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More of ONECUT2 Enhances Proliferation while less contribute to Apoptosis in Glioblastoma Cell Lines

Abstract

Glioblastoma multiforme (GBM) is a highly aggressive brain tumor associated with a high mortality rate, with an average survival time of less than two years. GBM treatment faces significant challenges due to its infiltrative nature, genetic diversity, protection by the blood-brain barrier (BBB), drug resistance, and post-treatment side effects. [11] Transcription factors (TFs) play a crucial role in regulating gene expression during cancer initiation and progression. [11] This study aimed to investigate the impact of altering the function of ONECUT-2 (OC-2) in GBM cells, focusing on metabolic activity, proliferation, cell cycle, and apoptosis. [48] To confirm the successful reduction of OC-2 expression in U251 and U87 cells compared to the control cells (wild types; WT), [103] quantitative real-time polymerase chain reaction (qPCR) was performed. [48] The downregulation of OC-2 resulted in a significant decrease in metabolic activity (MTT) of U251 cells by 47% (P = 0.0056) and U87 cells by 36.4% (P = 0.0003) compared to WT cells. [11] In U251 cells, OC-2 downregulation caused cell cycle arrest in both the G0/G1 phase (13.6%) and the S/G2 phase (52.52%) compared to WT cells. [11] Similarly, in U87 cells, OC-2 downregulation led to cell cycle arrest in the G0/G1 phase (90.5%) and the G2 phase (76.61%) compared to WT cells. Furthermore, OC-2 downregulation significantly affected early-stage apoptosis in U251 cells (95.46%, P=0.0005) and U87 cells (19.64%, P=0.0004). Conversely, upregulating OC-2 significantly increased the metabolic activity (MTT) of U251 cells by 94% (P = 0.0067) and U87 cells by 58% (P = 0.0028) compared to WT cells. These findings highlight the essential role of OC-2 in regulating the progression of GBM cells. [97]

Consequently, OC-2 represents a potential therapeutic target for inhibiting GBM.[45] Overall, this study demonstrates that OC-2 plays a significant role in the regulation of GBM cell progression, suggesting its potential as a therapeutic target for GBM treatment.

Keywords: transcriptional factor; ONECUT2; glioblastoma multiforme (GBM); tumor; brain; carcinogenesis; cell cycle; apoptosis.

1. Introduction

Glioblastoma multiforme (GBM), or grade IV astrocytoma, is the most aggressive form of nervous system tumor. GBM is characterized by cytological atypia, anaplasia, mitotic division, microvascular proliferation and/or necrosis, and the prognosis tends to be poor (Kabel et al., 2018, Alswailem et al., 2022, Wu et al., 2021). GBM accounts for more than 50% of all diagnosed malignant brain cancers and approximately 20% of all primary intracranial tumors (Alrfaei et al., 2020, Van Meir et al., 2010). In the United States, the prevalence of GBM increases with age and is greatest in people aged 75 to 84 years (Wu et al., 2021). Annually, approximately 15,000 new cases of GBM and central nervous system (CNS) malignancies are diagnosed (Alrfaei et al., 2020). Even with the highest level of care, the average patient survival time is less than two years (Alrfaei et al., 2020, Kruchko et al., 2018). In Saudi Arabia, there is an increased incidence of cancer, according to Saudi Cancer Registry (SCR) estimates, which reported 3% of total cancer patients are brain cancer cases (Moin et al., 2021, Al-Rawaji et al., 2018).

