RESEARCH ARTICLE

Editorial Process: Submission:11/18/2021 Acceptance:10/21/2022

Mesenchymal Stem Cells Treatment Aggravates Tumor Growth Regardless Its Route of Administration: An In vivo Study

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Abstract

Objectives: to clarify the effect of MSCs in cancer growth and to detect whether the rout of administration (either locally inside the tumor tissue or systemic)could affect the outcome of treatment or not. **Methods:** Eighteen female mice were involved in the study. All mice were subcutaneously inoculated with Ehrlich tumor cells into the right flank. After three week of tumor growth; the mice were divided randomly in to three groups six mice for each ; group I: untreated Erlish tumor group; group II: Erlish tumor treated by local injection of 1 x106 MSCs/week inside the tumor tissue, group III: Erlish tumor treated by systemic injection of 1 $x10^6$ MSCs iv in tail vein/week. Tumor growth was recorded .After 4 weeks of stem cells injection, all rats were sacrificed by cervical dislocation and tumor tissues were collected for histopathological study. inflammatory cytokine TNF was assessed by ELISA, lncRNA MALAT ,NFKB and MMP2 genes expression were assessed by Quantitative RT-PCR. **Results:** Erlish tumor was developed as a well-defined capsule composed by connective tissue infiltrated by inflammatory and neoplastic cells surrounded the tumors. The tumor growth regarding size and weight of tumor tissue was significantly aggravated after both local and systemic treatment MSCs (p value =0.007, 0.001) respectively. Inflammatory cytokines TNF and NFKB were significantly elevated (p value <0.0001), lncRNA MALAT, MMP2 expressions were significantly induced (p value <0.0001), after MSCs treatment with more significant increase in those treated by local intratumor injection of MSCs compared to those treated by systemic MSCs(p value <0.0001). **Conclusion:** Ehrlich tumor model is feasible and easily monitored tumor model. Although MSCs have anti-inflammatory effect and the ability to regenerate the damaged tissue; it could aggravate tumor growth as it exploited by cancer cells for behave of tumor cells.

Keywords: Erlish- MSCs- lncRNA MALAT- MMP2- TNF

Asian Pac J Cancer Prev, **23 (10)**, 3309-3315

Introduction

Cancer is considered as one of the most life-threatening diseases, which is responsible for a quarter of human mortalities (Siegel et al., 2016) Tumor microenvironment interacts with tumor cells, providing an atmosphere to contribute or suppress the tumor development. Mesenchymal stem cells (MSCs) have been demonstrated to possess the ability to determine the fate of tumor cells (Javan et al., 2019).

Several researches of long non-coding RNAs (lncRNAs) have been developed for the discovery of abundant new lncRNAs and extensive investigation of their roles in various diseases, especially in cancers. Metastasis associated in lung adenocarcinoma transcript 1 (MALAT1) is a lncRNA that emerges as a hotspot, which has been reported to be involved in dysregulation of cell signaling and correlated with cancer development, progression, and response to therapy (ZX Li et al., 2018).

Thus, there is a big controversy about the effect of MSCs on cancer; wither progress or regress tumor proliferation, our previous in vitro study revealed that MSCs inhibit cancer cells proliferation and reduce the inflammatory cytokines in culture media (Hassan AS et al., 2021)these finding were supported by a previous study (Ramasamy et al., 2007), on the other hand another previous study reported that MSC engulfment by tumor cells enhances epithelial mesenchymal transition (EMT), stemness, invasion, and metastasis of breast cancer in an in vivo study (Chen et al., 2019). MSCs have been considered as cells with double-edged effects, having either tumorigenic or anti-tumor activity (Javan et al., 2019).

That is why we aimed to investigate the cause of this controversy, and the cause of difference between the invitro and in vivo effects of MSCs on cancer cells, and if we use different routes of MSCs, it could affect its impact on tumor growth or not. That is why; It is critical strategy to unveil the pathophysiology of cancer to develop novel therapeutic agents against cancer. Despite different types

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of tumor models of human cancer have been developed (Ruggeri et al., 2014); Ehrlich tumor model could be considered as a good experimental cancer model due to its higher efficiency in producing an evident tumor mass that could be monitored grossly by easy methods; that could provide an efficient tool to investigate antineoplastic agents (Jaganathan et al., 2010).

