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2D and 3D *in vitro* assays to quantify the invasive behavior of glioblastoma stem cells in response to SDF-1α

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ABSTRACT

Invasion is a hallmark of cancer and therefore *in vitro* invasion assays are important tools in cancer research. We aimed to describe *in vitro* 2D transwell assays and 3D spheroid assays to quantitatively determine the invasive behavior of glioblastoma stem cells in response to the chemoattractant SDF-1α. Matrigel was used as a matrix in both assays. We demonstrated quantitatively that SDF-1α increased invasive behavior of glioblastoma stem cells in both assays. We conclude that the 2D transwell invasion assay is easy to perform, fast and less complex whereas the more time-consuming 3D spheroid invasion assay is physiologically closer to the *in vivo* situation.

METHOD SUMMARY

We describe 2D transwell invasion assays and 3D spheroid invasion assays for the investigation of effects of the chemoattractant SDF-1α on human glioblastoma stem cells *in vitro* in a quantitative manner using image analysis. In both *in vitro* invasion assays, Matrigel was used as the matrix that glioblastoma stem cells invade. The 2D assay is easy to perform, fast and less complex whereas the 3D assay models the *in vivo* situation more closely.

KEYWORDS:

2D transwell invasion assay ● 3D spheroid invasion assay ● cancer cell ● cellular invasion ● glioblastoma stem cells

Invasive behavior is a hallmark of cancer and a major reason why the disease is hard to eradicate, causing poor patient prognosis [1–3]. Invasive cancer cells migrate through the extracellular matrix (ECM), enabled by the secretion of proteases to degrade the ECM and alterations in the cytoskeleton [4–6].

Multiple *in vitro* assays and models are available to study cellular migration and invasion, such as the scratch assay, 3D bioscaffolds and microfluidic co-cultures [3,7–11]. Frequently used invasion assays are the transwell invasion assay (2D invasion assay) [3,8,12,13] and the spheroid invasion assay (3D invasion assay) [5,12,14–16]. The latter two types of invasion assays are discussed in this article. Both types of *in vitro* invasion assays are relatively easy to perform, fast, reproducible and cheap. Although the 3D invasion assay is more complex than the 2D invasion assay, it is physiologically more relevant because it represents the *in vivo* situation in solid tumors more accurately. In 3D invasion assays, cancer cells not only invade through a matrix, but are also affected by other cancer cells in their direct surroundings. Cancer cells need to change and modify their surrounding environment in order to invade, which is also the case in the *in vitro* invasion assays [1,17]. *In vitro* invasion assays are generally preferred over *in vivo* invasion experiments using fluorescently-labeled cells and intravital imaging in animal models because of ethical concerns and the high costs of animal models. Furthermore, the *in vitro* invasion assays are more controllable, more flexible and easier to modify [1,18,19].

In this article, we describe the *in vitro* 2D transwell invasion assay and the 3D spheroid invasion assay in detail with the use of a brain tumor patient-derived glioblastoma stem cell (GSC) line. GSCs have been used instead of differentiated glioblastoma cells, because they have a higher invasive potential than differentiated glioblastoma cells [20,21]. The presence of cell surface protein L1CAM and the intracellular PI3K/AKT and Notch signaling pathways are involved in the highly elevated invasive potential of GSCs compared with differentiated glioblastoma cells [21,22]. Therefore, GSCs are a suitable cell type to demonstrate cellular invasion. GSCs as well as differentiated glioblastoma cells express CXCR4, a specific receptor for the chemoattractant SDF-1α, which we found to be highly expressed in hypoxic peri-arteriolar GSC niches in glioblastoma patient samples where GSCs are maintained in the tumors and protected from chemotherapy and radiotherapy because of their quiescence [23–25]. Interactions between SDF-1α and CXCR4 have been reported

