Cellular and Molecular Neurobiology
https://doi.org/10.1007/s10571-019-00771-8

ORIGINAL RESEARCH

Glioblastoma Stem Cell-Derived Exosomes Enhance Stemness and Tumorigenicity of Glioma Cells by Transferring Notch1 Protein

Zhen Sun[1] · Li Wang[1] · Yueling Zhou[1] · Lihua Dong[2] · Weichao Ma[3] · Liang Lv[3] · Jie Zhang[1] · Xiujie Wang[1]

Received: 23 September 2019 / Accepted: 3 December 2019

Abstract

Exosomes contain plenty of bioactive information, playing an important role in intercellular communication by transfer their bioactive molecular contents to recipient cells. Glioblastoma stem cells (GSCs) and non-GSC glioma cells coexist in GBM microenvironment; GSC-released exosomes contain intracellular signaling molecules, which may affect the biological phenotypes of recipient cells. However, whether GSC exosomes could affect the biological phenotype of non-GSC glioma cells has not yet been defined. To explore whether GSC exosomes could reprogramme non-GSC glioma cells into GSCs and its possible mechanism involved, non-GSC glioma cells were treated with GSCs released exosomes; the potential mechanisms of action were studied with RNA interference, Notch inhibitors and Western blot analysis. The proliferation, neurosphere formation, invasive capacities, and tumorigenicity of non-GSC glioma cells were increased significantly after GSC exosome treatment; Notch1 signaling pathway was activated in GSCs; Notch1 protein was highly enriched in GSC exosomes; Notch1 signaling pathway and stemness-related protein expressions were increased in GSC exosome treated non-GSC glioma cells and these cell generated tumor tissues; Notch1 protein expression in GSCs and their exosomes, and the neurosphere formation of GSCs were decreased by Notch1 RNA interference; Notch1 signaling pathway protein and stemness protein expressions were decreased in GSC exosome treated non-GSC glioma cells by Notch1 RNA interference and Notch inhibitors. The findings in this study indicated that GSC exosomes act as information carriers, mediated non-GSC glioma cell dedifferentiation into GSCs by delivering Notch1 protein through Notch1 signaling activation, and enhanced stemness and tumorigenicity of non-GSC glioma cells.

Keywords Glioblastoma stem cells · Exosomes · Glioma cells · Dedifferentiation · Notch1

Abbreviations

GBM	Glioblastoma
GSCs	Glioblastoma stem cells
EVs	Extracellular vesicles
Exo	Exosome
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
bFGF	Basic fibroblast growth factor
EGF	Epidermal growth factor
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
SDS	Sodium dodecyl sulfate
TEM	Transmission electron microscopy
CSL	CBF-1/RBP-Jκ, Su(H), Lag-1

Zhen Sun and Li Wang are co-first authors.

✉ Xiujie Wang
xiujiewang@scu.edu.cn

[1] Laboratory of Experimental Oncology, State Key Laboratory of Biotherapy/Collaborative Innovation Center for Biotherapy, West China Hospital, West China Clinical Medical School, Sichuan University, No. 1 Keyuan Road 4, Gaopeng Avenu, Hi-tech Zone, Chengdu 610041, China

[2] Human Anatomy Department, School of Preclinical and Forensic Medcine, Sichuan University, Chengdu 610041, China

[3] Neurosurgery Department, West China Hospital, Sichuan University, Chengdu 610041, China

Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults, characterized by extreme aggressiveness, notorious treatment resistance, and poor prognosis

Published online: 18 December 2019

(Gusyatiner and Hegi 2018). Despite of the progress of multimodal treatment strategies, almost all GBM patients eventually develop local tumor recurrence at the original site (Bambury and Morris 2014; Dahan et al. 2014). As GBM hides a subpopulation of glioblastoma stem cells (GSC), which are responsible for tumor initiation, progression, and chemoradiotherapy resistance (Lan et al. 2017; Kim et al. 2018). It seems that target eliminating GSCs and/or induce them differentiation might be an optimistic opportunity for GBM therapy, but the efficacy of differentiation therapy by inducing GSCs differentiation is limited in preclinical and clinical studies (Cheng et al. 2019).

Cancer stem cells (CSCs) are not stayed in static status hierarchically, but in a highly dynamic equilibrium status. The phenotype and stemness of CSCs are plastic and influenced by the specific microenvironment for the individual tumor, the CSCs subpopulation can differentiate into non-CSC tumor cells (Kim et al. 2018; Andriani et al. 2016), likewise, differentiated non-CSC tumor cells may also have the potential to dedifferentiate into CSCs and acquire stemness phenotype in response to tumor microenvironmental stress and chemotherapeutic agents (Dahan et al. 2014; Kim et al. 2018; Andriani et al. 2016; Sun et al. 2018; Lee et al. 2016a, b; Lopez-Bertoni and Laterra 2015).

In glioblastoma, more and more evidences confirmed that there is an active phenotypic and functional conversion of non-GSC cells to GSC-like cells (Dahan et al. 2014; Kim et al. 2018; Lee et al. 2016a, b; Auffinger et al. 2014; Shi et al. 2015); the equilibrium between undifferentiated GSCs and differentiated non-GSCs was maintained dynamically in tumor microenvironment through bidirectional interconversion (Shi et al. 2015). Understanding the mechanisms of maintaining the dynamic equilibrium between GSCs and non-GSCs is crucial to the research and development of new therapeutic modalities that focus on targeting and depleting GSCs for GBM therapeutics. But, what mediated the conversion of non-GSC cells to GSC-like cells is poor understood (Lee et al. 2016a, b; Auffinger et al. 2014; Shi et al. 2015) and how the plasticity and dynamic equilibrium between GSCs and non-GSCs are regulated is unclear (Andriani et al. 2016; Shi et al. 2015).

Previous investigations indicated that Notch1 signaling pathway played an important role in the maintenance of the cancer stem-like phenotype (Safa et al. 2015), tumorigenic Notch signaling is activated in brain tumor-initiating cells (BTIC) in GBM (Konishi et al. 2016; Bayin et al. 2017), the expressions of Notch receptor (Notch1) and its target gene, Hes-1 were markedly increased in GSCs, which led to tumor invasion and recurrence of GBM (Cenciarelli et al. 2017; Sarkar et al. 2017); blocking Notch signaling pathway with inhibitors inhibited GSCs growth in vitro and in vivo (Yu et al. 2016).Thus, exploring novel anti-cancer agents targeting the Notch1 signaling pathway might be a promising strategy for GBM eradication. However, how Notch1 signaling pathway regulates GSC self-renewal, differentiation, tumorigenesis, and non-GSCs dedifferentiation in maintaining the dynamic equilibrium between GSCs and non-GSCs is elusive (Konishi et al. 2016; Bayin et al. 2017; Sarkar et al. 2017; Yu et al. 2016; Fan et al. 2010; Cenciarelli et al. 2017).

Exosomes, the nanovesicles originated from the endosomes, which are 30–100 nm extracellular vesicles (EVs) released by all types of cells, containing a lot of bioactive molecules, play an important role in intercellular communication by transfer their molecular contents between different types of cells (Hessvik and Llorente 2018). Exosomes derived from cancer cells carry malignant information in the form of proteins, DNA, mRNA, miRNA and lipids that can reprogram recipient cells (Pitt et al. 2016; Zhang et al. 2015). GSC-released exosomes contain tetraspanins, TSG101, Alix, Hsp70, etc. as the exosomes derived from glioma cells and other cells contain, and these proteins might serve as the specific markers of exosomes (Zhang et al. 2015; Gourlay et al. 2017). Besides, GSC exosomes also contain a variety of components including, VEGF-A, STAT3 (Treps et al. 2017; Gabrusiewicz et al. 2018) and other intracellular signaling proteins, standing for a unique signaling pathway of cellular export and cell-to-cell deliver of insoluble molecular regulators, such as membrane receptors and signaling proteins, etc. These signaling proteins and molecular regulators may reflect and affect the balance of the stem cell hierarchy, and consequently influencing the biofunctional integration and phenotype of cancer cells (Luhtala et al. 2017; Nakano et al. 2015; Tűzesi et al. 2017).

As mentioned above, Notch signaling is activated and Notch receptor protein expression (Notch1) is elevated in GSCs, which was associated with stem-like phenotype of GSCs (Konishi et al. 2016; Bayin et al. 2017; Sarkar et al. 2017; Yu et al. 2016). GSC exosomes are enriched with membrane receptors and signaling proteins that can reprogram recipient cells (Zhang et al. 2015; Luhtala et al. 2017; Nakano et al. 2015; Tűzesi et al. 2017). However, the implications of intercellular communication between GSCs and non-GSC glioma cells in the GBM tumor microenvironment, facilitated by the exchange of exosomes, are not fully elucidated. There is no clue to whether GSC derived exosomes contain Notch1 protein and play a part in the conversion of non-GSC cells to GSC-like cells until now. On account of that GSCs and non-GSC glioma cells coexist in GBM microenvironment, it could be assumed that GSC-released exosomes contain Notch1 receptor protein, and act as malignant information carriers, delivering Notch1 to non-GSC glioma cells, making them reprogrammed and dedifferentiate into GSCs. To test this hypothesis, this experimental study was therefore designed to investigate whether GSC

exosomes could reprogramme non-GSC glioma cells into GSCs, and to explore its potential mechanism involved.

Materials and Methods

Chemicals and Reagents

DMEM, DMEM/F12 media, fetal bovine serum (FBS) and Accutase were purchased from Gibco/BRLInvitrogen (Shanghai, China), Trypsin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), DMSO and other chemicals and reagents were obtained from Sigma-Aldrich (Shanghai, China).

Cell Lines and Cell Culture

Human GBM (WJ1) cell line was established in our laboratory (Wang et al. 2007), GSC cell line (WJ2) was isolated and characterized from WJ1 cell line (Wang et al. 2008; Guo et al. 2016), and both cell lines were kept in our laboratory for regular use. Human glioma cell lines, U251, U87, GFP transfected U251, and U87 were obtained from Shanghai Cell Biology Institute of Chinese Academy of Sciences (Shanghai, China). Human glioma cell lines (WJ1, U251, U87) were cultured in DMEM supplemented with 10% FBS; at 37 °C in a humidified atmosphere of 5% CO_2. The glioblastoma stem cells (GSCs) were cultured with DMEM/F12 serum-free medium supplemented with B27 (100×, Gibco/BRL Invitrogen), 20 ng/mL bFGF, 20 ng/mL EGF (PeproTech), 5 μg/mL insulin, 1% BSA, and 4 μg/mL heparin (Sigma-Aldric) in nonadhesive culture system (Chen et al. 2012).