Recent data explained that inflammation is a critical component of tumor progression as well as many cancers arise from sites of infection, chronic irritation, and inflammation. tumor microenvironment has been orchestrated by inflammatory cells, that could induce malignant cells proliferation, survival and migration (Coussens et al., 2002)that's why we assessed that levels of inflammatory cytokines TNF and NFKB to detect the effect of MSCs treatment by the different routes.

Materials and Methods

Isolation and preparation of BM-derived MSC

The tibiae and femurs of white albino rats' were flushed with 10% fetal bovine serum (GIBCO/BRL) and Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) to harvest the bone marrow. cells were incubated at 37°C' for 10— 14 days; in 5% humidified CO_2 until reach (80-90% confluence), after that, cultured cells were washed and then trypsinized with 0.25% trypsin in 1mM EDTA (GIBCO/BRL). The harvested cells were centrifuged (at 2400 rpm for 20 minutes) and the cell pellet was stored at -80 until usage (Farouk et al., 2018).

The Ehrlich tumor cells inoculation

The Ehrlich carcinoma cells were maintained in vivo in albino mice by intraperitoneal injection of cancer cells every 10 days. The ascitic fluid from the host animals was collected, washed in phosphate-buffered saline (PBS), then centrifuged for 10 min at 200g (Tennant, 1964).

Animals

Eighteen female mice were involved in the study. Sample size was calculated using G-power software. As regarding the primary outcome (tumor growth) based on (Campos et al., 2013; Calixto-Campos et al., 2013) we found that 6 mice per group were appropriate sample size with total sample size 18 mice (3 groups). the power is 80%, alpha probability is 0.05.

8–10 weeks of age mice were obtained from the animal house faculty of medicine. Cairo university. Mice were housed under standardized conditions (12/12 h dark/light cycle , 22 ± 1°C, and 45–65% air humidity), mice were allowed free access to food and water. All mice were subcutaneously inoculated with 1×10^6 cells in 100 µL of saline, into the right flank (Calixto-Campos et al., 2013). After three week of tumor growth; the rats were randomly divided into 3 groups of six mice each; group I: un treated Erlish tumor group; group II: Erlish tumor treated by local injection(intratumor) of 1 $x10^6$ MSCs/week, group III: Erlish tumor treated by systemic injection of 1 $x10^6$ MSCs iv in tail vein/week (Albarenque et al., 2011). Tumor growth was recorded with a digital caliper (Sagyma Plus, 0–150 mm) every two days, all measures were taken from the same examiner to minimize bias. After 4 weeks of stem cells injection, blood samples were collected from retroorbital vein under anesthesia (Ketamine and Xylazine cocktail, 5:1, 0.1 ml/20 g body weight). The plasma was separated using centrifugation at 1,000g for 15 min and stored at − 80°C until analysis. all rats were sacrificed by cervical dislocation and tumor tissues were collected for histological and molecular studies.

Estimation of serum levels of TNF

The serum levels of TNF from all studied groups were estimated according to kit instruction; TNF-alpha ELISA kit, Cat: SEK50349. Sino Biological US Inc.

Quantitative RT-PCR for studied genes

Twenty five µg of tumor tissues from all studied groups were homogenized in 300µl of lysis buffer for total RNA extraction according to kit instruction Gene JET Kit (Thermo Fisher Scientific Inc., Germany, #K0732). for reverse transcription ; about 5µl from the total RNA from each sample were used with subsequent amplification with Bioline, amedian life science company,U.K. (SensiFASTTM SYBR R Hi-ROX)One-step Kit (catalog number PI-50217 V) in a 48-well plate using the Step-one instrument (Applied Biosystems, U.S.A.). Thermal profile was as follows: 45◦C for 15min in one cycle (for cDNA synthesis), 10 min at 95◦C for reverse transcriptase enzyme inactivation, followed by 40 cycles of PCR amplification. Each cycle was continued for: 10 s at 95◦C, 30 s at 60◦C, and 30 s at 72◦C. the expression of studied genes (were normalized relative to the mean critical threshold (CT) values of GAPDH as the housekeeping gene by the Ct method. Primers' sequences for studied genes are listed in Table 1.

