Novel weapon to conquer human glioblastoma: G-quadruplexes and neuro-inducers

Galina Pavlova (lkorochkin@mail.ru)
Institute of Higher Nervous Activity and Neurophysiology of RAS https://orcid.org/0000-0002-6885-6601

Varvara Kolesnikova
Institute of Higher Nervous Activity and Neurophysiology of RAS https://orcid.org/0000-0002-2637-4524

Nadezhda Samoylenkova
Institution «N. N. Burdenko National Medical Research Center of Neurosurgery» of the Ministry of Health of the Russian Federation

Sergey Drozd
Institution «N. N. Burdenko National Medical Research Center of Neurosurgery» of the Ministry of Health of the Russian Federation

Alexander Revishchin
Institute of Higher Nervous Activity and Neurophysiology of RAS

Dmitry Usachev
Institution «N. N. Burdenko National Medical Research Center of Neurosurgery» of the Ministry of Health of the Russian Federation

Alexey Kopylov
Belozersky Institute of Physical Chemical Biology Moscow Sate University

Article

Keywords: Aptamers, Glioblastoma Recurrence, Tumor Resistance, Stem Cells

Posted Date: January 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-145388/v2

Novel weapon to conquer human glioblastoma: G-quadruplexes and neuro-inducers

Galina Pavlova*, Varvara Kolesnikova*, Nadezhda Samoylenkova, Sergey Drozd, Alexander Revishchin, Dmitry Y. Usachev and Alexey Kopylov

Abstract

Cancer cell reprogramming based on aptamers with antiproliferative properties in combination with small molecules that are used for conversion iPSCs into neurons represents a new approach to reduce the probability of glioblastoma recurrence and tumor resistance to therapy. In this research we tested several combinations of factors on whole cell cultures, derived from tumor tissue after surgical resection, and on cell cultures divided in CD133 enriched and depleted populations, as CD133 marker is believed to be characteristic for glioblastoma stem cells. We showed that $CD133^+$ and $CD133^-$ cells have a different response to tested combinations of factors and CD133-positive cells are more stable and possess stemness properties. Thus, affecting these cells will lead to decrease of therapy resistance. Moreover, we found a combination of factors that is able to inhibit proliferation of both $CD133^+$ and $CD133^-$ cells. Our results reveal a promising strategy to improve treatment of patients with glioblastoma.

Introduction

Glioblastoma is one of the most severe forms of tumor genesis in a human body. Unfortunately, to date there are no effective ways of treatment for this malignant brain tumor. All medical treatments known and available today (chemotherapy, radiotherapy, surgical operations) provide only slight prolongation of a patient's lifespan. Perhaps, the approach to glioblastoma therapy should be reconsidered and changed. A commonly-known fact that glioma has a heterogenic structure with an aberrant regulation of cell proliferation and differentiation should be primarily considered[1]. Such a feature of glioblastoma cell population makes it insensitive to various therapies, and the unaffected tumor recurs. Some cells of the tumor die under the influence of therapeutical factors, however, other cells with a different set of mutations appear to be resistant to these effectors, and their sustained proliferation promotes further tumor growth. Thus, the tumor is capable to actively increase in size and to withstand various types of therapies[2]. It is important to be specified, that the main goal of all approaches to glioma treatment is to make tumor cells die or to induce a toxic effect. Yet, this approach has not helped to cope with the problem. We suggest that other possible variants of glioma treatment should be considered. For example, an attempt to stimulate tumor cells' "maturation" may therefore promote the loss of their proliferative potential. Such an approach is beginning to attract scientists' attention in terms of different cancers therapy, for instance, a similar experience was described for rhabdomyosarcoma, rhabdoid tumors[3] and leukemia[4]. In the current work we attempt to generate a cocktail of a factor, which suppresses tumor proliferation, and neuro-inducers, which, we hope, can stimulate the maturation of tumor cells. While estimating the impact of non-toxic factors, it is extremely important to understand how exactly the proliferation state of tumor cells changes. It is generally accepted, that, according to one of the theories of glioma origin, the tumor derives and eternally extends from tumor stem cells. Several studies demonstrated that extracted and isolated tumor stem cells are capable of a new tumor formation and microenvironment modulation by themselves[5,6]. Hence, the understanding of the properties and characteristics of these cells is important for working out the effective means to influence and affect them. The researchers consistent with this approach concentrate on the biomarkers of cancer stem cells. It is worth to note, that despite the fact that all cancer

