

**Original research****REGULATION OF BREAST CANCER CELL PROLIFERATION AND IMMUNITY VIA TARGETING AUTOPHAGIC PROCESS USING siRNA ANTAGONIST *Atg-5* IN MCF-7 CELLS**

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**Abstract: Background:** Breast cancer initiates when normal breast epithelial cells begin to replicate unusually. These cells divide more rapidly than normal cells and continue to be accumulated and formed a lump or mass. Cells may migrate (metastasis stage) from breast tissues to lymph nodes or other human body parts. Cellular autophagy is one of the most critical mechanisms in breast cancer development. Autophagy plays a dual function as a tumor support process or suppressor mechanism. Accordingly, we aimed to highlight the molecular role of autophagy in breast cancer development and cancer therapy using RNA interference (RNAi).

**Material and Methods:** MCF-7 cell line was used to investigate the direct connection between breast cancer and the autophagy pathway using a respective siRNA antagonist, *Atg-5*. **Results:** MCF-7 cell morphology was markedly influenced by transfection with siRNA antagonist *Atg-5* compared with control-transfected and untreated cells. Likewise, the number of living MCF-7 cells reduced in transfected cells with siRNA that antagonist *ATG-5*, while the relative lactate dehydrogenases (LDH) production significantly increased compared with control-transfected cells. Notably, the knockdown efficiency of *Atg-5* gene expression was almost 60% inhibition when the MCF-7 was transfected with the siRNA antagonist *Atg-5*. Furthermore, the relative gene expression of interleukin -8 (IL-8) and transforming growth factor-beta (TGF- $\beta$ ) downregulated in response to depleted *Atg-5* in transfected cells.

**Conclusion:** Our findings demonstrate that downregulation of *Atg-5* leads to sufficient disturbance of autophagic machinery and subsequently regulates breast cancer cell proliferation.

**Keywords:** Breast cancer, Autophagy, RNAi, Cytokine

**INTRODUCTION** Breast cancer is the most common and antagonistic tumor type affecting women. The representative characteristics of the majority of patients with breast cancer are that they express receptors for estrogen (ER) and progesterone receptor (PR) and respond to hormonal therapy [1]. There plays a critical role in autophagy in cancer. Three main autophagy types are acknowledged: micro, macro, and chaperone dependent. These processes are made possible due to specific

enzymes and autophagy-related genes (ATGs) [2]. Autophagy refers to macroautophagy, the process through which dysfunctional cellular components are removed via the union of lysosomes and autophagosomes to produce autolysosomes. Autophagy can only be induced widely by internal or external stimulation. It can be significantly inhibited by knocking down ATGs which leads to the damaged proteins and cell organelles, such as the mitochondria, not being removed, thus creating a harmless environment that can affect the survival of normal cells; autophagy may defend the anticancer resistance response of breast cancer cells and maintain the activity of breast cancer [3]. The inhibition of autophagy can increase cancer cell death [4]. In addition, autophagy can affect tumor initiation, proliferation, and progression [5]. Cytokines are small signaling molecules produced by virtually all nucleated cells, especially

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endothelial/epithelial cells, and resident macrophages, in response to microbes or tumor antigens. They mediate intracellular signaling and regulate the homeostasis of the immune system. Dysregulated cytokine secretion from sustained inflammation can oblige oncogenesis [6]. The role of pro-inflammatory cytokines in arsenic carcinogenesis has become more evident recently. Increased secretion of IL8 and IL6 has been shown to correlate positively with arsenic-induced malignant transformation of urothelial [7] and bronchial epithelial cells [8]. In this way, the current study aimed to highlight the role of autophagy in cancer development and immunity by targeting one of the early events of the autophagy gene by specific siRNA antagonist Atg5 in MCF-7 cells.

## MATERIALS AND METHOD

### Cell lines

Breast cancer cells (MCF-7 cell line) were obtained from (VACSERA, Giza, Egypt) and regularly checked for mycoplasma contamination. The cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 media, containing 25 mM HEPES, four mM L-glutamine, 10% of heat-inactivated bovine serum albumin (BSA) and incubated at 37°C and relative humidity of 95% [3].

### Transfection protocol

MCF-7 cells were maintained in a complete RPMI medium and were overnight cultured in 6-well plates with confluency of 70-80%. MCF-7 cells were then transfected with a respective siRNA antagonist Atg-5 gene (5'-AAACAGCUUCUGAAUGAAAGGUCUU-3') or anti-Luciferase (5'-AACUUACGCUGAGUACUUCGA-3') which served as control (Khalil et al., 2016). According to the manufacturer's protocol, the cells were transfected with 500 ng of siRNA/well using 20µl HyperFect transfection kits (Qiagen, USA) prepared in 500 µl optimum media. Transfected cells were incubated for two days. Knockdown efficiency of Atg-5 expression and relative expression of IL-8 and TGF-β genes was assessed using qRT-PCR [9].