Exosome Isolation and Characterization

Exosomes were isolated from glioma cell and GSC conditional media by using total exosome isolation (from cell culture media) reagent (Invitrogen), according to the manufacturer' instruction (Zeringer et al.2013; Tang et al. 2017). Exosomal protein was quantified with Pierce™ BCA protein assay kit. The morphology of exosomes was observed by transmission electron microscopy; the size and quantity of particles isolated were examined using a Nanosight NS300 (NanoSight Ltd., Amesbury, UK) and the marker expressions were evaluated by Western blot (Zeringer et al. 2013; Tang et al. 2017; Sharma et al. 2018).

Exosome Uptake Assay

For fluorescence labeling, GSC exosomes (GSC-exo) were incubated with 10 μL CM-Dil dye (Sigma-Aldrich) for 5 min at 37 °C, and then for an additional 15 min at 4 °C. After labeling, the labeled exosomes were washed with PBS for 3 times and suspended in PBS. For uptake assay, U251 and U87 GBM cells were treated with 20 μg/ml GSC exosomes for 6 h at 37 °C, and observed under confocal microscopy (CLSM; TCS SP5-II, Leica Microsystems, Nanterre, France).

Cell Proliferation Assay

MTT assay was used to determine the effect of GSC-exo on the proliferation of non-GSC glioma cells. Briefly, U251 and U87 glioma cells were seeded into 96-well plates at the density of 1000 cells per well. After overnight incubation, the cells were then treated with various concentrations of GSC-exo (0, 5, 10, 20, and 40 μg/ml) for 1, 2, 3, 4, and 5 days. Then 10 μl of MTT (0.5 mg/ml) was added to each well and cultured for another 4 h at 37 °C. Finally, 100 μl of 10% SDS in 0.01 M HCl was added into each well and incubated overnight at 37 °C to dissolve the formazan. The absorbance was measured at 570 nm (Wedel et al. 2011). Three independent experiments were performed.

Neurosphere Formation Assay

Both U251 and U87 glioma cells were treated with 20 or 40 μg/ml of GSC-exo, respectively, for 3 cycles, each for 2 days. GSC-exo treated and untreated glioma cells were digested into single cells and seeded in 6-well plates coated with 0.5% agarose at a density of 1×10^4/well, and cultured in serum-free DMEM/F12 media supplemented with growth factors. The sphere number of each well was counted after 12 days of culture; sphere-forming efficiency was calculated as the number of spheres formed divided by the initial number of single cells seeded and expressed as a percentage (Guo et al. 2016) and the diameters of spheres were measured with Image-Pro plus 6.0 software. Three independent experiments were performed.

Cell Invasion Assay

GSC-exo treated and untreated U251 and U87 non-GSC glioma cells were harvested and re-suspended as single cell solutions. The cells were plated in 24-transwell upper chambers (Corning, Costar, USA), with 8 μm pores, at a density of 20,000 cells in 200 μl serum-free medium, 500 μl DMEM with 10% FBS was added into the lower chamber as a chemoattractant (Chen et al. 2017). After 24 h incubation at 37 °C, the cells on the upper surface of the insert were removed using tampons, and the cells that penetrated through the filter were fixed with 4% paraformaldehyde and stained with DAPI. Images were taken under fluorescent microscope (Nikon ECLIPSE Ti-U, Japan). The penetrated

cells in 5 non-overlapping random fields per well were counted. Three independent experiments were performed.

3D-Spheroids Invasion Assay

GFP transfected U251 and U87 glioma cells were treated with GSC-exo for 3 days, and harvested, 600 cells in 20 µl medium were cultured as hanging drops for 3 days. The spheroids were transferred into 24-well plate coated with 0.5% agarose for 3 days. Then, they were embedded into drops of 50 µl matrigel (BD Bioscience) in a 12-well plate, incubated for 30 min at 37 °C, and then covered with 1 ml DMEM medium (Del Duca et al. 2004; Motaln et al. 2015). The images were taken under fluorescent microscope (Nikon ECLIPSE Ti-U, Japan) at 1st, 2nd and 3rd Day. The spheroid diameters and cell invasion distances were quantified using Image-Pro plus 6. Three independent experiments were performed.

Tumorigenicity Assay

All animals were maintained under standard conditions according to the guidelines of the Institutional Animal Care and Use Committee of Sichuan University; the animal experiments were performed with an approved protocol by this committee (Permit Number: 2016039A). For tumor growth assays, 5×10^5 of GSC-exo treated and untreated U251 and U87 glioma cells were inoculated into 5-week-old male Balb/c nude mice ($n=4$). 10 days later, tumor volumes were measured with reading vernier caliper every two days, tumor volumes were calculated by the formula $V\ (mm^3)=a\times b^2/2$ (Zhao et al. 2017). At the end of experiment, the mice were sacrificed by carbon dioxide asphyxiation and dissected; the tumor masses were taken out and weighed.

Notch1 RNA Interference

The experiments of Notch-1 RNA interference were performed using shRNA LV**.** shRNA that co-expressed EGFP and shRNA against human Notch-1 (LV-GFP-shNotch1#15275-1) and a scrambled shRNA used as a negative control (LV-GFP-sh-Con) were synthesized by Genechem (Serial No: V20170602025; Shanghai, China). The target sequence of Notch1 is CTGCCTGGACAA GATCAAT. Briefly, to modify GSCs to express the exosomal siNocth1, GSCs were transfected with shNotch1-RNA [LV-NOTCH1-RNAi (15275-1)] or shCon-RNA (LVCON077) lentivirus vector, respectively, and cultured in serum-free medium for 4–6 h, then, in new serum-free medium for 48 h. Consequently, the transfected GSCs were cultured with puromycin (5 µg/ml) to screen puromycin resistant Notch1-directed stable cell clones. The stable shNotch1-RNA (shNotch1-GSC) and shCon-RNA transfected (shCon-GSC) GSCs were expanded in serum-free medium, and used for siNocth1 modified exosome isolation. The isolated shCon-RNA and siNocth1RNA modified exosomes (shCon-GSC-Exo, shNotch1-GSC-Exo, 40 µg/ml) were used to treat GSCs, GSC-exo treated and untreated non-GSC glioma cells, U251 and U87, for further experiments. Neurosphere formation assay and Western blotting with the method as described in this section.

Notch Signaling Inhibition Assay

For blocking Notch signaling, GSC-exo treated and untreated U251 and U87 non-GSC glioma cells were seeded into 6-well plates at density of 1×10^5 cells/well, cultured overnight, and then treated with Notch inhibitors (Astudillo et al. 2016; Katsushima et al. 2016), IMR-1 (1 µM) or RO4929097 (6 µM) (Selleck Chemicals, USA), respectively. After 48 h, the IMR-1 or RO4929097 treated and untreated glioma cells were harvested, and proteins were prepared for western blot analysis.

Western Blotting

The cells, exosomes, and tumor tissues were lysed with RIPA lysis buffer to extract proteins. 20 µg denatured protein samples were separated by 12% SDS-PAGE and transferred on PVDF membranes. The membranes were then blocked with 5% non-fat milk and immunoblotted using the following primary antibodies: Anti-CD133 (1:500), anti-Nestin (1:500), anti-GFAP (1:500), anti-Oct4 (1:500), anti-Sox2 (1:500), anti-CD63 (1:500), anti-Tig101 (1:500), anti-HSP70 (1:500), anti-Alix (1:500), anti-Notch1(Rabbit anti-Notch1 transmembrane protein antibody, 1:500) was derived from human C-terminal sequence of Notch 1 extracellular truncation and Notch 1 intracellular domain: 2101-2300/2555 (www.bioss.com.cn/prolook_03.asp?id=AF08169606001217&pro37=1), it shares structural characteristics including an extracellular domain and an intracellular domain (www.ncbi.nlm.nih.gov/gene?cmd=Retrieve&dopt=Graphics&list_uids=4851), anti-β-actin (1:1,000) (Beijing Biosynthesis Biotechnology Co., Ltd., China), anti-Notch1 (1:500), anti-DLL1 (1:1000) (Abcam), anti-HEY1 (1:1000), anti-HES1 (1:2000)(CST), respectively. Immuno-reactive proteins were detected with peroxidase-conjugated goat anti-rabbit IgG (H + L) secondary antibody directed to the corresponding primary antibodies. The bands were visualized with chemiluminescence system (Millipore). The densitometry of the western blot bands were quantified by Image J software, and normalized to β-actin.

Statistical Analysis

All results were presented as mean ± SED, statistical significance was determined with two-tailed Student's *t* test or 1-way ANOVA for multiple comparisons using GraphPad Prism. $p < 0.05$ was considered significant.

Results

Characteristics of GSC Stem Cells and Glioma Cells

Non-GSC glioma cells (WJ1, U251, and U87 cells) cultured in DMEM medium supplemented with 10% FBS grew as adherent monolayer; While GSCs cultured with serum-free DMEM/F12 medium supplemented with growth factors in nonadhesive culture system grew as suspensions and formed neurospheres (Fig. 1a).The expression levels of stemness-associated proteins, CD133, Nestin, Oct4, and Sox2 were significantly increased, while, the expression level of GFAP were significantly decreased in GSCs, compared with parental adherent non-GSC glioma cells, respectively (Fig. 1b, c).

Characterization of GSC Exosomes and Glioma Cell Internalization

Exosomes from GSC and non-GSC glioma cell culture medium were isolated using total exosome isolation reagent, and characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis and Western blot analysis. As shown in Fig. 1, the isolated exosomes exhibited a prototypical round shape morphology under TEM (Fig. 2a), average diameter of 106.2 nm (Fig. 2b), and expressed exosome special marker including CD63, TSG101, Alix and HSP70 but absence of CytC (Fig. 2c).

To determine whether GSC-exo could be actively uptaken by non-GSC glioma cells, GSC exosomes were labeled with CM-Dil and added to non-GSC glioma cell culture media. As expected, GSC exosomes were rapidly internalized in the cytoplasm of non-GSC glioma cells (Fig. 2d).