stem cells are characterized by various biomarkers[7], the proper identification of these cells remains an unsolved problem. Consequently, it becomes obvious that the investigations of this type of cells are of great importance for target cancer therapy development[8,9]. One of the markers arousing interest is CD133, which is found in glioblastoma (glioma Grade IV) cancer stem cells[10]. This protein has two names, CD133 and prominin-1 (Prom1): it is called CD133 when localized on the cell membrane surface, and Prom1 is used for its intracellular form. The role of CD133 protein in stem cells as well as its correlation with the properties of stem cells are unclear so far, which is striking, as the protein has been studied for quite a long time. It should be noticed, that CD133 is also a surface biomarker of normal brain stem cells[6]. Interestingly, the surface CD133 protein of neural stem cells is different from that of brain cancer stem cells in its structure. Kemper and coauthors demonstrated that the presence of non-glycosylated epitope of CD133, which is located in the second loop of the protein they named AC133, is characteristic of cancer stem cells only[11]. The same authors showed that the presence of the AC133 epitope on the cell surface correlates with clonogenicity[11]. It was also found that $CD133^+$ stem cells, which possess an increased mutability and a predisposition to a higher mutation rate, are the main risk factor for tumor development not only in the brain, but also in many other organs[12]. Several researchers demonstrated that the decrease of $CD133^+$ cells content in a cell culture led to a decrease in this culture's ability of self-renewal and tumor potential[13]. This suggests the necessity of careful investigation of $CD133^+$ glioblastoma cells. In addition, some studies evidence that, while CD133 is a biomarker of glioblastoma cancer stem cells, it functions as a protein associated with the maintenance of stem cells phenotype. For example, it was demonstrated that the tyrosine residue located in the C-terminus of CD133 interacts with the regulatory subunit of phosphoinositide 3-kinases (PI3K) p85 and activates PI3K-AKT signaling pathway in glioblastoma cells[14]. However, the data about CD133 significance are contradictory. For instance, several researchers disprove the correlation between CD133 expression and patients' survival[15]. In another study it was shown that CD133 expressed on the glioma cell surface was poorly immunoreactive for anti-AC133 antibodies, that are used to detect an epitope on CD133[16]. At the same time, these authors demonstrated that the concentration of CD133 in the membranes of glioma cells varies during the cell cycle. Based on this fact, they insist that the interpretation of AC133 epitope presence or absence in glioma cells should be done with caution[16]. Such contradictory results require careful investigation of $CD133^+$ cells and the analysis of their significance for glioblastoma tumor genesis. In the current study we made and attempt to answer two questions: (i) whether the approach to the therapy of glioma can be changed via the application of proliferation-decreasing factors and neuro-inducers; and if it is possible, (ii) how significant the role of $CD133^+$ cells is in providing cell population stability under a similar influence and their ability to preserve the "immature" state, which is based on their proliferation activity.

Results

The search for glioblastoma cell culture characterized by CD133 high expression rate

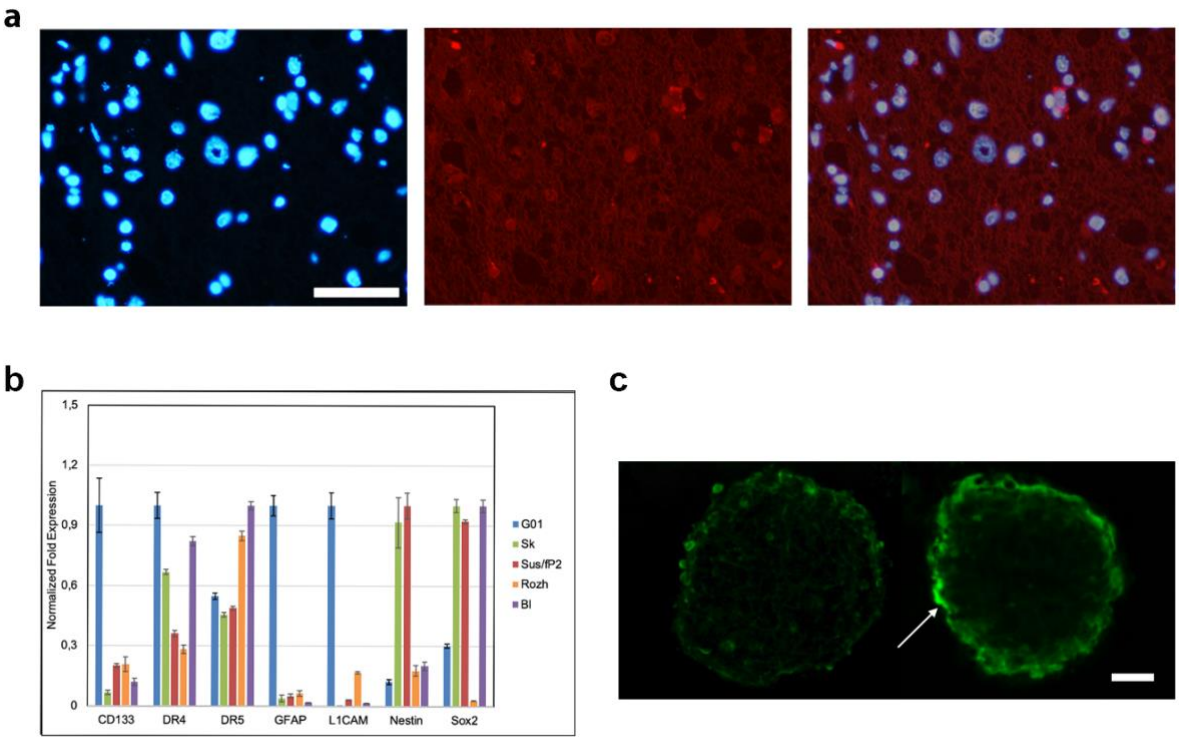
Out of the 20 cell cultures obtained from surgical samples of tumor tissues, for the further investigation we selected a cell culture designated G01, which demonstrated the maximum expression level of CD133. G01 cell culture was derived from glioblastoma cancer tissue (Grade IV) from 37-old female patient with the tumor localized in the left frontal lobe. Figure 1a represents immunohistochemical staining of tumor transections for CD133, which demonstrates the enrichment of the tumor tissue in G01 $CD133^+$ cells.

The cell culture G01 derived from glioblastoma tissue also had an elevated CD133 expression level. Figure 1b represents the comparative analysis of expression of genes known to correlate with the properties of neural stem cells. G01 cell culture possesses several characteristic features attractive for investigation, as this cell culture demonstrated high expression of DR4, GFAP, L1CAM in addition to the stable high CD133 expression.