[43] Transcription factors belonging to the ONECUT (OCs) family play crucial roles in central nervous system (CNS) development and have been extensively studied across various species, including zebrafish, sea urchins, ascidians, Drosophila species, frogs, and

mice (Francius and Clotman, 2010, Espana and Clotman, 2012). These factors are involved in the regulation of neuronal differentiation in some of these organisms (Espana and Clotman, 2012). ONECUT2 (OC-2) is the second member of the OCs family (Yu et al., 2020, Lu et al., 2018) , and it was initially identified by Jacquemin et al. in 1999 (Choi et al., 2022b, Jacquemin et al., 1999). The OC-2 coding gene is located on chromosome 18 in humans and consists of two exons and one intron. The OC-2 protein binds to specific DNA sequences to stimulate the expression of target genes. It plays a regulatory role in cell proliferation, migration, and differentiation (Choi et al., 2022b). In humans, OC-2 is predominantly expressed in the liver, gallbladder, duodenum, and small intestines, with lower expression levels in the brain, testes, stomach, and pancreas (Jacquemin et al., 1999, Yu et al., 2020). OC-2 has also been implicated in angiogenesis and epithelial-mesenchymal transition (EMT) (Lu et al., 2018, Zhang et al., 2018, Sun et al., 2014). Furthermore, OC-2 is involved in the regulation of pancreatic cell identity, retinal horizontal cells, and liver cell differentiation (Vanhorenbeeck et al., 2007, Francius and Clotman, 2010, Wu et al., 2012, Ma et al., 2019). Overexpression of OC-2 activates oncogenic pathway genes in neurons, influencing development, angiogenesis, extracellular matrix organization, as well as cell locomotion, migration, and proliferation. Conversely, downregulation of OC-2 primarily impacts genes associated with the inflammatory response and chemotaxis (Ma et al., 2019).

OC-2 is a significant player in cancer, as it is involved in various processes such as tumor cell proliferation, cancer cell manifestation, metastasis, tumorigenesis, angiogenesis, EMT, and potentially tumor stemness (Sun et al., 2014, Zhang et al., 2018, Yu et al., 2020). There have been specific studies on OC-2's involvement in bladder cancer, where it showed

high specificity and sensitivity in an epigenetic analysis (Lu et al., 2018). Additionally, OC-2 plays critical roles in neuroendocrine prostate cancer (NEPC), metastatic castration-resistant prostate cancer, and ovarian cell adenocarcinoma (Lu et al., 2018, Vanhorenbeeck et al., 2007, Wu et al., 2012, Ma et al., 2019, Yu et al., 2020). In NEPC, OC-2 interacts with hypoxic conditions to inhibit androgen signaling and induce neuroendocrine plasticity. It also activates the SMAD3 gene, resulting in NEPCs exhibiting higher levels of hypoxia compared to prostate adenocarcinomas (Guo et al., 2019).

However, the specific role of OC-2 in the progression of glioblastoma (GBM) remains unclear. Therefore, the objective of this study was to explore the pathological relationship between OC-2 and the development of brain tumors, specifically GBM. To accomplish this objective, we conducted experiments to investigate whether OC-2 is implicated in the apoptosis and/or proliferation of human glioblastoma U251 and U87 cells.

2. Results

2.1.Efficiency of OC-2 transduction

Cells named U251 and U87, which are derived from aggressive glioblastoma tumors, were modified by introducing OC-2 through the use of lentiviruses, as shown in Fig 1. To estimate how effectively the cells were modified, the level of green fluorescent protein (GFP) expression in infected cells was assessed, and it was found to be approximately 99%. The cells were viewed using an EVOS XL Core Imaging system.

2.2.Validation of OC-2 down-regulation in GBM cells

To confirm the successful reduction of OC-2 in the GBM cells, the researchers employed a technique called Reverse Transcription Polymerase Chain Reaction (RT-PCR). The results, depicted in both Fig 2A and Fig 2B, revealed a significant decrease in OC-2 expression in both the U251 cells (with a remarkable 98.93% decrease; P 0.0001) and the U87 cells (with a 17.17% decrease; P = 0.0207) when compared to the control group consisting of wild-type cells.[95] These findings provide strong evidence for the successful manipulation of OC-2 expression levels in the U251 and U87 cells, validating the researchers' efforts in constructing cells with reduced OC-2 expression.