Histological analysis

the tumor tissues were dissected, weighted and fixed in 10% buffered formalin for 24 hours. Serial 5-µm paraffin sections were prepared and stained with hematoxylin and eosin (H&E) for histological structure analysis

Statistical method

Data were analyzed using SPSS computer software version 22. Data were expressed as mean ± SD. Comparison between groups were done using ANOVA and post hoc tests for multiple comparisons. All tests are two tailed. Results were considered significant at $p<0.05$.

Results

Histopathological results

The light microscopic examination of tumor tissues revealed that all studied group showed tumor necrotic cells interspaced the tumor tissues with extensive necrosis in locally treated tumor tissues by MSCs in compared to systemically treated and untreated ones; more over locally treated tumor showed extensive muscular infiltration by tumor cells as well as systemically treated, but higher than the untreated tumor tissues Figure 1.

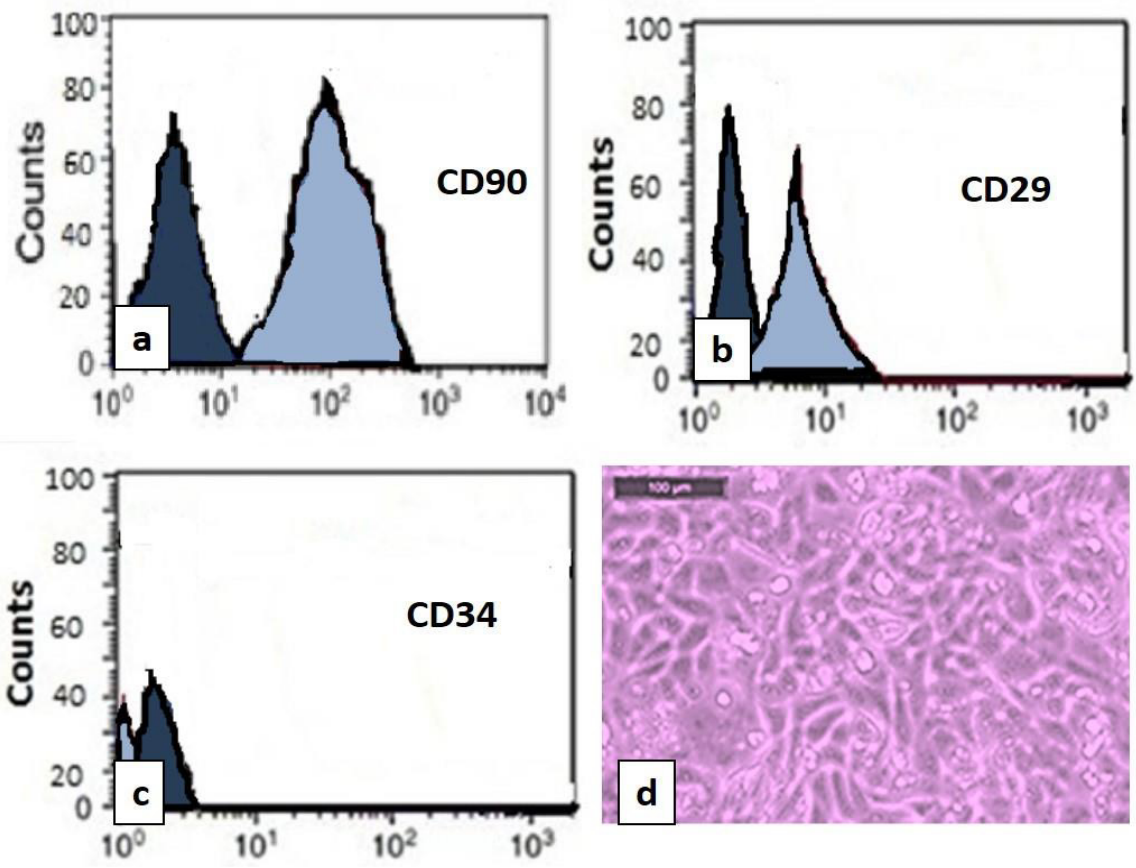

Figure 1. Flow Analysis Cytometry of the Isolated Bone Marrow Derived Mesenchymal Stem Cells. MSCs were positive for CD 90 (a) , CD 29(b) and negative for CD34 (c). MSCs were identified by their spindle shape under light microscope (d)

MSCs treatment aggravate tumor growth