Despite the fact that GFAP is known to be a biomarker of glial cells, it is also found in neural stem cells[17]. Therefore, the increased expression of both GFAP and CD133, as well as L1CAM, that is known to be characteristic for glioma cells[18], should be considered as a feature of stem cells. An interesting fact to be discussed is the relatively low expression of Nestin, which is believed to be the main biomarker of neural stem cells.

For the analysis of the ability of G01 cell culture to form neuro-spheres, it was cultivated in a serum-free medium. G01 was shown to actively develop into neuro-spheres (Figure 1c). It is possible that self-organizing of cells into a neuro-sphere is an attempt of a heterogenous population to make up a tumor with a structure similar to a natural tumor structure *in vivo*.

Fig. 1: Characterization of glioblastoma cell culture with CD133 high expression rate.



a Immunohistochemical staining with anti-CD133 antibodies (left); bisbenzimide staining of cell nuclei of the patient's biopsy samples (middle); a and b merge (right); scale bar is 50 µm.
b Real-time quantitative PCR. The expression of neural stem cells' genes in tested cell cultures. Data are represented as mean ± SEM. n=3 for each group.
c Immunohistochemical staining of the neuro-spheres with anti-CD133 antibodies. CD133+ cells (arrow) are located in the outer layer of the neuro-spheres; scale bar is 20 µm.

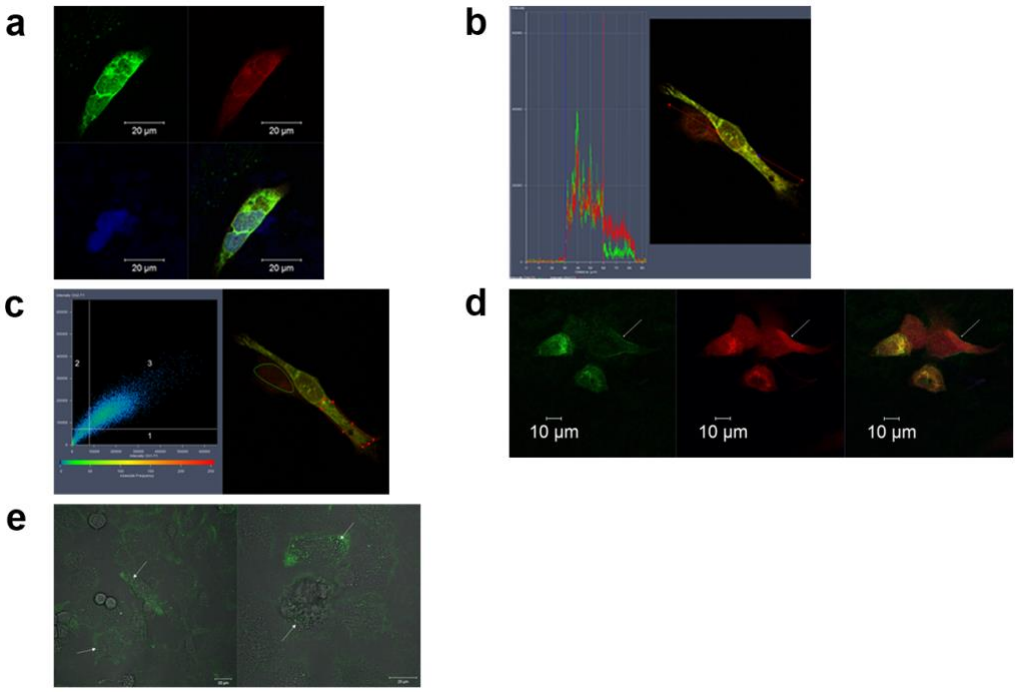
This result demonstrates that CD133+ cells are located in the outer layer of the neurospheres, which is quite similar to the distribution of cancer stem cells in a developed brain tumor[19,20]

The estimation of CD133 distribution in the cell membranes of G01 glioblastoma cells using CD133fr/peGFP-c1 recombinant DNA

The estimation of the presence of cells with combined green fluorescence (GFP) and positive staining for anti-CD133 antibodies resulted in the observation of cells, which were intensively stained with both antibodies (Figure 2a). Interestingly, in these cells a combined fluorescence was observed both in cell cytoplasm and on the cell membrane. A colocalization of green (GFP) and red (anti-CD133 antibodies) fluorescence was proven by using a diagram, which demonstrated the distribution of the signals along the axis (Figure 2b). The graph (left) in the Figure 2b demonstrates the distribution of both proteins along the axis (right). As it can be observed from the Figure 2b, both CD133fr and GFP (red and green) are located together, i.e. glioblastoma cell culture transfection with the CD133fr/peGFP-c1 recombinant DNA succeeded. The same result was shown comparing signals from both proteins (Figure 2c).

Because CD133 is a transmembrane isoform of Prom1, it was necessary to estimate the exact membrane colocalization of CD133fr and GFP (red and green). Figure 2d demonstrates that the CD133fr/peGFP-c1 recombinant DNA provides the visualization of colocalization of these proteins on the cell membrane.

Fig.2: Confocal visualization of CD133 distribution on the cell membrane of G01 glioblastoma cells using CD133fr/peGFP-c1 recombinant DNA.



a Micrographs of fixed glioblastoma cell culture cells stained with anti-CD133 antibodies (dilution 1:25), anti-GFP antibodies (FITC) (dilution 1:200). GFP (green), CD133 (red), DAPI (blue).
b Diagram of signal location along the axis (red) (right) through the areas of CD133fr localization (red) and CD133fr and GFP (red and green) (left).
c Diagram of colocalization of CD133fr and GFP in fixed glioblastoma cells. To the left, signals from GFP and CD133fr are on the axes of both proteins (area 3). To the right, the area outlined in red is the co-location of CD133fr and GFP.

d Micrographs of membrane localization of CD133fr/peGFP-c1 recombinant DNA, the arrows indicate the colocalization of GFP (green), CD133fr (red) and CD133/GFP (yellow) **e** Micrographs of the distribution of microbeads in glioblastoma cell culture transfected with peCD133fr/peGFP-c1. The arrows indicate magnetic beads.