2.3. Effect of OC-2 on GBM cell viability[36]

To assess the impact of OC-2 on the viability (MTT) of GBM cells, we conducted experiments involving the downregulation and upregulation of OC-2 in U251 and U87 cells. The baseline control was established by measuring the levels of OC-2 expression in wild-type U251 and U87 cells. We observed a significant decrease in cell viability when OC-2 was downregulated, with a 47% decrease in U251 cells (P = 0.0056) and a 36.4% decrease in U87 cells (P = 0.0003). These findings suggest that downregulating OC-2 may effectively inhibit the viability of GBM cells (Fig 3A and B). Conversely, upregulating OC-2 led to a significant increase in cell viability, with a 94% increase in U251 cells (P = 0.0067) and a 58% increase in U87 cells (P = 0.0028). These results indicate that upregulating OC-2 significantly enhances both OC-2 expression and cellular activity (Fig 3C and D).

2.4. Proliferation Assay

The downregulation of OC-2 caused a significant decrease in cell proliferation, with a 65.5% reduction in U251 cells ($P \leq 0.0001$) and a 71.7% reduction in U87 cells ($P \leq$

0.0001) when compared to wild-type (WT) cells, as illustrated in Figure 4 (A and B). Conversely, upregulating OC-2 resulted in a substantial increase in cell proliferation, with a 183.2% increase in U251 cells ($P \leq 0.0001$) and a 123.5% increase in U87 cells ($P \leq 0.0001$) when compared to WT cells (Fig 4C and D).

2.5. Flow cytometry analysis

2.5.1. Effect of OC-2 on cell cycle of GBM cell

To examine the impact of decreasing OC-2 expression on apoptosis in GBM cells, a cell cycle analysis was conducted with flow cytometry. The Flowing software program generated DNA histograms, which enabled determining the distribution of cells across the various stages of the cell cycle. The histograms presented in Figure 5 illustrate the impact of knocking down OC-2 in U251 cells and U87 cells on the distribution of cells across different phases of the cell cycle. In the U251 cells, reducing OC-2 resulted in a decrease in the proportion of cells in the G1 phase (13.6% compared to control cells), as well as a reduction in the number of cells in the S phase (43.99% compared to control cells) and the G2/M phase (52.52% compared to control cells), as depicted in Figure 5. Similarly, knocking down OC-2 in U87 cells led to a decrease in the proportion of cells in the G1 phase (90.5% compared to control cells), while the fraction of cells in the G2/M phase increased (76.61% compared to control cells), and the proportion of cells in the S phase remained unchanged compared to control cells, as shown in Figure 5. These results indicate that reducing OC-2 expression can impede the progression of cells at the S/G2 phase of the cell cycle.

2.5.2. Effect of OC-2 on apoptosis of GBM cell

To evaluate the effect of OC-2 downregulation on apoptosis in GBM cells, we analyzed the levels of annexin V-FITC/PI dual staining using the Flowing software program v.2.5.1. Annexin V-FITC staining was performed to detect early-stage apoptosis, and its relationship to OC-2 downregulation was examined. The proportion of pre-apoptotic cells in the population was determined, and the resulting histograms are presented in Figure 6. Our findings revealed that OC-2 downregulation led to a significant increase in apoptotic cells (with degraded DNA), with 95.46% more apoptotic cells in OC-2-downregulated U251 cells (P = 0.0005) and 19.65% more apoptotic cells in OC-2-downregulated U87 cells (P = 0.0004) compared to WT cells (Fig 6A–D). These results strongly indicate that OC-2 has an apoptotic effect on GBM cells.

3. Discussion

Hepatic nuclear factor 6 (Hnf6) is a member of the OCs family of transcription factors.[43] It has been shown to be involved in organogenesis, cell fate, and tumorigenesis (Choi et al., 2022b, Kropp and Gannon, 2016).[43] Another OCs family member, OC-2, has been shown to be elevated in several different cancers, such as hepatocellular carcinoma, NETs, lung adenocarcinoma, and gastric, colorectal, prostate, and ovarian cancer (Choi et al., 2022b, Guo et al., 2019, Lu et al.,[67] 2018, Chen et al., 2020b, Sun et al., 2021, Zhang et al., 2015, Ma et al., 2019).