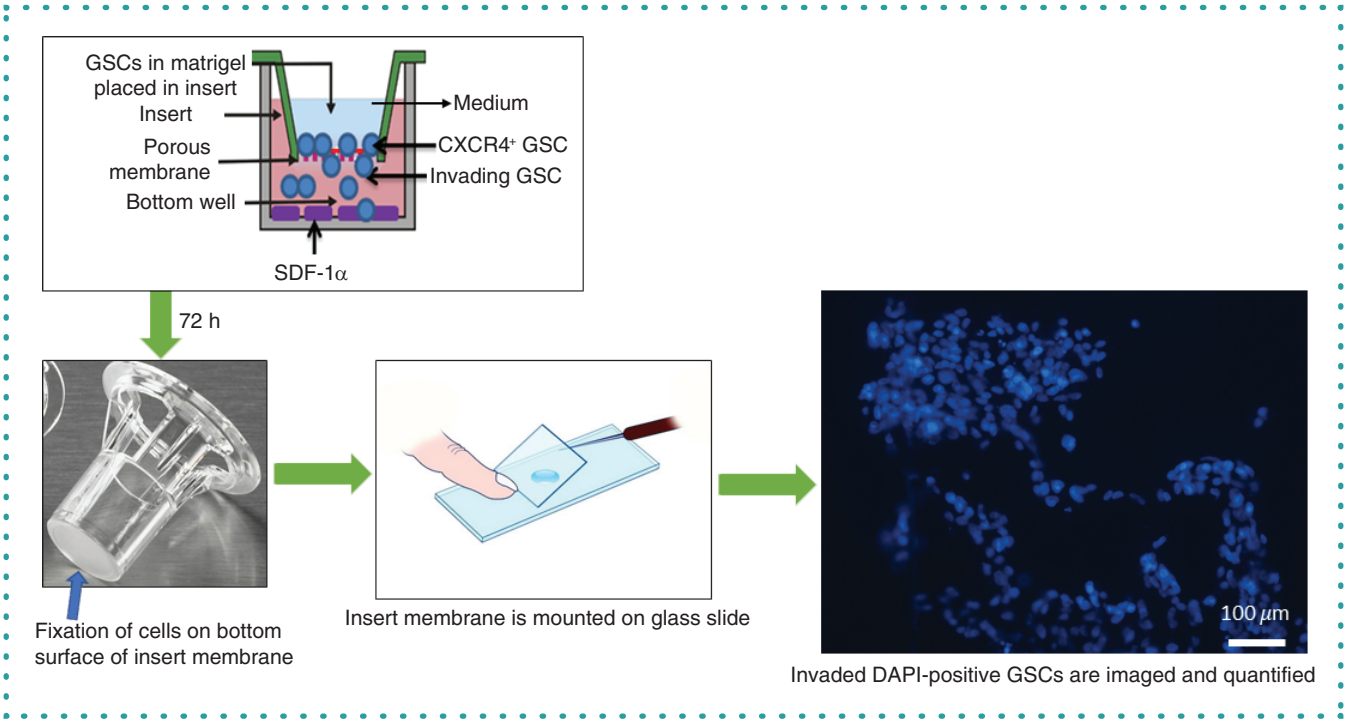

Figure 1. Schematic overview of the 2D transwell invasion assay. CXCR4-positive GSCs in Matrigel-containing medium are placed in the top wells (inserts) in the presence or absence of chemoattractant SDF-1α in the medium in bottom wells. Invaded GSCs accumulate at the bottom surface of the insert membrane (blue arrow). After 72 h, cells on the bottom surface of insert membranes are fixed and the membrane is cut out of the insert. The membrane is mounted on a microscopic slide, followed by imaging and quantification of DAPI-positive GSCs using image analysis. Scale bar = 100 μm. DAPI: 4′,6-diamidino-2-phenylindole; GSC: Glioblastoma stem cell.

to be involved in cellular invasive behavior in glioblastoma [26–29] and the SDF-1α–CXCR4 axis enables invasion of CXCR4-positive glioblastoma cells into SDF-1α-rich niches and transformation into GSCs [23–25].

In the 2D and 3D invasion assays, Matrigel is used as a matrix. In this study we aimed to quantitatively determine the invasive cellular behavior of CXCR4-expressing GSCs in the presence and absence of SDF-1α. In addition, we aimed to assess the advantages and disadvantages of both types of *in vitro* invasion assays.

