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In vitro study to evaluate the effect of granulocyte colony stimulating factor on colorectal adenocarcinoma and on mesenchymal stem cells trans differentiation into cancer stem cells by cancer cells derived exosomes

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Abstract

Background Colorectal cancer (CRC) is a common and lethal malignancies with poor prognosis. CRC cells release extracellular vesicles called exosomes to facilitate tumor progression by passing bioactive molecules such as proteins and nucleic acids between cells of the tumor and their microenvironment. Granulocyte colony stimulating factor (G-CSF) is a hematopoietic growth factor which mainly affects the lineage of neutrophil and exerts direct anti-tumor effects on various tumor types. The purpose of our study is to investigate the effect of G-CSF on CRC cells and to evaluate its capability to attenuate the potentiality of CRC cells derived exosomes to induce bone marrow-derived mesenchymal stem cells (BM-MSCs) malignant transformation into cancer stem cells (CSCs).

Results The level of both lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) ($p=0.014$) & β-catenin ($p=0.01$) was significantly decreased, whereas programmed cell death 4 (PDCD4) ($p=0.018$) was increased in CRC exosomes pre-treated with G-CSF compared to untreated CRC exosomes. Additionally, there was a significant decrease in the cell proliferation in CRC cells pre-treated with G-CSF compared to untreated CRC cells ($p=0.008$). Flow cytometric analysis of BM-MSCs showed that G-CSF could attenuate their transformation into CSCs.

Conclusion G-CSF can be a promising therapeutic agent for CRC treatment.

Keywords CRC, Exosomes, G-CSF, MALAT and CSCs

1 Background

Colorectal cancer (CRC) is one of the main causes of cancer deaths as there is about 1.4 million new cases yearly and one million new deaths yearly worldwide [1]. The microenvironment of the tumor is made up of a variety of cells, such as cancer stem cells (CSCs). CSCs are a group of cells exhibiting a stemness trait, such as the ability to self-renew and differentiate into multiple cell types. These cells are considered to be the cause of

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recurrence of the tumor and the failure of traditional cancer therapy [2].

Cancer cells release exosomes which are microscopic vesicles that transport biologically active molecules such as deoxyribonucleic acids (DNAs), long non-coding RNAs (LncRNAs), micro RNAs (miRNAs), and proteins [3, 4]. According to research, these exosomes have role in apoptosis, cell cycle progression, cell signaling, as well as cancer initiation and progression [5, 6] and could play a crucial role in transforming BM-MSCs into a tumor-like cells [7].

Overexpression of the metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1)-LncRNA in CRC cells and their derived exosomes was later linked to the cell proliferation of CRC cell, invasion, migration, and metastasis via activation of the beta catenin (β-catenin) gene [8, 9].

Programmed cell death 4 (PDCD4) is a tumor suppressor whose expression is reduced in a variety of malignancies, including CRC. PDCD4 has a role in the inhibition of the cell proliferation PDCD4 as its overexpression inhibits cell proliferation [10].

CRC is often diagnosed at late stage. Chemotherapy and other therapies provide a limited increase of the survival for these patients [11], so there is a big need for discovery of novel therapeutic strategies in order to increase the survival rate of CRC. Granulocyte colony-stimulating factor (G-CSF) can stimulate differentiation and proliferation of myeloid precursor cells into granulocytes so that, it was used to treat neutropenia following cancer chemotherapy [12]. Surprisingly, some studies demonstrated that G-CSF could inhibit the progression of cancer in various cancer types [13–15].

This work aims to investigate the effect of G-CSF on CRC cells and to evaluate its capability to prevent CSCs formation induced by CRC derived exosomes.

2 Methods

Our study was an observational study that aimed to investigate the effect of G-CSF on CRC cells and to evaluate its capability to prevent BM-MSCs malignant transformation into CSCs induced by CRC exosomes.

