EMD Millipore, Billerica, MA, USA), CD34 (dilution, 1:25; catalog no. 030848; United States Biological, Salem, MA,

USA), CD90 (dilution, 1:25; catalog no., C2441-60; United States Biological, Salem, MA, USA), CD11b (dilution, 1:50; catalog no., 11-0113-42; Thermo Fisher Scientific, Inc.), CD105 (dilution, 1:50; catalog no., MA5-11854; Thermo Fisher Scientific, Inc.), CD14 (dilution, 1:25; catalog no., 033460; United States Biological, Salem, MA, USA) and CD45 (dilution, 1:25; catalog no., 040667; United States Biological) antibodies. Any unbound antibodies were removed by washing the cells in Flow Cytometry Staining Buffer (R&D Systems, Minneapolis, MN, USA). The suspended cells were centrifuged at 300 x g for 5 min at 4˚C and the buffer was decanted. Cells were then resuspended by adding 2 ml of Flow Cytometry Staining Buffer. Isotype-matched normal mouse immunoglobulin G (IgG) molecules were used as controls. Flow cytometry was performed on a fluorescence-activated cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) in samples from 5 donors from whom ASCs were obtained. Briefly, ASCs were positive for CD90 (96.23±2.89%), CD44 (98.17±1.35%) and CD105 (97.62±1.56%), and negative for CD14 (0.62±0.15%), CD11b (0.38±0.18%), CD34 (1.25±0.26%) and CD45 (0.59±0.21%). This major phenotype is consistent with the identified features (positive for CD90, CD44 and CD105; negative for CD14, CD11b, CD34 and CD45) of human ASCs (19,20). This result also indicated that the ASCs used in the present study were primarily fibroblasts and myofibroblasts (8,9), thereby validating the usage of WI-38 cells as controls. Data analysis was conducted using FCS Express 5.0 software (De Novo software, Glendale, CA, USA).

Cytokine antibody array. An antibody-based cytokine array system was used to detect the levels of growth factors and cytokines in the supernatants from co-culture media. In order to minimize the effect of exogenous cytokines and growth factors present in FBS, the FBS concentration in the co-culture medium was reduced to ~1% (v/v). The experiments were performed using the RayBio G-Series Human Cytokine Antibody Array kit (catalog no., AAH-CYT-G2000; RayBiotech, Norcross, GA, USA) to detect the expression of 174 cytokines according to the manufacturer's protocol. The cell-free supernatant was used undiluted. The signal intensity was quantified by light densitometry. ASC/MCF-7 co-culture media and ASC/MDA-MB-231 co-culture media were examined once. WI-38/MCF-7 co-culture media and WI-38/MDA-MB-231 co-culture media were used as positive controls to normalize the results.

Anti-CXCL5 treatment. Co-cultured ASCs and tumor cells at the density of $1x10^4/cm^2$ were incubated at 37˚C with an anti-human CXCL5 monoclonal antibody (catalog no., MAB 254, R&D Systems) diluted to 2.5 μg/ml to neutralize CXCL5 or with a mouse monoclonal IgG1 isotype control diluted to 2.5 μg/ml (catalog no., MAB 002; R&D Systems, Minneapolis, MN, USA) for 4 days, which were placed in the upper and lower sides of the chamber to inhibit all possible functions of CXCL5. This CXCL5 neutralization treatment started immediately following co-culturing, and tumor cells were observed under microscope (Model, CX22RF; Olympus, Tokyo, Japan) at magnification, x10, every 24 h, 5-10 visual fields were randomly selected, and the cell numbers in every visual field were counted.

Statistical analysis. Data were analyzed with the paired Student's t-test for comparisons between two independent groups, and with analysis of variance (ANOVA) for comparisons between three groups. Data were expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference. Data analysis was conducted using SPSS 17.0 software (IBM SPSS, Armonk, NY, USA).

