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Interaction of human adipose tissue-derived mesenchymal stromal cells with breast cancer cells

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Human adipose tissue was shown to be a very attractive source of mesenchymal stromal cells that have a wide scale of potential applications in reconstructive plastic surgery and regenerative medicine. However, these cells were described to have profound effects on biological behaviour of tumour cells. The aim of this study was to analyze the influence of adipose tissue-derived human mesenchymal stromal cells (AT-MSC) on the proliferation of breast cancer cells. We have tested proliferation of three different human breast cancer cell lines under the influence of AT-MSC derived soluble factors as well as in the direct cocultures. These data were supplemented with the expression analysis of cytokines and their cognate receptors on the target cells. We have observed stimulation of proliferation in breast cancer cells MDA-MB-361, T47D and EGFP-MCF7. AT-MSC were found to secrete wide scale of cytokines, chemokines and growth factors, thus we concluded that this pro-proliferative effect was a result of their synergistic action. These data bring out a need to evaluate whether primary breast tumour derived human cells would respond to these type of stimuli in a similar manner in order to exclude any potential clinical risk related to the application of human mesenchymal stromal cells under the context of patient with history of breast cancer malignancy.

Key words: human mesenchymal stromal cells, adipose tissue, breast cancer, proliferation, cytokine profile

Over the last 30 years there has been continuous interest in breast augmentation via autologous fat transplantation frequently used as a routine procedure due to its simplicity and reproducibility [1-2]. It is offered to patients as a final perfecting procedure for breast reconstruction, irrespective of technique used for the initial reconstructive procedure [3]. For cosmetic purposes, some surgeons use this procedure for breast augmentation as a sole treatment or for correction of the capsular deformity [4-5]. However, position paper published by American Society of Plastic and Reconstructive Surgeons in 1987 predicted that fat grafting would compromise breast cancer detection [6]. Randomized prospective multicenter trial (GRATSEC) began in 2010 in France with objective to compare the impact of surgical treatment by fatty tissue transfer to a simple oversight on the radiological monitoring in patients who underwent conservative surgery for breast cancer (http://clinicaltrials.gov/ct2/show/NCT01035268). There are still many unanswered questions regarding the use of fat graft in breast reconstruction, breast contour deformity correction or even its use in breast augmentation [7-10].

Grafting of heterogeneous mixture of cellular components carries potential oncological risk of de novo breast cancer and/or its recurrence. Although adipose tissue is the most abundant stromal constituent in the breast and a rich source of mesenchymal stromal cells (MSC), little is known about the involvement of this resident stem cell population in

Abbreviations: bFGF – basic fibroblast growth factor, cMET – hepatocyte growth factor receptor, CXCR4 – SDF-1α (CXCL12) receptor, EGF- epidermal growth factor, EGFR – epidermal growth factor receptor, FBS – foetal bovine serum, GAPDH – glyceraldehyde 3-phosphate dehydrogenase, G-CSF – granulocyte-colony stimulating factor, GM-CSF- granulocyte monocyte-colony stimulating factor, HGF – hepatocyte growth factor, IFN-g – interferon γ, IL – interleukin, MCP-1 (CCL2) – monocyte chemoattractant protein-1, chemokine CCL2, MIP-1a (CCL3) – macrophage inflammatory protein-1alpha, MIP-1b (CCL4)- macrophage inflammatory protein-1beta, PDGF-BB – platelet-derived growth factor, RANTES (CCL5)- Regulated on Activation, Normal T-cell Expressed and Secreted, chemokine CCL5, SCF – stem cell factor, SDF-1α – stroma-derived factor 1α, chemokine CXCL12, TGF-β- transforming growth factor beta, TNF-a – tumor necrosis factor α, VEGF- vascular endothelial growth factor, VEGFR – vascular endothelial growth factor receptor

mammary carcinogenesis. Primary mammary cancers tend to develop in close proximity to adipose tissue [11]. The interaction between epithelial cells and stromal fibroblasts plays a fundamental role in the development of both organs and tumors [12]. This cross-talk is bidirectional and usually paracrine in nature, which means that cells of the stromal compartment produce certain growth factors, cytokines or chemokines, which act on neighboring epithelial cells and *vice versa* [13]. The plasticity of MSC, combined with their migratory potential and their preference for injured tissue, makes these cells an ideal tool for therapeutic tissue regeneration [14]. However, MSC also enter tumours because they apparently "mistake" the tumours for normal wounds [15]. This response occurs because cancer cells secrete chemokines along with other proteins that attract MSC, and increase their migratory activity [16-17]. In the tumor, MSC may alter the behavior of the cancer cells. In addition, MSC may differentiate to carcinoma-associated fibroblasts (CAF), which are known to be involved in cancer progression [18]. Adipose tissue-derived MSC (AT-MSC) are an attractive and abundant stem cell source with therapeutic potential to be used in diverse fields of medicine for tissue repair or regeneration [19]. Due to their characteristics, AT-MSC have rapidly advanced into clinical trials for treatment of a broad range of different conditions. Some experimental studies in vitro and preclinical studies using animal models have demonstrated that both MSC and adipocytes could favor tumor growth [20-22]. Three recent studies similarly reported that MSC (abdominal- and/or breast adipose tissue-derived) promoted growth, invasion, and metastasis of fully malignant breast epithelial cells [23-25]. Zhao et al. hypothesized that multipotent adipose tissue derived MSC (AT-MSC) directly altered breast microenvironment favoring the transition from premalignancy to malignancy [11]. AT-MSC diminished activity of T lymphocytes and increased neoangiogenesis via endothelial cells. All of these elements could have promoted proliferation of breast tumors. On the contrary, Martin et al. found no significant increase in breast cancer cell proliferation upon coculture with MSC but stimulation of transition from epithelial to mesenchymal phenotype correlating with aggressiveness increase as described previously for sulforaphane [26-27]. These effects in breast cancer cells were also attributed to response to TGF-β that was described to be produced by MSC [28-29]. Even so, some studies suggested that MSC may have antitumor activities as well [30-33].