In this research study, our primary focus was to examine the impact of OC-2 function gain or loss on GBM cells. To achieve this, we employed a range of assays to thoroughly investigate this phenomenon. Notably, this study is the first of its kind to explore the influence of OC-2 on the progression of GBM. To begin our investigation, we utilized qPCR techniques to validate the upregulation and downregulation of OC-2 in both

transfected and transduced U251 and U87 cells. The results of the validation process were highly significant, providing compelling evidence that OC-2 expression was downregulated in U251 cells by an impressive 99% (P 0.0001), as well as in U87 cells by 18% (P = 0.0207) (as depicted in Fig 2A and B). Interestingly, despite multiple attempts, we encountered challenges in achieving a significant downregulation of OC-2 in U87 cells, with the average reduction amounting to just 19%. This resistance to OC-2 inhibition can likely be attributed to the presence of PTEN deletion in the U87 cells, resulting in diminished effectiveness of OC-2 downregulation.

In our study, we also investigated the impact of knocking down OC-2 on the metabolic activity of U251 and U87 cells. The results were striking, with a significant decrease of 47% (P = 0.0056) in U251 cells and 36.4% (P = 0.0003) in U87 cells (Fig 3A and B).[11] These findings suggest that OC-2 plays a crucial role in GBM cells, potentially influencing cell survival beyond its effect on metabolic activity.[11] Conversely, when OC-2 was overexpressed in U251 and U87 cells, a noteworthy increase in metabolic activity was observed compared to wild-type cells. Specifically, there was a 94% (P = 0.0067) increase in U251 cells and a 58% (P = 0.0028) increase in U87 cells (Fig 3C and D). This indicates that OC-2 plays a significant role in promoting cell proliferation.[11] Interestingly, previous studies have indicated that OC-2 is overexpressed in various cancers such as liver, colon, prostate, and lung cancers, which are known to exhibit increased cell proliferation (Lu et al., 2018, Ma et al., 2019, Sun et al., 2014, Zhang et al., 2015). The fact that similar findings have been reported in different types of cancer suggests that OC-2 likely performs similar functions across different organs.[90]

To further understand the impact of downregulating OC-2 on cell proliferation, we conducted real-time assessments using U251 and U87 cells, with wild-type (WT) cells serving as controls.[67] The results revealed a significant reduction of 65.5% (P 0.0001) in U251 cells and 71.7% (P 0.0001) in U87 cells (Fig 4A and B). These findings suggest that OC-2 may play a role in limiting the propagation of GBM cells in vitro.[95] In line with our results, similar studies have reported reduced cell proliferation in gastric cancer and ovarian cancer upon the downregulation of OC-2 (Lu et al., 2018, Chen et al., 2020b). In ovarian cancer, the Akt/Erk signaling pathway, which is associated with cell proliferation, migration, and angiogenesis, has been investigated for its relationship with ONECUT2 expression (Lu et al., 2018). Additionally, in neuroendocrine prostate cancer, ONECUT2 was found to regulate cell proliferation and tumor growth (Choi et al., 2022a). Extra, ONECUT2 has been shown to control the expression of specific genes involved in cell proliferation. In gastric cancer, ONECUT2 was found to control the expression of ROCK1, a gene associated with tumor proliferation (Chen et al., 2020a). This data supports the notion that OC-2 may have a broader influence on cell proliferation across different cancer types.[77] Conversely, when OC-2 was overexpressed in U251 and U87 cells, a significant increase in proliferation was observed compared to WT cells.[77] Specifically, there was a 183.2% (P 0.0001) increase in U251 cells and a 123.5% (P 0.0001) increase in U87 cells (Fig 4C and D). These findings suggest that OC-2 may stimulate cell proliferation pathways. Interestingly, our proliferation data indicated an initial spike in proliferation during the first 10 hours, where the population of OC-2 inhibited cells temporarily outgrew the control. This could be attributed to cellular stress, as the modified cells attempted to

compensate by increasing their rate of proliferation. Consequently, we conducted cell cycle assays to further investigate this phenomenon.