Results

Effects of co-culturing human ASCs with cancer cells on the proliferation of breast cancer cells. Human ASCs were isolated from adipose tissues and evaluated for their ability to induce the proliferation of estrogen receptor (ER)-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cells. As a comparison, the human WI-38 fibroblast cell line, which had been previously demonstrated to stimulate tumor growth, was also included (21). The negative control was set by adding regular growth medium into the chambers to monitor potential changes in the number of breast cancer cells. At day 6 after co-culture, the average number of tumor cells was different between different groups (Fig. 1A), namely $6.4\pm0.7x10^5$, $4.4\pm0.5x10^5$, $3.8\pm0.4x10^5$ and $3.0\pm0.5x10^5$ MCF-7 cells when these were co-cultured with ASCs, WI-38 cells, HMECs and growth medium, respectively. Similarly, the numbers of MDA-MB-231 cells (Fig. 1B) co-cultured with ASCs ($44.0\pm6.1x10^5$) were significantly increased compared with those observed following co-culture with WI-38 cells ($30.2\pm3.5x10^5$) and with the negative control ($24.9\pm2.9x10^5$) ($P<0.001$). By contrast, the number of proliferated breast cancer cells only modestly increased in upon co-culturing with HMECs or WI-38 cells, while the negative controls exerted no significant effect on breast cancer cell proliferation. These results suggest that the effect of ASCs was independent of estrogen, since both ER-positive and ER-negative tumor cells increased their cell number significantly following co-culture with ASCs.

Effects of co-culture medium of ASCs on the proliferation of breast cancer cells. The chambers with ASCs and cancer cells were separated by a filter, and the pore size of the filter was smaller than the diameter of either ASCs or cancer cells, therefore, there was almost no direct contact between breast cancer cells and ASCs under the current experimental conditions. The ASC-secreted factors were most likely responsible for the stimulatory effect on tumor cell proliferation. To evaluate this hypothesis, conditioned co-culture medium was used instead of ASCs to stimulate breast cancer cell proliferation. ASCs and breast cancer cells were first co-cultured for 6 days, and then the supernatant was collected and used as growth medium to stimulate the proliferation of MCF-7 (Fig. 2A) and MDA-MB-231 (Fig. 2B) cells. The results demonstrated that the numbers of MCF-7 ($4.7\pm0.4x10^5$) and MDA-MB-231 cells ($8.9\pm0.6x10^5$) in ASC-conditioned medium were significantly ($P<0.001$) higher compared with those in their corresponding controls of regular culture medium. This result supports the aforementioned hypothesis. It is also notable that the cancer cells cultured in this growth medium proliferated at a slower rate than the cancer cells co-cultured with ASCs.

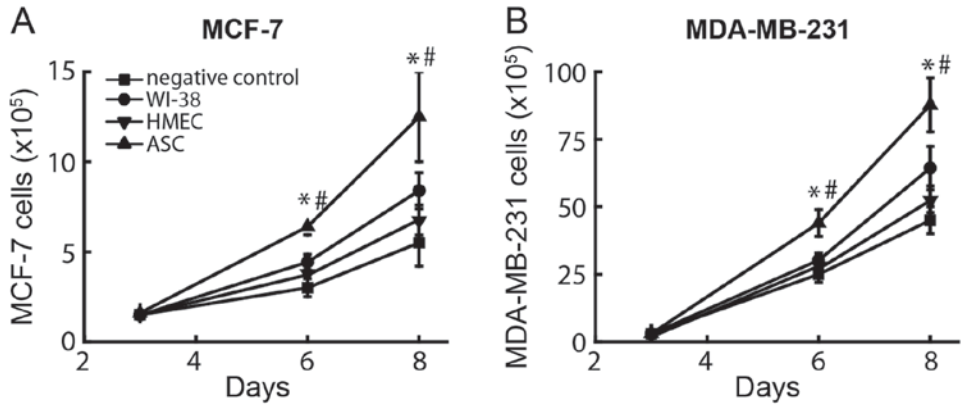

Figure 1. ASCs promote the proliferation of breast cancer cells. Comparison of the effects of ASCs, WI-38 fibroblasts, HMECs and regular growth medium alone (negative control) on the proliferation of (A) MCF-7 and (B) MDA-MB-231 cells. ASCs isolated from breast and abdominal adipose tissues were loaded in the lower chamber of the plates, while the tumor cells were placed in the upper side. The number of tumor cells was counted following co-culture on days 3, 6 and 8. All experiments were repeated 5 times; #P<0.01 vs. negative control, *P<0.01 vs. WI-38 cell co-cultured system and &P<0.01 vs. HMEC co-cultured system by analysis of variance. ASC, adipose tissue-derived stem cell; HMEC, human mammary epithelial cell.