We also examined the localization of microbeads that we used for $CD133^+$ cells isolation and demonstrated that magnetic beads are indeed specific to CD133 localized in the cell membrane(Figure 2e) that is concordant with the data of P. Müller et al.[21].

Therefore, we conclude that the anti-CD133 antibodies effectively bind to this protein located in the cell membranes. We also state that a significant amount of protein, which, nevertheless, does not tend to localize in the cell membrane, is situated in the cell cytoplasm.

G01 CD133- and G01 CD133+ cell cultures' analysis

Using magnetic beads with the immobilized anti-CD133 antibodies (Miltenyi Biotec, Germany) we obtained two cell fractions from G01 glioblastoma cell culture, that we conventionally named G01 $CD133^+$ and G01 $CD133^-$. We underscore that the names are given by conventionality, as there is no unambiguous data so far whether this CD133 can be considered as a true biomarker of stem cells or cancer stem cells. If this is the case indeed, then the percentage of these cells will be constantly decreasing in a cell culture as a result of an asymmetric cell division, and such a cell culture should be considered as «enriched in G01 $CD133^+$ cells». During our investigation we also found that it is impossible to completely deplete the cell culture of G01 $CD133^+$ cells, as there were always some G01 $CD133^+$ cells left in it, hence, the culture resulting from the depletion should be considered as «G01 $CD133^+$ depleted». To simplify the terms, we introduce a standard designation of the cultures as G01 $CD133^+$ and G01 $CD133^-$ correspondingly. G01 $CD133^+$ and G01 $CD133^-$ cell cultures demonstrated different proliferation activity; at the beginning of the experiment G01 $CD133^-$ cells proliferation rate was higher than that of G01 $CD133^+$ both in 10 days and in 20 days following the separation (Figure 3a). Low proliferation activity of G01 $CD133^+$ was observed as expected, as, firstly, the initial number of cells was quite small, and, secondly, if these cells possess the properties of stem cells indeed, the latter are known to lack high proliferation rate characteristic to immature progenitor cells.

As mentioned above, G01 $CD133^-$ cell culture, on the contrary, possessed high proliferation activity. However, it is important to note that both cell cultures equaled in their proliferation rates in 30 days after separation, and by the 70^{th} day G01 $CD133^-$ cell culture stopped proliferating and eventually died, while G01 $CD133^+$ cells continued their growth and were passaged without a decrease in their proliferation activity. Therefore, we suggest that $CD133^-$ cell culture has limited proliferation potential compared to $CD133^+$ cell culture, which preserves stable proliferation rate 30 days after increasing the cell mass in culture. This experiment was repeated three times.

Using RT-qPCR analysis we investigated the changes in the expression of genes associated with the properties of stem cells in glioblastoma G01 $CD133^-$ and G01 $CD133^+$ cell cultures. Gene expression was estimated in 7, 21, 35 and 42 days after cell separation. The initial cell culture G01 (before separation to G01 $CD133^-$ and G01 $CD133^+$) was taken as a reference sample. DR4 and DR5 receptors of TRAIL-dependent apoptotic pathway were analyzed, however, the papers to date discussing whether this pathway is characteristic for stem cells are contradictory. At the same time, there are studies evidencing that DR5 also induces anti-apoptotic pathway and is characteristic for a number of tumor cells, possibly, stem cells, thus targeting this pathway may provide a

new approach for cancer therapy[22]. Another gene in our panel was GFAP, which is a generally accepted biomarker of stem cells and a marker of mature glial cells at the same time. Interestingly, this gene is actively expressed in neural stem cells, both cancer and normal, while in daughter progenitor cells its expression disappears completely. Further expression of GFAP reappears in mature glial cells[17]. Hence, this marker should be estimated in a combination with others. Other genes in our panel, known to be associated with stem cells and their properties, included Nanog, Oct4, Sox2[17,23]. In addition, we included Notch2, as signal transduction via Notch-receptor family proteins is frequently reported in gliomas, and the increased signaling via Notch correlates with poorer prognosis. Several studies point to an important role of Notch2 in carcinogenesis and the development of the most malignant gliomas[24]. Recently, there appeared a great number of studies showing the significance of Notch2 as a marker of neural stem cells[25]. Further, we included L1CAM in the gene expression analysis, as it is also typical for neural stem cells and glioma stem cells[26]. Finally, we chose, as it may be expected, Nestin, which is one of the main biomarkers of neural stem cells[27].

During the analysis of the expression of the panel of genes described above in $CD133^-$ cell culture, it was shown that the expression of stem cell markers was either decreased (CD133, GFAP, Nanog, Oct4, Sox2, L1CAM, Notch2, Nestin) or did not change significantly (DR4 and DR5) (Figure 3b).