Risk of cancer development or cancer recurrence related to fat grafting has not been addressed in the recent clinical studies [3, 34]. Breast cancer can recur years or even decades after the original treatment [35-36]. Dormant tumor cells can be in a state of cell cycle arrest or micrometastases having balanced apoptosis and proliferation [37]. These can be transformed into active metastasis by autocrine and paracrine stimulating factors. Inability to trigger neovascularization can be an important factor in maintaining dormancy with VEGF having a specific role in the initial tumor growth [2, 38]. The introduction of AT-MSC, abundantly present in fat grafts, as a source of VEGF and other paracrine factors might represent a stimulus to release these cells into full tumor development, as previously demonstrated for melanoma [39]. Therefore it is necessary to further develop methods to unravel determinants of grafted adipose tissue or AT-MSC and the host local environment interaction in order to render fat grafting safer with less potential risks to patients.

In this basic research study we have evaluated effects of AT-MSC on several breast cancer cell lines in order to determine their effect on tumor cell proliferation. Our data clearly show the capability of AT-MSC to increase proliferation of breast cancer cells depending on the specific receptor-ligand interaction and identify several potential signalling axes to be involved in this process.

Materials and methods

Cells and chemicals. Human mammary gland adenocarcinoma cell line T47D (ATCC® Number: HTB-133™), MCF7 (ATCC® Number: HTB-22™), MDA-MB-231 (ATCC® Number : HTB-26™) and MDA-MB-361 (derived from brain metastasis, ATCC® Number: HTB-31™) were cultured in high-glucose (HG; 4,500 mg/ml) Dulbecco's modified Eagle medium (DMEM, PAA Laboratories GmbH) supplemented with 5% fetal bovine serum (FBS, Biochrom AG), 2 mM glutamine and Antibiotic-antimycotic (GIBCO BRL, Gaithesburg, MD).

EGFP-MCF7 cell line stably expressing enhanced green fluorescent protein (EGFP) was prepared according to recently published [40] with modifications. Briefly, 2 μg of recombinant retrovirus vector pAP-EGFP was transfected using Effectene transfection reagent (Qiagen, Hilden) and G418 selected to produce GP+E-86/pAP-EGFP ecotropic virus producing cells. Amphotropic GP+envAm12 packaging cells were infected in the presence of Polybrene (8 μg/ml) and G418 selected as above. Virus-containing medium was collected from 90% confluent cultures of GP+envAm12/pAP-EGFP cells, filtered through 0.45 μm filters and used either fresh or kept frozen until use at -80°C. To prepare MCF7 cells expressing EGFP, sub-confluent MCF7 cell cultures were transduced once with virus-containing media supplemented with 5 μg/ml protamine sulphate. Cells were cultivated in selected media containing 0.5 mg/ml G418 for 10 days. EGFP expression in transduced EGFP-MCF7 cells was verified by fluorescence microscopy and flow cytometry.

AT-MSC were derived by plastic adherence technique as previously described [40]. Briefly, AT-MSC were expanded from adherent cells obtained by collagenase type VIII digestion of lipoaspirate obtained from healthy persons, who provided an informed consent. Cells were expanded in low-glucose DMEM (LG; 1,000 mg/ml) with GlutamMAX™ (GIBCO Invitrogen Life Technologies) supplemented with 10% HyClone® AdvanceSTEM™ Mesenchymal Stem Cells Growth Supplement (Thermo Scientific) and Antibiotic-antimycotic (GIBCO

Invitrogen Life Technologies). AT-MSC were characterized by surface marker expression as CD44+, CD73+, CD90+, CD105+, CD14-, CD34-, CD45- population. Conclusions were drawn from similar results of experiments performed with two different isolates if not specified otherwise.

To prepare growth arrested AT-MSC, cells were treated with 10 µg/ml Mitomycin C (Mit-C, Kyowa, Hakko Ltd UK) for 3 hours in culture medium.

Cell-free AT-MSC conditioned medium (CM) was collected from 80% confluent AT-MSC cultures after 24 hrs cultivation with fresh culture medium and filtered through 0.45 µm filters. All cells were kept in humidified atmosphere and 5% CO2 at 37°C. All chemicals were purchased from Sigma (St. Louis, MO) if not stated otherwise.

AT-MSC and tumor cell co-cultures. Conditioned medium (CM) from AT-MSC was prepared fresh by culturing AT-MSC in LG DMEM culture medium for overnight one day prior to experiment start. On the same day, triplicates of 2.000 MDA-MB-361, T47D and EGFP-MCF7 tumor cells per well were seeded in 96-well plates. Filtered conditioned medium was diluted with fresh culture medium (undiluted AT-MSC CM, 1:2, 1:4, 1:8, 1:16, 1:32, unconditioned culture medium). Tumor cell medium was aspirated and breast cancer cells were cultured in given conditioned medium dilutions to determine the effect of AT-MSC secreted factors on cell proliferation. Fresh conditioned medium was provided every 2 days and relative proliferation was evaluated on day 3, 6 and 9. Experiments were performed at least twice with similar results and representative experiment result is shown, SD did not exceed 5% and was omitted from figures for clarity.