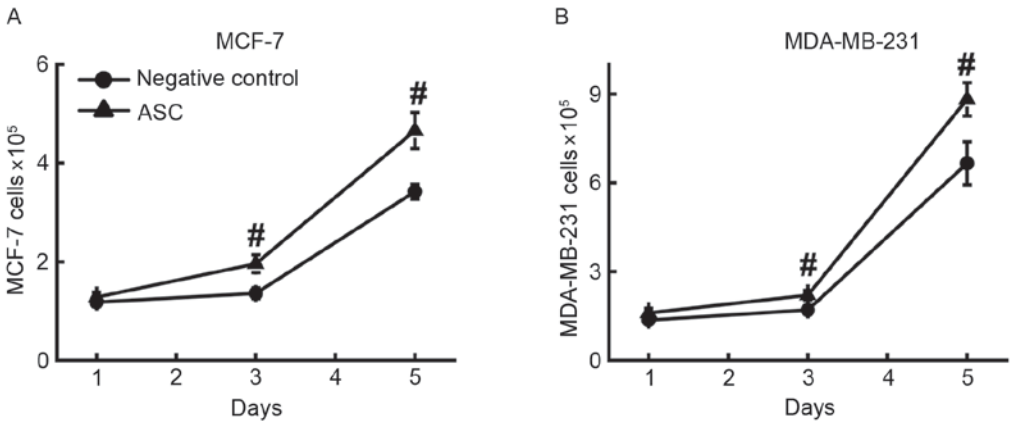

Figure 2. ASC-conditioned medium enhances the proliferation of breast cancer cells. Effects of ASC-conditioned medium on the proliferation of (A) MCF-7 cells and (B) MDA-MB-231 cells. ASC-conditioned medium was the supernatant of the culture medium of ASCs co-cultured with breast cancer cells for 6 days. The number of tumor cells was counted at days 1, 3 and 5 after co-culture. All experiments were repeated 5 times. #P<0.01 vs. regular (non-conditioned) medium by Student's t-test. ASC, adipose tissue-derived stem cell.

Chemokines in ASC-conditioned medium. In order to identify the factors that promoted the proliferation of breast cancer cell lines, an antibody-based cytokine array that is capable of detecting 174 different growth factors and cytokines was used to analyze the differences in factors between control medium and ASC-conditioned medium. Considering high concentration of FBS would generate high background noise, the FBS concentration in the culture medium was reduced to 1%. As demonstrated in Fig. 3A, a number of typical cytokines and growth factors, including CXCL5, also known as epithelial cell-derived neutrophil-activating peptide-78 (ENA-78), monocyte chemotactic protein (MCP) 2, MCP3 and regulated on activation, normal T cell expression and secreted (RANTES), were significantly upregulated, at least ≥2-fold vs. control levels, in ASC-conditioned medium. Among all the detected factors, increases in the protein level of CXCL5 were most significant in ASC-conditioned medium. By contrast, the levels of CXCL5, MCP2, MCP3, ENA-78 and RANTES were not changed significantly in WI-38/breast cancer cell co-cultures, thus supporting a specific expression of CXCL5 in ASCs.

Consequences of CXCL5 neutralization on the stimulatory effect of ASCs. CXCL5 is a chemokine that has been implicated in the chemotaxis of inflammatory cells (22). CXCL5 may contribute to tumor metastasis and recurrence of intrahepatic cholangiocarcinoma (23), and may serve critical roles in bladder tumor growth and progression (24). To explore its potential on promoting breast cancer cell proliferation in a paracrine manner, CXCL5 was neutralized with a CXCL5-specific antibody, and then the effect of co-culture of WI-38 cells, HMECs or ASCs on the number of breast cancer cells was observed. In the present study, non-specific monoclonal IgG1 was used as a control antibody, and WI-38 cells and HMECs were used as additional controls to highlight the specific effect of ASC-secreted CXCL5. All experiments were repeated 5 times. The results demonstrated that at day 4 after co-culture (Fig. 3), CXCL5 depletion by the above CXCL5-specific antibody blocked the proliferation-promoting activity of ASCs significantly (P<0.001 by ANOVA) in MCF-7 ($4.2\pm0.6\times10^5$ vs. $2.6\pm0.5\times10^5$) and MDA-MB-231 ($25.6\pm4.3\times10^5$ vs. $15.8\pm4.9\times10^5$) cells. Conversely, 4 days after co-culture, the anti-CXCL5 antibody did not significantly (P>0.05) affect