2.1 Colon adenocarcinoma cell culture

Human colorectal adenocarcinoma cell line (CACO-2) as colon cancer cell line was purchased from (Sigma-Aldrich Chemical Co., St. Louis, MO, USA, Cat. No MTOX1000P24). It was cultured in Park Memorial Institute (RPMI -1640) culture media with glutamine 2 mM (bio west, Nampa, cat n L0498-500), 10% Fetal Bovine Serum (FBS) (PAA, Pasching Austria, Cat nA11-151) and Penicillin with Streptomycin 1% (Lonza, Verviers, Belgium, CatN DE17-602E). CaCo-2 cells were grown in 5% CO_2, 95% air at 37 °C (NEW BRUNSWICK SCIENTIFIC-Innova co-170). Cells were washed with cold phosphate buffer saline (PBS), trypsinized, harvested and centrifuged to form cell pellets. Then cells were divided into two groups; 1st group: untreated CRC cells, and the 2nd group: CRC cells treated with G-CSF (sigma-Aldrich Chemical Co., St. Louis, MO, USA, MDL number: MFCD00166475) at a single dose of 20 ng/ml at a dose of 20 ng/ml [16]. CRC cells from both groups were suspended for further MTT assay and exosomal extraction; then exosomes from both groups were harvested for molecular studies, and further application on BM-MSCs.

2.2 MTT cell proliferation assay

In order to investigate the anti-proliferative effect of G-CSF on CACO-2 cells (4,5-dimethylthiazol, 2,5-diphenyltetrazolium bromide (MTT) assay was used. We fixed CACO-2 cells (for 24 h) with the frequency of (1×10^6) cells into 96 well plates. The plate was divided equally into two groups as mentioned before. MTT reagent was added to the wells in line with the manufacturer's instructions (Biospes, China, Cat n#BAR1005-1), detergent reagent was added (100 µl per well) when the purple precipitate was visible clearly, in order to make the formazan dye soluble. In the dim, plates were left with cover for 2 to 4 h. The cover of the plate was detached and we assessed the absorbance in each well at a range from 490 to 630 nm by using ELISA plate reader (Stat Fax 2200, Awareness Technologies, Florida, USA). Amount of absorbance was proportional to cell number.

2.3 Isolation, identification, and quantitation of exosomes derived from cultured cancer cells

Exosomes from both groups were isolated through differential centrifugation at 3000*g* to get rid of cells then 10,000*g* for 20 min to remove the debris, at 4° ultracentrifugation was done at 100,000g to cell-free supernatants (Beckman Coulter Optima L-90K ultracentrifuge) for 90 min, the supernatant was removed carefully, and crude exosome-containing pellets were re-suspended in 1 mL of ice-cold PBS and pooled. A second ultracentrifugation in the same conditions was preformed [17]. The exosomes' protein content was quantified by Bradford method (BioRad, Hercules, CA, USA).

2.4 Extraction of exosomal RNAs from both studied CRC cells groups

A total RNA was extracted from exosomes derived from both groups by miRNeasy mini kit provided by Qiagen, Germany (Cat no: 217084). By using Beckman dual spectrophotometer (USA) at 260 nm, the total RNA obtained was determined.

2.5 Quantitative reverse transcriptase—polymerase chain reaction (qRT-PCR)

Reverse transcription and PCR amplication for mRNA expression was done using kit provided by Vivantis, Vi Prime PLUS One Step thermus aquaticus (Taq) RT-qPCR Green Master Mix I with ROX (SYBR Green Dye) (cat no #QLMM14-R) according to the manufacturer's protocol. One Step Taq qRT-PCR Green Master Mix I with ROX Kit was compatible with three-step cycling, reverse transcription occurs for 10 min at 55 °C as one cycle, enzyme activation for 8 min at 95 °C as one cycle, denaturation occurs for 10 s at 95 °C and both annealing and extension for 60 s at 60 °C for forty cycles. The data were expressed in Cycle threshold (Ct) after the run of the RT-PCR. According to the calculation of delta-delta Ct (ΔΔCt), the quantification of the RQ of each target gene is done by normalization against house-keeping gene glyceraldehyde 3 phospate dehydrogenase for (mRNAs and LncRNA MALAT-1). Table 1 presented the sequence of the primer of all studied genes and LncRNA.