In an independent experiment, increasing numbers of either AT-MSC or growth-arrested AT-MSC (0, 31, 62, 125, 250, 500, 1.000, and 2.000 AT-MSC) were added to the tumour cells on day 0. Usually sixplicates of 4.000 MDA-MB-361, T47D or EGFP-MCF7 tumour cells/well were seeded in 96-well plate or in black-walled 96-well plate (for EGFP expressing cells) (Greiner Bio-One Intl. AG) for overnight. Appropriate dilutions of AT-MSC and growth-arrested AT-MSC were prepared in low serum LG DMEM (2% FBS). Tumor cells MDA-MB-361 and T47D were co-cultured with Mit-C arrested AT-MSC only, EGFP expressing tumour cells were co-cultured both with AT-MSC and arrested AT-MSC. Duplicates with corresponding number of arrested AT-MSC alone were seeded in parallel 96-well plate for a background control. LG DMEM (2% FBS) was replaced every 2 days. Relative proliferation for MDA-MB-361 and T47D was evaluated using MTS proliferation assay. Relative proliferation of EGFP-MCF7 was evaluated using both MTS proliferation assay and relative fluorescence measurements.

Proliferation assay. Relative proliferation of tumor cells was assessed by CellTiter® 96 AQueous One Solution Cell Proliferation Assay (MTS Assay, Promega). 10 µl of 5 mg/ml MTS diluted in 90 µl DMEM was added to each well and incubated for 2 hrs at 37°C. Absorbance values were read out spectrophotometrically at 492 nm by PolarStar OPTIMA reader (BMG Labtechnologies, Offenburg, Germany). Results were expressed as relative proliferation, where the proliferation of cells in standard culture medium DMEM without AT-MSC cells or CM was set to 100% by default. Absorbance values were corrected for the contribution of AT-MSC by subtracting these background values from the absorbances in cocultures. Results were expressed as means of sixplicates.

Relative proliferation of EGFP expressing cells was evaluated by determining relative green fluorescence on PolarStar OPTIMA reader (BMG Labtechnologies, Offenburg, Germany). Culture plates were carefully washed by PBS and fluorescence of EGFP-MCF7 was determined in 100 µl PBS. Values were expressed as means of relative fluorescence ± SD, where EGFP-MCF7 tumor cell florescence in culture medium DMEM without AT-MSC or arrested AT-MSC was set to 100% by default. It was previously determined, that there was a linear correlation between fluorescence intensity and number of EGFP expressing cells under these experimental culture conditions and cell densities.

Analysis of gene expression. Total RNA was isolated from $5x10^6$ cultured cells collected by trypsinization by Qiagen RNeasy kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen, Hilden, Germany). Total RNA was subjected to control PCR for human actin to exclude potential contamination with undigested DNA. RNA was reverse transcribed with RevertAid™ H minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD). 200 ng of cDNA was subject to standard PCR performed in 1x PCR Master Mix (Fermentas, Hanover, MD) with respective specific primers [41] for 35 cycles and gel resolved on 2% agarose or 4 % MetaPhor® Agarose (Lonza, Rockland, ME, USA) for qualitative analysis.

Cytokine secretion analysis. Duplicates of 50.000 AT-MSC cells/well were cultured in 1ml of complete culture medium for three days. Cell-free supernatants were collected, filtered and subjected to human Bio-Plex™ 27-plex Cytokine Assay (Bio-Rad Laboratories Inc, Hercules, CA). Measurements were performed on Luminex 100 System (Luminex Corporation, Austin, TX) in duplicates with four different AT-MSC isolates at passages 1 to 3. Results were expressed as means ± SD.

Results

AT-MSC have pro-proliferation effect on EGFP-MCF7 cells. In order to distinguish proliferation of tumor cells in the coculture with adipose tissue derived mesenchymal stromal cells (AT-MSC), we prepared stable cell line EGFP-MCF7 expressing marker transgene EGFP by retroviral transduction. We have previously employed this strategy to determine pro- and anti-proliferative effect of our therapeutic approach [39, 41]. By determining fluorescence intensity proportionally corresponding to the number of proliferating cells it is possible to evaluate proliferation outcome. We were able to confirm successful gene transfer and stable EGFP expression in EGFP-MCF7 cells by standard flow cytometric analysis (Figure 1A). When