Materials & methods

Cell culture

NCH421k GSCs [30,31] were a generous gift from Christel Herold-Mende (Heidelberg University, Heidelberg, Germany) and were cultured as non-adherent 3D spheroids in Neurobasal™ medium (Gibco, Life Technologies, CA, USA) containing 1% penicillin/streptomycin (Sigma, MO, USA), 1% L-glutamine (Sigma), 2% B27 (Gibco), 0.08% bFGF (Gibco), 0.01% EGF (Gibco) and 0.01% heparin (Sigma) at 37°C in a 5% CO_2 incubator.

2D transwell invasion assay

The method of the transwell invasion assay is described step by step, to be performed chronologically to reproduce the experiment. NCH421k GSCs growing as 3D spheroids were mechanically resuspended to obtain single cells. Transwell invasion assays as shown in Figure 1 were performed using inserts with 8.0-μm pores (Corning Life Sciences, NY, USA). NCH421k GSCs (80,000 cells/insert) were used that express CXCR4 as described elsewhere [12,24]. Cells were mixed in 0.5 mg/ml Matrigel (Corning) and plated in inserts in a total volume of 50 μl, and the inserts were then placed in 24-well plates (Corning). After incubation for 30 min at 37°C in a 5% CO_2 incubator, 50 μl complete Neurobasal medium was added to the inserts to obtain a total volume of 100 μl. For the experimental condition, 600 μl complete Neurobasal medium containing 10 ng/ml SDF-1α (catalog #: 300-28A; Peprotech, NJ, USA) was added to the bottom wells. As a control, complete Neurobasal medium without SDF-1α was added to the bottom wells. After 72 h, invaded cells that accumulated on the bottom surface of the insert membranes were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 20 min at room temperature, washed with 1× phosphate-buffered saline (PBS; Gibco) for 10 min and the cell nuclei were stained with the DNA dye 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 5 min, followed by a washing step with 1× PBS for 10 min. After removing the cells and Matrigel out of the inserts with cotton swabs, the membranes were cut out from the inserts with a scalpel and mounted on microscopic slides using Prolong Gold mounting medium (Life Technologies, CA, USA) for quantification of invaded DAPI-positive cells (Figure 1).

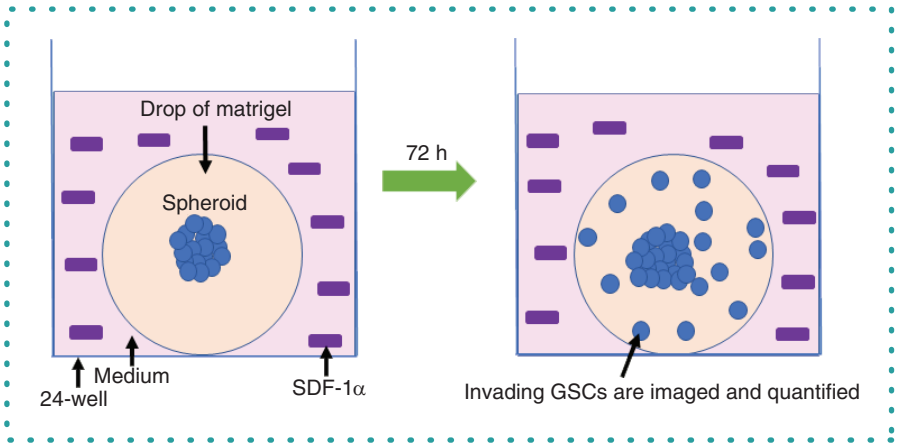

Figure 2. Schematic overview of the 3D spheroid invasion assay. After spheroid preparation in U-bottomed 96 wells, single GSC spheroids are transferred into 24-wells and embedded in a drop of Matrigel. The spheroids are covered with Neurobasal medium in the presence or absence of SDF-1α. After 72 h of incubation at 37°C, images of the spheroids are captured and the GSCs that invade from the spheroid into the Matrigel-containing medium are quantified using image analysis.
GSC: Glioblastoma stem cell.

Images of the membranes were captured using a Nikon Eclipse Ti-E fluorescence inverted microscope and the NIS-Elements AR 4.13.04 software (Nikon, Tokyo, Japan). The total number of cells in ten individual fields per condition was counted using image analysis and the ImageJ software [32,33]. Three experiments were performed, each in triplicate.