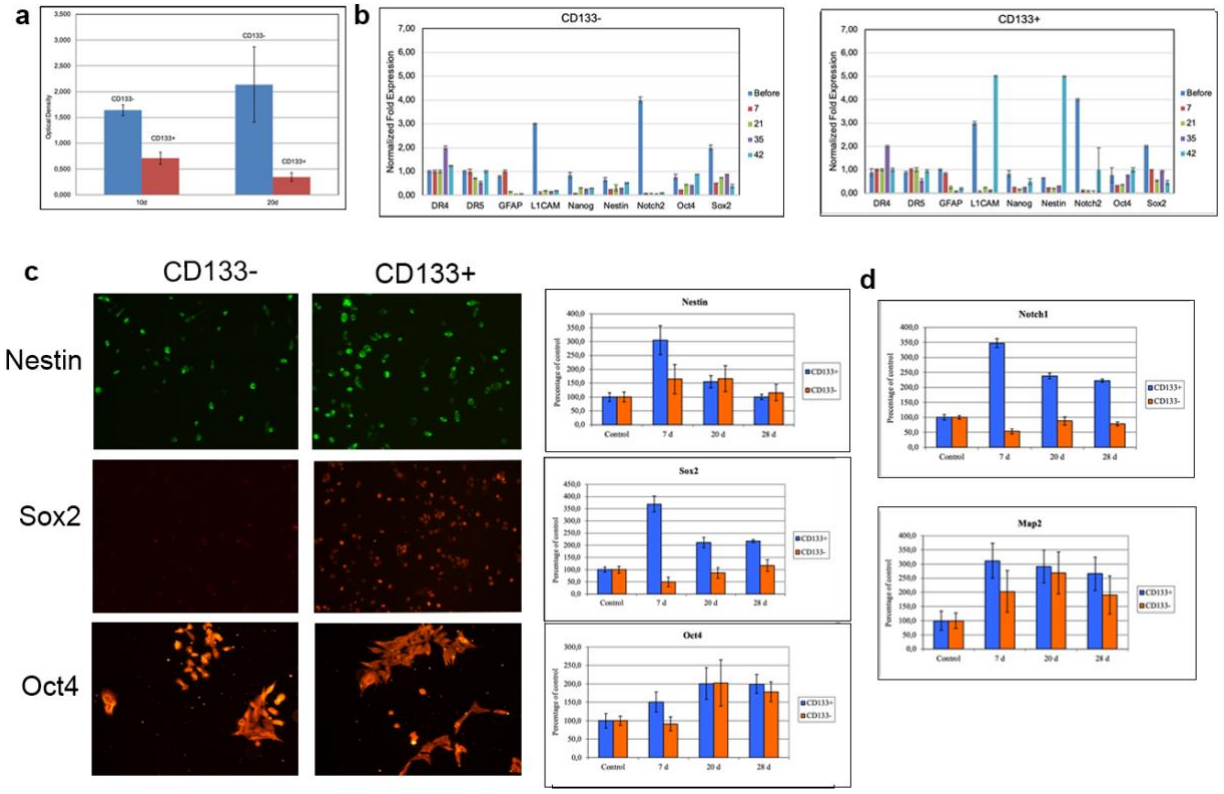
We suggested that it would be interesting to estimate the expression of the same biomarkers in $CD133^+$ cell culture and to compare it to $CD133^-$ data. We noticed that the separation of the cell culture into $CD133^+$ and $CD133^-$ populations did not significantly affect the expression of DR4 and DR5. We also did not see a significant difference in the expression of GFAP, Nanog, Oct4, and Sox2. Notch2 demonstrated a slightly different expression profile in $CD133^+$ cells: its expression started increasing by the 42 day of culture, which was not observed in $CD133^-$ cells. $CD133^+$ and $CD133^-$ cell cultures differed significantly in the expression of L1CAM and Nestin (Figure 3b). After separation of the cultures, L1CAM expression decreased severely, but by the 42 day of culture it was restored, moreover, it became significantly more intense in G01 $CD133^+$ cell culture. The analysis of the expression of Nestin, one of the main markers of neural stem cells, also demonstrated intriguing results: it increased after separation of the cultures and was gradually rising as the culture proceeded

Thus, in $CD133^+$ cell culture we observe an obvious and expected increase in the expression of Nestin, the stem cell biomarker, and L1CAM, which also can be considered as a marker of glioblastoma stem cells. The difference between the initial G01 cell culture and G01 $CD133^+$ is evidenced by Nestin expression, while the change of other markers of stem cells was insignificant. It is possible that there exist at least two populations of stem cells characterized by different patterns of the expression of the considered biomarkers.

Immunohistochemical analysis of the corresponding proteins in glioblastoma cell cultures G01 $CD133^-$ and G01 $CD133^+$ demonstrated that the cultures did not differ in the expression of Nestin and Oct4. At the same time, the content of Sox2 protein was higher in $CD133^+$ cells (Figure 3c). We also observed a significant intensifying of the staining for Notch2 protein, as well as the increasing of Notch2 mRNA expression, in $CD133^+$ cell culture compared to $CD133^-$ cells.

Moreover, immunohistochemical analysis of Notch2 protein in G01 $CD133^+$ and G01 $CD133^-$ cell cultures showed the prevalence of this protein in G01 $CD133^+$ cells starting from the 7^{th} day after separation, although there was no significant difference for Map2 content in the cultures from the 20^{th} day after separation (Figure 3d).