2.6 Preparation and isolation of human BM-MSCs

Under aseptic conditions, bone marrow (20 mL) was aspirated from the iliac crest and kept in heparinized tubes. A density gradient (Ficoll-Paque; GE Health-Care, Waukesha, WI) was used to separate nucleated cells and then re-suspended in culture medium (Delbecco's Modified Eagle's Medium) which was supplemented with 10% FBS and 1% penicillin–streptomycin (10,000 g/mL). Then the culture of the cells was done at 37 °C for 14 days in 5% CO_2. The culture media was replaced every 2 to 3 days When the cells had reached 80–90%, the cultures were washed using PBS twice and trypsinized at 37 °C for 5 min with 0.25% Trypsin in 1 mM EDTA. Cells were re-suspended in media and subcultured for 10 days after centrifugation, so that an average count of 15 × 106 cells was obtained.

Table 1 Primers sequence of studied genes

Gene symbol	Primer sequence from 5′–3′ F: Forward primer, R: Reverse primer	Gene bank accession number
LncRNA MALAT-1	F: GTCATAACCAGCCTGGCAGT R: CGAAACATTGGCACACAGCA	NR_002819.4
PDCD4	F: AACCCTGCAGAAAATGCTGG R: GGATCGCCTATCCAGCAACC	NM_001199492.2
β catenin	F: CTGAGGAGCAGCTTCAGTCC R: ATTGCACGTGTGGCAAGTTC	NM_001098209.2
GAPDH	F: AATGGGCAGCCGTTAGGAAA R: TTCCCGTTCTCAGCCTTGAC	NM_001256799.3

2.7 Co-culture of BM-MSCS with CRC derived exosomes

BM-MSCS were divided as following into three groups: **Group 1:** untreated BM-MSCs (1×10^6) as a control group; **Group 2:** BM-MSCs (1×10^6) co-cultured with 100 µg/ml of exosomes derived from untreated CRC cells [18]; **Group 3**: BM- MSCs (1×10^6) co-cultured with 100 µg/ml of exosomes derived from CRC cells pre-treated with G-CSF [18]. BM-MSCs from the three studied groups were incubated for 24 h. BM-MSCs malignant transformation into CSCs was assessed by immunophenotyping analysis for CSCs markers (CD44 and 133) expression.

2.8 Statistical analysis

IBM® SPSS® Statistics Version 25 was used for statistical analysis. The mean and standard deviation of numerical data were presented. The paired sample t-test was used to compare G-CSF-treated and untreated CRC cells. One-way ANOVA was used to compare the three BM-MSC groups, followed by a Tukey post hoc test for multiple group comparisons [19]. The significance level was set at $P \leq 0.05$ within all tests.

3 Results

3.1 Co-culture of CRC cells with G-CSF

The morphological observations using inverted light microscopy in untreated CRC cells group revealed adherent growth with an increase in cell population (Fig. 1A), while there was a decrease in cell population, cell rounding and detachment in CRC cells pre-treated with G-CSF (Fig. 1B).

3.2 Identification of CRC derived exosomes from both studied groups

Exosomes from both untreated CRC cells and CRC cells pre-treated with G-CSF were identified by TEM according to their size (100 nm) and their morphology which is cup shaped as shown in (Fig. 1C, D).

3.3 G-CSF significantly inhibits CRC cell proliferation by MTT assay

The cell proliferation in CRC cells pre-treated with G-CSF was significantly decreased compared to untreated CRC cells ($P = 0.008$) (Fig. 2A).

3.4 G-CSF represses the expression of LncRNA MALAT-1 and β catenin and induces the expression of PDCD4

Concerning the expression levels of LncRNA MALAT-1 and β- catenin gene there were a significant decrease in their expression in the exosomes derived from