Locally administrated MSCs significantly increase the tumor size and weight compared to untreated ones (p value =0.007, 0.001) respectively. While no significant difference between I.V MSCs treated cells and controls (p value >0.05), on the other hand significant increase in tumor size and weight in locally administrated ones compared to systemic ones (p value =0.03, 0.025). respectively, Figure 2.

Inflammatory cytokines and necrosis significantly flared up after MSCs treatment

NFKB gene significantly highly expressed after both local and systemic injection of MSCs in tumor tissue

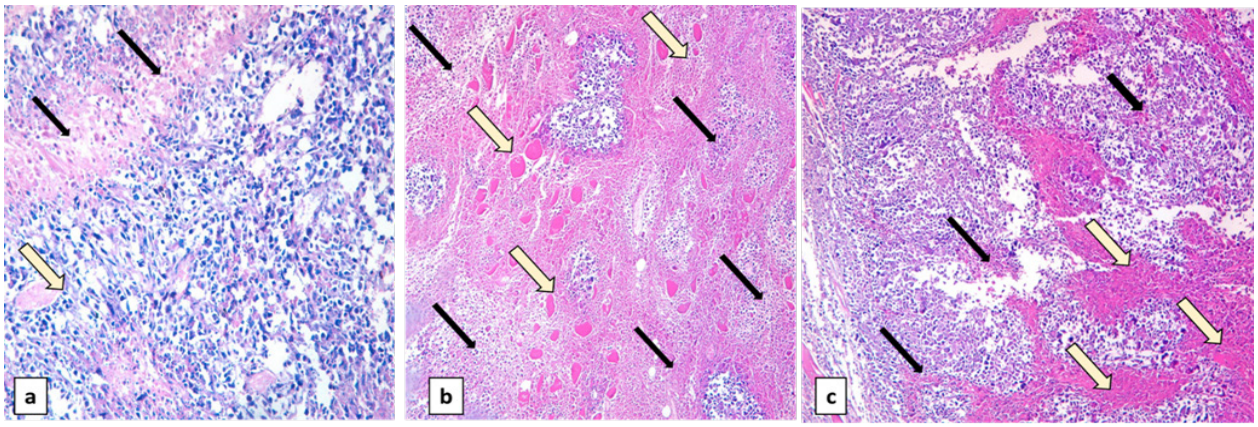

Figure 2. Light Microscopic Pictures from the Tumors of each Studied Groups (a: control, b: local MSCs treatment; c: systemic MSCs treatment.) stained with hematoxylin–eosin. The tumors of all groups are interspaced by necrotic zones infiltrated with inflammatory cells (black arrows) with extensive necrosis was observed in tumor tissues treated locally by MSCs (b) compared to untreated tumor (a) and those treated by systemic MSCs (c). Furthermore, the tumor tissues show the invasion of subcutaneous adipose tissue and skeletal muscles by the neoplastic cells in all groups (yellow arrows); In locally treated MSCs(b), several skeletal muscle fibers encompassed by neoplastic cells as well as the different types of cellular organizations within the tumor in addition to extensive muscle infiltration compared to other groups.