Fig. 3: Characterization of G01 CD133$^+$ and G01 CD133$^-$ cell cultures after cell separation.



a MTT assay of G01 CD133$^+$ and G01 CD133$^-$ glioblastoma cell cultures in 10 and 20 days after cell separation; Data are represented as mean ± SD. n=5 for each group.
b Real-time quantitative PCR of stem cell genes in G01 CD133$^-$ (left) and G01 CD133$^+$ (right) cells before and in 7, 21, 35, 42 days after cell separation. Data are represented as mean ± SEM. n=3 for each group.
c Micrographs of immunohistochemical staining of G01 CD133$^+$ and G01 CD133$^-$ cell cultures with anti-Nestin, anti-Sox2, anti-Oct4 antibodies. To the right: the diagrams representing the percentage of the marker-positive cells in the tested cell cultures. Data are represented as mean ± SD.
d Diagrams representing the content of Notch1 and Map2 positive cells in G01 CD133$^+$ and G01 CD133$^-$ cell cultures based on the data of immunohistochemical staining with anti-Notch1 and anti-Map2 antibodies. Data are represented as mean ± SD.

During the analysis of CD133 in the cell cultures under investigation we noticed that the expression of CD133 was enough for detection, still low in G01 CD133$^-$ cell culture. This fact suggested the need to determine Prom1 distribution inside the cell and its potential abundance in the cell membrane, as it is observed for CD133.

G01 CD133$^-$ and G01 CD133$^+$ exposure to an aptamer and a single neuro-inducing factor

With the analysis of molecular properties of the glioblastoma G01 CD133$^-$ and G01 CD133$^+$ cell cultures derived from G01 cell culture completed, from the mixture of previously tested DNA aptamers we selected two DNA aptamers demonstrated the most effective suppressing of glioblastoma cell cultures proliferation. It was shown that these aptamers decreased cell proliferation rates in a dose-dependent manner (Figure S1a). One of them is designated biHD1 and reported by a group of scientists from MSU, and

another one, currently being developed, is called bi-(AID-1-T). Both DNA aptamers were kindly given to us by the group of professor A. Kopylov. The aptamers arouse interest as they possess several advantages, namely, they are able to reduce proliferation of cancer cells, they are non-toxic and transiently acting, and they have a cytostatic effect.

10 days after the exposures to the aptamers in combination with a single neuro-inducer no significant effect on G01 $CD133^+$ cell culture proliferation activity was observed. The proliferation rates of cells treated with biHD1, biHD1+SB, biHD1+BDNF, biHD1+PRM, bi-(AID-1-T), bi-(AID-1-T)+SB, bi-(AID-1-T)+BDNF, bi-(AID-1-T)+PRM were similar to that of the control (non-treated) cell culture. At the same time, glioblastoma G01 $CD133^-$ cell culture demonstrated higher sensitivity in response to the exposures (Figure 4a). The aptamer bi-(AID-1-T) used alone and the combination of the bi-(AID-1-T) and BDNF were shown to significantly suppress cells proliferation. Interestingly, the cells did not die under the exposures, but stopped dividing, and the culture growth ceased. The different effects of the exposures on the two cell cultures were as expected, as $CD133^+$ cell culture was believed to contain more stem cells, so that the response to the treatments, we thought, would be insignificant. However, we were inspired by the fact that the certain combinations of factors were able to substantially alter the state of tumor cells, at least, $CD133^-$ cells.

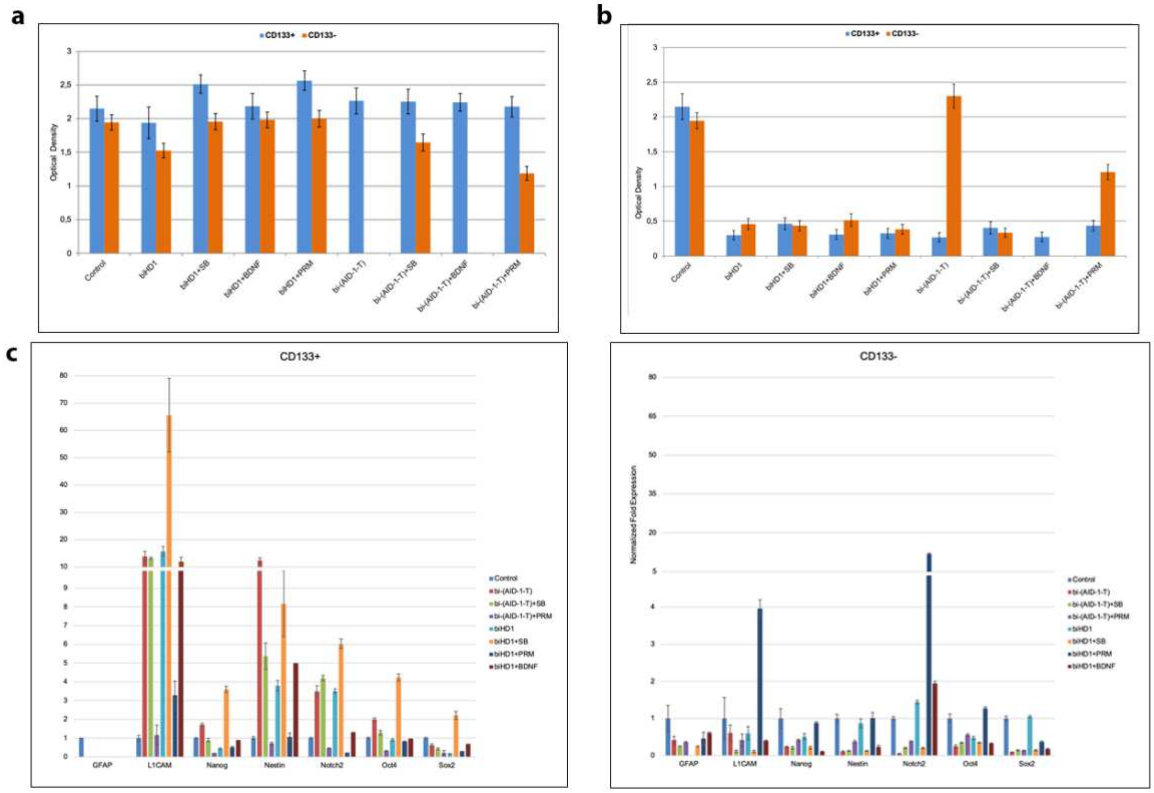
The effect of the exposures changed dramatically after 20 days of culture. We observed significant changes in the proliferation rates of the G01 $CD133^+$ cell culture treated with biHD1, biHD1+SB, biHD1+BDNF, biHD1+PRM, bi-(AID-1-T), bi-(AID-1-T)+SB, bi-(AID-1-T)+BDNF, bi-(AID-1-T)+PRM, and all of the options reduced the ability of the cells to divide compared to the non-treated cells. This means that $CD133^+$ cells demonstrated sensitivity to the exposures only 20 days after the addition of the factors to the medium, i.e. the effect was deferred in time. As for the analysis of G01 $CD133^-$ cell culture, several changes in the effects of the exposures were also detected 20 days after the initial treatment (Figure 4b). It should be noted that by the 20^{th} day G01 $CD133^-$ cell culture exposed to the combination of bi-(AID-1-T)+BDNF died completely and was not analyzed. At the same time, G01 $CD133^-$ cell culture exposed to the aptamer bi-(AID-1-T) alone, which had demonstrated a significant decrease of the growth rates before, restored its ability to divide (apparently, when the aptamer's effect ceased), and thus, it was included in the test on the 20^{th} day along with the others. Since the significant recovery by the 20^{th} day for the G01 $CD133^-$ cells treated with bi-(AID-1-T) alone was demonstrated, the importance of using the aptamer in a combination with a neuro-inducer became obvious. G01 $CD133^-$ cell culture proliferation rates were significantly reduced in 20 days after the exposures to the biHD1, biHD1+SB, biHD1+BDNF, biHD1+PRM and bi-(AID-1-T)+SB, while bi-(AID-1-T)+PRM did not affect much the cell growth and division.