Image analysis was performed using ImageJ software and the following tools: → Analyze → Cell counter. Cells were counted by clicking on all DAPI-positive nuclei (Figure 1).

Ki67 expression to determine cell proliferation in transwell invasion assays

In order to determine whether invasion of GSCs is affected by cell proliferation during the transwell invasion assays, transmembranes were stained using the proliferation biomarker Ki67. After fixation of the cells with 4% paraformaldehyde, cell membranes were permeabilized using PBS containing 1% bovine serum albumin and 0.1% Triton-X for 30 min at room temperature. Afterward, transmembranes were incubated with anti-Ki67 antibodies conjugated with fluorescein isothiocyanate (Miltenyi Biotec, CA, USA; catalog number: 130-117-691) in a dilution of 1:50 in PBS at room temperature for 1 h. After one washing step with PBS, transmembranes were stained with DAPI for 5 min, followed by a washing step with 1× PBS for 10 min. Transmembranes were cut out of the inserts and mounted on microscopic slides using Prolong Gold mounting medium for quantification of invaded DAPI-positive and Ki67-positive cells. Images of the transmembranes were taken using a Nikon Eclipse Ti-E inverted microscope and the NIS-Elements AR 4.13.04 software. The total number of cells in ten individual fields per transmembrane was counted using image analysis and Image J software [32,33]. Three experiments were performed, each in triplicate.

Three-dimensional spheroid invasion assay

The method of the spheroid invasion assay is described step by step, to be performed chronologically to reproduce the experiment. NCH421k GSCs were seeded in complete Neurobasal medium containing 4% methylcellulose in U-bottomed 96-well plates with 3000 cells/well (BD Biosciences, CA, USA). The 4% methylcellulose was prepared as follows: 6 g of pure methylcellulose powder (Sigma, catalog # m-0512) was autoclaved and dissolved in 250 ml pre-heated complete Neurobasal medium (60°C) for 20 min using a magnetic stirrer. Then 250 ml complete Neurobasal medium (room temperature) was added, to a final volume of 500 ml, and this solution was mixed for 2 h at 4°C. The final stock solution was aliquoted and cleared by centrifugation (5000×*g*, 2 h, room temperature). Only the clear highly viscous supernatant – approximately 90–95% of the stock solution – was used for the spheroid invasion assay. The cells in U-bottomed 96-well plates were centrifuged at 850 × *g* and 31°C for 90 min and incubated at 37°C in a 5% CO_2 incubator for 72 h to form a single spheroid of similar size in each well. Spheroids were transferred into 24-well plates (Corning) and embedded in 6.0 mg/ml Matrigel (Figure 2). After 30 min of incubation at 37°C in a 5% CO_2 incubator, spheroids were covered with complete Neurobasal medium containing SDF-1α (10 ng/ml). As a control, complete Neurobasal medium without SDF-1α was used to cover the spheroids. Microscopical images were captured after 72 h using a Nikon Eclipse Ti inverted fluorescence microscope and NIS-Elements AR 4.13.04 software. The number of GSCs that invaded from the spheroids into the Matrigel was determined using image analysis and the NIS-Elements software (Figure 2) [32,33]. GSC spheroids with a minimum diameter of 40 μm were considered as spheroids [11]. Three experiments were performed, each in triplicate.