### Proliferation and cytotoxic assay

For the proliferation assay of transfected MCF-7 cells with anti-Atg-5, cells were cultured in duplicate in a 6-well plate at 10X10<sup>4</sup> cells per well. Cell morphology was monitored using the inverted microscope. The number of living cells upon transfection was counted by using a hemocytometer. In brief, the old media was discarded, and the cells were washed twice with phosphate buffer saline (PBS) before trypsinizing by 3 min incubation at 37°C. Then a suitable volume of complete RPMI media was added to the trypsinized cells, and the number of cells was manually counted [10, 11].

### Lactate dehydrogenase (LDH) production

The cytotoxic potential of transfection on MCF-7 cells was assessed using an LDH production kit. Briefly, the released LDH within the fluid medium of the transfected cells was monitored in a 96-well plate using the LDH production kit. Following the manufacturing procedures, 100 µl of each sample was incubated with 40 µl LDH buffer and 20 µl substrate for one hour. Then the relative production of LDH was calculated by dividing the mean absorbance values of treated cells by the mean absorbance values of the mock, which was indicated by the fold change [12].

### Detection of relative gene expression

To detect the relative gene expression, qRT-PCR was used to perform cDNA construction and amplification in one step using the purified total RNA as a template. Total RNA from transfected MCF-7 cells was extracted 48 hrs post-transfection using TRIzol and purified using the RNeasy Mini Kit (Qiagen, USA). The relative expression of Atg-5, IL-8, and TGF-β in transfected MCF-7 cells was detected using the QuantiTect SYBR Green PCR Kit (Qiagen, USA). The oligonucleotides in Table 1 have been used as specific primers for indicated genes. Level of amplified GAPDH was used for normalization. The following reagents were prepared for each reaction; 10µl SYBR green, 0.2 µl RNase inhibitor (20 U/µl), 0.25µl reverse transcriptase (50 U/µl), 1µl purified total RNA (100 ng/µl) and 0.5 µl from each primer up to a final volume of 20 µl using RNase free water. According to the manufacturer's protocol, the following PCR parameters were used to construct and amplify cDNA, in one step, from a total RNA template: 50°C for 30 min, 95°C for 3 min, 40 cycles (95°C for 30 sec, 60°C for 15 sec, 72°C for 30 sec) and 72°C for 10 min.

### ELISA assay

For quantitative measurement of individual interleukin, sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of IL-8 and TGF-β using human ELISA kits (Abcam, 46032) and (Abcam, 100647), respectively. Accordingly, as previously described, MCF-7 cells were seeded in a 96-well plate and transfected with siRNA antagonist ATG5 or Luciferase. Then the transfected cells were incubated for different time points, including 6, 12, 24, and 48 hrs post-transfection. Standards and samples were transferred into a new 96-well plate, and the produced interleukin was bound to the wells by the immobilized antibody. After washing, a biotinylated interleukin antibody was added, followed by an HRP-conjugated streptavidin antibody. Finally, a TMB substrate solution was added to the wells, and color developed according to the amount of

interleukin bound. The intensity of the color was measured at 450 nm [11, 13].

#### Data analysis

For quantification of the cycle threshold (Ct) of each investigated gene expression, delta-delta-Ct equations were used as previously described: (1) delta-Ct = Ct value for gene - Ct value for GAPDH, (2) (delta-delta-Ct) = delta-Ct for experimental-delta-Ct for control, (3) relative expression of targeted gene =  $(2^{-\text{delta-delta-Ct}})$  [14]. Statistical analysis was done using the student's t-test between two groups. P-value  $\leq 0.05$  was considered statistically significant.

targeted *Atg5* compared with other treated cells (Figure 1C). This result indicates that the transfection of siRNA antagonist *Atg5* can induce programmed cell death in transfected cells and change cell morphology in transfected MCF-7 cells.