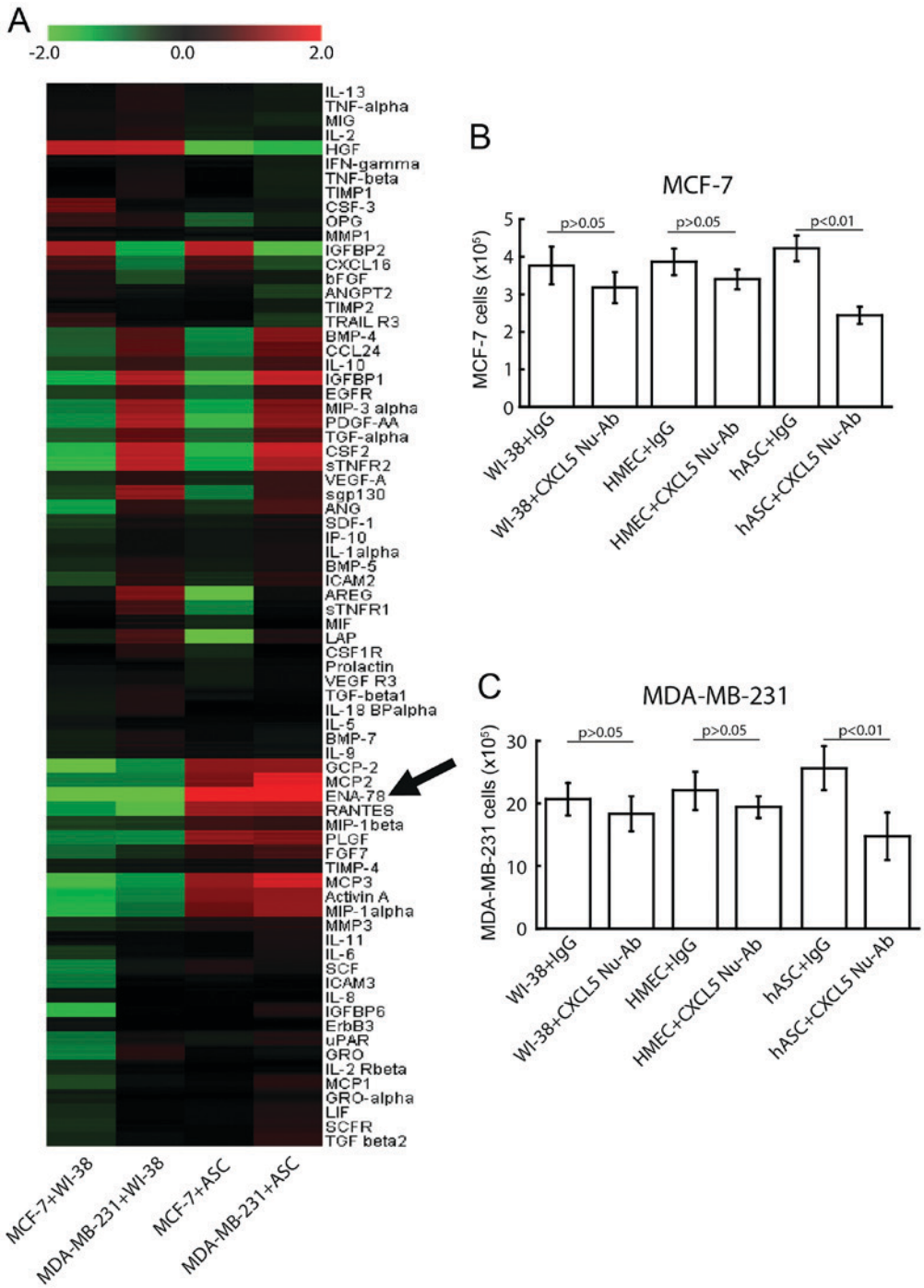

Figure 3. CXCL5 neutralization reduces the proliferation-promoting effect of ASCs. (A) Representative cytokine array analysis of the expression of 174 cytokines in the media from co-cultures of tumor cells with WI-38 cells (left columns) or ASCs (right columns). Arrow denotes the CXCL5 (also known as ENA-78) band. (B and C) Effects of neutralization of CXCL5 on the ASC-stimulated proliferation of (B) MCF-7 and (C) MDA-MB-231 cells. CXCL5 was neutralized with CXCL5 Nu-Ab, and a non-specific monoclonal IgG1 was used as the control antibody. WI-38 cells and HMECs were used as additional controls to highlight the specific effect of ASC-secreted CXCL5. All experiments were repeated 5 times. CXCL5, C-X-C motif ligand 5; CXCL5 Nu-Ab, CXCL5-specific neutralizing monoclonal antibody; Ig, immunoglobulin; ASC, adipose tissue-derived stem cells; HMEC, human mammary epithelial cell; ENA-78, epithelial cell-derived neutrophil-activating peptide-78.

breast cancer cell proliferation in the WI-38 cells co-cultured with MCF-7 ($3.8\pm0.7\times10^5$ with control IgG vs. $3.2\pm0.6\times10^5$ with CXCL5 neutralizing antibody) or MDA-MB-231 ($20.7\pm3.6\times10^5$ with control IgG vs. $18.3\pm3.8\times10^5$ with CXCL5 neutralizing antibody) cells. Similar results were also observed when cancer cells were co-cultured with HMECs. These data are illustrated in Fig. 3B for MCF-7 cells and Fig. 3C for MDA-MB-231 cells. These results support the hypothesis that the neutralizing effect of the anti-CXCL5 antibody was specific to the ASC-conditioned medium.

Discussion

In the present study, it was revealed that ASCs may promote breast tumor proliferation by releasing specific cytokines. Among the numerous cytokines secreted by ASCs, the increase

in CXCL5 levels was the most marked, and its neutralization reversed the proliferation effect of ASCs on breast tumor cells. To the best of our knowledge, the present study indicates for the first time that CXCL5 is a key factor of the promotion of breast tumor cell proliferation by ASC secretion through paracrine and endocrine effects.