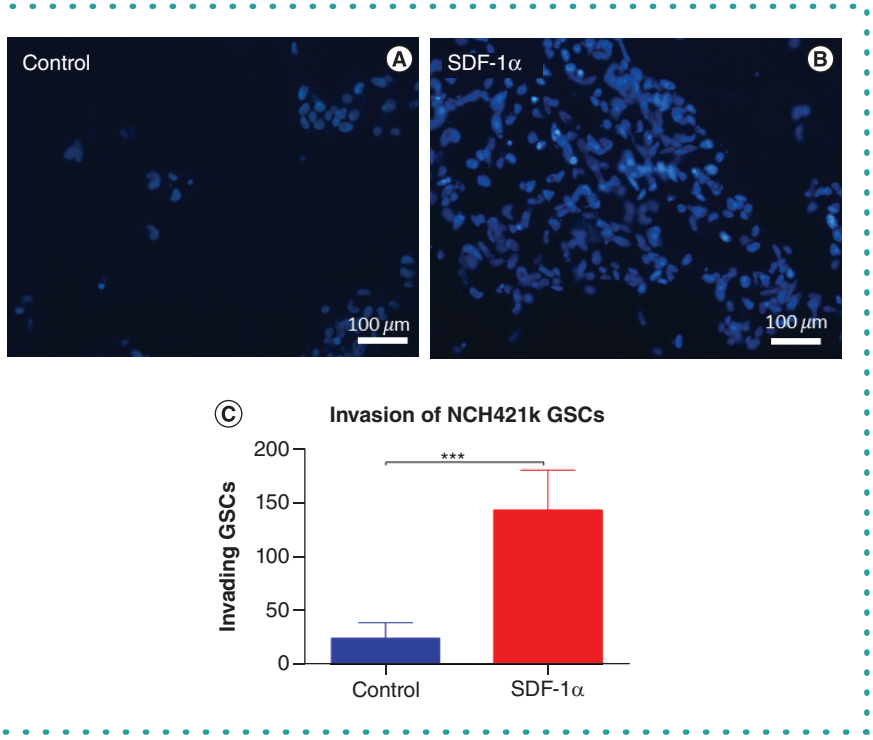

Figure 3. 2D transwell invasion data of CXCR4-positive NCH421k glioblastoma stem cells. GSCs that accumulated on the bottom surface of the insert membranes were imaged at 72 h. DAPI staining was used to show cell nuclei. In the presence of the chemoattractant SDF-1α in bottom wells, a significant increase in the average number of invading GSCs was observed as compared with the control condition without SDF-1α. Bars represent the mean number (± standard error of the mean) of invading NCH421k GSCs after 72 h of incubation in control conditions (empty medium) or in medium containing 10 ng/ml SDF-1α.
Scale bar = 100 μm. ***$p < 0.001$.
DAPI: 4′,6-diamidino-2-phenylindole; GSC: Glioblastoma stem cell.

Statistical analyses

Data was processed in Excel 2013 (Microsoft, WA, USA) and GraphPad Prism 6 (GraphPad, CA, USA) for statistical analyses using one-way analysis of variance; p-values lower than 0.05 were considered to indicate significant differences.

Results & discussion

CXCR4-positive GSCs are attracted to SDF-1α in 2D transwell invasion assays

Transwell invasion assays were performed using CXCR4-positive NCH421k GSCs in Matrigel-containing medium in inserts in the presence or absence of the chemoattractant SDF-1α in the bottom wells. Imaging of DAPI-positive GSCs on the bottom surface of insert membranes was performed at 72 h (Figure 3). Our data demonstrate a significant (sevenfold) increase in the average number of invading GSCs in the presence of SDF-1α (Figure 3B & C) as compared with the control condition, where empty medium was added to the bottom wells (Figure 3A & C). Only cells that had accumulated on the bottom surface of insert membranes were included as no cells were found in the bottom wells.

CXCR4-positive GSCs invade into Matrigel in the presence of SDF-1α in 3D spheroid invasion assays

In the 3D invasion assay, spheroids of CXCR4-positive NCH421k GSCs were embedded in Matrigel and covered with medium with or without SDF-1α (Figure 4). At 72 h, imaging was performed and the number of GSCs that invaded out of the spheroid was quantified. Our data demonstrate that the presence of SDF-1α resulted in a 1.8-fold higher number of GSCs invading from the spheroid into the Matrigel-containing medium (Figure 4B & C), as compared with the control condition (Figure 4A & C).

Proliferation of GSCs is not affected during invasion assays

To determine whether or not proliferation of GSCs is affected during invasion assays, antibodies against the proliferation biomarker Ki67 were used to detect proliferating cells on the membrane of inserts in 2D transwell invasion assays (Figure 5A). Our data demonstrate that proliferation of NCH421K GSCs was not affected; the percentage of Ki67-positive GSCs did not differ between control conditions and the wells where SDF-1α was added (Figure 5A). In the 3D spheroid invasion assays, we assessed that cells that invaded out of spheroids were always single cells and not clusters of cells (Figure 5B), confirming that GSC proliferation did not take place during invasion.