In order to gain further insights into the effects of downregulating OC-2 on cell cycle progression, we performed cell cycle analysis on U251 and U87 cells. The results revealed that U251 cells with downregulated OC-2 showed that the cells arrested at G2 phase 52.5% (Fig 5A). Also, downregulation of OC-2 in U87 cells was found to arrest cells at the G2 76.61% and failed to initiate G1 phase 90.5% (Fig 5B). Furthermore, S phase was significantly low 43.99% in U251 modified cells only. We concluded that the two type of cell lines probably use two different mechanisms when responding to OC-2 inhibition. [43] Cellular arrest at the S/G2 phase is often associated with the initiation of apoptosis, a programmed cell death process. [43] In light of this, we decided to perform an apoptosis assay to investigate the potential involvement of apoptosis in the observed effects of OC-2 downregulation. [43] By analyzing the cell cycle and apoptosis responses, we aimed to uncover the underlying mechanisms through which OC-2 influences cell cycle progression and cell fate decisions in U251 and U87 cells.

[43] Apoptosis analysis of U251 cells with downregulated OC-2 revealed a significant activation of the pre-apoptotic pathway by 95.46% (P=0.0005) (Fig 6 A). This indicates that OC-2 plays a crucial role in promoting cell survival. On the other hand, downregulation of OC-2 in U87 cells resulted in a less pronounced activation of the pre-apoptotic pathway by 19.65% (P=0.0004) (Fig 6 B). This suggests that OC-2 is important for the survival of U87 cells. It's worth noting that U87 cells have a PTEN mutation, which might contribute to the reduction of apoptotic initiation by inhibiting OC-2-associated apoptosis. [11] These findings are consistent with the results of our proliferation assay. In the

case of U251 cells, proliferation plateaued after 20 hours when OC-2 was inhibited. Conversely, U87 cells did not plateau at 20 hours but rather after 60 hours of proliferation (Fig 4A and B). This further confirms that both cell lines employ different mechanisms or routes to ensure their survival. Taken together, the apoptosis analysis and proliferation assay highlight the importance of OC-2 in promoting cell survival and provide evidence for distinct mechanisms employed by U251 and U87 cells in response to OC-2 downregulation.

Interestingly, both cell lines experienced arrest at the G2 phase and a decrease in the G1 phase (P value is 0.0005), although this effect was more pronounced in the U87 cells. [48] The results were particularly surprising in the case of the U87 cells, which showed almost complete elimination of the G1 phase. Despite the arrest at the G1 phase, apoptosis still occurred at a rate of approximately 20%. This suggests that the cells may be in a quiescent state or that the deletion of PTEN is causing a delay in the apoptosis process.

[11] This study provides evidence that downregulating OC-2 leads to a notable decrease in metabolic activity and proliferation in GBM cells. [11] Conversely, upregulating OC-2 results in increased cell proliferation and metabolic activity. [28] These findings highlight the role of OC-2 in GBM and suggest its potential as a therapeutic target in GBM treatment. [28] It is worth noting that this study is the first to demonstrate the impact of OC-2 on GBM cell lines. [45] The involvement of OC-2 in tumor progression suggests its potential as a therapeutic target for GBM. [11] To further understand the role of OC-2, future studies should investigate its contribution to angiogenesis and metastasis, two crucial processes in tumor development and spread. [45] Additionally, it is important to assess the in vivo toxicity of OC-2 inhibition to evaluate its suitability for systemic treatment in GBM.

[8 4]
4. Materials and Methods

Ethical approval for this study is waived as per institutional regulations' stating that commercial cell lines are exempt from mandatory IRB application.

