RESEARCH ARTICLE

Analysis of pluripotency marker expression in human glioblastoma multiforme cells treated with conditioned medium of umbilical cord-derived mesenchymal stem cells

[version 1; peer review: 2 not approved]

Novi Silvia Hardiany ¹, Purnamawati Huang ², Syarifah Dewi¹, Reni Paramita¹, Septelia Inawati Wanandi ¹

¹Department of Biochemistry & Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

²Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

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Abstract

Background: Glioblastoma multiforme (GBM) is the most aggressive form of malignant glioma and is also known as grade IV astrocytoma. This might be due to the presence of cancer stem cells with high pluripotency and ability of self-renewal. Recently, it has been reported that tumor stroma cells, including mesenchymal stem cells (MSCs), secrete factors that affect cancer cell growth. Until now, the role of MSC secretomes in cancer stem cell pluripotency remains unclear. The aim of this study was to analyze the effect of MSC secretomes in conditioned medium (CM) on the expression of pluripotency markers of GBM cells.

Methods: Umbilical cord-derived MSCs (UCSCs) were grown on serum-free alphaMEM for 24 hours to prepare the UCSC-CM. Human GBM T98G cells were treated with UCSC-CM for 24 hours. Following this treatment, expression of pluripotency markers SOX2, OCT4 and NANOG genes was analyzed using quantitative RT-PCR.

Results: SOX2 and OCT mRNA expression was 4.7-fold ($p=0.02$) and 1.3-fold ($p=0.03$) higher in CM-treated cells compared to the control. However, there was no change in NANOG mRNA expression. This might be due to there being other factors regulating NANOG mRNA expression.

Conclusions: UCSC-CM could affect the expression of SOX2 and OCT4 in human glioblastoma multiforme T98G cells. Further research is needed to elucidate the mechanism by which pluripotency markers are expressed when induced by the UCSC secretome.

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Approval Status

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1. Jie Sun , Shanghai Jiao Tong University, Shanghai, China		
2. Isabele C. Iser , Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, Brazil		
Any reports and responses or comments on the article can be found at the end of the article.		

Keywords

conditioned medium, mesenchymal stem cells, glioblastoma
multiforme, pluripotency expression

Corresponding author: Septelia Inawati Wanandi (septelia@gmail.com)

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Introduction

Glioblastoma multiforme (GBM) is a primary brain tumor that arises from glial cells (glioma), and is the most aggressive form of malignant glioma. According to the WHO, GBM is also known as grade IV astrocytoma¹. The life expectancy of patients with GBM is very low, usually less than 1 year despite available options such as surgery and chemoradiation. Failure of therapy might be due to the presence of cancer stem cells with high pluripotency and ability of self-renewal². Cancer stem cells have an improved the ability to repair their own DNA³, and can be identified by the analysis of transcription factors found in embryonic stem cells such as OCT-4 (octamer-binding transcription factor 4), SOX2 [SRY (sex determining region Y)-box 2] and NANOG. Those transcription factors play an essential role in sustaining the pluripotency and self-renewal ability of embryonic stem cells^{4,5}.

Recently, it has been reported that tumor stroma cells, including mesenchymal stem cells (MSCs), secrete factors that affect cancer cell growth. Previous studies have demonstrated that MSCs were recruited from bone marrow and that they home around the cancer cells to support tumor growth and metastasis⁶. Other studies have shown that MSCs can trigger cancer growth by inducing angiogenesis, suppressing the immune system, forming cancer-associated fibroblasts (CAF) that contributing to the tumor growth, epithelial mesenchymal transition (EMT) and metastasis⁷. The role of MSCs in cancer growth has been widely investigated. Nevertheless, the role of MSC secretomes in cancer stem cell pluripotency remains unclear. The aim of this study was to analyze the effect of MSC secretomes in conditioned medium (CM) on the expression of pluripotency markers of GBM cells.

Methods

Cell culture

The Human Glioblastoma Multiforme T98G cell line was grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) on T-25 cm² culture flasks (Corning). Medium was added with sodium bicarbonate, 10% fetal bovine serum (FBS, Biowest), 1% streptomycin - penicillin and 1% amphotericin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The medium was changed 2 times in a week. T98G cells were sub-cultured after being 70–80% confluent⁸.