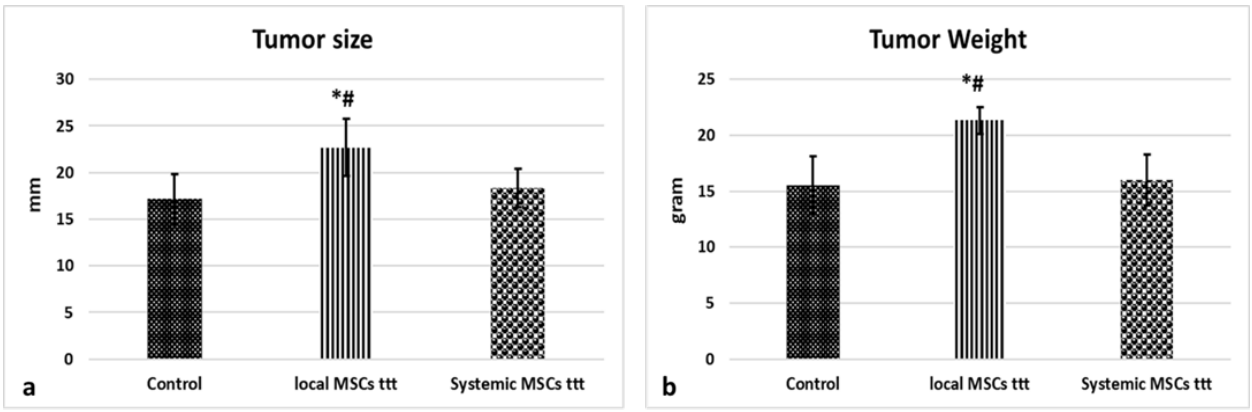

Figure 3. Data were Expressed as mean ± SD. *, denotes significant difference versus control group; #, denotes significant difference versus systemic MSCs treatment group.

compared to untreated tumor tissues (p value <0.001, 0.002) respectively; in addition significant increase in its expression after local MSCs treatment compared to those treatment by systemic treatment of MSCs (p value <0.001), Figure 3a.

The serum levels of TNF-α significantly elevated in both local and systemic injection of MSCs in tumor tissue compared to untreated ones (p value <0.001); and significant increase in its level after local MSCs treatment compared to systemic treatment of MSCs (p value <0.001), Figure 3b.

LncRNA MALAT and MMP2 expressions are significantly increased after MSCs treatment

Both LncRNA MALAT and MMP2 expressions are significantly increased after both local and systemic injection of MSCs in tumor tissue compared to untreated tumor tissues (p value <0.001); more over the locally treated tumor tissues by MSCs showed higher expression compared to those treated by systemic MSCs (p value <0.001), Figure 3c andd.

Table 1. Primers Sequence for Studied Genes

Gene	Primer sequence from 5′- 3′
MALAT	Forward: GAAGGAAGGAGCGCTAACGA
	Reverse: TACCAACCACTCGCTTTCCC
MMP2	Forward: GTCTTCCCCTTCACTTTTCTG
	Reverse: CGGAAGTTCTTGGTGTAGGTG
NFKB	Forward: GAAATTCCTGATCCAGACAAAAAC
	Reverse: ATCACTTCAATGGCCTCTGTGTAG
GAPDH	Forward: ACAGTCCATGCCATCACTGCC
	Reverse: GCCTGCTTCACCACCTTCTTG