4.1. [25] Cell culture

The human GBM cell line U251 was obtained from Merck (Darmstadt, Germany), and the U87 cell line (characterized by a homozygous mutation in PTEN) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA)[75]. Cells from both lines were cultured to form monolayers in Dulbecco's Modified Eagle's Medium (DMEM, Cat No. /ID: [11] 11995-065) containing 10% fetal bovine serum (FBS, Cat No. /ID: 10100147) and 1% penicillin-streptomycin (Cat No. /ID: [25] 15140122), obtained from Gibco (Carlsbad, CA, USA)[36]. Cells were incubated at 37 °C in a humidified atmosphere (95% air and 5% CO2). The cell culture conditions have been published previously (Akiel et al., 2022). The two cell lines used possess two different mutational background affecting which probably affect different pathways. This approach allows for a more comprehensive understanding of the molecular mechanisms involved in diseases or biological processes under investigation.

4.2. Transduction and Transfection

4.2.1. [2 8] In vitro transient transfection of OC-2

For DNA transfection, U251 and U87 cells were seeded (3,000 per well) onto 96-well tissue culture plates in culture medium and incubated overnight at 37 °C[28]. The next day, the medium was replaced with starvation culture medium, and the cells were incubated for 30–60 min at 37 °C[28]. Then, the cells were transfected with the OC-2 (NM_004852) Human Tagged ORF Clone expression plasmid (Cat No. /ID:

RC211951, Origene, Rockville, MD, USA) using the GenMute™ DNA Transfection Reagent (Cat No. /ID: SL100568, SignaGen Laboratories, Gaithersburg, MD, USA) according to the manufacturer's instructions. The delivery system was incubated with the cells for 5 h to mediate transfection. After the 5-h incubation, the medium was replaced with culture medium containing 10% FBS, and the cells were incubated for 48 h. After this time, the medium was replaced with selection culture medium containing 200 µg of neomycin (Cat No. /ID: PHR1491, Sigma-Aldrich, Darmstadt, Germany). After two passages, the cells in the selection culture medium were ready for further experiments. The cells were visualized using the EVOS XL Core Imaging system (Cat No. /ID: AMEX1000, ThermoFisher Scientific, Waltham, MA, USA).

4.2.2. In vitro permanent transduction of OC-2

To perform lentivirus transduction, U251 and U87 cells were seeded at a density of 3,000 cells per well in 96-well tissue culture plates with culture medium. The plates were then incubated overnight at 37 °C. The following day, the culture medium was replaced with starvation culture medium, and the cells were incubated for 30 minutes at 37 °C. Subsequently, the cells were transduced with the knockdown OC-2 Human shRNA Lentiviral Particle (Locus ID 9480; Cat No. /ID: TL311023V, Origene, Rockville, MD, USA) at a concentration of 9,000 lentivirus particles per well. The cells were then incubated overnight at 37 °C. The next day, the culture medium was replaced with culture medium containing 10% FBS, and the cells were incubated for 48 hours. After this period, the culture medium was replaced with selection culture medium containing 1 µg puromycin (Cat No. /ID: A1113803, ThermoFisher Scientific,

Waltham, MA, USA) to eliminate untransduced cells. The efficiency of transduction was evaluated by observing the presence of GFP-positive cells.[11] After undergoing two or three passages, the culture medium was replaced with regular culture medium, and the cells were prepared for further experiments.

4.3.Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)

4.3.1. RNA Isolation

To extract total RNA, the pellets of both transduced and untreated U251 and U87 cells were processed using the MagNA Pure Compact RNA Isolation Kit (Cat No. /ID: 04802993001, Roche Life Sciences, Mannheim, Germany) following the manufacturer's instructions.[103] The RNA concentration and purity were measured using a NanoDrop™ 8000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).[103] The extracted RNA samples were then stored at -80°C for future use.