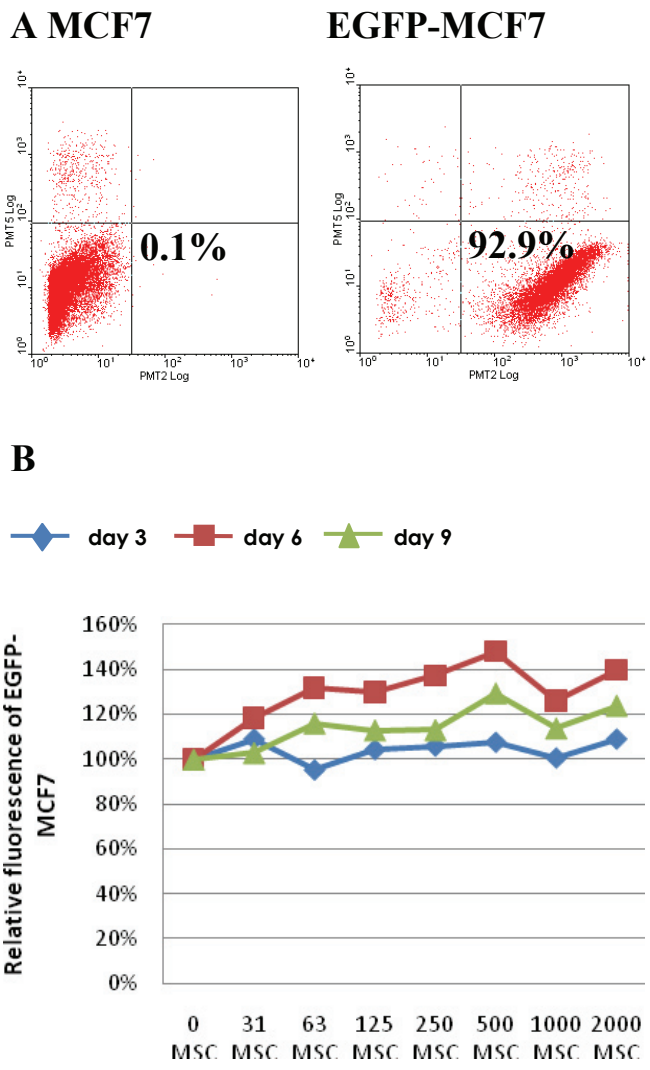

Figure 1A. Parental MCF7 cell were retrovirus transduced to stably express EGFP. Flow cytometric analysis has shown that 92.2% of EGFP-MCF7 were highly positive for fluorescent label (PMT2). Viability was determined by 7-aminoactinomycin dye (PMT5). 1B. Proliferation of EGFP-MCF7 cells co-cultured with increasing numbers of AT-MSC. Proliferation of EGFP-MCF7 cells was evaluated by determining relative fluorescence in co-cultures with increasing AT-MSC numbers on day 3, 6 or 9. AT-MSC supported the proliferation of EGFP-MCF7 cells.

these cells were directly cocultured with increasing numbers of AT-MSC, we were able to detect dose dependent increase in relative fluorescence in the cocultures corresponding to the pro-proliferation effect of AT-MSC on EGFP-MCF7 cells (Figure 1B). Relative fluorescence of EGFP-MCF7 when co-cultured with 2.000 AT-MSC increased by 9%, 40% and 24%, on day 3, 6 and 9, respectively. Decrease of tumor cell proliferation at later time-points was present in lower concentrations on day 9 than on day 6 probably due to depletion of growth factors by high number of proliferating AT-MSC.

Effects of growth arrested AT-MSC on breast cancer cells. Next, we decided to extend our findings on two more cell lines and evaluate the effect of growth arrested Mitomycin C-treated AT-MSC on breast cancer cell proliferation. Under these experimental conditions the input number of AT-MSC remains stable and its contribution to the final absorbance values can be subtracted, therefore the relative proliferation changes reflect effects on tumor cells. We tested the effect of arrested AT-MSC on MDA-MB-361, T47D and EGFP-MCF7 tumor breast cell proliferation.

Increasing number of arrested AT-MSC led to an increased proliferation rate of MDA-MB-361 and T47D. Increasing numbers of arrested AT-MSC increased the proliferation of T47D on day 3, 6 and 9 by 4%, 9% and 19%, respectively (Fig. 2A). Proliferation rate of MDA-MB-361 in medium containing 2.000 arrested AT-MSC on day 3, 6 and 9 increased by 43%, 58% and 95%, respectively when compared to control (Fig. 2B).

Same effect on proliferation was observed when co-culturing arrested AT-MSC with EGFP-MCF7. In a preliminary experiment, we have confirmed that the effects in EGFP-MCF7 can be determined by both MTS proliferation assay and relative fluorescence determination with corresponding results and same tendencies as with parental MCF7 cells (data not shown). These data further verified our fluorescence method for determination of relative proliferation in these cells expressing marker transgene. Output fluorescence was proportional to the number of EGFP-MCF7 cells and the number of admixed AT-MSC cells did not interfere with output fluorescence. For EGFP-MCF7, relative proliferation in medium containing 2.000 arrested AT-MSC increased by 9%, 68% and 34% on day 3, 6 and 9, respectively in comparison to medium without arrested AT-MSC (Fig. 2C). Maximum ratio of EGFP-MCF7 was present on day 6. On day 9, percentage of EGFP-MCF7 was lower than on days 6 although increasing numbers of arrested AT-MSC had still pro-proliferative effect. This is suggested to be a result of growth factor depletion and a plateau concentration achieved on day 6 followed by cell culture apoptosis.

Effects of cell-free AT-MSC conditioned medium on tumor cells. In order to examine, whether direct coculture and cell contact is necessary for observed pro-proliferation effects, we examined the effect of cell-free AT-MSC CM on T47D, MDA-MB-361 and EGFP-MCF7 cell proliferation. There was a significant increase in proliferation with increasing concentration of AT-MSC CM observed in all breast cancer cell lines. Proliferation of T47D in 100% cell-free AT-MSC CM on day 6 and 9 increased by 118% and 140%, respectively when compared to standard culture medium (Fig. 3A). For MDA-MB-361, maximum stimulation was achieved in 1:2 diluted CM, when proliferation increased by 56% on day 3, and by 205% on day 6 in comparison to proliferation in standard culture, medium, respectively (Fig. 3B). MDA-MB-361 proliferation in 100% AT-MSC CM increased by 30% on day 6. Proliferation in 100% AT-MSC CM dropped to 61% on day 9, this could have been caused by depletion or breakdown