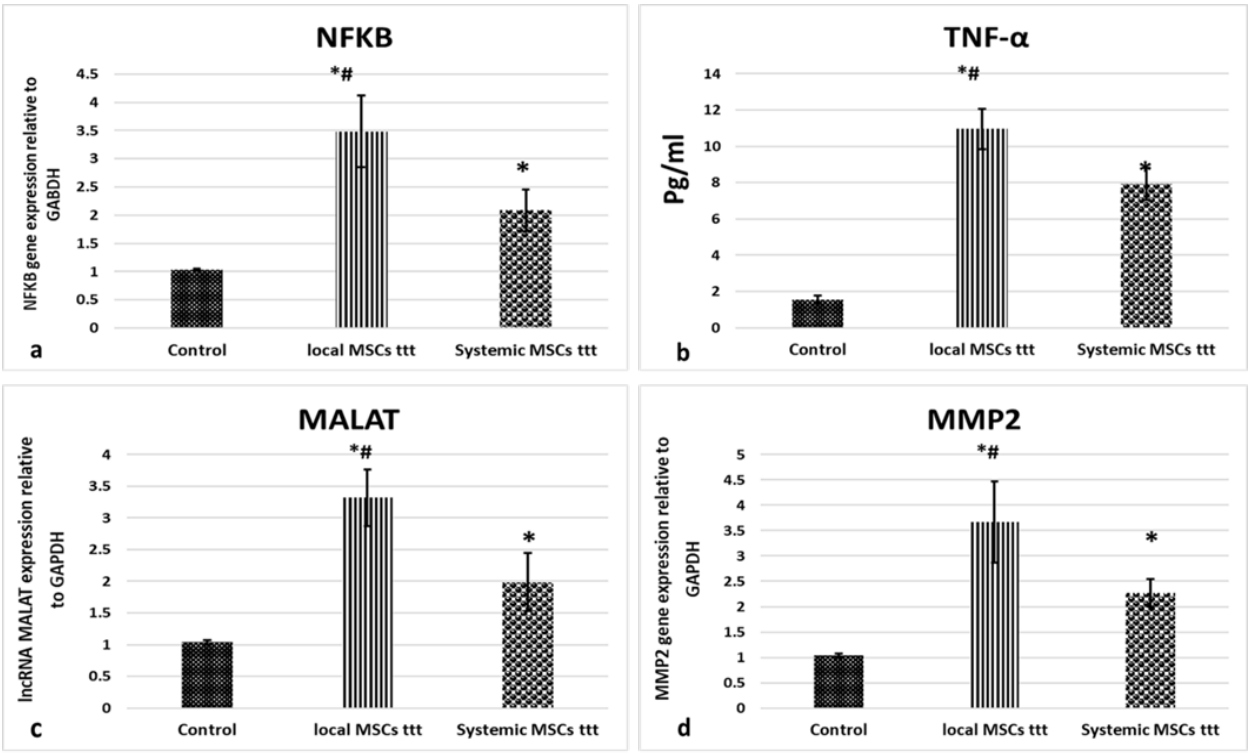

Figure 4. Data were expressed as mean ± SD. *, denotes significant difference versus control group; #, denotes significant difference versus systemic MSCs treatment group.

Discussion

The therapeutic potential of MSCs is mediated by their ability to migrate toward the damaged tissues. In addition to secretion of bioactive mediators, such as growth factors, cytokines and extracellular vesicles that could induce anti-apoptotic, anti-fibrotic, immunosuppressive, , angiogenic, and anti-inflammatory effects (Nakamizo et al., 2005). However, the ability of MSCs to migrate toward cancerous tissue is also controlled by multiple factors as , inflammatory cytokines, growth factors , and extracellular matrix molecules such as matrix metalloproteinase 2 (MMP-2). As MSCs reach the tumor niche, MSCs interact with cancer cells via direct and indirect mechanisms that affect tumor development (GC Li et al., 2016).

In this study we found significant increase in tumor growth regarding its size and weight in both locally and systemically treated groups by MSCs compared to untreated ones. Previous studies examined the effect of stem cells therapy on hepatocellular carcinomas of mice and reported that MSCs support tumor angiogenesis, that is essential process in cancer progression (Mishra et al., 2008).

Moreover , it has been reported that the soluble factors secreted from cancer cells induce the transformation of MSCs and differentiation into cancer-associated fibroblast (CAFs),CAFs are cell type within the tumor microenvironment that are capable of promoting tumorigenesis (Mishra et al., 2008). The newly formed CAFs could secrete immune-modulating agents, pro-angiogenic factors (VEGF, TGFβ, PDGF), pro-survival factors (Hepatocyte Growth Factor, Insulin like Growth Factor 1, Interleukin 6), and extracellular matrix modulators (MMP) ; all of these factors contribute to tumor progression (Kalluri, 2016).