Preparation of conditioned medium of umbilical cord-derived MSCs (UCSC-CM)

Umbilical cord-derived MSCs (UCSCs) were kindly provided by Prof. Jeanne Adiwinata Pawitan (Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital, Jakarta)⁹. 125,000 UCSCs were cultured in Minimum Essential Medium alpha (αMEM, Gibco) supplemented with 10% FBS (Biowest)/Glutamax (Gibco), 1% streptomycin-penicillin and 1% amphotericin on T-25 cm² culture flasks (Corning) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After the cells were 70–80% confluent, medium was removed and cells were washed 3 times using 1x Phosphate Buffer Saline (PBS). Then, cells were grown on serum free- αMEM for 24 hours to prepare

the UCSC-CM. After 24 hours, UCSC-CM were collected, centrifuged to remove cell debris and passed through 0.22 μm filter. Concentration of UCSC-CM was 50%, diluting UCSC-CM in freshly high glucose DMEM.

UCSC-CM treatment of T98G cells

400,000 T98G cells were seeded in triplicate on a 12-well-plate in high glucose DMEM/10% FBS/1% streptomycin-penicillin/1% amphotericin and allowed to adhere overnight. The following day, medium of T98G cells was replaced by 50% (v/v) UCSC-CM and incubated for 24 hours.

Analysis of pluripotency marker expression

SOX2, OCT4 and NANOG genes expression was detected using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). First, total RNA was extracted from CM-treated T98G cells using Tripure RNA Isolation kit (Roche). Total RNA was amplified using qPCR (PCRmax Eco48, United Kingdom) with KAPA SYBR FAST One step qRT-PCR (KAPA Biosystem) and primers for SOX2, OCT4, and NANOG (Table 1). Reaction protocol was as follows: cDNA synthesis for 10 minutes at 42°C, inactivation with iScript Reverse transcriptase for 5 minutes at 95°C, then 40 PCR cycles for 10 seconds at 95°C, followed by 30 seconds at 57°C for OCT4, 59°C for SOX2, 60°C for NANOG; then 30 seconds at 72°C. Following the PCR cycles, the protocol was subsequently continued with the melting curve analysis, i.e. 1 minute on 95°C; 1 minute on 55°C; 10 seconds on 55°C (80 cycles, increase 0.5°C every cycles).

Statistical analysis

All data were presented as means ± SD from triplicate experiments. Statistical analysis was performed using Student's t-test using PASW 18 software, with p < 0.05 as a cut-off for determining a significant difference.

Results

Cell morphology

Cell morphology was observed under an inverted microscope (100X magnification) after 24 hours of the CM treatment. There was no difference in morphology between control and CM-treated T98G cells, as shown in Figure 1. Both control T98G cells and CM-treated T98G cells appear with fibroblast morphology, adhering to the plate. There was no change in cell shape and the cell membrane was still intact.

Table 1. Primer sequences.

Human	Sense:	5'-GAGGAGTCCCAGGACATCAAA-3'
OCT4	Antisense:	5'-AGCTTCCTCCACCCACTTCT-3'
Human	Sense:	5'-GGAGAGTAAGAAACAGCATGGA-3'
SOX2	Antisense:	5'-GTGGATGGGATTGGTGTCT-3'
Human	Sense:	5'-ACAGAAATACCTCAGCCTCCAGCA-3'
NANOG ¹⁰	Antisense:	5'-CTCCAGGTTGAATTGTTCCAGGTC-3'

Pluripotency marker expression

Pluripotency marker expression was analyzed by measuring SOX2, OCT4 and NANOG mRNA expression. Relative mRNA expression was calculated using the Livak method ($2^{-\Delta\Delta CT}$) with 18S rRNA as the reference gene. SOX2 mRNA expression was significantly higher in CM-treated T98G cells compared to control (Figure 2).

SOX2 mRNA expression was up-regulated 4.7-fold in CM-treated T98G cells. Moreover, OCT4 mRNA expression was also significantly increased in CM-treated T98G cells compared to the control (Figure 3). OCT4 mRNA expression increased 1.2 fold in CM-treated T98G cells. Nevertheless, there was no significant change in NANOG mRNA expression (Figure 4).

Figure 1. T98G cell morphology. About 4×10^5 cells were plated triplicate in a 12-well plate and grown in high glucose Dulbecco's Modified Eagle's Medium supplemented with 10% FBS/1% penicillin-streptomycin/1% amphotericin at 37°C, 5% CO₂ for 1 day. Afterwards, the medium of treated cells was replaced with 50% (v/v) conditioned medium of umbilical cord stem cells, while medium of control cells was replaced with 50% (v/v) Minimum Essential Medium alpha. After 24-hour incubation, cell morphology was observed under inverted microscope (100X magnification). (A). Control cells; (B). Conditioned Medium-treated cells.