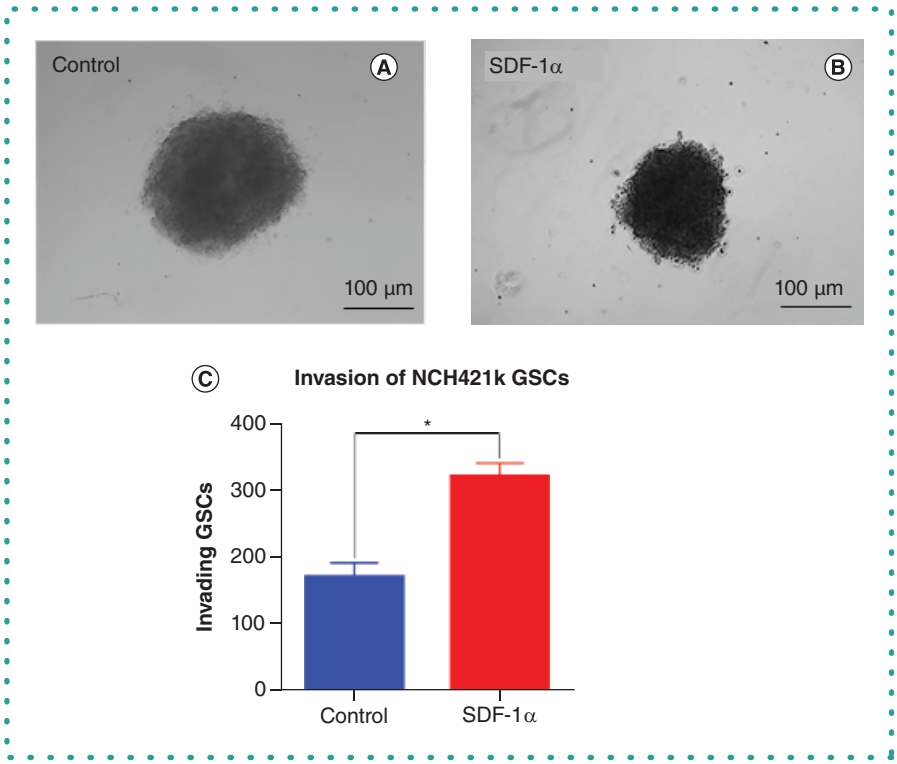

Figure 4. 3D spheroid invasion data of CXCR4-positive NCH421k glioblastoma stem cells. GSC spheroids were embedded in a drop of Matrigel that was covered with empty medium **(A)** or medium containing SDF-1α **(B)**. **(C)** Higher numbers of GSCs invaded from the spheroid in the presence of SDF-1α as compared with the control condition. Bars represent the mean number (± standard error of the mean) of invading NCH421k GSCs after 72 h from the spheroids into the Matrigel in control conditions (empty medium) or in medium containing 10 ng/ml SDF-1α. Scale bar = 100 µm.
*$p < 0.05$.
GSC: Glioblastoma stem cell.

Taken together, our data indicate that SDF-1α attracts CXCR4-positive GSCs in 2D transwell invasion assays and induces invasion of CXCR4-positive GSCs into the Matrigel in 3D spheroid invasion assays. Proliferation is not affected during invasion of GSCs in either type of *in vitro* invasion assay.

In this article, we describe two quantitative assays for the evaluation of GSC invasion *in vitro* – the 2D transwell invasion assay and a 3D spheroid invasion assay – and assess the advantages and disadvantages of each.