#### Knockdown of *Atg5* stimulates gene expression of *IL-8* and *TGF- $\beta$* in transfected cells.

The expression profile of IL-8 and TGF- $\beta$  was achieved in MCF-7 in response to the downregulation of *Atg5*. First, to confirm the knockdown efficiency of siRNA antagonist *Atg5*, the expression profile of *Atg5* was

Description	Primer sequences (5'-3')
ATG5-sense	CGTGTATGAAAGAAGCTGATGC
ATG5-anti-sense	ACGAAATCCATTTTCTTCTGGA
IL-8-sense	AAGAGAGCTCTGTCTGGACC
IL-8-anti-sense	GATATTCTTTGGCCCTTGG
TGF- $\beta$ -sense	CCGATGGGTTGTACCTTGTC
TGF- $\beta$ -anti-sense	GGGCTGGGTAGAGAATGGAT
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-anti-sense	TGGATGCAGGGATGATGTTCT

**Table 1.** Oligonucleotides sequence used for detection of steady state mRNA of indicated genes.

## Results

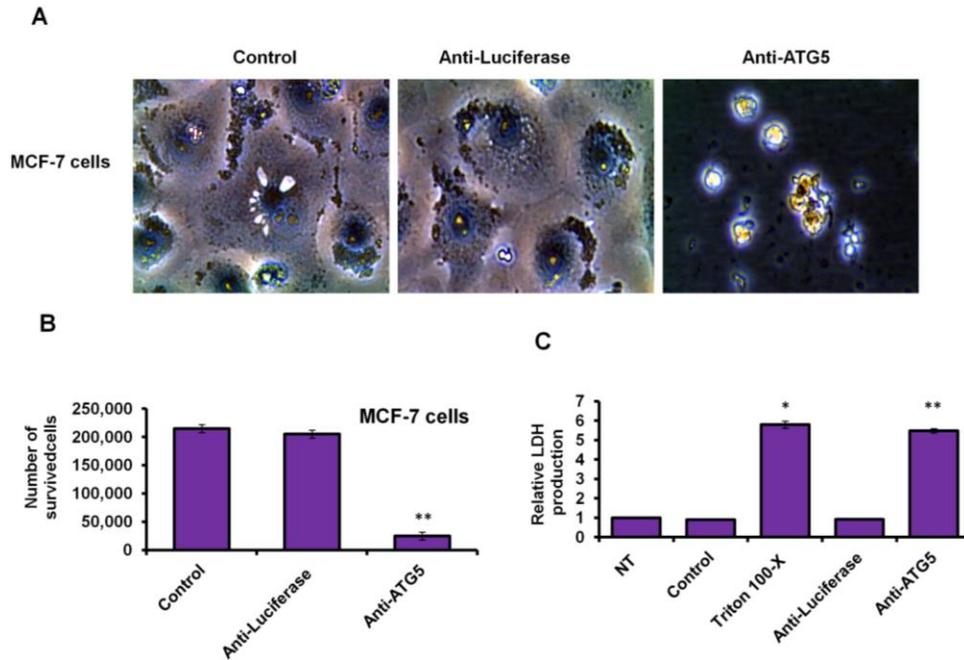
### siRNA antagonist *Atg5* induces alteration in cell morphology and the number of living MCF7 cell.

To investigate the cytotoxic effect of siRNA antagonist *Atg5*, MCF7 cells were cultured in 6 wall plates and incubated for 24 hours. The cultured cells were then transfected with either siRNA antagonist *Atg5* or siRNA specifically targeted *luciferase* gene, which served as control. Interestingly, the result showed marked alternation in cell morphology of MCF-7 transfected with the specific siRNA targeted *Atg5* when compared with anti-luciferase transfected and control-transfected cells (Figure 1A). In addition, as presented in Figure 1B, after transfection, the number of living MCF-7 cells transfected with siRNA antagonist *Atg5* showed a significant reduction in the number of survived cells compared with other transfected cells. Likewise, the relative LDH production was increased to reach the induced level by Triton-100X in MCF-7 transfected with the specific siRNA-