Figure 2. SOX2 mRNA expression. 100 ng of total RNA was amplified using quantitative reverse transcriptase polymerase chain reaction to detect SOX2 mRNA expression. The expression was relatively calculated using Livak formula with 18S rRNA gene as a reference gene. All values are means $\pm$ SE, n = 9. Significant differences at *(p<0.05). SOX2 expression was 4.7-fold (p=0.02) higher in the conditioned medium-treated T98G cells compared to the control.

Figure 3. OCT4 mRNA expression. 100 ng of total RNA was amplified using quantitative reverse transcriptase polymerase chain reaction to detect OCT4 mRNA expression. The expression was relatively calculated using Livak formula with 18S rRNA gene as a reference gene. All values are means $\pm$ SE, n = 9. Significant differences at *(p<0.05). OCT4 expression was 1.3-fold (p=0.03) higher in the conditioned medium-treated cells compared to the control.

Figure 4. NANOG mRNA expression. 100 ng of total RNA was amplified using quantitative reverse transcriptase polymerase chain reaction to detect NANOG mRNA expression. The expression was relatively calculated using Livak formula with 18S rRNA gene as a reference gene. All values are means $\pm$ SE, $n = 9$. Significant differences at $^*(p < 0.05)$. There was no significant difference in NANOG expression between control and conditioned medium-treated T98G cells.

Dataset 1. 18S rRNA Cq values

<http://dx.doi.org/10.5256/f1000research.13154.d191296>

18S rRNA Cq was used to calculate SOX2, OCT4 and NANOG mRNA expression using the Livak formula (K: control cells; CM: condition medium-treated cells).

Dataset 2. SOX2 Cq values

<http://dx.doi.org/10.5256/f1000research.13154.d191297>

SOX2 Cq was used to calculate SOX2 mRNA expression using the Livak formula (K: control cells; CM: condition medium-treated cells).

Dataset 3. OCT4 Cq values

<http://dx.doi.org/10.5256/f1000research.13154.d191298>

OCT4 Cq was used to calculate OCT4 mRNA expression using the Livak formula (K: control cells; CM: condition medium-treated cells).

Dataset 4. NANOG Cq values

<http://dx.doi.org/10.5256/f1000research.13154.d191299>

NANOG Cq was used to calculate NANOG mRNA expression using the Livak formula (K: control cells; CM: condition medium-treated cells).

Dataset 5. Raw unedited images for Figures 1A and 1B

<http://dx.doi.org/10.5256/f1000research.13154.d191300>

Discussion

Similar to normal stem cells, stem cell-like properties such as pluripotency in glioma CSCs are maintained by a core set of transcription factors, including SOX2, OCT4, and NANOG. Up-regulation of this set of genes is associated with poor outcome in terms of tumor malignancy, recurrence and metastasis¹¹. Here, we demonstrated that the pluripotency markers SOX2 and OCT4 were up-regulated in UCSC-CM-treated cells. This indicates that UCSCs secrete certain factors that support the self-renewal capacity of GBM cells. Liu *et al* showed that bone marrow-derived MSCs produce a cytokine meshwork that stimulates CSCs¹².

There is accumulating evidence suggesting that the regulation of stem cell-like properties requires a two-way interaction between CSCs and their microenvironment, particularly the MSCs. For instance, secretion of interleukin-1 (IL-1) in colon cancer cells could stimulate MSCs to produce prostaglandin E2 (PGE2). Then, PGE2 collaborated with IL-1 to produce other cytokines and chemokines such as IL-6, CXCL1 & CXCL8 by MSCs, leading to enhancement of the cancer stem cell population¹³. Wu *et al* proved that cytokines (IL-6, CXCL-8) were detected in conditioned media from MSCs. Those cytokines stimulated the expression of pluripotency markers (SOX2, OCT4, cMyc), as well as NF- κ B & AMPK/mTOR signaling pathways in colon cancer cells¹⁴. In another study by Luo *et al.*, CCL5 secreted by recruited BM-MSCs were found to induce prostate CSCs via androgen receptor signaling¹⁵. Investigating the secreted components of our UCSC-CM could allow us to determine more targeted CSC therapy in GBM.

Unlike SOX2 and OCT4 expressions, NANOG mRNA expression in T98G cells was not affected by UCSC-CM in our study. This might be due to the abundance of NANOG pseudogenes^{16,17} and also due to the way NANOG mRNA undergoes unique N⁶-methyladenosine (m⁶A) posttranscriptional modification as part of its regulation¹⁸. Furthermore, microRNA-134 has been reported to suppress proliferation and invasion of T98G cells by reducing NANOG expression¹⁹. Further studies are required to elucidate the involvement of UCSC secretomes in inducing the differential expression of pluripotency markers.