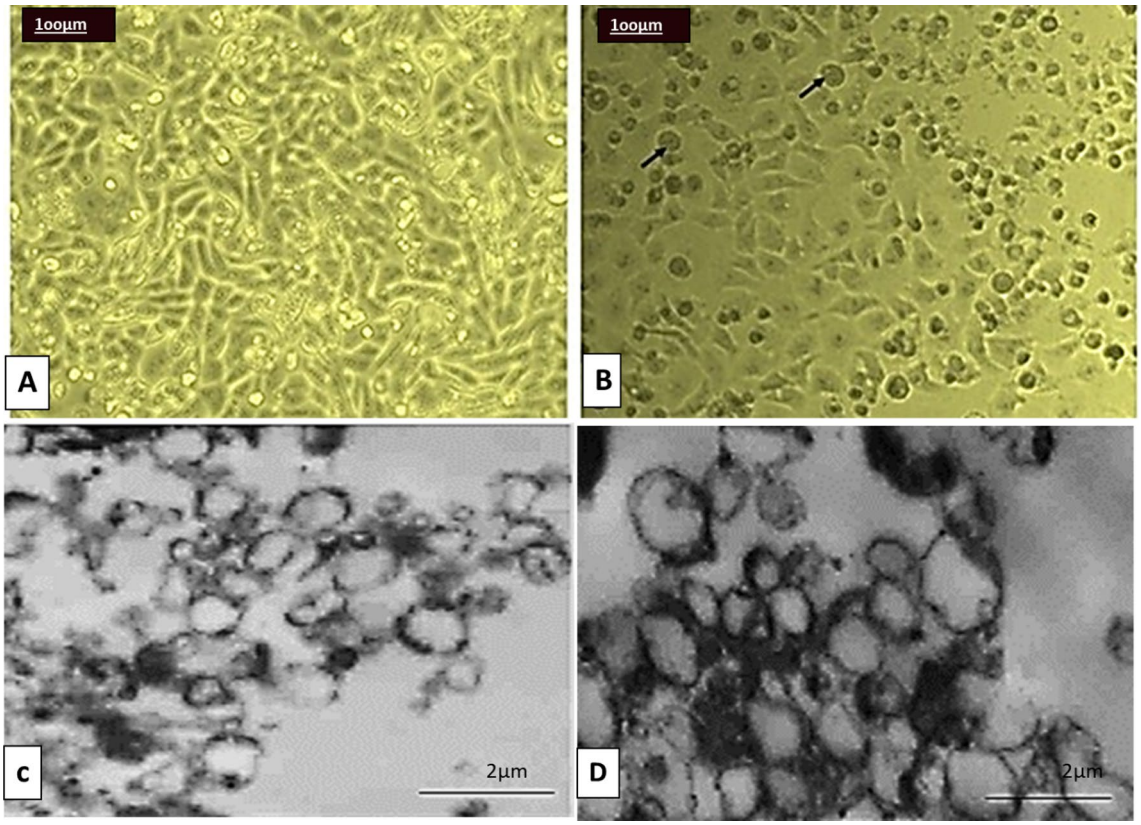

Fig. 1 **A** untreated CRC cells; **B** CRC cells treated with G-CSF; showing decrease in the number of CRC cells, black arrows indicate cell rounding and detachment which are signs of apoptosis, **C** exosomes from untreated CRC cells, **D** exosomes from G-CSF treated CRC cells by TEM showing their cup shaped morphology and size (100 nm), scale bar (2 µm)

CRC cells pre-treated with G-CSF compared to those derived from untreated CRC cells group ($P=0.014$ and 0.01, respectively). (Fig. 2B, C).

Regarding PDCD4 gene expression there was a significant increase in its expression in exosomes derived from CRC cells pre-treated with G-CSF compared to exosomes derived from untreated CRC cells ($P=0.018$) (Fig. 2C).

3.5 G-CSF could attenuate BM-MSCs transformation into CSCs

As shown by Flow cytometric analysis for CD markers of CSCs (CD44 and 133) in BM-MSCs studied groups, the CSCs markers CD44 and CD133 were significantly increased in BM-MSCs co-cultured with exosomes from untreated CRC cells group compared to other groups ($P=0.001$, $P=0.001$, respectively), whereas no significant difference was found between BM-MSCs co-cultured with exosomes from CRC cells pre-treated with G-CSF and BM-MSCs control group ($P=0.8$, $P=0.99$) (Fig. 3A, B).

3.6 The anti-proliferative effect of G-CSF is demonstrated by MTT cell proliferation in BM-MSCs

The cell proliferation in BM-MSCs co-cultured with exosomes from untreated CRC cells group was significantly increased compared to both control group of BM-MSCs and BM-MSCs co-cultured with exosomes from CRC cells pre-treated with G-CSF ($P=0.005$; $P=0.002$, respectively), whereas no significant difference was found in cell proliferation between BM-MSCs control group and BM-MSCs co-cultured with exosomes from CRC cells pre-treated with G-CSF ($P=0.85$) (Fig. 3C).

4 Discussion

Throughout the years, CRC has been considered the third most diagnosed cancer and the second cause of death related to cancer globally [20]. Despite the current therapy available for CRC, the cancer prognosis for patients has not been satisfying, especially for patients with metastatic tumors [21]. Thus, it is crucial to have new alternative effective treatment strategies for CRC patients.

