



Antiproliferative Effect of Mesenchymal Stem Cells on Human Breast Carcinoma: New Insight on FOXO/LncRNA-AF085935 Axis

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Abstract

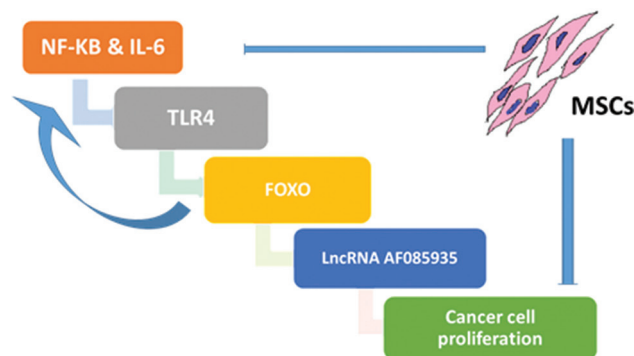
AIM: Cancer breast is one of the most common cancers in women leading to death; that is why we are in urgent need to develop new modalities of treatment. Mesenchymal stem cells (MSCs) have an anti-inflammatory effect due to capability to regenerate the damaged tissues.

METHODS: MCF7 breast cancer cells were divided into two groups; group 1: untreated cancer cells, group 2: cancer cells cocultured with MSCs; after 24 incubation the cells from the two groups were collected to assess cell proliferation, Interleukin-6 (IL-6) levels and genes expression of Nuclear factor-kappa B (NF-KB), FOXO, and LncRNA AF085935.

RESULTS: Statistically significant decrease in cancer cell proliferation and all other studied parameters in cancer cells after coculture with MSCs.

CONCLUSION: Breast carcinoma once initiated; it runs in a vicious circle due to stimulation of FOXO/LncRNA AF085935 axis by the inflammatory mediators released from cancer environment. FOXO/LncRNA AF085935 induces cancer proliferation and survival; furthermore, FOXO once induced, it produces further induction of inflammatory cytokines IL-6 and NF-KB and so on, MSCs due to its anti-inflammatory role could break this circle and thus inhibit cancer cell proliferation.

Graphical abstract



Introduction

Breast carcinoma is one of the leading causes of cancer death in women. Recently approved that there is a strong relation between inflammation and breast cancer [1].

Toll-like receptors (TLRs) are key receptors in innate immunity and inflammation. TLRs in addition expressed in different cancer cells. Recent evidence suggests that TLRs play an important role in cancer initiation and progression [2]. TLRs are stimulated by endogenous danger-associated molecular patterns

(DAMPs), which are released in cancer development followed by successive stimulation of inflammatory pathways [3].

NF- κ B is an important tumor-promoting transcription factor. TLRs signaling can intrinsically and extrinsically upregulate the inflammatory cytokines such as interleukin-6 (IL-6), IL-1b, and tumor necrosis factor α (TNF- α) through NF- κ B-dependent pathways [4].

TLR signaling has also been shown to regulate cell death and increase the expression of the anti-apoptotic proteins. In addition, TLR4 signaling induces tumor escape from the immune system and enhances tumor cell metastasis [5].

Forkhead box (FOX) proteins are a superfamily of transcriptional regulators, that control a wide range of biological processes, therefore, a disturbance of Fox activity can alter cell development, initiate tumorigenesis and cancer metastasis [6].

Long non-coding ribonucleic acids (LncRNAs) are non-coding RNAs, shorter than 200 nucleotides in length. Several lncRNAs are involved in crucial signaling pathways of tumorigenesis. Such as lncRNA-AF085935 [7], [8].

Mesenchymal stem cells (MSCs) are progenitor cells, which can be isolated from different types of tissues like bone marrow, adipose tissue, umbilical cord. MSCs possess a tropism to damaged tissues and tumor sites makes them a promising agent in cancer therapy [9].

Materials and Methods

Human umbilical cord MSCs isolation

Human umbilical cord specimens were obtained after approval of the ethical committee of faculty of Medicine. MSCs were isolated by collagenase II enzyme (IgG, C. histoliticum, Biological life science, USA), digested and maintained in 2% fetal bovine serum (FBS) and 1× Pen/Strep (Invitrogen, CA, USA). Cells were incubated at 5% CO₂, 37°C until cells will reach 70%–80% confluency, then trypsinized with 0.25% trypsin for 5 min at 37°C. After centrifugation, cell pellets were resuspended with medium for further coculture with MCF7 cancer cells [10].