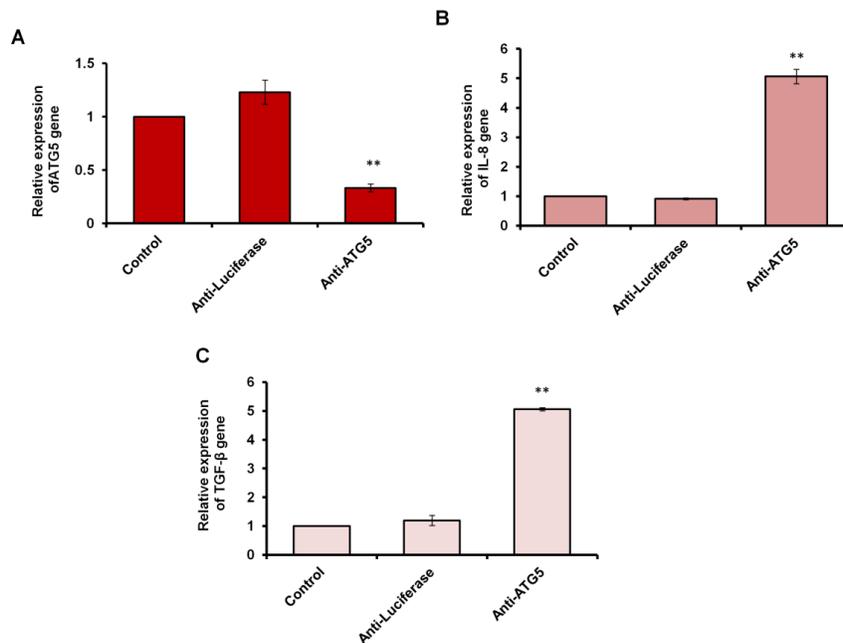
assessed in transfected cells using qRT-PCR. Notably, the relative expression of *Atg5* significantly downregulated in cells transfected with siRNA antagonist *Atg5* with more than 50% inhibition compared with other transfected cells (Figure 2A). Accordingly, the relative expression of IL-8 and TGF- $\beta$  significantly upregulated in cells transfected with siRNA antagonist *Atg5* compared with other transfected cells (Figure 2B and C). These results indicate that targeting *Atg5* in MCF-7 cells stimulates the relative gene expression of IL-8 and TGF- $\beta$  in MCF-7 transfected cells.

### Downregulation of *Atg5* increases the production of *IL-8* and *TGF- $\beta$* in MCF-7 cells.

ELISA assay monitored the level produced from transfected cells to confirm the influence of *Atg5* gene expression in IL-8 and TGF- $\beta$  expression profiles. Interestingly, the delivered level of both cytokines was increased by more than 300 pm/ml at 48 hours post-transfected with siRNA antagonist *Atg5*. Notably, their levels were constant in the early events following



**Figure 1. Cytotoxic potential of transfection protocol.** (A) MCF-7 cell morphology indicated by inverted microscope upon 48 hrs of transfection with the siRNA antagonist *Atg-5* compared with control-transfected and untreated cells. (B) The number of living MCF-7 cells upon 48 hrs post-transfection. (C) Relative LDH production from transfected cells with anti-*Atg-5* upon two days of transfection compared with the control-transfected cells. Error panels indicate the standard deviation (STD) of three independent experiments. Student two-tailed t-test used for statistical analysis, (\*) indicates  $P$  values  $\leq 0.05$ , (\*\*) indicates  $P \leq 0.01$ .

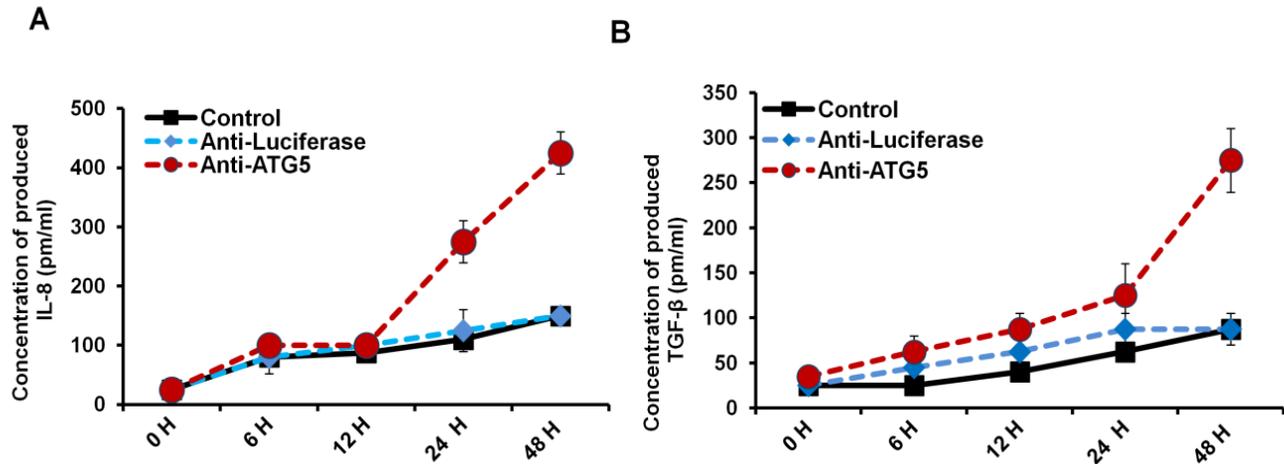


**Figure 2: Knockdown efficiency of *Atg5* and expression profile of IL-8 and TGF**

(A) Relative gene expression of *Atg5* in transfected MCF-7 cells compared with control-transfected and nontreated cells using qRT-PCR. (B and C) Relative gene expression of IL-8 and TGF- $\beta$  in transfected MCF-7 cells compared with control-transfected cells using qRT-PCR. Error bars indicate the STD of three independent experiments. Student two-tailed t-test used for statistical analysis, (\*) indicates  $P$  values  $\leq 0.05$ , (\*\*) indicates  $P \leq 0.01$ .