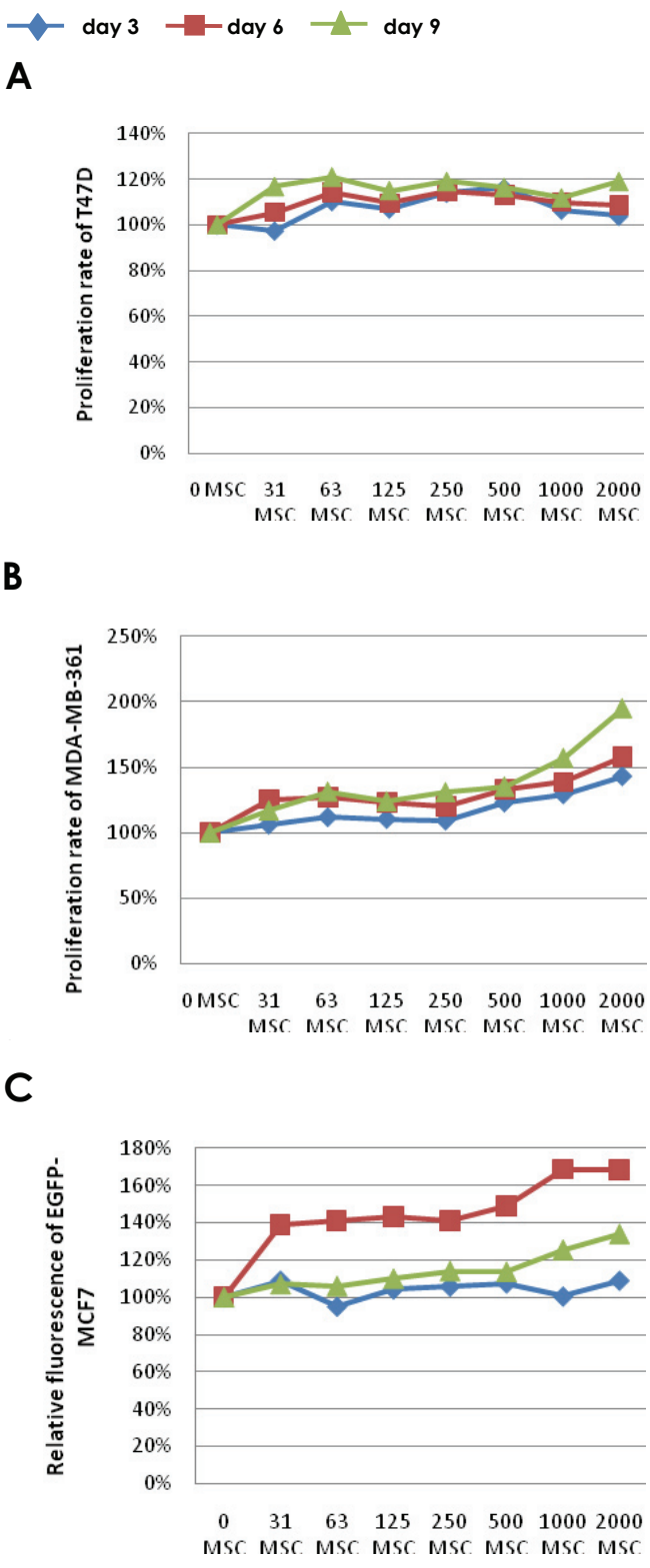

Figure 2. Proliferation of T47D (A), MDA-MB-361 (B) and EGFP-MCF7 (C) cells co-cultured with increasing numbers of growth arrested AT-MSC. Relative proliferation was determined by MTS proliferation assay (A, B) or relative fluorescence determination (C) on day 3, 6 and 9. Mitomycin C treated AT-MSC supported proliferation of tumour cells.