MCF7 cancer cells isolation

Human breast adenocarcinoma (MCF7) cells were obtained from (VACSERA, Egypt). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in 5% CO₂ at 37°C. By day 3, cells were trypsinized, centrifuged and resuspended with fresh medium.

Coculture of MCF7 cancer cells and hucMSCs

MCF7 cancer cells were divided in 96 well plate for 3-[4, 5-dimethylthiazol-2yl]-2, 5- diphenyl-tetrazolium bromide (MTT) assay and two culture flasks for molecular studies then cocultured with MSCs incubated for 24hrs

Cells proliferation MTT assay

The measurement of cell proliferation was done by MTT cell proliferation kit (Trevigen Inc., Wiesbaden-Nordenstadt, Germany). MCF7 cancer cells were seeded in 96-well microtiter plates at the required concentration of 1×10^4 cells/well and after overnight incubation half of plate was cocultured with 1×10^4 MSCs. After 24 h cells incubation, 10 mL of tetrazolium compound, MTT was added to the wells and the cells were incubated 2–4 h at 37°C. MTT was reduced by metabolically active cells to insoluble purple formazan dye crystals. When purple precipitate was clearly visible under the microscope, 100 µL of detergent reagent was added to all wells. The covered plate was left in the dark at 18–24°C for overnight. The absorbance was measured at 570 nm with a reference wavelength of 650nm. Cell proliferation was assessed as the percentage of cell proliferation compared to untreated MCF7 as control cells.

IL-6 estimation from culture medium by ELISA technique: IL-6 level was detected according to kit instructions (Invitrogen Corporation, Camarillo, CA, USA).

lncRNA-AF085935, TLR4, Nuclear factor-kappa B (NF-KB) and FOXO genes expression by quantitative reverse transcription polymerase chain reaction (qRT PCR)

Isolation of total RNA

Total RNA was extracted from the two studied groups by RNA isolation system (Promega, Madison, WI).

Table 1: primers sequence for studied genes

TLR4	Forward primer: TGGATACGTTTCCTTATAAG Reverse primer: GAAATGGAGGCACCCCTTC 18S
NF-KB	Forward primer: TACCATGCTCTTTTGGTTAC Reverse primer: TCCCTCTTTTCTATTGATTGGA
FOXO	Forward primer: TGCATCCATGGACAACAACA Reverse primer: CGAGGGCGAAATGTACTCCAGTT
lncRNA-AF085935	Forward primer: CAGGGCAGCAAGGTGTTTTC Reverse primer: TTGGTGGGTTGCCTGATACC

One step RT PCR

5 µL of extracted RNA were added to qRT-PCR SYBR™ green master mix (Abgene, Hamburg, Germany) and the appropriate primers (Table 1) then filled with deionized water to 25 µL. qRT-PCRs in an Applied Biosystems Step One™ RT-PCR system (Applied Biosystems, Foster City, CA, USA) with 2 min as initial stage at 50°C to activate the DNA polymerase, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 1 min. $\Delta\Delta Ct$ were calculated from: $\Delta\Delta Ct = \Delta Ct$ sample assessed gene (CT sample assessed gene–CT reference home gene GAPDH (glyceraldehyde phosphate dehydrogenase))– ΔCt calibrator

(CT control gene– CT reference home gene GAPDH). Then, $2^{-\Delta\Delta Ct}$ gives the relative quantification gene expression compared to the control; Standardization was performed by quantification of the GAPDH gene as an endogenous control.

Table 2: correlation between studied parameters

	NFKB	IL6	TLR4	FOXO	AF085935	Cell proliferation
NFKB						
r	1	.961**	.948**	.947**	.911**	.939**
p value		<0.001	<0.001	<0.001	0.002	0.001
IL6						
r	.961**	1	.876**	.950**	.969**	.897**
p value	<0.001		0.004	<0.001	<0.001	0.003
TLR4						
r	.948**	.876**	1	.880**	.813*	.837**
p value	<0.001	0.004		0.004	0.014	0.01
FOXO						
r	.947**	.950**	.880**	1	.962**	.894**
p value	<0.001	<0.001	0.004		0	0.003
AF085935						
r	.911**	.969**	.813*	.962**	1	.836**
p value	0.002	<0.001	0.014	<0.001		0.01
Cell proliferation						
r	.939**	.897**	.837**	.894**	.836**	1
p value	0.001	0.003	0.01	0.003	0.01	

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Cancer cell proliferation is significantly correlated with inflammatory markers IL-6, NFKB, TLR4 (P value= 0.003, 0.001, 0.01), (r= 0.897, 0.939, 0.837) respectively ; all previous markers are significantly correlated with FOXO and lncRNA-AF085935 (p value <0.001); cancer cell proliferation is significantly correlated with FOXO and lncRNA-AF085935(p value =0.003,0.01)(r=0.894, 0.836) respectively.