4.3.2. Reverse Transcription (RT) into cDNA

To generate complementary DNA (cDNA), the isolated RNA samples were subjected to reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Cat No. /ID: 00795574, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The RNA (1 µg) was converted into cDNA using a polymerase chain reaction (PCR) thermocycler (GeneAmp™ PCR System 9700, Cat No. /ID: 4413750, ThermoFisher Scientific, Waltham, MA, USA).[57] The resulting cDNA samples were stored at 4°C for further use.

4.3.3. Real-Time (Quantitative) PCR

The quantitative real-time polymerase chain reactions (qRT-PCRs) were conducted using a 7900HT fast real-time PCR system (Cat No./ID: 4351405, ThermoFisher

Scientific, Waltham, MA, USA). An IDT Prime time™ gene expression 2X master mix (Cat No. /ID: 1055771, Integrated DNA Technologies, Coralville, Iowa, USA) was used to identify and quantify OC-2 gene expression (amplification) in transduced and untreated U251 and U87 cells. As an endogenous control, an 18S rRNA (DQ) oligo mix (20×) housekeeping gene (Cat No. /ID: 4352655, Applied Biosystems, Foster City, California, USA) was employed. The qRT-PCR was performed using PrimeTime Std qPCR Assay OC-2 primers obtained from Integrated DNA Technologies (Cat No. /ID: NM_004852, Coralville, Iowa, USA). The relative expression was determined by subtracting the target cycle threshold (Ct) from the 18S Ct, and then subtracting the resulting value from the Ct of the control samples (untreated). The final values were calculated using the formula 2^(-delta delta Ct). The detailed calculation process has been previously described (Rao et al., 2013, Alrfaei et al., 2020).

4.4.Metabolic activity Assay

Cell viability was measured using the PrestoBlue™ Cell Viability Reagent (Cat No. /ID: A13262, Invitrogen, Carlsbad, CA, USA). [25] Transfected U251 and U87 cells were plated onto 96-well plates at a density of 3,000 cells/well. [48] Untreated cells were included as controls. [48] The cells were incubated in a 5% CO_2 incubator at 37 °C overnight. [59] The next day, the medium was replaced with starvation culture medium, and the cells were incubated overnight. [59] The next day, the starvation medium was removed, and the cells were washed with 1× phosphate buffered saline (PBS; Cat No. /ID: 15374875, ThermoFisher Scientific, Waltham, MA, USA). [25] According to the manufacturer's protocol, fresh medium was added to each well, and the cells were incubated for 1 h. [90] Then, the PrestoBlue Cell Viability Reagent was added to all wells, and the wells were

completely covered to protect the cells from light.[25] The cells were then incubated for 1 h.[75] Fluorescence was measured using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA); the excitation wavelength was 560 nm, and the emission wavelength was 590 nm.[48] The cell viability was calculated as previously described (Alswailem et al., 2022).

4.5.Proliferation assay

To assess the proliferative capacity of both transduced U251 and U87 cells and untreated cells, real-time cell analysis (RTCA) was performed using a real-time cell analyzer (xCELLigence, ACEA Biosciences, Inc, CA, USA). Impedance measurements of the cell index (CI) were conducted following the manufacturer's protocol.[25] The cells were suspended in culture media and adjusted to a density of 10,000 cells per well.[11] Subsequently, 100 µL of the cell suspension was added to each well (8 wells per group) of 16-well E-plates (Cat No. /ID: 20180618, Agilent Technologies, ACEA Biosciences Inc., San Diego, USA). The RTCA system monitored the cells every 15 minutes for a duration of 70 hours. This cell proliferation experiment was repeated two times to ensure reproducibility of the results.

4.6.Flow cytometry analysis

4.6.1. Cell cycle

The cell-cycle protocol has been previously described (Asiri et al., 2021).[36] After being cultured for 72 hours, the cells (U251 and U87 cells that were either transduced with OC-2 or left untreated) were subjected to trypsinization.[36] Following trypsinization, the cells were washed twice with ice-cold 1× PBS and then fixed in ice-cold methanol for a duration of 20 minutes.[25] To further analyze the cells, the samples were treated with

RNase/ribonuclease obtained from Worthington (Cat No. /ID: 52B13247, USA). Subsequently, the cells were exposed to propidium iodide (PI) obtained from ThermoFisher Scientific (Cat No. /ID: R37108, Waltham, MA, USA). Finally, the cells were analyzed using a BD FACs Canto II instrument.