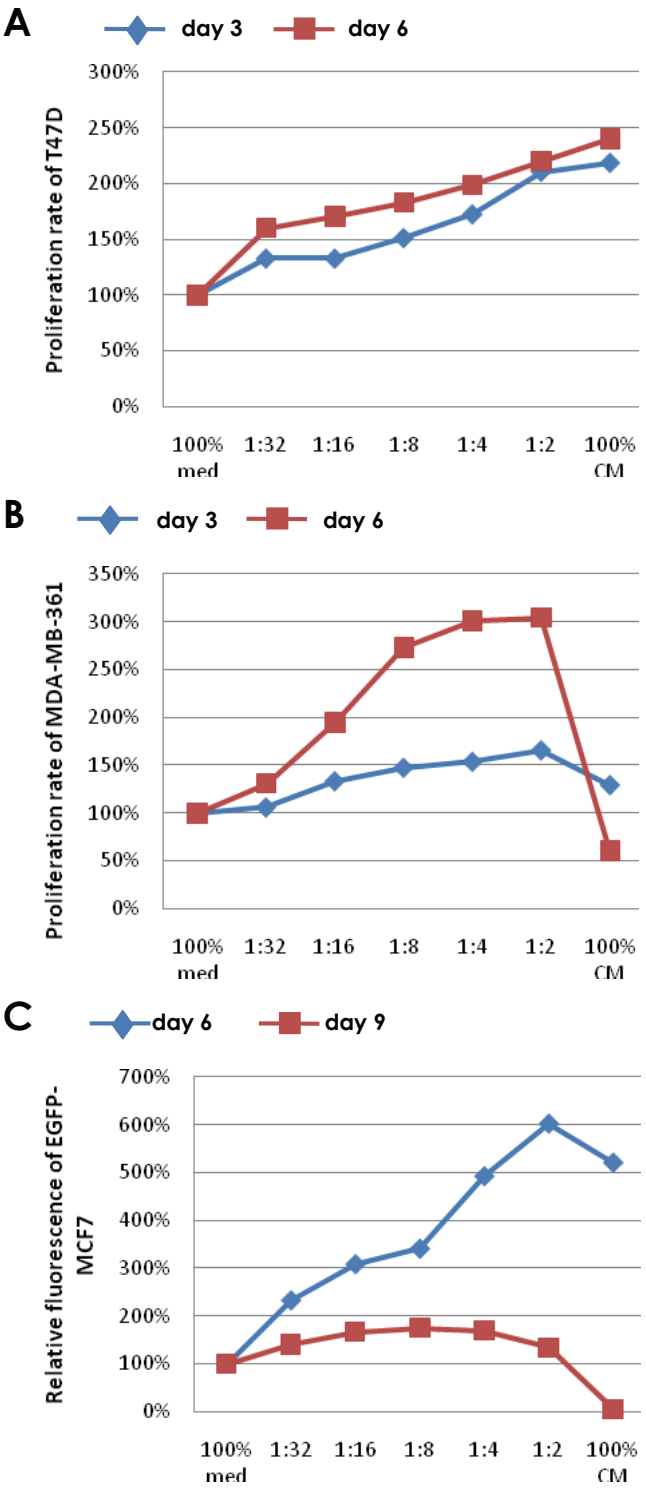

Figure 3. Proliferation of T47D (A), MDA-MB-361 (B) and EGFP- MCF7 (C) cells when cultured in medium containing AT-MSC-secreted factors. Proliferation assay with MTS was performed on day 3 and 6. For EGFP expressing cells EGFP-MCF7, the relative fluorescence increase was measured on day 6 and 9. AT-MSC-secreted factors present in conditioned medium significantly supported tumour cell proliferation in a dose dependent manner. This effect was higher in comparison to proliferation support mediated by growth arrested AT-MSC.

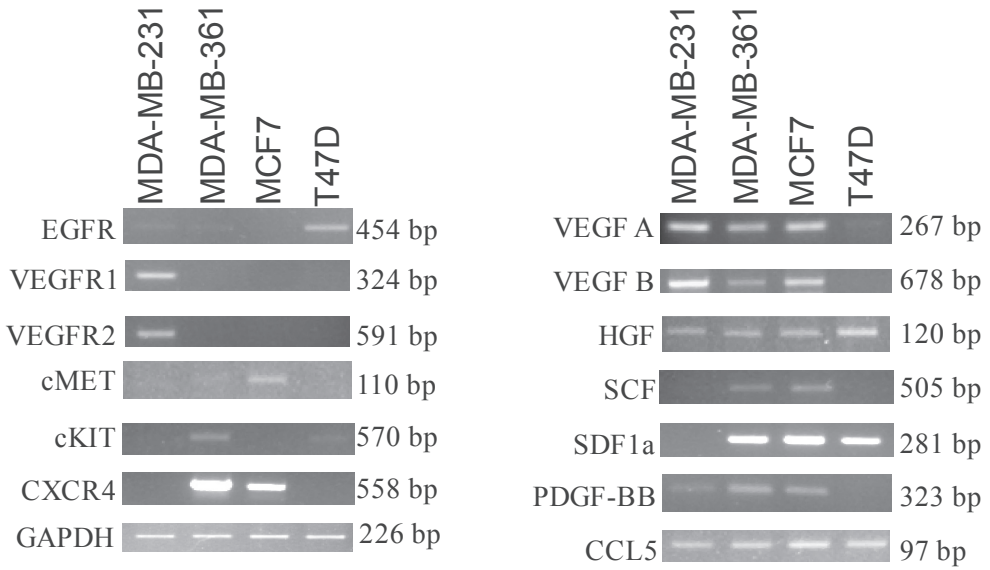

Figure 4. Growth factor and their cognate receptor expression in human breast cancer cell lines. RT-PCR was performed on total RNA isolated from cultured parental MDA-MB-231, MBA-MB-361, T47D and MCF7 cells. MDA-MB-231 cell line was used as a control to illustrate detection of VEGFR expression and its absence in other cell lines. Expression analysis has shown the absence of VEGFR1,2 receptors expression in MBA-MB-361, T47D and MCF7, but sustained expression of cKIT, CXCR4 in MDA-MB-361; cMET and CXCR4 in MCF7; EGFR and cKIT in T47D. Furthermore, we have identified expression of several growth factors in breast cancer cells such as VEGF A, VEGF B, HGF, SCF, SDF-1α, PDGF-bb, CCL5.

of growth factors. Simultaneously, a plateau concentration in these wells was achieved, which resulted in inhibition of cell culture and its apoptosis. Similarly, in EGFP-MCF7 cell cultures, relative fluorescence of cells in 1:2 diluted CM increased by 496%, and in 100% AT-MSC CM increased by 421%, which is more than five times increase on day 6 (Fig. 3C). On day 9, we could still observe the pro-proliferative effect of diluted AT-MSC CM on EGFP-MCF7, however, relative abundance was lower than on day 6. Relative fluorescence dropped to 120% in diluted CM 1:2 and to 5% in undiluted AT-MSC CM. We suggest that after day 6, when the plateau concentration was achieved, cells reached confluence, insufficient nutrition status and growth arrest accompanied by loss of EGFP fluorescence and perhaps decreased abundance of EGFP-MCF7 was also caused by cell apoptosis.

Taken together, AT-MSC conditioned medium has a pro-proliferative effect on MDA-MB-361, T47D and EGFP-MCF7 cells in vitro. These effects can be attributed to the presence of secreted soluble factors produced by AT-MSC that could synergistically stimulate breast cancer cell proliferation in vitro.

Differential expression of growth factor receptors in breast cancer cell lines. Although we have observed same pro-proliferation tendency in all cell lines examined, the extent of proliferation support differed between them. In order to unravel underlying molecular mechanism we have examined the expression of several potentially relevant growth factor receptors expression in the unstimulated parental tumor cells of interest. These were described to play important role in the mesenchymal stromal cell and tumor cell interactions previously [39, 42]. We were able to detect expression of cKIT (stem cell factor receptor) and chemokine SDF-1α receptor CXCR4 in MDA-MB-361 cells, cMET (hepatocyte growth factor receptor) and CXCR4 in MCF7 cells, and EGFR (epidermal growth factor receptor) and cKIT in T47D cells (Figure 4). No VEGFR-1 and -2 expression was detected in given cell lines in contrast to the control cell line MDA-MB-231, which was used to illustrate the absence of VEGFR expression in MDA-MB-361, T47D and MCF7 cells. Right panel of the Figure 4 shows abundant growth factor expression in breast cancer cell lines examined, that may contribute to the autocrine stimulation of proliferation and also have profound effects on the stromal tumor compartment as well.