In summary, the experiment of the treatments of the cell cultures with different combinations of the DNA aptamers and neural differentiation inducing molecules demonstrated that the most interesting combination for further investigations was the bi-(AID-1-T)+BDNF.

The analysis of the expression of stem cells associated genes conducted in 20 days after the exposure to the factors showed that the surviving cells of the G01 $CD133^+$ cell culture were characterized with the increased stem cells properties (increased expression of L1CAM, Nestin, Notch2 genes). In other words, those cells, which possessed the properties of stem cells, were able to survive. Therefore, we should search for the combinations of factors targeting the expression of stem cells biomarkers (Figure 4c). In addition to bi-(AID-1-T)+BDNF, a combination of bi-(AID-1-T)+PRM and biHD1+PRM might be used for this purpose.

During the analysis of G01 CD133$^-$ cell culture we observed rather low expression rates of the genes associated with stem cells, with the increase in L1CAM and Notch2 expression after the exposure to biHD1+PRM being the only exception (Figure 4c).

Fig. 4: G01 CD133$^-$ and G01 CD133$^+$ exposure to an aptamer and a single neuro-inducing factor.



a MTT assay of G01 CD133$^+$ cells and G01 CD133$^-$ cells after the exposure to aptamers biHD1 and bi-(AID-1-T) and neural differentiation inducers SB431542, purmorphamine, BDNF in 10 days after the exposure. Data are represented as mean ± SD. n=5 for each group.
b MTT assay of G01 CD133$^+$ cells (left) and G01 CD133$^-$ cells (right) after the exposure to aptamers biHD1 and bi-(AID-1-T) and neural differentiation inducers SB431542, purmorphamine, BDNF in 20 days after the exposure. Data are represented as mean ± SD. n=5 for each group.
c Real-time quantitative PCR of stem cell genes in G01 CD133$^+$ (left) and G01 CD133$^-$ (right) cell cultures in 20 days after the exposure to the aptamers biHD1 and bi-(AID-1-T) and neural differentiation inducers SB431542, purmorphamine, BDNF. Data are represented as mean ± SD. n=3 for each group.
SB - SB431542, PRM - purmorphamine

In review, our results suggest that it is possible to make up a combination of factors able to reduce proliferation activity of glioblastoma tumor cells and even to make them switch from their immature state, which is in charge with the active cell division. To date, the most promising combinations, yet needed further investigations, are bi-(AID-1-T)+BDNF and bi-(AID-1-T)+PRM.

G01 CD133$^-$ and G01 CD133$^+$ cell cultures exposure to the bi-(AID-1-T) aptamer and a cascade of neural inducers commonly used for iPSCs

The results obtained inspired us to further test the combinations of anti-proliferative and neuro-inducing factors, which are capable of stable negative affecting glioblastoma cells ability to divide. However, we failed to get any worthy results in regard to CD133-positive cells. Obviously, the application of the BDNF neuro-inducer alone was enough to block cell division in the more mature CD133-negative cell culture, while for G01 $CD133^+$ cells it appeared to be inappropriate. That is why next we tested a combination of the bi-(AID-1-T) DNA aptamer and the whole set of molecules used for neural differentiation of induced pluripotent stem cells (iPSCs). Therefore, we first applied bi-(AID-1-T) for primary reducing cell proliferation, and then SB431542, LDN-193189, Purmorphamine and finally BDNF (N cocktail) were added one by one with one-day interval between each of the treatments. This experiment included a wide panel of controls: a standard control of non-treated cells, and intermediate controls with the addition of either the aptamer alone or the aptamer in combination with one the factors from the cocktail. Table 1 represents the scheme of the treatments.