Within adipose tissue, ASC is increasingly recognized as one of the most promising cell types responsible for a number of important functions (25), including the secretion of chemokines (8) and the formation of a microenvironment that tightly controls the proliferation of cells (9). However, it remains unknown how ASC facilitates breast tumor proliferation. The results of the present study suggest that ASCs provide a potent stimulus for tumor cell proliferation *in vitro*. As the ASCs used in the present study were obtained from cancer-free individuals and had never been exposed to any tumor milieu, the present data suggest that ASCs possess an inherent ability to enhance breast tumor proliferation.

Using cytokine array analysis, it was demonstrated that ASC secreted a number of factors known to promote tumor cell proliferation. As the ASCs were from breast and abdominal tissues, their promotion of breast tumorigenesis is attributable to paracrine and endocrine effects, a conclusion consistent with previous studies (3,4). In fact, adipose tissue has been known to actively participate in endocrine processes by secreting numerous cytokines and growth factors (26). ASCs release high levels of epidermal growth factor, bFGF, platelet-derived growth factor, hepatocyte growth factor, vascular endothelial growth factor, transforming growth factor-β, insulin-like growth factor and brain-derived neurotrophic factor (26-29). It has also been indicated that ASCs may secrete cytokines such as granulocyte colony-stimulating factor, macrophage colony-stimulating factor, tumor necrosis factor-α, IL-6, IL-7, IL-8, IL-11, IL-12 and leukemia inhibitory factor (26,27). It is considered that these growth factors and cytokines are released in bioactive levels by ASCs, and that their secretion increases significantly under certain conditions such as hypoxia or tumorigenesis (27,30). In the present study, it was additionally identified that the release of CXCL5 from ASCs was the most significant incidence in the co-culture medium, and that CXCL5 is a key factor in the ASC promotion of tumor cell proliferation.