Chemokine and cytokine production in AT-MSC. Human AT-MSC express VEGF A, VEGFB, PDGF-BB, SCF, SDF-1α HGF and CCL5 as shown on Figure 5A. However, these cells produce significant amount of various soluble factors into media as demonstrated by multiplex cytokine assay (Figure 5B). Their synergistic paracrine action on target tumor cells together with the specific pattern of respective receptors expressed on tumor cells gives the final outcome and extent of pro-proliferation effect of human adipose tissue derived mesenchymal stromal cells on breast cancer derived cells.

Discussion

In this study, we have investigated whether human adipose tissue-derived mesenchymal cells might provide breast cancer cells with an advantageous microenvironment for prolifera-

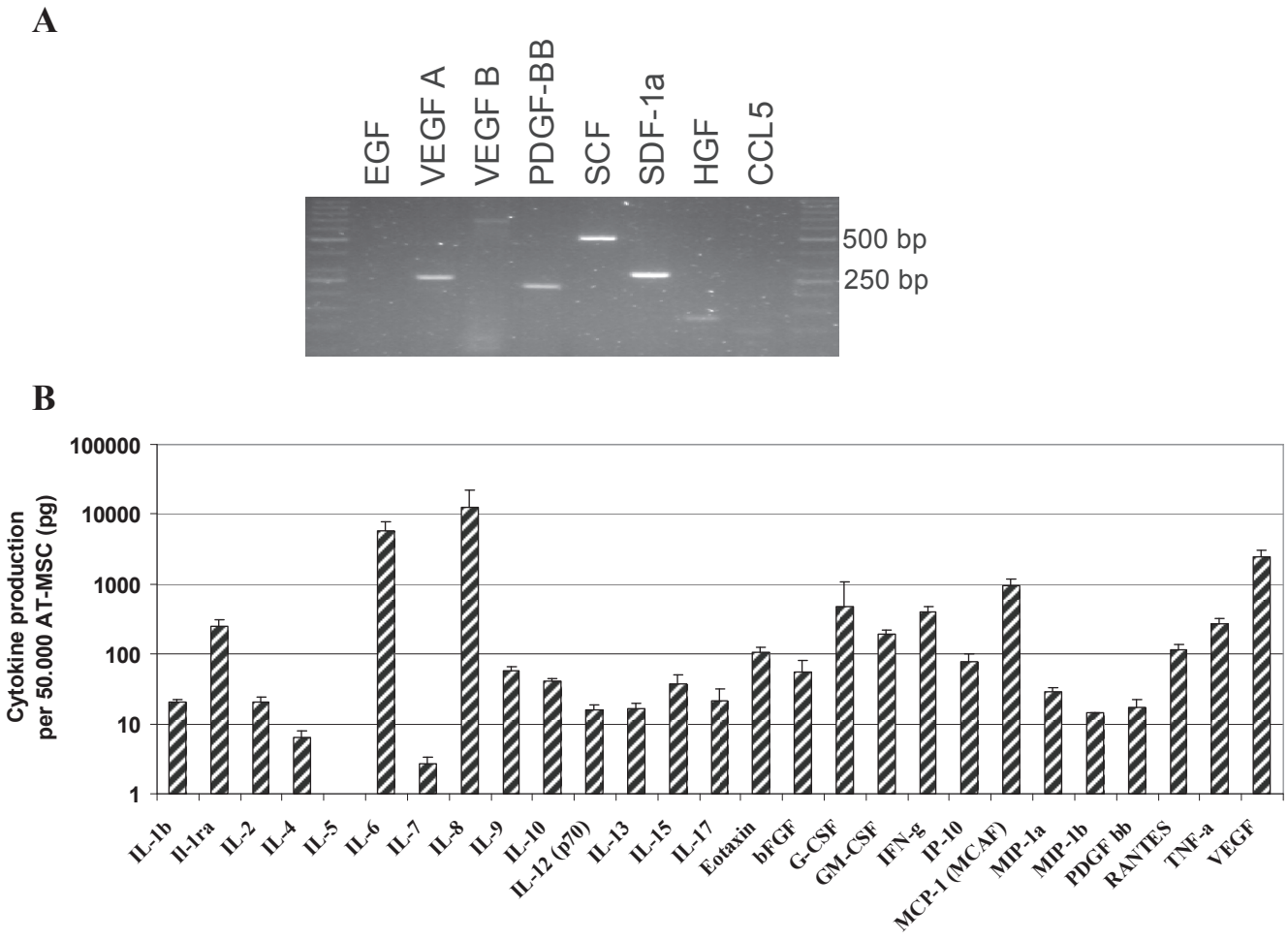

Figure 5. Adipose tissue derived human mesenchymal stromal cells produce abundant levels of cytokines. A. RT-PCR was performed on total RNA isolated from AT-MSC and confirmed expression of VEGF A, VEGF B, PDGF-bb, SCF, SDF-1α, HGF and CCL5. B. Multiplex analysis of cytokine production from four different isolates of AT-MSC at passage 1 to 3 revealed high production of interleukins IL-6 and IL-8, other interleukines, chemokines and growth factors.