Statistical analysis

Data were coded as mean and standard deviation and analyzed by SPSS software. Paired sample t-test was used to compare between untreated cancer cells and cancer cells cocultured with MSCs. Pearson correlation was done to detect the correlation between studied parameters. $p < 0.05$ is considered significant (Table 2).

Result

Coculture of MCF7 and MSCs

MSCs were identified by their spindle shape that then form clusters attacking cancer cells with marked reduction in a number of cancer cells (Figure 1).

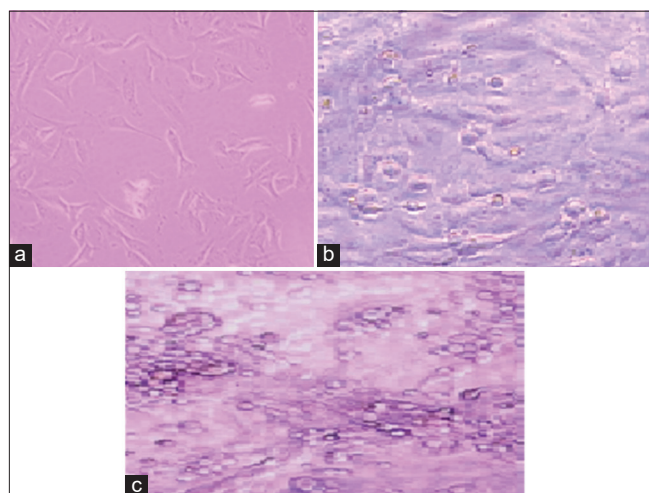


Figure 1: (a) Spindle-shaped MSCs (b) MCF7 cancer cells (c) coculture of both MCF7 cancer cells and MSCs showing cluster of MSCs around MCF7 with marked decrease in number of cancer cells

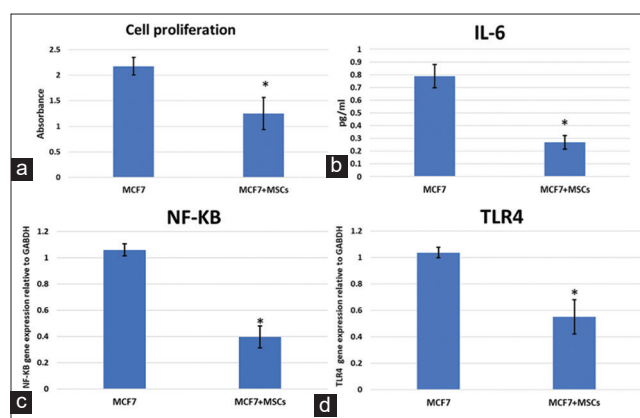


Figure 2: Significant decrease in cell proliferation and inflammatory markers IL6, NF-KB, and TLR4 after coculture of MCF7 with MSCs (p < 0.05)

MSCs significantly inhibits cancer cells proliferation

Statistically significant decrease in proliferation of cancer cells cocultured with MSCs compared to untreated cancer cells ($p = 0.016$) (Figure 2a)

MSCS inhibit the inflammation in tumor environment

Statistically significant decrease in IL6 level in the media derived from cultured cancer cells with MSCs compared to untreated cells ($p = 0.004$) (Figure 2b), and downregulation of inflammatory genes NF-KB, TLR4 ($p = 0.002, 0.008$) (Figure 2c and d).