Conclusions

The conditioned medium of umbilical cord-derived mesenchymal stem cells could affect the expression of SOX2 and OCT4 as pluripotency markers in human glioblastoma multiforme T98G cells.

Data availability

Dataset 1: 18S rRNA Cq values. 18S rRNA Cq was used to calculate SOX2, OCT4 and NANOG mRNA expression using the

Livak formula (K: control cells; CM: condition medium-treated cells). DOI, [10.5256/f1000research.13154.d191296](https://doi.org/10.5256/f1000research.13154.d191296)²⁰

Dataset 2: SOX2 Cq values. SOX2 Cq was used to calculate SOX2 mRNA expression using the Livak formula (K: control cells; CM: condition medium-treated cells). DOI, [10.5256/f1000research.13154.d191297](https://doi.org/10.5256/f1000research.13154.d191297)²¹

Dataset 3: OCT4 Cq values. OCT4 Cq was used to calculate OCT4 mRNA expression using the Livak formula (K: control cells; CM: condition medium-treated cells). DOI, [10.5256/f1000research.13154.d191298](https://doi.org/10.5256/f1000research.13154.d191298)²²

Dataset 4: NANOG Cq values. NANOG Cq was used to calculate NANOG mRNA expression using the Livak formula (K: control cells; CM: condition medium-treated cells). DOI, [10.5256/f1000research.13154.d191299](https://doi.org/10.5256/f1000research.13154.d191299)²³

Dataset 5: Raw unedited images for Figures 1A and 1B. DOI, [10.5256/f1000research.13154.d191300](https://doi.org/10.5256/f1000research.13154.d191300)²⁴

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Isabele C. Iser

Department of Basic Health Sciences, Laboratory of Cell Biology, Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, Brazil

In the study titled “Analysis of pluripotency marker expression in human glioblastoma multiforme cells treated with conditioned medium of umbilical cord-derived mesenchymal stem cells”, the authors investigated the effects of UCSC on the expression of pluripotency factors in GBM cells. The authors showed an increased expression of SOX4 and OCT4 in GBM cells treated with CM when compared to untreated cells.

The work presents numerous limitations, and does not have scientific relevance that justify its indexing. For example:

- The authors evaluated the gene expression only at the RNA level, however, the ideal would be to evaluate also at the protein level in order to obtain more conclusive results. In addition, the authors evaluated a limited number of genes related to CSCs. I suggest evaluating a greater number of genes to confirm the results presented here.
- The authors also should access other parameters in the cells besides gene expression, such as resistance to therapy, proliferation and viability.
- It is also very important analyze which factors in the CM could be responsible for the effect presented in this work.
- I strongly suggest that the authors use a positive control for qPCR reactions, such as GBM cells treated with CSC inducers.
- In the discussion the authors say that “the up-regulation of this set of genes is associated with poor outcome in terms of tumor malignancy”, so in my opinion it would be interesting to perform an *in vivo* model to testing the effects of these treated cells in terms of tumor formation and malignancy.

In view of these considerations, I do not consider the work apt to be published in F1000 Research.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

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No

Are sufficient details of methods and analysis provided to allow replication by others?

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If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 26 March 2018

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Jie Sun

Department of Urology, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

The present study mainly showed the effects of MSC conditioned medium in glioblastoma multiforme cells. Authors found that MSC-CM promote the expression of pluripotency markers in T98G cells, like SOX4 and OCT4, but not NANOG. The research has limited novelty and integrity. Main concerns are listed below:

1. Serum free medium were used to acquire the MSC-CM for 24 hours in this study, authors should test the cell viability of MSC in this state for a better assess the quality of CM.
2. All the gene markers were tested in mRNA level in the manuscript, protein levels should be added.
3. Only a small number of cells were regarded as cancer stem cells, especially in commercial cell lines. MSC-CM increases the original stem cells to express pluripotency markers or it could induce non-stem cells to express these markers still unclear. What is more, whether CM would affects cell functions after the increase of these markers still unclear. All these

date are needed to be completed or discussed in the manuscript.

4. As authors mentioned in the discussion, various researches has been tested to clarify the interactions between MSC and cancer cells. Based on the existed results in this paper, limited novelty also decrease the quality of the manuscript.
5. The background of the pictures in Figure 1 should be changed and the scale bar should be added.
6. The statistical analysis should not be used on its own, as the study is not good and qualified enough/

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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