GSC Exosomes Promote Non-GSC Glioma Cell Proliferation and Neurosphere Formation

After GSC-exo treatment, the proliferative activity of non-GSC glioma cells, U251 and U87 were increased significantly in a dose and time-dependent manner (Fig. 3a, b, $p < 0.05$ or $p < 0.01$); The neurosphere formation rates of untreated U251 and U87 non-GSC glioma cells were

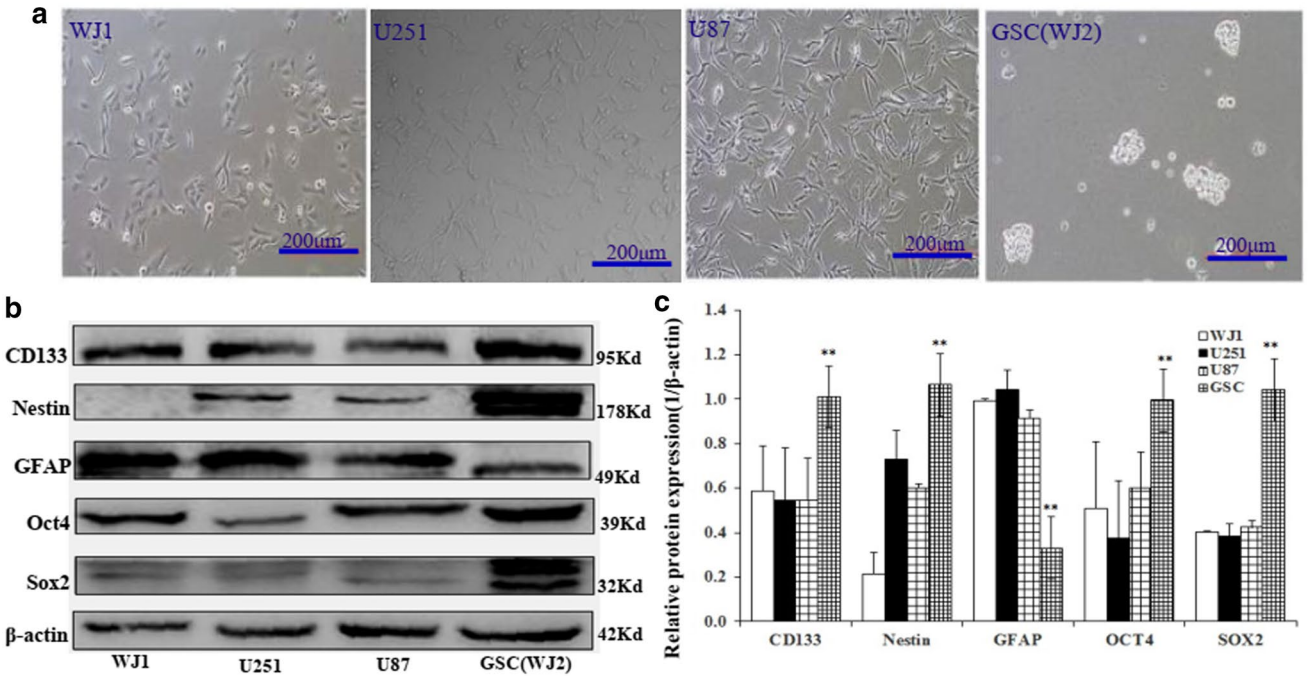

Fig. 1 Morphological characteristic and stemness-related protein expressions of GSCs and non-GSC glioma cells. **a** Morphology of WJ1, U251, U87 cells, and GSCs. Glioma cells cultured in DMEM supplemented with 10% FBS grew as adherent monolayer, GSCs cultured in serum-free DMEM/F12 medium supplemented growth factors formed neurospheres. **b** Western blot analysis of stemness-related protein expressions in non-GSC glioma cells and GSCs. **c** The histogram shows that there was a significant difference of stemness-related protein expressions in non-GSC glioma cells and GSCs. (*$p < 0.05$, **$p < 0.01$)

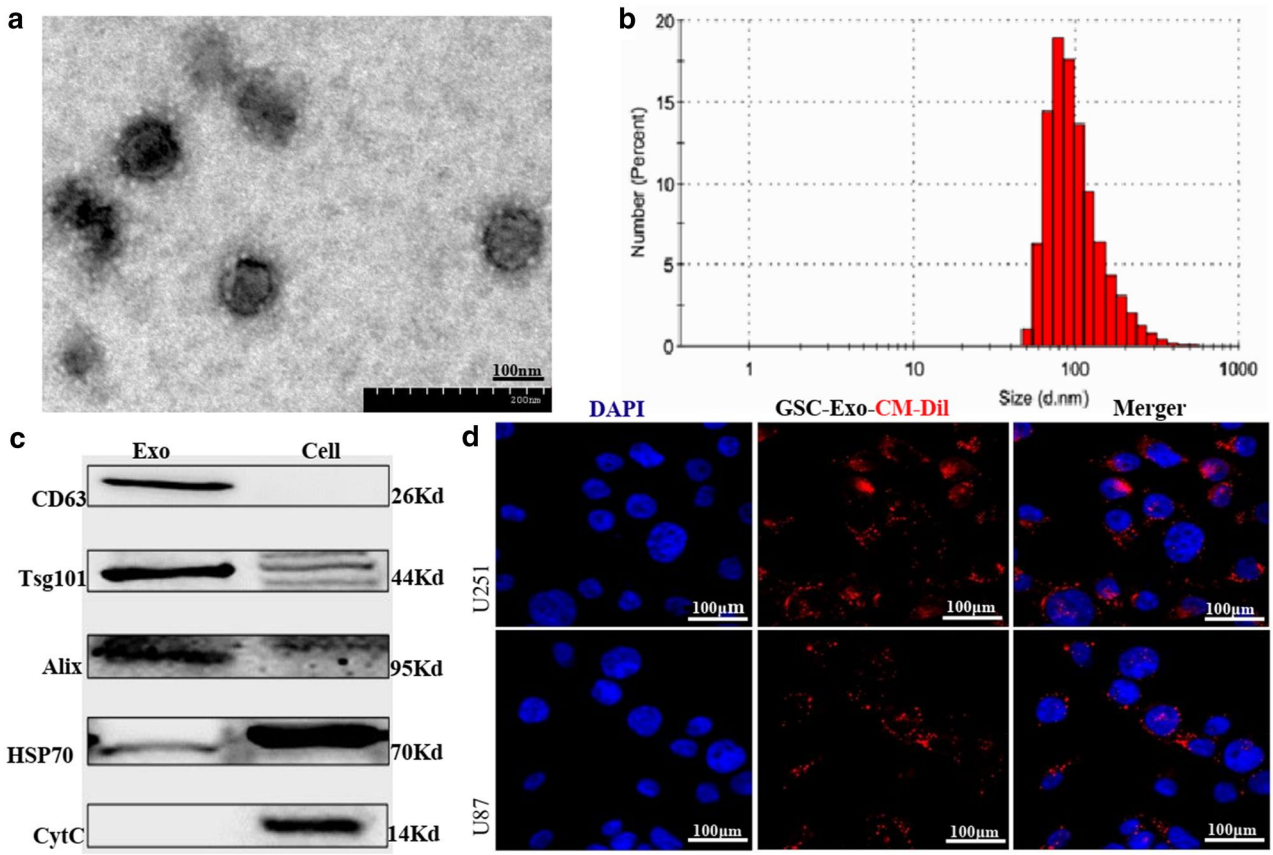

Fig. 2 Characterization of GSC exosomes and internalization in non-GSC glioma cells. **a** Transmission electron microscopy image of GSC exosomes. **b** size distribution of GSC exosomes. **c** Western blot analysis of CD63, TSG101, Alix, HSP70, and CytC of GSC exosomes and cells. **d** U251 and U87 non-GSC glioma cells were incubated with 20 μg/ml of CM-Dil-labeled GSC-exo for 6 h at 37 °C. Exosome internalization was observed by confocal microscopy

$1.14 \pm 0.27\%$ and $0.67 \pm 0.19\%$, respectively, and the average sphere diameters were 56.1 ± 10.8 μm and 94.1 ± 34.0 μm, respectively, after treatment with 20 or 40 μg/ml GSC-exo, neurosphere formation rates and sphere diameters of U251 and U87 non-GSC glioma cells were increased significantly (Fig. 3c, d, $p < 0.05$ or $p < 0.01$).

GSC Exosomes Promote Glioma Cell Invasion

Transwell and 3D-spheroid invasion assays revealed that the number of cells penetrated transwell membrane and the invasion distances of U251 and U87 non-GSC glioma cells were increased significantly in a dose and time-dependent manner after GSC-exo treatment (Fig. 4a–f, $p < 0.05$ or $p < 0.01$).

GSC Exosomes Enhance Tumorigenicity of Non-GSC Glioma Cells

GSC-exo treated and untreated U251 and U87 non-GSC glioma cells were inoculated subcutaneously into immunodeficiency mice, the tumor growth speed, average tumor volume and mean tumor weight of GSC-exo treated U251 and U87 non-GSC glioma cells were increased significantly, compared with those of the untreated glioma cells (Fig. 5a–f, $p < 0.05$ or $p < 0.01$).

Notch1 Signaling Protein Expressions in GSCs, Non-GSC Glioma Cells and Their Exosomes

Western blot analysis displayed that Notch1 signaling related protein were expressed in GBM cells, the expressions of receptor protein Notch1, ligand protein DLL1, and target gene proteins, HES1 and HEY1were upregulated significantly in GSCs, compared with non-GSC, WJ1, U251 and U87 glioma cells (Fig. 6a, b, $p < 0.05$ or $p < 0.01$); More interestingly, Notch1 protein was enriched in GSC exosomes, while exosomes of non-GSC glioma cells, including WJ1, U251 and U87 contain little Notch1 protein (Fig. 6c, $p < 0.01$). It is suggested that Notch1 protein in GSC exosomes might be involved in transferring stemness information to non-GSC glioma cells.