Another mechanism of interaction between MSCs and cancer cells is cell engulfment. A recent study demonstrated that breast cancer cell engulfment of MSCs leads to changes in the transcriptome profile of tumor cells that directed toward the oncogenic pathways. This MSC engulfment enhances epithelial-to-mesenchymal transition, stemness, invasion, and metastasis of breast cancer (Chen et al., 2019).

However our recent invitro study regarding the effect of MSCs on cancer cells proliferation revealed that cancer cells treated by MSCs showed significant decrease in its proliferation together with suppression of the expression of molecular markers involved in tumor progression (IL-6, NF-KB, TLR4 and FOXO/LncRNA AF085935) (Hassan AS et al., 2021). The same results was obtained by Ramasamy et al., who reported that MSC inhibit the proliferation of malignant cells of hematopoietic and non-hematopoietic origin (Ramasamy et al., 2007). More study coincides with these result is the study of Milena et al., who reported decreased proliferation and viability of prostate cancer cell line at cell-to-cell interaction with MSCs (Milena K. et al., 2020).

Thus, there is a big controversy about the effect of MSCs on cancer; either progress or regress tumor proliferation; we can conclude from this study and our previous studies that the impact of MSCs on cancers depend on tumor environment; not on MSCs by itself. MSCs have anti-inflammatory and regenerative ability in addition to the capability to regenerate the damaged tissues. In case of invitro study the MSCs have the opportunity to directly attack the cancer cells without any other confounding factors of tumor microenvironment ; thus in that case the MSCs could attack the cancer cells as cell by cell with subsequent inhibition of cancer cells proliferation ; but in case of in vivo application of MSCs; here cancer cells are not alone but within tumor matrix and growth factors that help the cancer cells to attract the MSCs by its chemotactic properties and exploit it for its own. Cancer cells could engulf MSCs as mentioned before and reuse the genetic materials for its own thus could repair any damage within the tumor construction by the regenerative properties of MSCs. Thus, MSCs execute its beneficial effects but for the favor of cancer cells

That's what happens in the current study, wither the MSCs is locally injected within the tumor tissue or systemically administrated by IV injection, resulted in tumor progression and proliferation. Locally administrated MSCs produced more tumor progression and growth than systemically administrated; this could approve our theory as the locally administrated MSCs could be easily and rapidly engulfed by tumor cells rather than in systemic administration that MSCs travel through the blood stream and could be obstcaled by the immune cells

lncRNAs are a type of non-coding RNAs, longer than 200 bp, It has been reported that lncRNAs are involved in normal cell and tissue development and differentiation as well as in the initiation and progression of various pathological conditions, including cancer (Hrdlickova et al., 2014). dysregulated expression of lncRNAs was observed in many types of malignancies (Van Grembergen et al., 2016).

MALAT1 is a highly conserved lncRNA that was found to be upregulated in lung cancer with a high tendency to metastasize (P Ji et al., 2003). Matrix metalloproteinase 2 (MMP2) is a key regulator in cancer development. It participates in the tumor neovascularization, tissue penetration by cancer cells (Quintero-Fabián et al., 2019; Xu et al., 2005).

We found significant increase in MALAT1 and MMP2 expression in both locally and systemically treated mice by MSCs in compare to untreated ones with more expression in locally treated ones

A previous study showed that MALAT1, and NEAT1 lncRNAs are dysregulated in breast cancer and suggested poor prognosis (Arshi et al., 2018). More study revealed that overexpression of MALAT1 could promote cancer cells proliferation and migration in vitro, and promote tumor growth and metastasis in colorectal cancer mice (Q Ji et al., 2014). And breast cancer model (Mendes et al., 2007).