FOXO gene and lncRNA-AF085935 are downregulated in MSCs treated cancer cells

Statistically significant decrease in the expression of FOXO gene and lncRNA-AF085935 in MCF7 cocultured with MSCs compared to untreated cells ($p = 0.005, 0.009$ respectively) (Figure 3a and b)

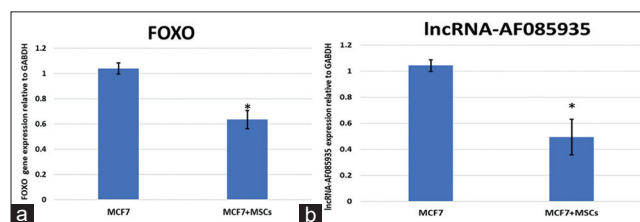


Figure 3: Significant decrease in FOXO gene and lncRNA-AF085935 expression after coculture of MCF7 with MSCs (p < 0.05)

Cancer cell proliferation significantly correlated with inflammatory markers, FOXO and lncRNA-AF085935

Cancer cell proliferation is significantly correlated with inflammatory markers IL-6, NFKB, TLR4 ($p = 0.003, 0.001, 0.01$), ($r = 0.897, 0.939, 0.837$) respectively; all previous markers are significantly correlated with FOXO and lncRNA-AF085935 ($p < 0.001$); cancer cell proliferation is significantly correlated with FOXO and

lncRNA-AF085935 ($p = 0.003, 0.01$) ($r = 0.894, 0.836$) respectively.

Discussion

Breast cancers represent a major health risk for women throughout the world. Previous researches reported a significant relation of chronic inflammation at the local and/or systemic level in breast tumor pathobiology. Inflammation could induce breast cancer progression, metastasis through a variety of cytokines and hormones. This provides a novel area for the development of new strategies for prevention [11].

IL-6 is a pleiotropic cytokine that plays a crucial role in regulating the inflammatory process [12]. NF- κ B is an inflammatory cytokine that plays a role in human cancer initiation, development, metastasis, and resistance to treatment [13].

hMSC are undifferentiated cells capable of self-renewal and proliferation; Due to their ability to migrate, hMSCs have an anti-inflammatory role and re-creation of tissue integrity. Inflammation and tissue damage are strong chemoattractants for hMSC, Cancer tissue release inflammatory cytokine as inflamed tissue; hence, there is a strong tropism of hMSC towards tumors [14] in this study we found significant decrease in inflammatory markers IL6 and NF κ B in cancer cells treated by MSCs; it could be due to the inflammatory effect of MSCs. We aimed in this study to detect the molecular mechanism by which MSCs inhibit cancer cells proliferation; as we found significant decrease in cell proliferation in cancer cells cocultured with MSCs compared to untreated cells.

TLRs play a role in both breast cancer cells and the microenvironment. TLRs are mainly expressed in macrophages, dendritic cells, and other innate immune cells [15]. In coincide to previous studies; we found that TLRs are highly expressed in breast cancer cells. It has been suggested that NF- κ B induces TLR activation that promotes tumor cell survival in several types of cancer [16]. TLR signaling can promote tumor progression and immune evasion in addition to resistance of tumor cells to cell death [17]. We found significant decrease in TLR4 expression in cultured cancer cells with MSCs compared to untreated cells; thus we could say that TLR4 is the effector of inflammatory cytokines such as IL6 and NF- κ B; furthermore due to the anti-inflammatory effect of MSCs could block the stimulation of TLR4, thus breast cancer cells could not survive and proliferate; but the question now is there is another molecular mediators are involved in the mechanism by which MSCs inhibit cancer cells proliferation; based on previous studies that investigated factors that control cancer cell proliferation we found that FOXO/lncRNA AF085935 axis is one of the major pathways that control cancer cell survival [18]. We found that FOXO/lncRNA AF085935 are highly expressed in cancer cells

but after coculture with MSCs we found downregulation of FOXO/lncRNA AF085935, but the question now is there is a relation between FOXO/lncRNA AF085935 and TLR4? In this study, we found significant correlation between TLR4 and both FOXO/lncRNA AF085935.

The transcriptional factor FoxO is a key regulator of cell metabolism, cell cycle, and cell death. Previous study reported that FoxO can promote inflammation by enhancing TLR4 signaling [19], furthermore; another study reported that Foxo up-regulates the expression of inflammatory cytokines IL-6 [20]. Moreover, another recent study reported that FOXO is an up regulator of lncRNA AF085935 thus promoting cancer survival [18].

Conclusion

We could conclude that chronic inflammation is a crucial element for cancer initiation and progression through subsequent activation of other molecular mediator that promotes cancer cell survival and proliferation; MSCs due to its anti-inflammatory effect could turn of this signaling cascade

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