Furthermore, CRC cells release high amounts of nanovesicles (exosomes), enclosing proteins, DNA, miRNA,

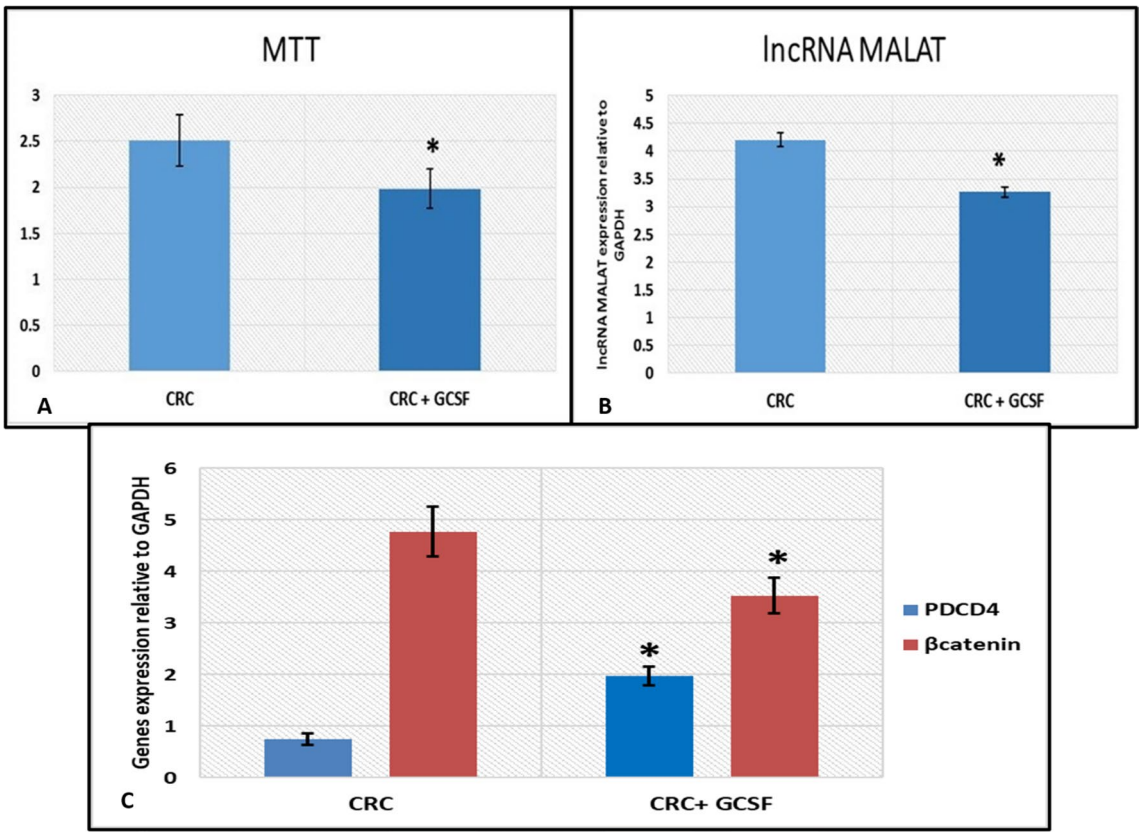

Fig. 2 **A** MTT assay of both studied groups. **B** comparison of LncRNA MALAT expression between CRC studied groups. **C** comparison of PDCD4 and β catenin genes expression between CRC studied groups (*) Denotes significant difference compared to untreated CRC cells