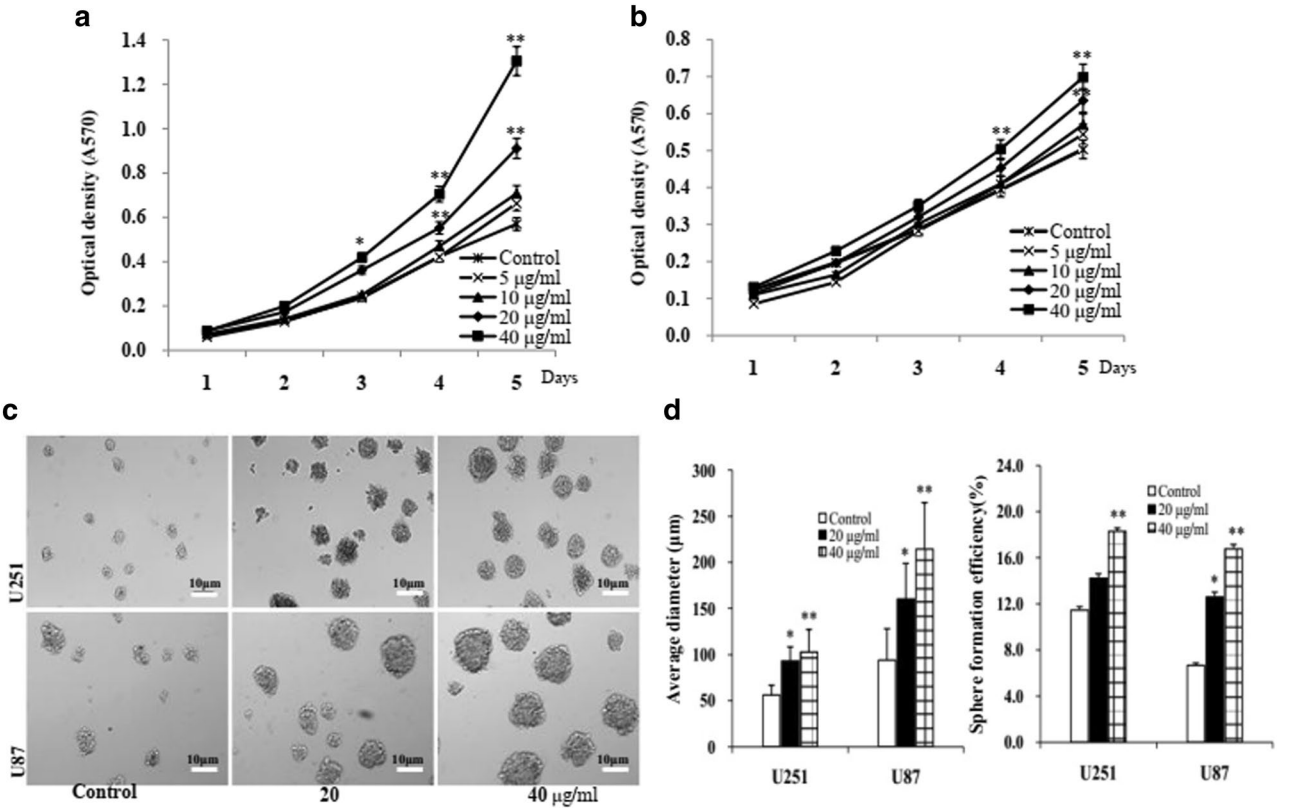

Fig. 3 GSC exosomes promoted non-GSC glioma cell proliferation and neurosphere formation. **a** GSC exosomes promoted U251glioma cell proliferation**. b** GSC exosomes promoted U87 glioma cell proliferation**. c** GSC exosomes promoted U251and U87 glioma cell neurosphere formation. **d** The histogram shows the significant increase of neurosphere formation of U251 or U87 glioma cells treated with GSC exosomes. (*$p<0.05$, **$p<0.01$)

GSC Exosomes Enhance Notch1 Signaling and Stemness Protein Expressions

GSC exosome treatment enhanced Notch1 signaling protein expressions, including receptor protein Notch1, ligand protein DLL1, and target gene proteins, HEY1 and HES1 (Fig. 7a–f, $p<0.05$ or $p<0.01$) and stemness protein expressions, including CD133, Nestin, Oct4, and Sox2 (Fig. 8a–f, $p<0.05$ or $p<0.01$), both in GSC-exo treated U251 and U87 non-GSC glioma cells and in these cells generated tumor tissues.

Notch1 RNA Interference Decrease Notch1 Protein Expression in GSCs and Their Exosomes and Neurosphere Formation of GSCs

ShNotch1-GSC-Exos were taken in and internalized in GSCs (Fig. 9). Notch1 protein expression in shNotch1-GSC-Exo treated GSCs and their exosomes were decreased significantly, compared with those of shCon-GSC-Exo treated GSCs and their exosomes (Fig. 10a, b, $p<0.01$); shNotch1-GSC-Exo treated GSCs formed less and smaller nerurospheres than shCon-GSCs (Fig. 10 c, d, $p<0.01$).

Notch1-Directed shRNA Downregulates Notch1 Signaling and Stemness Protein Expressions

Both GSC-exo treated U251 and U87 non-GSC glioma cell were further treated with shNotch1-GSC-Exo and shCon-GSC-Exo (40 μg/ml), respectively. The expressions of Notch1 signaling proteins including, Notch1, HEY1 and HES1 in shNotch1-GSC-exo treated non-GSC glioma cells were decreased (Fig. 11a–d, $p<0.01$), compared with those in shCon-GSC-exo treated non-GSC glioma cells; Similarly, the expressions of stemness proteins, including CD133, Nestin, Oct4, and Sox2 were significantly downregulated in shNotch1-GSC-exo treated non-GSC glioma cells, compared with those in shCon-GSC-exo treated non-GSC glioma cells (Fig. 12a–d, $p<0.01$).

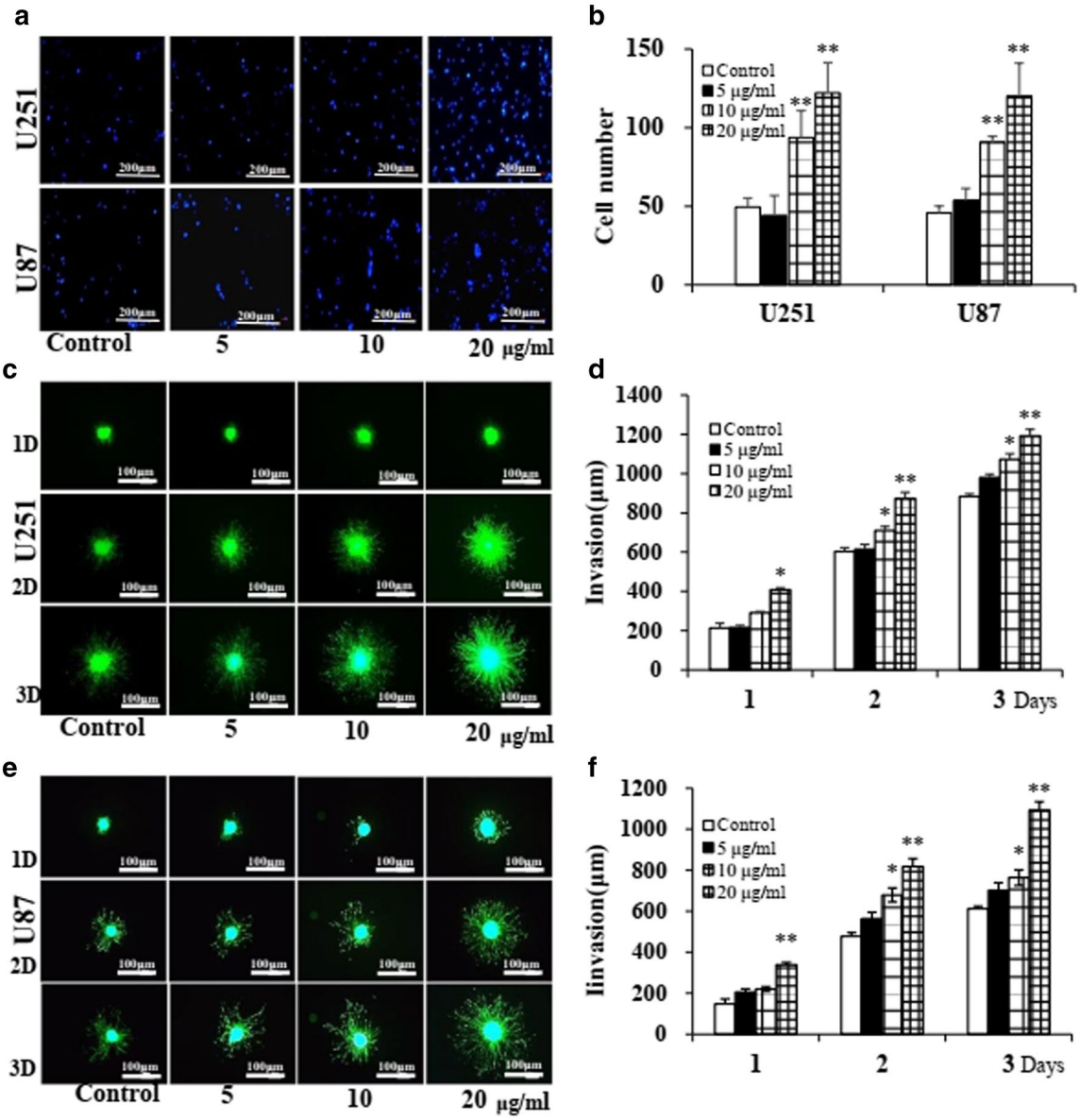

Fig. 4 GSC exosomes promoted non-GSC glioma cell invasion. **a** U251 and U87 non-GSC glioma cells were treated with GSC exosomes for 3 days, and then, cell invasion was evaluated by transwell assay. **b** The histogram shows the significant increase of invasion numbers of U251 or U87 non-GSC glioma cells treated with GSC exosomes. **c** and **e** U251 and U87 non-GSC glioma cells were treated with GSC exosomes for 3 days, and then, cell invasion was measured by 3D-spheroid invasion assay. **d** and **f** The histogram shows the significant increase of invasion distance of U251 or U87 non-GSC glioma cells treated with GSC exosomes. (*$p < 0.05$, **$p < 0.01$)

Notch Inhibitors Reverse the Enhancement of GSC Exosomes on Notch1 Signaling and Stemness Protein Expressions

Notch1 target gene protein and stemness protein expressions were significantly upregulated in the GSC-exo treated U251 and U87 non-GSC glioma cells; the expressions of Notch1 target gene proteins, including**,** HEY1 and HES1, and stemness proteins, including CD133, Nestin, Oct4, and Sox2 in GSC-exo treated U251 and U87 non-GSC glioma cells were significantly downregulated after Notch inhibitor, IMR-1, or RO4929097 treatment (Fig. 13, $p < 0.01$).

Discussion

Glioblastoma is a devastating and deadly malignant primary brain tumor in adults. It nearly always relapses after initial conventional treatment, and frequently exhibits resistance to current therapeutics (Gusyatiner and Hegi

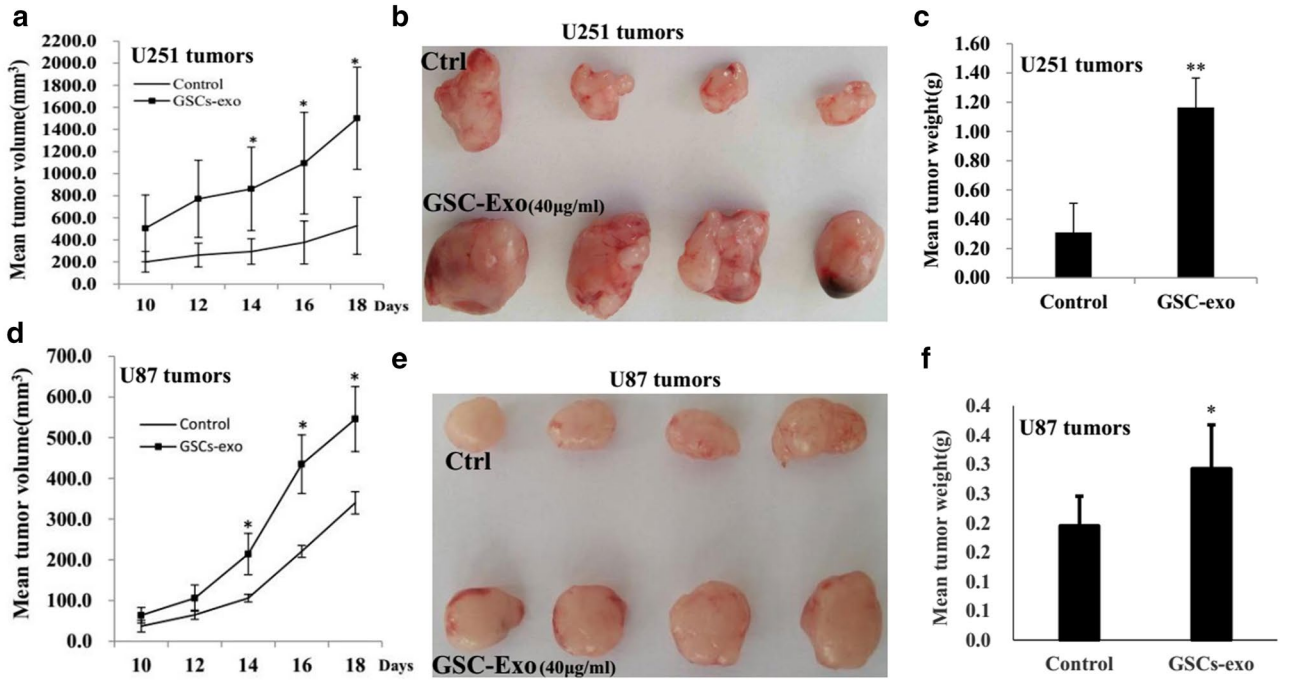

Fig. 5 GSC exosomes promoted non-GSC glioma cell tumorigenecity. 5×10^5 of the untreated and GSC exosomes treated U251 or U87 non-GSC glioma cells were inoculated into nude mice subcutaneously. **a**–**c** Tumor growth of the untreated and GSC exosomes treated U251 non-GSC glioma cells. **d**–**f** Tumor growth of the untreated and GSC exosomes treated U87 non-GSC glioma cells. (*$p<0.05$, **$p<0.01$)