4.6.2. Apoptosis assay

The extent of apoptosis was assessed using the Alexa FluorTM 488 Annexin V/Dead Cell Apoptosis Kit from Invitrogen (Cat No. /ID: 2127457, Carlsbad, CA, USA), following the manufacturer's protocol. After 72 hours of culture, the transduced U251 and U87 cells, as well as the untreated cells, were collected and washed twice with 1× PBS. The cells were then resuspended at a density of 30,000 cells/mL. For the transduced cells, annexin V and propidium iodide (PI) staining was performed in the dark for 15 minutes. Next, the cells were fixed by adding cold methanol in the dark for 15 minutes. The fixed cells were subsequently analyzed using a BD FACs canto II instrument, as previously described,(Alghamdi et al., 2020).

4.7.Statistical analysis

Each experiment was conducted a minimum of three times to ensure validation. The apoptosis and cell cycle assays were performed twice, unless specified otherwise. For statistical analyses, the GraphPad Prism (GraphPad Software v.5.03, Inc., San Diego, California) was utilized. The mean Ct values of the treated samples (knockdown and overexpressed) and the control samples (WT) were compared using an unpaired two-tailed t-test, assuming the data followed a normal distribution. Similarly, the mean relative proliferation values of the WT, OC-2 knockdown, and overexpressed OC-2 samples were compared using an unpaired two-tailed t-test. In the results,

asterisks (*) were used to indicate statistical significance, with $P \leq 0.05$ being the threshold for significance.

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Figure Legends

Fig 1. Estimation of Transduction efficiency by using GFP expression as indicator of successful transduction. A- Shows U251 transfected cells (Green). B- Shows U87 transfected cells (Green). The bottom images represent light microscope depiction showing the same areas of the Fluorescent micrograph on top (×10 magnification).

Fig 2. Confirmed inhibition of OC-2. A and B- show qRT-PCR results of the OC-2 inhibition in comparison to the expression in the WT cells. Knockdown of OC-2 was shown as 98.93% inhibition for U251 and 17.17% for U87.

Fig 3. Metabolic activities of OC-2 modified cells. A- and B- show OC-2 inhibitor reduction of MTT (activities) within U251 (50%) and U87 (40%) cells compared to the control. C- and D- show OC-2 overexpression of increase of MTT (activity) within U251 (21%) and U87 (40%) compared to the control. The overexpression of OC-2 on U251 and U87 was done at background of 200ug of neomycin to make stable OC-2 expressing cell lines.

4Fig Proliferation analysis by a real-time cell analyzer (RTCA; xCELLigence, E-Plate 16). A- show U251 cells (Green) with OC-2 down-regulation compared with the WT (Red). B- show U87 cells (Green) with OC-2 down-regulation compared with the WT (Red). C- show U251 cells (Green) with OC-2 up-regulation compared with the WT (Red). D- show U87 cells (Green) with OC-2 up-regulation compared with the WT (Red).

5Fig Cell cycle distribution analysis. A- Down-regulation of OC-2 in U251 results showed of the proportion (%) of the cells in each stage of cell cycle and comparison with WT. B- Down-regulation of OC-2 in U87 results showed of the proportion (%) of the cells in each stage of cell cycle and comparison with WT.

6Fig Pre-apoptosis analysis of OC-2 modified cells. A- Represent U251 WT showing minimal pre-apoptotic effect. B- Represent U251 cells down regulated with OC-2 inhibitor showing high pre-apoptotic effect. C- Represent U87 WT showing minimal pre-apoptotic effect. D- Represent U87 cells down regulated with OC-2 inhibitor showing high pre-apoptotic effect.