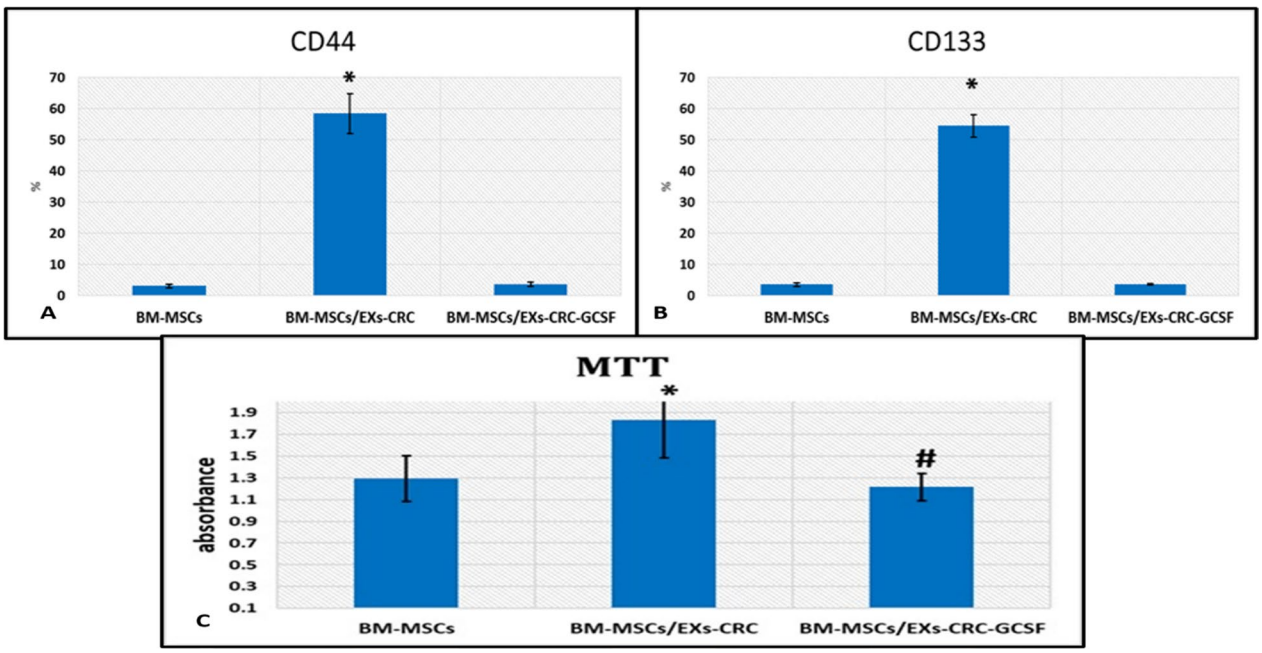

Fig. 3 **A** and **B** comparison of FACS analysis for CSC marker CD44&CD133 between BM-MSCs studied groups. **C** comparison of MTT between BM-MSCs studied groups. * Denotes significant difference compared to BM-MSCs group. # Denotes significant difference compared to BM-MSCs/EXs-CRC group

and LncRNA which play an important role in regulating tumor growth, angiogenesis, and metastasis [22]. Besides CRC cells derived exosomes have been shown to enhance malignant transformation of colonic stromal MSCs into CSCs and thus, promoting tumor growth and progression [23].

G-CSF, which is a key regulator of neutrophil production and activity, is commonly used to prevent and treat febrile neutropenia following chemotherapy and radiotherapy in cancer patients [24]. Surprisingly, recent studies have revealed that G-CSF has valuable direct antitumor effects on various types of cancers, including mammary adenocarcinoma [14] and lung cancer [15]. Despite these interesting studies, we have a limited knowledge about the G-CSF's effects on CRC.

Our study was conducted to investigate the G-CSF's effect on CRC cells and to evaluate its capability to prevent BM-MSCs malignant transformation into CSCs induced by CRC exosomes.

LncRNAs are considered a type of ncRNA and their length is more than 200 nucleotides whose aberrant expression has been linked to cellular proliferation, differentiation, autophagy, and metastasis [25, 26]. LncRNA MALAT-1 was highly expressed in CRC [27] and promote both tumor growth and metastasis through triggering the expression of β-catenin. Wnt/β-catenin signaling was shown to promote the CSCs' differentiation greatly [28], that can be progenitors of mature cancer cells, in addition to enhancing epithelial to mesenchymal transition (EMT) [29]. Therefore, targeting this pathway may attenuate the abnormal behavior of stem cell and EMT involved in carcinogenesis.

Interestingly, the results of our study showed a significant decrease in the expression levels of LncRNA MALAT-1 and β catenin in exosomes derived from CRC cells pre-treated with G-CSF in comparison with exosomes derived from untreated cells of CRC, which in turn suggest that G-CSF may inhibit tumor initiation and progression through downregulation of LncRNA MALAT-1/β-catenin axis.