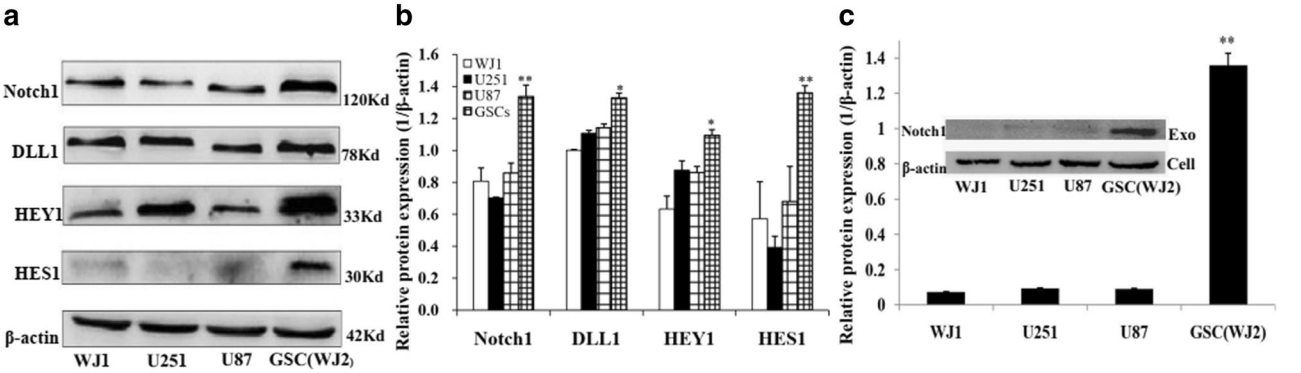

Fig. 6 Notch1 signaling protein expressions in GSC and glioma cells and their exosomes. **a** Western blot analysis of Notch1 signaling protein expressions in non-GSC glioma cells and GSCs. **b** The histogram shows the significant increase of Notch1 signaling protein expressions in GSCs. **c** Notch1 protein was highly expressed in GSC exosomes, little was found in glioma cells derived exosomes. (*$p<0.05$, **$p<0.01$)

2018; Mostovenko et al. 2018). The reason that may explain the recurrence and treatment resistance is the presence of glioma stem cells (GSCs) (Mostovenko et al. 2018). The approaches of target eliminating GSCs might be effective for GBM eradication. However, the strategies of identifying or targeting GSCs have been proved to be unsuccessful recently, the differentiated non-GSCs may dedifferentiate into GSCs and acquire stemness phenotype, and GSCs were maintained in a highly dynamic equilibrium state (Dahan et al. 2014; Kim et al. 2018; Andriani

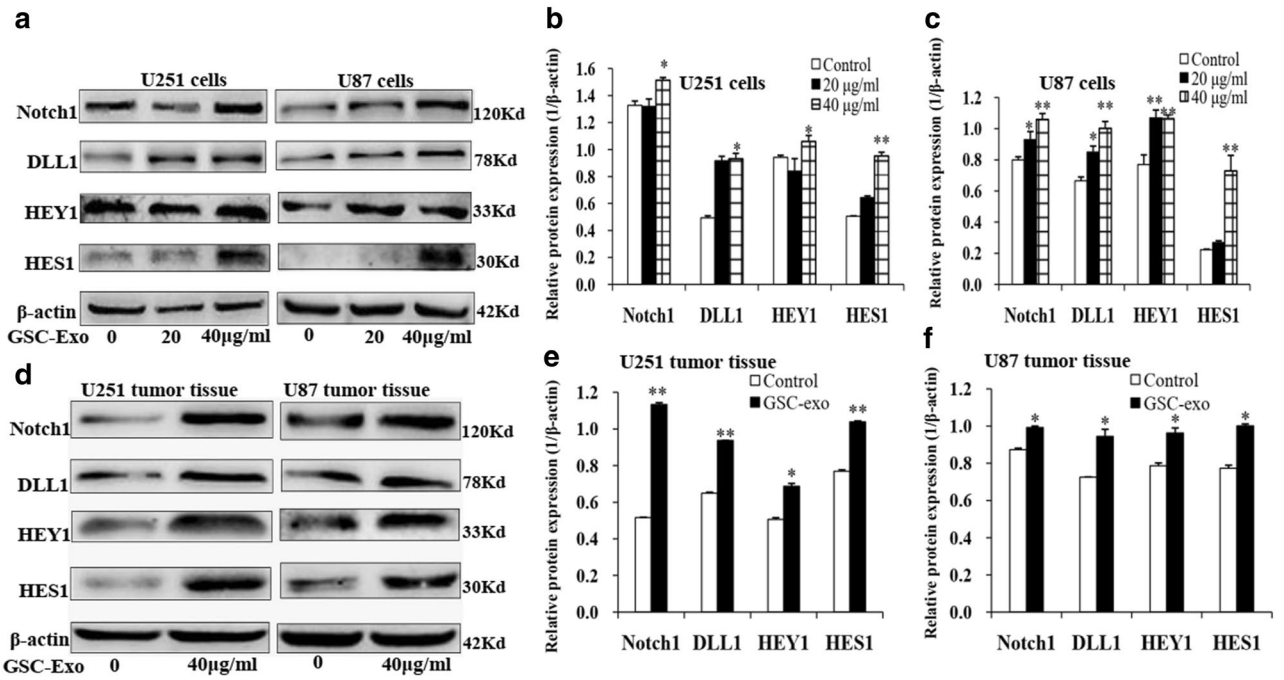

Fig. 7 Notch1 signaling protein expressions in GSC exosomes treated non-GSC glioma cells and these cell generated tumor tissues. **a** Western blot analysis of Notch1 signaling protein expressions in GSC exosomes treated U251 and U87 non-GSC glioma cells. **b** and **c** The histograms show the significant increase of Notch1 signaling protein expressions in GSC exosomes treated U251 or U87 non-GSC glioma cells, respectively. **d** Notch1 signaling protein expressions in GSC exosomes treated U251 and U87 non-GSC glioma cell generated tumor tissues. The untreated and GSC exosomes treated U251 or U87 glioma cells were inoculated into nude mice, the generated tumor tissues were used for western blot analysis. **e** and **f** The histograms show the significant increase of Notch1 signaling protein expressions in GSC exosomes treated U251 and U87 non-GSC glioma cell generated tumor tissues. (*$p < 0.05$, **$p < 0.01$)

et al 2016; Sun et al. 2018; Lee et al. 2016a, b). As the paracrine signaling networks in GBM microenvironment played crucial roles in the growth and maintenance of GSCs, therefore, to study the stemness of GSCs and their communication with other cells within their microenvironment is not only important to understand the biology of GSCs but also to discover new targets for GBM therapeutics (Sattiraju et al. 2017).

Exosomes are 30–100 nm extracellular vesicles (EVs) released by all living cells, including GBM cells and GSCs. These small vesicles contain lots of bioactive materials in the form of proteins, DNA, mRNA, miRNA and lipids, acting as information carriers, and playing important roles in intercellular communication by transferring their molecular contents to recipient cells (Pitt et al. 2016; Zhang et al. 2015; Luhtala et al. 2017; Nakano et al. 2015; Tűzesi et al. 2017). Exosomes derived from GSCs carried membrane receptors, signaling proteins and pro-tumorigenic factors that participate in glioblastoma progression (Treps et al. 2017; Zeng et al. 2017) and reprogram recipient cells, including differentiated glioma cells (Zhang et al. 2015; Luhtala et al. 2017; Nakano et al. 2015; Tűzesi et al. 2017), GBM cell released exosomes initiated the phenotype changes, even tumourigenicity of normal human astrocytes (Oushy et al. 2018), GSC-secreted exosomes affected the gene expression with a role in cell fate and tumorigenesis of received NSCs (Tűzesi et al. 2017).