The anti-CXCL5 antibody used in the present study was a monoclonal antibody that had been used by numerous other studies (31,32). As a monoclonal antibody, it will bind CXCL5 directly without interaction with other molecules such as C-X-C chemokine receptor 2 (CXCR2), which is the receptor of CXCL5 (33). Thus, according to the present ASC-breast cancer cell co-culture data, it can be proposed that CXCL5 directly acts on breast cancer cells to promote cancer proliferation. It is notable that CXCR2 is also the receptor for CXCL2, CXCL3 and IL8 (34). Among them, IL-8 derived from local tissue-resident stromal cells is suggested to promote breast cancer cell proliferation (14). Accordingly, results from CXCR2-blocking experiments may be due to the dysfunction of IL-8/CXCR2 instead of CXCL5/CXCR2. Based on the suppressive effect of the anti-CXCL5 antibody, it can be hypothesized that the effect of the other cancer-promoting factors from ASCs is either very weak or dependent on the action of CXCL5 on ASC-cancer cell interactions.

It has been well established that cancer cells exhibit the ability to recruit stem cells into the vicinity of tumors, and that this recruitment is important for the generation of a microenvironment that promotes cancer growth (35,36). In addition, evidence suggests that the chemokines produced by bone marrow-derived mesenchymal stem cells (BM-MSCs) serve an important role in tumorigenesis and tumor progression (37). Halpern *et al* (38) have demonstrated that BM-MSCs express chemokines that enhance the migration of CXCR2-positive cancer cells via the secretion of chemokine ligands such as CXCL1 and CXCL5. In this regard, it is notable that the cytokine profiles released from the ASCs (as shown in Fig. 3A) are similar to those displayed by MSCs (39). The present *in vitro* study clearly indicates the role of ASC-secreted CXCL5 in promoting breast cancer cell proliferation in ER-positive and ER-negative cell lines. This result is in accordance with a previous study demonstrating the growth-promoting effect of CXCL5 in the tunica intima and tunica adventitia of adipose tissue blood vessels (32). Additionally, high level of CXCL5 is a biomarker for poor prognosis in pancreatic cancer (40) and cholangiocarcinoma (41). Thus, it is conceivable that high CXCL5 level provides a microenvironment that is favorable to tumor growth and progression, which offers an explanation for the poor survival of patients with breast cancer who are obese (4).

The results of the present study do not completely exclude an additional effect of ASCs on guiding cancer cell proliferation through direct physical contact with the tumor cells *in vivo*. It was previously indicated that fibroblasts were capable of generating tracks and guide the movement of carcinoma cells when the two types of cells were in contact physically (42). Considering the highly migratory characteristics of ASCs, it is possible that the CXCL5-secreting and track-generating capabilities of ASCs contribute to their cancer proliferation-promoting effects *in vivo*.

It must be noted that there are differences in the mechanisms of promotion of breast cancer cell proliferation in fibroblasts (WI-38 cells) and ASCs. In the present study, CXCL5 did not significantly affect WI-38 cell- or HMEC-mediated breast cancer cell proliferation, thereby suggesting the existence of multiple mechanisms responsible for the induction of cancer proliferation. The present study primarily focused on the biological characteristics of cancer cells. The data demonstrated that CXCL5 may markedly affect cell proliferation independently of its expression levels. Certainly, the determination of the expression of the CXCL5 cytokine and its receptor in MDA-MB-231 and MCF-7 cells will also support the hypothesis of the present study.

The present study included ER-positive and ER-negative cells, in addition to WI-38 cells HMECs as controls. However, normal breast-associated fibroblast were not used as a control based on the following reason: The WI-38 cell line, which is a diploid human cell line composed of fibroblasts derived from lung tissue of an aborted Caucasian female fetus in the 1960s (43), has been widely used as a control to study breast cancer (17,44). In addition, normal breast-associated fibroblasts could inhibit epithelial growth (45). As a result, to the best of our knowledge, there are limited studies using normal breast-associated fibroblasts as controls. Therefore, in the present study, both WI-38 cells as HMECs were used as

controls instead of normal breast-associated fibroblasts, and the same conclusion was obtained, i.e., ASC-secreted CXCL5 is a key factor in promoting breast tumor cell proliferation.

In conclusion, CXCL5 is an important factor for the interactions between ASCs and breast cancer cells. The interactions between tumors and adipose tissues enhance CXCL5 expression, which is a key factor in breast tumorigenesis. CXCL5 may be a potential therapeutic target in breast cancer, and should be more extensively studied, in addition to other cytokines.

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