transfection compared with the control-transfected cells (Figure 3A and B). This result indicates that transfection of MCF-7 cells with siRNA antagonist Atg5 and autophagy

is formed, autophagy may contribute to cancer cell survival in response to metabolic stress or therapy. On the other hand, autophagy is induced during breast cancer



**Figure 3. Level of produce cytokines in transfected MCF-7.**

(A) The concentration of IL-8 (pm/ml) produced in the fluid media of transfected MCF-7 cells at the indicated time points. (B) The concentration of TGF-β in the culture media of transfected MCF-7 cells simultaneously post-transfection. Error bars indicate STD of four different replicates.

process leads to the production of the inflammatory cytokine (IL-8) and TGF-β.

### Discussion

Several studies evidenced that autophagy markedly controls cellular immune responses by regulating cytokines production and the functions of immune cells [15, 16]. Alternatively, other cytokines and immune cells significantly affect the autophagy process. Therapies targeting autophagy to enhance the immune responses and anti-tumor progress of immunotherapy have become the prospective strategy [17]. Furthermore, stimulation of autophagy may also benefit tumor cells' escape from immune surveillance and result in intrinsic resistance against anti-tumor immunotherapy [18]. Other studies have demonstrated the excellent use of either ATG inducers or inhibitors. This strategy can restrain tumor growth and progression by enhancing anti-tumor immune responses and overcoming anti-tumor immune resistance with several immunotherapeutic strategies [17].

RNAi is a biological process in which RNA molecules are involved in sequence-specific suppression of gene expression by double-stranded RNA through RNA degradation or translational inhibition [19]. Once a tumor

development to recycle the misfolded protein and avoid apoptosis [20]. In this way, the current work suggests that the knockdown of autophagy-related 5 (Atg5) showed sufficient regulation of breast cancer cell proliferation via high production of IL-8 and TGF-β, which are implicated in programmed cell death and cell migration. Recently, many studies have applied autophagy for cancer stem cell (CSC) targeted therapy; autophagy has been shown to play a dual role in cancer as a tumor suppressor and a tumor promoter [21]. Therefore, autophagy acts as a double-edged sword in tumor cells. Autophagy genes have frequently been found to be mono-allelically deleted, silenced, or mutated in solid tumors, resulting in increased oxidative stress conducive to DNA damage, genomic instability, and tumor progression [22]. In this way, we thought to control *Atg5* gene expression by using specific siRNA to confirm further the efficient role of Atg5 in breast cancer and possibly using siRNA as a potential therapeutic strategy. Interestingly, we further demonstrate the correlation between autophagy gene expression and breast cancer cell proliferation and immunity.

Tumor cells autonomously produce TGF-β and IL-8, synergistically attracting circulating tumor cells (CTCs) and promoting the self-seeding of breast, colon, and

melanoma tumors [23]. Furthermore, these cytokines enhance tumor cell migration through cell-autonomous paracrine mechanisms driven partly by the increase in local cell density [24]. Interestingly, this signaling is unique to tumorigenic metastatic cells but not the regular or non-metastatic cancer cells. However, autophagy restricts tumor alteration in normal cells via regulating DNA damage and oxidative stress; autophagy is required for tumor maintenance and cancer cell metabolism [25]. Indeed, the mechanism by which autophagy maintains tumorigenesis and sustains cancer cell metabolism varies in different conditions. In general, the critical role of autophagy in mammalian cells' survival, mitochondrial metabolism, and cancer cells usurp is observed by preventing p53 activation, growth arrest, senescence, and apoptosis [26, 27]. Likeminded, our findings revealed that the knockdown of *Atg5* showed sufficient regulation of breast cancer cell proliferation via high production of IL-8 and TGF- $\beta$ , which are implicated in programmed cell death.

#### Authors' contributions

Amira Alian performed the experiments. Adel A. Guirgis and Shaden Muawia helped conceptualize experiments, interpret data. Hany Khalil designed the research plan, supervised overall research, provided, and interpreted data, organized, and wrote the manuscript.

#### Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

#### Conflicts of interest

All authors declare that there are no conflicts of interest.

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