In the present experimental study, exosomes were isolated from GSC and non-GSC glioma cell culture media (Fig. 1a–c) and characterized (Zeringer et al. 2013; Tang et al. 2017; Sharma et al. 2018) (Fig. 2a–d). U251 and U87 non-GSCs glioma cells were treated with GSC exosomes and the exosomes were internalized in the cytoplasm of U251 and U87 non-GSC glioma cells (Fig. 2d). After various amount of GSC exosome treatment, the proliferation (Fig. 3a, b), neurosphere formation (Fig. 3c, d), invasive capacities (Fig. 4), and tumorigenicity ex vivo (Fig. 5) were increased significantly. These findings were matched with

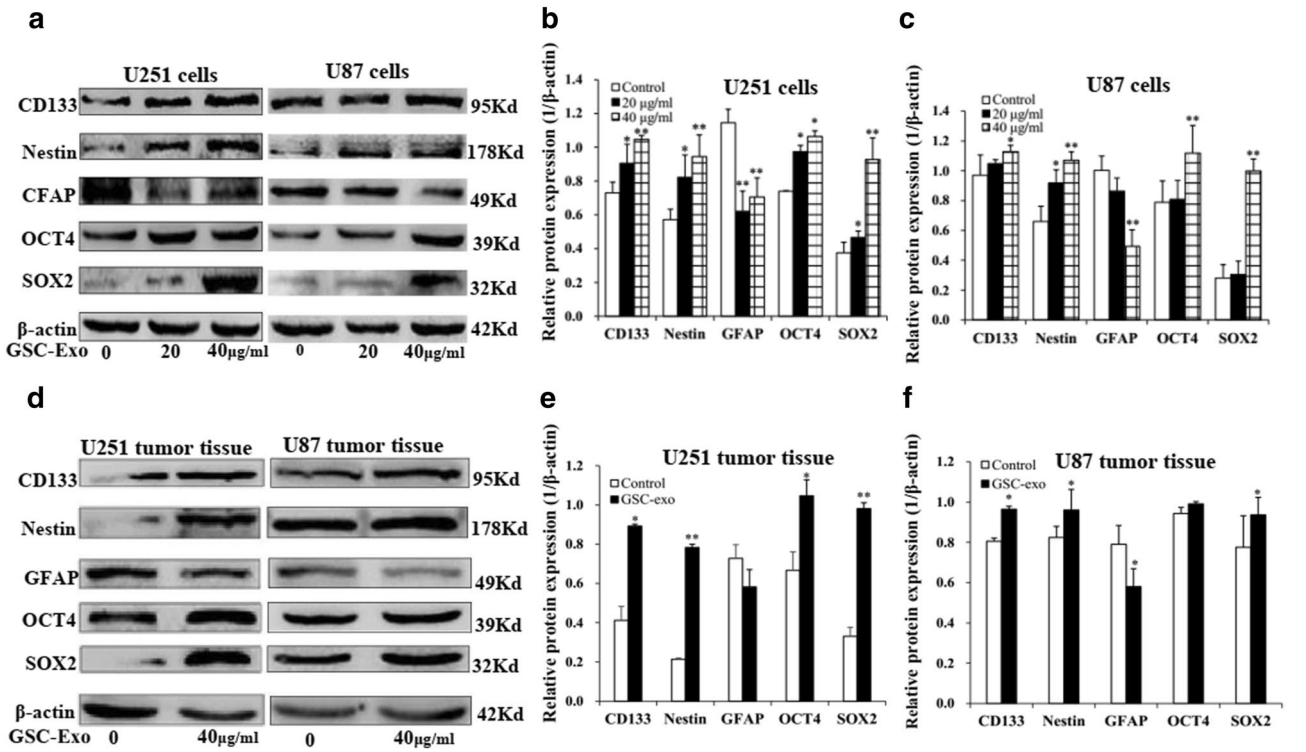

Fig. 8 Stemness protein expressions in GSC exosomes treated non-GSC glioma cells and these cell generated tumor tissues. **a** Western blot analysis of stemness protein expressions in GSC exosomes treated U251 and U87 non-GSC glioma cells. **b** and **c** The histograms show the significant increase of stemness protein expressions in GSC exosomes treated U251 or U87 non-GSC glioma cells, respectively. **d** The stemness protein expressions in GSC exosomes treated U251 and U87 non-GSC glioma cell generated tumor tissues. The untreated and GSC exosomes treated U251 or U87 non-GSC glioma cells were inoculated into nude mice, the generated tumor tissues were used for western blot analysis. **e** and **f** The histograms show the significant increase of stemness protein expressions s in GSC exosomes treated U251 and U87 non-GSC glioma cell generated tumor tissues. (*$p < 0.05$, **$p < 0.01$)

the stemness phenotype of GSCs (Gilbert and Ross 2009; Raysi Dehcordi et al. 2017; Gopalan et al. 2018). It is indicated that GSC exosome exposure increased the stemness and tumorigenicity of non-GSC glioma cells. However, whether the exosomes from GCS have the same effect in vivo needs to be determined further; what GSC exosomes carried and delivered to U251 and U87 non-GSC glioma cells and through what way that U251 and U87 non-GSC glioma cells were reprogrammed or dedifferentiated into cancer stem-like cells are still unknown.

The expressions of Notch1, its ligand, and target genes are pivotal to glioma cell survival and proliferation (Teodorczyk and Schmidt 2014; Purow et al. 2005; Li et al. 2018). Patient derived GBM cells can be dedifferentiated into CSC-like cells by expressions of Oct4, Sox2 and Nanog transcription factors; Notch1 signaling was activated in CSC-like cells (Olmez et al. 2015). Notch1 can directly induce CD133 expression (Konishi et al. 2016), and blocking Notch signaling pathway can deplete CD133-positive GBM cells (Fan et al. 2010); ShRNA targeting Notch ligand Delta-like 1 (DLL1) and Notch1 target Hes1 decreased CD133 and Nestin expression (Fan et al. 2010; Safa et al. 2016) [20,55]. Notch1 signaling, Notch1-SOX2 signaling and the expression of cancer stem cell markers, including CD133, Sox2, Oct4, Nanog, etc. contributed to maintain the stemness properties of GSCs (Konishi et al. 2016; Bayin et al. 2017; Sarkar et al. 2017; Li et al. 2018; Zhang et al. 2017).

In this experimental investigation, Western blotting analysis revealed that the expression levels of Notch1 signaling proteins, including, Notch1 transmembrane protein (Notch1), ligand protein DLL1, and target gene proteins, HEY1 and HES1 in GSCs were higher than those in non-GSCs, WJ1, U251 and U87 glioma cells (Fig. 6a, b); even, Notch1 protein was highly enriched in

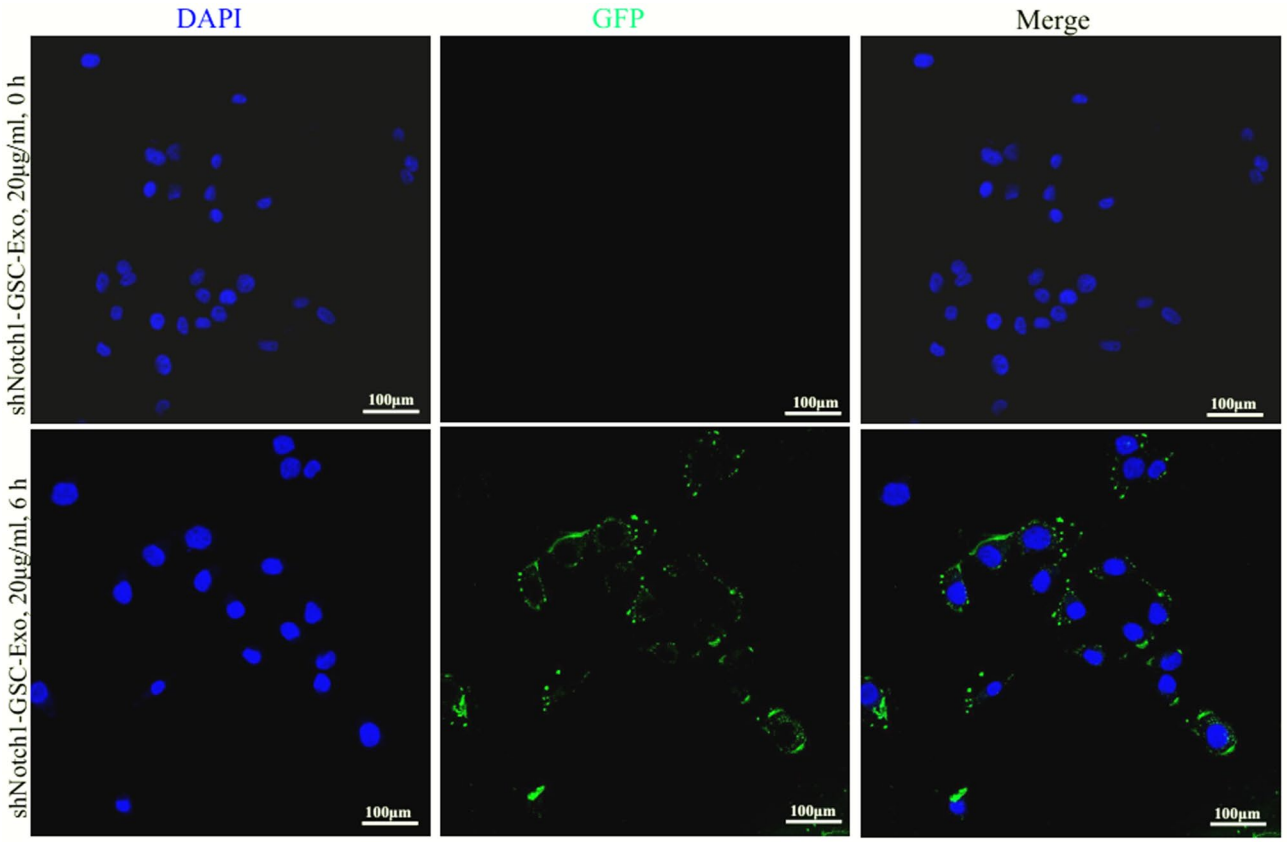

Fig. 9 shNotch1-GSC-exo internalization in GSCs. GSCs were incubated with 20 μg/ml of shNotch1-GSC-exo for 6 h at 37 °C, exosome internalization was observed by confocal microscopy

GSC exosomes, little was detected in the exosomes from non-GSCs, including WJ1, U251, and U87 glioma cells (Fig. 6c). Furthermore, GSC exosome treatment increased the expressions of Notch1 signaling proteins, including Notch1, DLL1, HEY1, and HES1 (Fig. 7), and stemness proteins**,** including CD133, Nestin, Oct4, and Sox2 (Fig. 8) in GSC-exo treated U251 and U87 non-GSC glioma cells as well as these cells generated tumor tissues. In addition, besides Notch1, Notch1 target gene proteins, HES1, and HEY1were upregulated significantly in GSCs, compared with non-GSC glioma cells, even more than Notch1 (Fig. 5a, b). It couldn't be excluded that HES1 and HEY1 play roles in enhancing stemness, proliferation, invasion, and tumorigenicity of non-GSCs. However whether GSC exosomes contain HES1 and HEY1 needs to be confirmed.

Notch signaling promotes st+emness phenotype of cancer stem cells (Saito et al. 2019); the signaling is activated by ligand binding to Notch receptor. Notch receptor protein was cleaved through a two-step proteolytic cleavage by ADAM family proteases and γ-secretase, releasing the Notch intracellular domain (NICD). Then, NICD is translocated to the nucleus where it binds to CSL and converts the complex from a repressor to an activator of Notch target genes, mediating transcriptional activation of Notch signaling (Venkatesh et al. 2018).

Based on the findings above, it might be speculated that GSC exosomes mediated the reprogramming or dedifferentiation of non-GSC glioma cells by transferring Notch1 protein through Notch1 signaling pathway.

To verify this speculation, Notch1 gene expression was knockdown with Notch1 RNA interference, Notch1 signaling pathway was blocked with Notch inhibitors.