The tumor suppressor, PDCD4 that slows down the benign and malignant tumors' progression by inhibiting AP-1 transactivation and the translation machinery, in addition to induction of apoptosis [30]. According to recent studies, it has been reported that in CRC, the expression of PDCD4 is decreased or even completely absent [31]. Accordingly, upregulation of PDCD4 expression can represent a promising therapeutic strategy in CRC' treatment.

Our present study's results demonstrated that expression of PDCD4 was significantly increased in CRC exosomes pre-treated with G-CSF compared to the exosomes from untreated CRC cells suggesting that G-CSF may exhibit direct antitumor effect on CRC through upregulating PDCD4 expression. Besides, MTT cell proliferation assessment showed that cell proliferation in CRC cells pre-treated with G-CSF was significantly decreased in comparison with the untreated CRC cells group, thus, emphasizing the anti-tumoral activity of G-CSF on CRC.

MSCs are multipotent cells, replicating as undifferentiated cells, in addition to their ability to differentiate into mesenchymal tissue lineages such as; adipocytes, osteocytes, and chondrocytes [32]. MSCs are recognized as vital constituents of the tumor microenvironment, that can augment cell proliferation, invasion and metastasis of the cancer, and also enhancing angiogenesis, and suppressing anti-tumor immune responses [33]. These diverse effects are the result of mutual interactions between MSCs and cancer cells via cancer cells derived exosomes [34].

Accumulating evidence indicates that CD44, 133 are colorectal CSCs markers and are crucial factors in regulating the properties of CSCs, including self-renewal, metastasis, and chemo-radio-resistance [35, 36].

Consistent with the previous findings, in our work the BM-MSCs' isolation was successful, confirmed by their positivity for CD90 and CD105 surface markers, besides, FACS analysis of BM-MSCs co-cultured with exosomes derived from untreated CRC cells presented significant increase in CSCs markers' expression; CD44 and CD133 compared to BM-MSCs control group and to BM-MSCs co-cultured with exosomes derived from cells of CRC pre-treated with G-CSF, thus emphasizing the capability of CRC derived exosomes to induce BM-MSCs malignant transformation into CSCs.

However, no significant difference was shown in the expression levels of CD44 and CD 133 between the BM-MSCs control group and BM-MSCs co-cultured with exosomes derived from cells of CRC pre-treated with G-CSF, indicating that the capability of CRC exosomes to induce BM-MSCs malignant transformation into CSCs could be attenuated by G-CSF.

Moreover, MTT cell proliferation assessment showed that the the cell proliferation in BM-MSCs co-cultured with exosomes derived from untreated cells of CRC group was significantly increased compared to BM-MSCs control group and to BM-MSCs co-cultured with exosomes from CRC cells pre-treated with G-CSF group, while there was no difference existed significantly in cell proliferation between BM-MSCs co-cultured with exosomes derived from CRC cells pre-treated with G-CSF group and BM-MSCs control group, thus confirming the capability of G-CSF to attenuate the ability of CRC cells derived exosomes to induce higher

proliferation rate and malignant transformation of BM-MSCs into CSCs.

5 Conclusion

In conclusion, our present study clearly demonstrated that GCSF could exhibit direct antitumor effects on colorectal adenocarcinoma cell line CaCo-2; moreover, G-CSF could attenuate the potentiality of exosomes derived from CRC cells to induce malignant transformation of BM-MSCs into CSCs, thus, providing a new approach for the CRC's treatment.

Abbreviations

CRC	Colorectal cancer
G-CSF	Granulocyte colony stimulating factor
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CSCs	Cancer stem cells
PDCD4	Programmed cell death 4
MALAT-1	Lung adenocarcinoma transcript 1
CACO-2	Human colorectal adenocarcinoma cell line

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Author contributions

Conceptualization: AM. Methodology and validation: AA, DS, MM and AM, Data curation: AM and MM. Writing—original draft preparation: MM, AA and AM Writing—review and editing: AA, and AM. Supervision, DS. All authors read and approved the final manuscript.

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Availability of data and materials

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

Declarations

Ethics approval and consent to participate

Our study was ethically approved by the Ethical Committee of Faculty of Medicine, Cairo University, Egypt, on 21/1/2019. Consent to participate is not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare there are no competing interests.

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