tion. Several recent reports including our work have described biological effects of mesenchymal stromal cells on tumor cells although with both stimulatory and inhibitory outcomes [15, 39, 43]. We have previously demonstrated AT-MSC stimulatory effect involving VEGF and SDF1-α/CXCR4 signalling on human melanoma cells both in studies *in vitro* and *in vivo* studies. However, same AT-MSC failed to support proliferation and growth of glioblastoma tumour cells. We have never observed tumour cell-triggered morphological changes, significant cell death or terminal differentiation in AT-MSC in contrast to response to genotoxic damage [44]. This study extends our observations to several breast cancer cell lines. Our results have clearly shown that changes in proliferation status of tumour cells were not solely induced by direct cell-to-cell contact with AT-MSC, but also by soluble ligands present in AT-MSC CM. Moreover, increased proliferation rate in EGFP-MCF7 cell line was observed in direct co-cultivation with arrested AT-MSC as well as with non-arrested AT-MSC. Our in vitro data correlate with report from Karnoub *et al.* [45], who described significant stimulation of MCF7 cells upon co-injection with bone marrow derived mesenchymal stromal cells (BM-MSC) *in vivo*. Moreover, they reported no significant effect of BM-MSC on proliferation of MDA-MB-231 cells in vivo well being consistent with our findings of no significant stimulation of MDA-MB-231 cells by AT-MSC in our experiments (data not shown). These findings further support hypothesis presented by Zhao et al. [11] that AT-MSC derived from abdominal sources are very similar to BM-MSC based on gene expression profile and biological behaviour.

According to results from this study, MDA-MB-361 and EGFP-MCF7 cells have higher proliferation activity than T47D cell line in response to AT-MSC (Figure 2). Proliferation increase was higher when tumor cells were cultured in medium containing AT-MSC-secreted factors than with AT-

MSC. When co-cultured together, AT-MSC and tumor cells shared the same growth factors and nutrients and the plateau concentration of tumor cells was achieved at their lower abundance. The result of AT-MSC co-cultivation with tumor cells is a steady state in cell culture and eventually apoptosis which is observed by decrease in tumor cells at longer proliferation. Mitomycin C-mediated abrogation of AT-MSC proliferation has no significant influence on tumor cell proliferation support because the proliferation rate of EGFP-MCF7 co-cultured either with arrested or non-arrested AT-MSC is very similar (Fig 1B and 2C).

Herein, we have reported breast cancer cell proliferation support in the presence of AT-MSC CM, AT-MSC or arrested AT-MSC, however, the extent of support had clearly differed when compared under the same culture conditions: proliferation increased 2.18-fold in T47D, 3-fold in MDA-MB-361 and 5-fold in EGFP-MCF7 on day 6 in 1:2 diluted AT-MSC conditioned medium (Figure 2). Although our data show proliferation support mediated by MSC on T47D (Fig. 2A and 3A) on day 3, it is not further increased by prolonged culture. We hypothesized that MSC-produced factors have exerted only short term initial stimulation effect on these cells with no further increase in later culture time. Analyzed breast cancer cell lines also differed in their proliferation properties and nutrients consumption, therefore timepoints for evaluation of stimulatory effects had to be adjusted accordingly. In a preliminary experiment we have found a time window in EGFP-MCF7 cells, when pro-proliferation effect was most prominent on day 6. At earlier time point by day 3, there was no significant stimulatory effect (data not shown). At later time point, the outcome was not that prominent although the tendency to stimulate proliferation was preserved (Fig. 2C and 3C). Our data as presented on Figure 3 might indicate that extensive proliferation in MDA-MB-361 and EGFP-MCF7 cells has lead to consumption of growth factors, reaching confluence and decrease of the relative proliferation at the highest CM concentrations.

Our experimental observations concurred with those of Fierro *et al.* [46], who had observed increased proliferation of MCF7 in co-culture both with AT-MSC CM or AT-MSC and Rhodes et al. [47] who had observed similar effects of BM-MSC on MCF7. Our results correlated with their findings of SDF-1α/CXCR4 signalling role in this process as suggested in their study, because we have shown abundant expression of CXCR4 receptor on MDA-MB-361 cells and SDF-1α expression in AT-MSC (Figure 4 and 5). This signalling has been recognized as an important axis for the crosstalk between tumor cells and their environment [39, 48-49] and both autocrine and paracrine signalling role was confirmed to be operating in the invasive breast carcinomas [50]. CXCR4 expression was detected in both MCF7 and MDA-MB-361 cells correlating to the extent of AT-MSC mediated tumor cell proliferation. Moreover we have detected cMET expression in MCF7 cells and its ligand HGF in AT-MSC that might act synergistically in strong tumor growth promotion by AT-MSC in MCF7 cells [38, 51]. Previously mentioned studies have described important role for VEGF/VEGFR signalling in tumor microenvironment, however it did not seem to be the case for these breast cancer cell lines. MDA-MB-231 express both VEGFR1,2 (Fig. 4), but there was no significant proliferation support (our unpublished data and [45]). On the contrary, other cell lines MDA-MB-361, MCF7 and T47D did not express these receptors although their proliferation was supported by AT-MSC. Interaction of AT-MSC and breast cancer cells is a rather complex process as obvious from cytokine production pattern, and includes both autocrine and paracrine signalling. We anticipate that other mechanisms and signalling axes except for those already mentioned are involved. Martin *et al.* [26] noted no significant increase in proliferative activity when T47D and MDA-MB-231 cells were grown under the influence of BM-MSC. Instead, they have reported changes in the expression pattern of genes involved in epithelial mesenchymal transition suggesting another effect of stromal cells on biological properties of breast cancer cells.

Based on our observations we can conclude that the in vitro interaction of tumor cells MDA-MB-361, MCF7 and T47D with AT-MSC or with AT-MSC soluble proteins (i.e., growth factors) results in increased tumor cell proliferation. This study demonstrated importance of further studies to dissect operating mechanisms in order for the therapeutic intervention. Furthermore it justifies further studies of primary breast tumor cells and their interactions with mesenchymal stromal cells derived from various sources.

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