ShNotch1 RNA downregulated Notch1 protein expression in shNotch1-GSCs and their exosomes significantly,

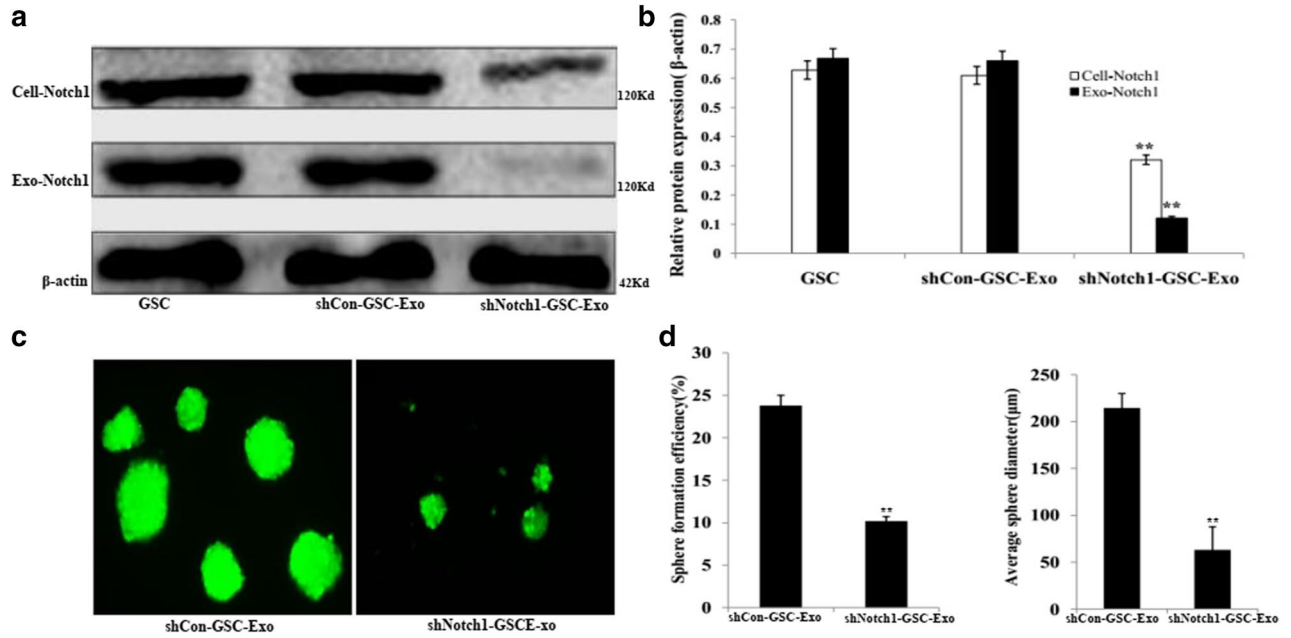

Fig. 10 Notch1 RNA interference decreased Notch1 protein expression in GSCs and their exosomes and neurosphere formation of GSCs. **a** Western blot analysis of Notch1 protein expression in shNotch1-GSCs and their exosomes. **b** The histogram shows the significant increase of Notch1 protein expressions in shNotch1-GSCs and their exosomes. **c** Nerurosphere formation of shCon-GSC-Exo and shNotch1-GSC-Exo treated GSCs. **d** The histograms show the significant decrease of nerurosphere numbers and size of shNotch1-GSC-Exo treated GSCs. (*$p < 0.05$, **$p < 0.01$)

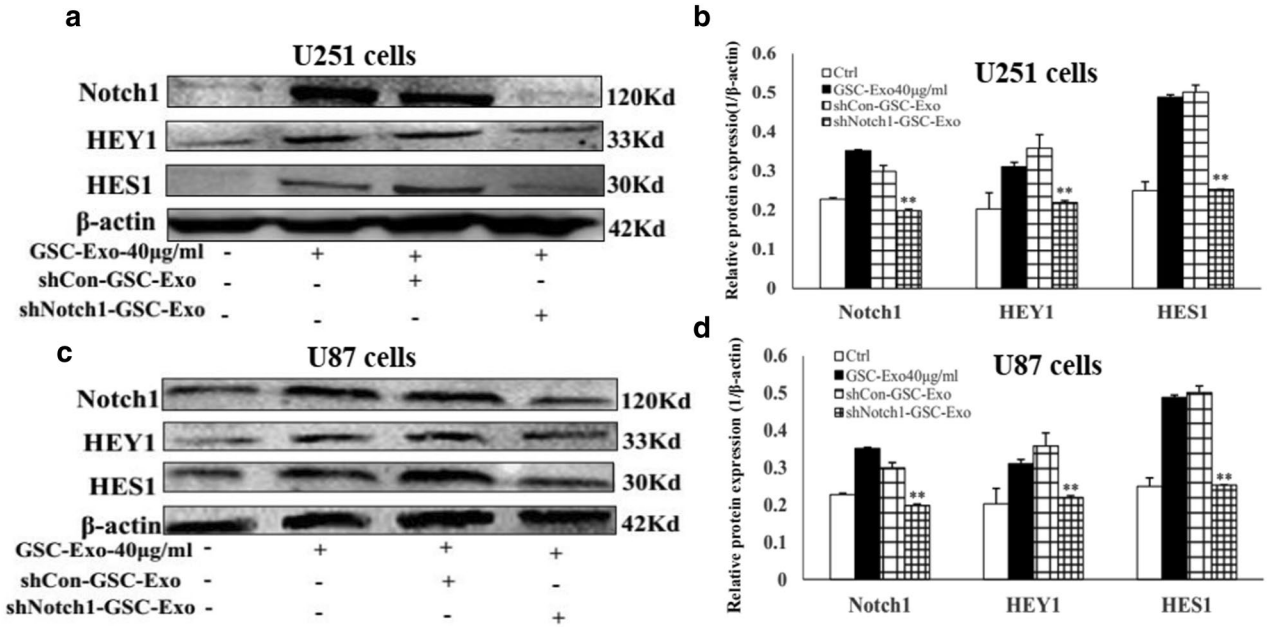

Fig. 11 Notch1 RNA interference downregulates Notch1 signaling protein expressions in GSC exosomes treated non-GSC glioma cells. U251 and U87 non-GSC glioma cells treated with GSC exosomes (40 μg/ml) for 3 days, were further treated with shCon-GSC-Exo and shNotch1-GSC-Exo (40 μg/ml), respectively, and then Western blotting was performed. **a** Notch1 signaling protein expressions in GSC-Exo, shCon-GSC-Exo and shNotch1-GSC-Exo treated U251 non-GSC glioma cells. **b** The histogram shows the significant decrease of Notch1 signaling protein expressions in GSC-Exo treated U251 non-GSC glioma cells by shNotch1-GSC-Exo. **c** Notch1 signaling protein expressions in GSC-Exo, shCon-GSC-Exo and shNotch1-GSC-Exo treated U87 non-GSC glioma cells. **d** The histogram shows the significant decrease of Notch1 signaling protein expressions in GSC exosome treated U87 non-GSC glioma cells by shNotch1-GSC-Exo. (*$p < 0.05$, **$p < 0.01$)

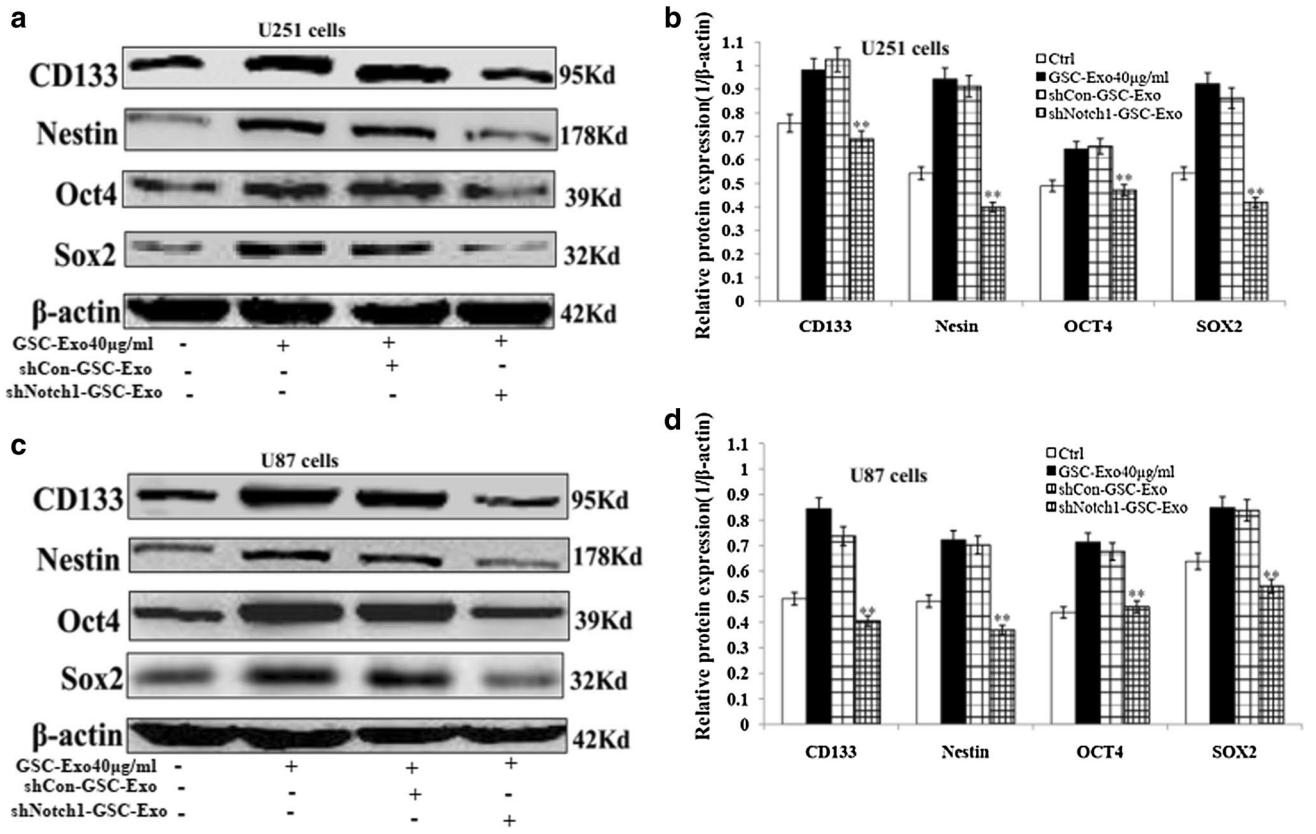

Fig. 12 Notch1 RNA interference downregulated stemness protein expressions in GSC exosomes treated non-GSC glioma cells. U251 and U87 non-GSC glioma cells treated with GSC exosomes (40 μg/ml) for 3 days, and further treated with shCon-GSC-Exo and shNotch1-GSC-Exo (40 μg/ml), respectively, and then Western blotting was performed. **a** Stemness protein expressions in shCon-GSC-Exo and shNotch1-GSC-Exo treated U251 non-GSC glioma cells. **b** The histogram shows the significant decrease of stemness protein expressions in GSC exosome treated U251 non-GSC glioma cells by shNotch1-GSC-Exo. **c** Stemness protein expressions in shCon-GSC-Exo and shNotch1-GSC-Exo treated U87 non-GSC glioma cells. **d** The histogram shows the significant decrease of stemness protein expressions in GSC exosome treated U87 non-GSC glioma cells by shNotch1-GSC-Exo. (*$p < 0.05$, **$p < 0.01$.)

compared with those of shCon-GSCs and their exosomes (Fig. 10a, b); the expressions of Notch1 signaling proteins, including, Notch1, HEY1, and HES1 (Fig. 11) and stemness proteins, including CD133, Nestin, Oct4, and Sox2 were decreased in GSC-exo treated U251 and U87 non-GSC glioma cells (Fig. 12) significantly, after shNotch1-GSC-exo treatment. These findings indicated that Notch1 signaling activated by Notch1 carried in GSC exosomes was attenuated by shNotch1 RNA.