A previous study agreed with our RT PCR results that revealed MMP 2 and MMP 9 mRNA expression levels were significantly higher in MDA MB 231 and MCF 7 cells and in breast cancer tissues compared to tumor adjacent normal tissues. MMP 2 and MMP 9 expression levels in breast cancer tissues were correlated with lymph node metastasis and tumor staging (H Li et al., 2017).

Another study evaluated the expression of matrix metalloproteinase 2 gene (MMP2) in CRC revealed that the expression of MMP2 was significantly higher in CRC tissues than in adjacent normal colorectal tissues. In addition, high levels of MMP2 protein were positively correlated with the status of tumor size, lymph node metastasis, distant metastasis, Dukes' stage, and tumor invasion. Moreover, patients with higher MMP2 levels had markedly shorter overall survivals than those with low MMP2 levels (Dong et al., 2011).

Regarding our finding of increased the expression of lncRNA MALAT and MMP after MSCs treatment ; a previous study agreed with our results and revealed that transplantation of human MSCs for induced myocardial stroke produced greater neurological recovery and decreased infraction volume. The elevation of MMP-2 activity and the increase in vascular density after MSC treatment suggest that MSCs might help promote angiogenesis and lead to neurological improvement during the recovery phase (Nam et al., 2015).

Another study reported that MSCs saturate their environment with both MMPs and TIMPs. Since they bind and activate MMPs expression (Lozito et al., 2014).

Inflammation has been recognized as a hallmark of cancer that plays fundamental role in the development and progression in spite of absence of obvious signs of inflammation and infection. Nuclear factor-κB (NF-κB), a transcription factor essential for the development of inflammatory responses, NF-κB is one of the most important molecules linking chronic inflammation to cancer (Taniguchi et al., 2018). It has been reported that NF-kB activation was observed in many solid tumors, NF-kB activation is a result of underlying inflammation or the consequence of formation of an inflammatory microenvironment during malignant progression. NF-kB could induce up-regulation of the expression of tumor promoting cytokines, such as IL-6 or TNF-a, and tumor survival genes, such as Bcl-XL (Karin, 2009).

Tumor necrosis factor alpha (TNF-alpha), is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as in inflammation and immunity (van Horssen et al., 2006). TNF-α promotes colon cancer cell migration and invasion by upregulating TROP-2 (Zhao et al., 2018).

In this study we found significant increase in NFKB expression and TNF in both locally and systemically treated rats by MSCs in compare to untreated ones with more expression in locally treated ones.

Previous study reported that Inflammation and other signaling pathways, including NF-κB and STAT3, stimulates cancer stem cells proliferation, survival, maintenance and expansion (Vazquez-Santillan et al., 2015).

NF-κB induces MMP-2, -3, and -9 that degrade the basement membrane and remodel the extracellular matrix, which facilitates cell migration and favors either angiogenesis (endothelial cells) or metastasis (cancer cells) in different microenvironment (John et al., 2001)

Inflammatory factors can induce homing of circulating MSCs and MSCs in adjacent tissues into tumors, to be involved by the tumor microenvironment to support tumor growth. T-MSCs could recruit more immune cells into the tumor microenvironment, increase the proportion of cancer stem cells and promote tumor angiogenesis, further supporting tumor progression (Sun et al., 2014).

Thus we could conclude that cancer cells tumor matrix and growth factors help the cancer cells to attract the MSCs by its chemotactic properties and exploit it for its own. MSCs execute its beneficial effects but for the favor of cancer cells.

Author Contribution Statement

Conceptualization: AM, Methodology, Software: AM, DM, W.S, SM: Data curation, Writing- Original draft preparation. AM: Visualization, Investigation. AM, DM, W.S, SM: Writing- Reviewing and Editing: AM, DM, W.S, SM

Acknowledgments

All authors offer sincere appreciation for the experimental animal unit, Faculty of medicine-Cairo University.

Ethics statement

The animal study was reviewed and approved by Ethics and Scientific Committee, Department of medical biochemistry and molecular biology, Kasr Al Ainy, Faculty of Medicine, Cairo University, Egypt.

Availability of data

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Conflict of interest

No conflict of interest.

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