Our data demonstrate that both *in vitro* invasion assays are feasible experimental methods to investigate the cellular invasive behavior of cancers with invasive properties, such as glioblastoma. As an example, we demonstrate that CXCR4-positive GSCs are attracted toward SDF-1α in bottom wells in the 2D transwell invasion assays (Figure 3) and in Matrigel-containing medium around spheroids in the 3D spheroid invasion assay (Figure 4). GSCs invaded out of spheroids as single cells (Figures 4 & 5B), as reported in our previous study [12]. We also demonstrate that cell proliferation is not affected in either type of *in vitro* invasion assay (Figure 5). This is in line with studies that reported the 'go or grow' hypothesis, which states that cancer cells either invade or proliferate, because cellular invasion and proliferation do not occur simultaneously [34,35].

The major advantages of the 2D transwell and 3D spheroid invasion assays for the *in vitro* evaluation of GSC invasion are that they are technically easy to perform, relatively cheap and reproducible [3]. They can also be applied to other types of cancer (stem) cells and are now also used for drug testing, to determine whether drugs can inhibit the invasive behavior of cancer (stem) cells and their spheroid formation ability [36–41]. Chemotaxis, such as attraction between CXCR4 and SDF-1α, can be studied in an accurate manner using both assays [12]. In both assays, the presence of SDF-1α clearly results in increased invasive behavior of CXCR4-positive GSCs (Figures 3 & 4). Another advantage of 2D transwell and 3D spheroid invasion assays is that qualitative data are obtained by imaging (differences in numbers of invading GSCs can be clearly observed microscopically) and quantitative data are obtained using image analysis, which is relatively easy to perform and accurate at the same time. Alternatively, a quantitative microscopic analysis has been described for the 3D spheroid assay in which the distance of invading cells from the spheroid or invasive area is determined [15,42].

Both *in vitro* invasion assays can be performed in more complicated experimental settings by using multiple cell types – for example, CXCR4-positive GSCs in the inserts and SDF-1α-producing cells in the bottom wells, such as mesenchymal stem cells (MSCs) that are known to infiltrate glioblastoma tumors and are major SDF-1α producers [24]. Compared with experiments with exogenous SDF-1α

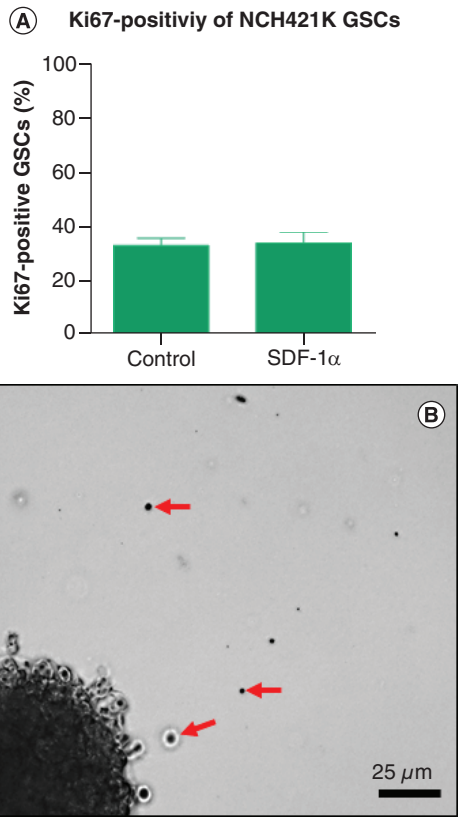

Figure 5. Proliferation of NCH421k glioblastoma stem cells was not affected during invasion assays. In the 2D transwell invasion assay, Ki67 staining was performed on GSCs that accumulated on the bottom surface of insert membranes to detect proliferating GSCs in transwell invasion assays. Imaging was performed after 72 h. **(A)** No differences were found between the percentages of proliferating Ki67-positive GSCs in control conditions and in the presence of SDF-1α in bottom wells. **(B)** NCH421k GSCs that invaded out of the spheroid in 3D spheroid invasion assays were single cells, which confirms that invading GSCs are not proliferating. Bars represent the mean number (± standard error of the mean) of invading NCH421k GSCs in the 2D transwell invasion assay after 72 h of incubation in control conditions (empty medium) or in medium containing 10 ng/ml SDF-1α. Scale bar = 25 μm. GSC: Glioblastoma stem cell.