Moreover, Notch inhibitors, IMR-1, or RO4929097 treatment inhibited the expressions of Notch1 target gene proteins, including, HEY1 and HES1, and stemness protein expressions, including CD133, Nestin, Oct4, and Sox2 in GSC-exo treated U251 and U87 non-GSC glioma cells (Fig. 13a–d). This result suggested that Notch1 signaling pathway activation in GSC-exo treated U251 and U87 non-GSC glioma cells was abated.

RO4929097, a specific γ-secretase inhibitor, exhibited growth inhibition effect on malignant gliomas through Notch pathway inhibition in experimental studies and Phase I clinical study, as it has toxicity in clinical study, RO4929097 is no longer being produced (Yahyanejad et al. 2016; Pan et al. 2016). Based on the compelling scientific findings in this experimental investigation, it is deserved to explore some

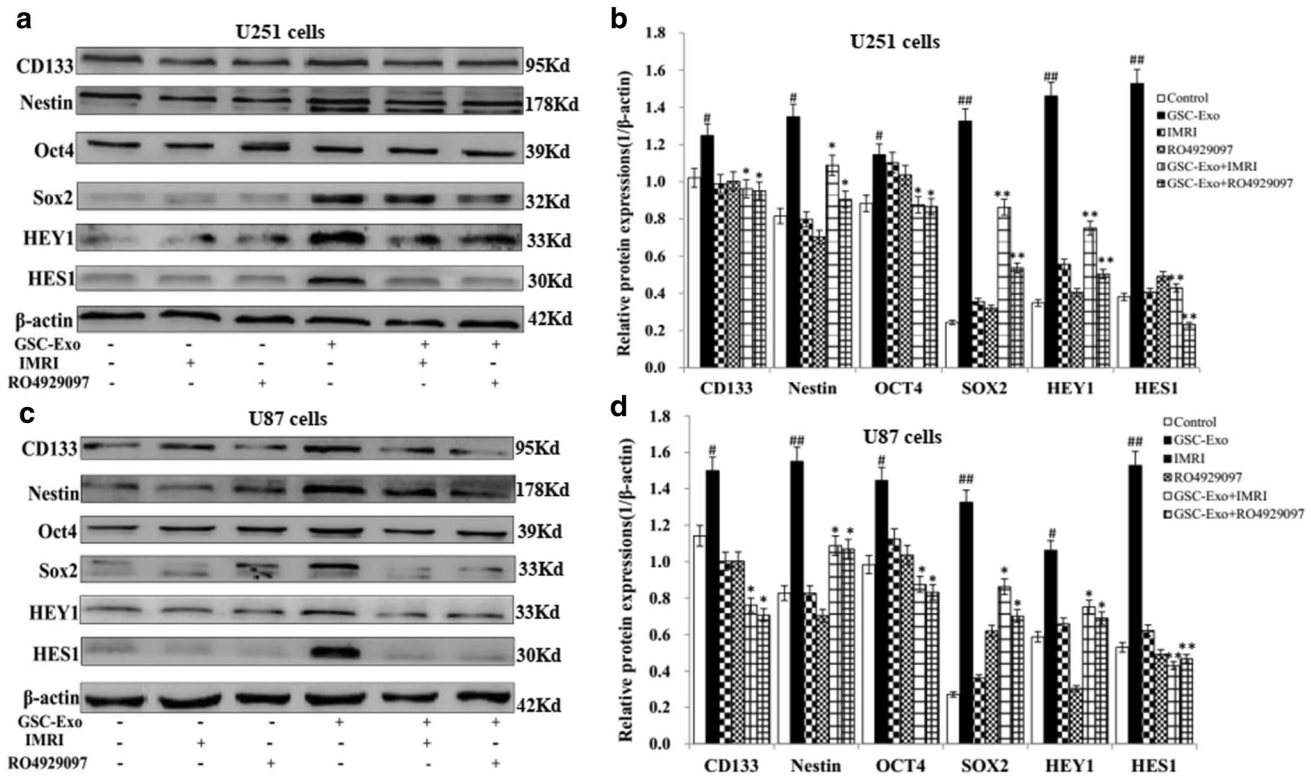

Fig. 13 Notch inhibitors decreased stemness and Notch1 target gene protein expressions in GSC exosomes treated non-GSC glioma cells. U251 and U87 non-GSC glioma cells were treated with GSC exosomes (40 μg/ml) for 3 days, and further treated with Notch inhibitor, IMR-1 or RO4929097, respectively, and then Western blotting was performed. **a** Stemness and Notch1 target gene protein expressions in GSC exosome treated U251 non-GSC glioma cells. **b** The histogram shows the significant decrease of stemness and Notch1 target gene protein expressions in GSC exosome treated U251 non-GSC glioma cells by Notch inhibitor, IMR-1 and RO4929097, $^{*}p<0.05$, $^{**}p<0.01$, compared with the control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with GSC exosome treated U251 non-GSC glioma cells. **c** Stemness and Notch1 target gene protein expressions in GSC exosome treated U87 non-GSC glioma cells. **d** The histogram shows the significant decrease of stemness and Notch1 target gene protein expressions in GSC exosome treated U87 non-GSC glioma cells by Notch inhibitor, IMR-1 and RO4929097, $^{*}p<0.05$, $^{**}p<0.01$, compared with the control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with GSC exosome treated U87 non-GSC glioma cells

other new small molecules and strategies targeting Notch1 signaling pathway for GBM therapeutics.

In the light of the findings in this experimental study, it could be assumed that GSC released exosomes containing Notch1 protein; once these exosomes were taken into by non-GSC glioma cells, the stemness and tumorigenicity of these non-GSC glioma cells were enhanced by Notch1 transferred from GSC exosomes through Notch1 signaling pathway activation (Fig. 14).

Taken together, GSC exosomes act as information carriers, mediated non-GSC glioma cells dedifferentiating into GSCs through delivering Notch1 protein and Notch1 signaling activation, and played crucial role in maintaining the dynamic equilibrium state of CSCs in tumor microenvironment. Targeting GSC exosomes and Notch1 signaling pathway to damage GSCs might be a novel strategy for GBM eradication, which needs to be investigated further.

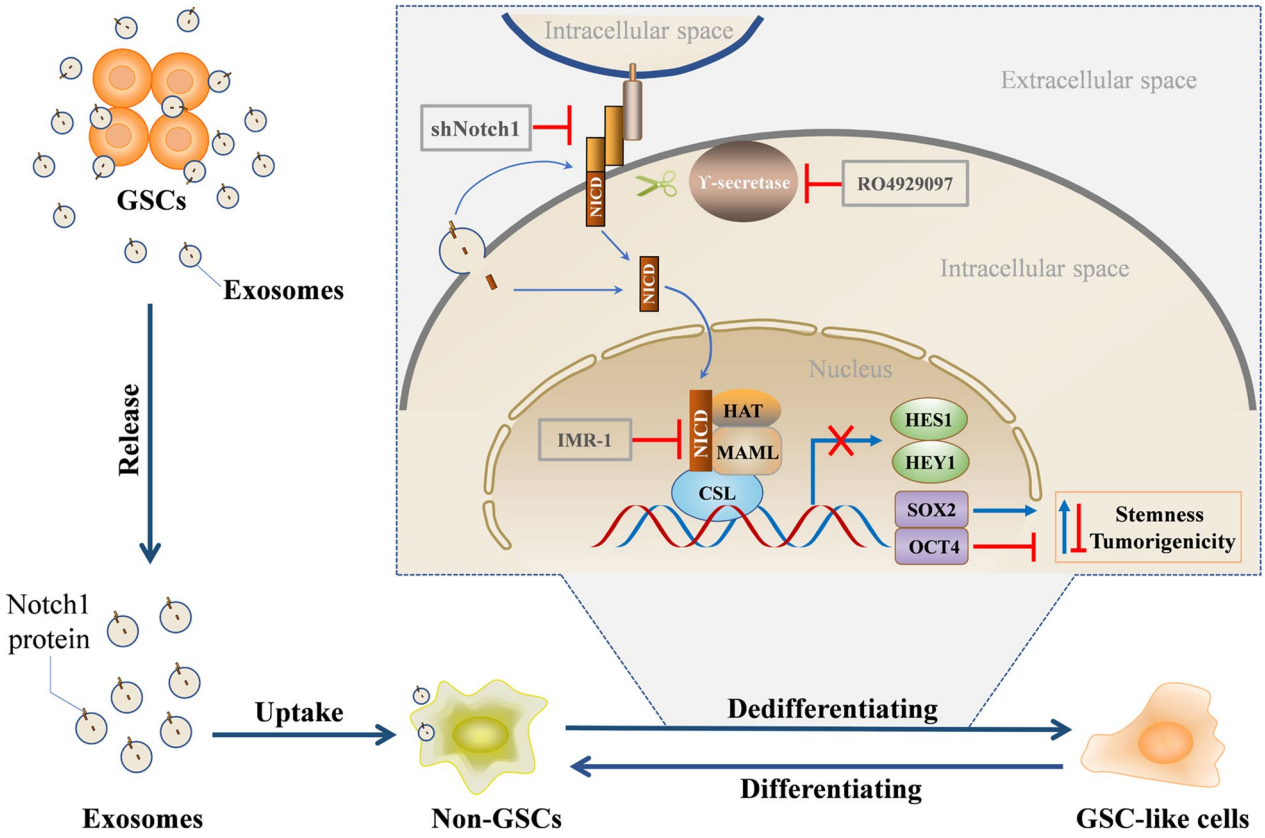

Fig. 14 schematic illustration demonstrates that GSC exosomes enhance the stemness and tumorigenicity of non-GSC glioma cells by transferring Notch1 protein through Notch1 signaling pathway activation

Author Contributions XW conceived and designed the experiments. ZS, LW performed the experiments, analyzed the data, and wrote the manuscript draft. YZ, LD, WM, LL performed some experiments. JZ performed project administration. All authors reviewed the manuscript and approved submission of the manuscript.

Funding This work was supported by National Natural Science Foundation of China (Grant No. 31371148).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethics Committee of Sichuan University.

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