present in the bottom wells, such experiments are both more biologically relevant (because SDF-1α is produced by MSCs in GSC niches *in vivo*) and cheaper (fewer reagents are needed because MSCs produce SDF-1α). However, it should be noted that MSCs produce a plethora of chemokines, not only SDF-1α [12,43]. Thus, to determine whether the obtained effect was caused by MSC-secreted SDF-1α, control experiments need to be performed using selective CXCR4 inhibitors like plerixafor [44]. In the 3D spheroid invasion assays, spheroid co-cultures of different fluorescently-labeled cell types can also be used to study cancer cell invasion into the 3D matrix [14]. A disadvantage of these two invasion methodologies is that the tumor microenvironment is not accurately reproduced [3,8]. More advanced and expensive *in vitro* 3D invasion methods are now available, such as 3D microfluidic co-culture systems that allow the construction of a 3D tumor microenvironment, real-time cell tracking, evaluation of interactions between different cell types, mimicking of blood flow and studies of phenomena such as cell proliferation and invasion [3,8,11,45–47]. The most true-to-nature systems are the 3D tumor organoids, in which the tumor complexity (i.e., tumor structures including blood vessels, stroma, ECM) is intact and tumor heterogeneity is present. Organoids can be used for drug testing as well for the investigation of phenomena such as cellular invasive behavior [48–55]. In future research, we aim to introduce 3D tumor organoids as a model to study glioblastoma invasion and interactions between glioblastoma cells/GSCs with other cell types in the tumor microenvironment, but both 2D transwell invasion assay and 3D spheroid invasion assay will continue to be used as well because of their own advantages. The 2D transwell invasion assay is a faster method and is less complex than the 3D spheroid invasion assay. However, it is more expensive than the 3D invasion assay because of the costs of inserts. Therefore, we suggest that the 2D transwell assay is applied first to determine whether the hypothesized chemoattractant-receptor interactions are involved in the process of cellular invasion. Afterward, cellular invasion can be studied using the more time-consuming 3D spheroid invasion assays that are more complex and closer to the *in vivo* situation. A limitation of the present study is that both *in vitro* invasion assays have been performed with the use of a single GSC line only. The application of this protocol to additional patient-derived GSC lines in the future may enable the optimization of this methodology for individual cell lines.

Besides the SDF-1α–CXCR4 axis, various other signaling axes are involved in the invasive behavior of GSCs, such as the intracellular PI3K/AKT and Notch signaling pathways [21,22]. In addition, proteases such as matrix metalloproteinases (e.g., MMP-2, MMP-9 and MMP-13), membrane-type matrix metalloproteinases and cathepsins, which degrade the ECM, are also involved in increased cancer cell invasion [5,6,56–58]. Pro-invasion soluble factors secreted from non-cancerous cells such as endothelial cells and immune cells in the tumor microenvironment [25,56,59–61] have also been reported to be involved in the invasive behavior of GSCs.

In conclusion, both 2D transwell invasion assays and 3D spheroid invasion assays are accurate and reproducible *in vitro* methodologies to study GSC invasion and are also applicable to other types of cancer. We conclude that the 2D transwell invasion assay is faster and less complex and is therefore more suitable for the initial experimental determination of specific chemoattractant receptor-mediated cellular invasion, which can then be studied using the more time consuming and complex 3D spheroid invasion assay that is physiologically closer to the *in vivo* situation than the 2D transwell invasion assay.

Future perspective

The described *in vitro* invasion assays are excellent experimental tools for translational research to increase our understanding of glioblastoma biology and to facilitate the development of novel therapies that inhibit invasion of GSCs. The methodological principles are not exclusive for glioblastoma and can also be applied to other types of cancer (stem) cells. In addition, novel therapies designed on the basis of these methodologies can work synergistically with current therapeutic approaches such as chemotherapy, radiotherapy and/or immunotherapy.

Author contributions

Study conception: V Hira, B Breznik, R Molenaar. Experiments: V Hira, B Breznik. Analysis: V Hira, B Breznik. Manuscript drafting: V Hira, B Breznik, R Molenaar. Manuscript editing: all authors. Supervision: C Van Noorden, T Lah, R Molenaar. Funding: T Lah, R Molenaar. Final approval of the manuscript: all authors.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (approval no. 0120-190/2018/4).

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