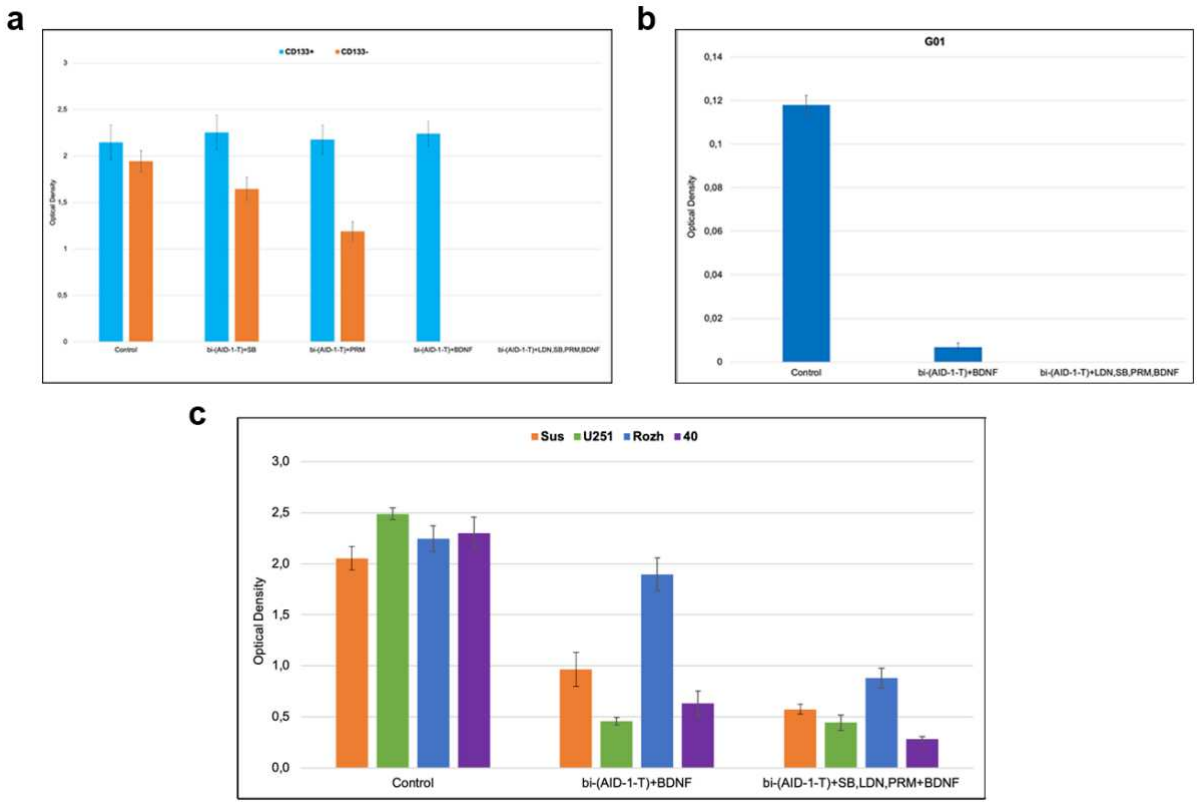
The effectiveness of the treatments was analyzed 10 days after the addition of the final factor (i.e. BDNF) via the MTT assay estimating cells proliferation activity. During the investigation of the effects of the N cocktail on the G01 $CD133^-$ cell culture we observed a complete stopping of cells proliferation. The same effect was observed in bi-(AID-1-T)+BDNF treated cells, which is in concordance with the results of the previous experiment (Figure 5a). An inhibiting effect on the cell growth and division was also demonstrated for the combinations of bi-(AID-1-T)+SB, and bi-(AID-1-T)+BDNF, but these treatments only slightly reduced cell proliferation.

When we analyzed the effects of the treatments on G01 $CD133^+$ cell culture, it occurred that only N cocktail blocked cell proliferation (Figure 5a), while the rest of the combinations did not alter the culture's proliferation potential. Thus, we demonstrated that the N cocktail (bi-(AID-1-T)+SB431542, LDN-193189, Purmorphamine, BDNF) can significantly reduce the ability to proliferate of both G01 $CD133^-$ and G01 $CD133^+$ cell cultures.

Next, we repeated the experiment with the initial G01 cell culture, in which we compared the treatment of cells with the N cocktail and the combination of bi-(AID-1-T)+BDNF Figure 5b). The results of the treatments of the initial G01 cell culture demonstrated that bi-(AID-1-T) in combination with BDNF significantly reduced glioblastoma cell proliferation, however, did not completely suppress the tumor growth, while the N cocktail entirely blocked the ability of the cells to divide.

We also tested N cocktail on whole cell cultures including cell line U251 (Figure 5c) and showed that all cultures response to such treatment by decreasing their proliferation.

Fig. 5: Cell cultures' exposure to the bi-(AID-1-T) aptamer and a cascade of neural inducers.



a MTT assay for G01 $CD133^+$ and G01 $CD133^-$ in 10 days after the exposure to the bi-(AID-1-T) aptamer and the neural differentiation inducers. Data are represented as mean ± SD. n=5 for each group.
b MTT assay for G01 cells in 10 days after the exposure to the bi-(AID-1-T) aptamer and the successive addition of the neural differentiation inducers; Data are represented as mean ± SD. n=5 for each group.
c MTT assay for Sus, U251, Rozh and 40 cell cultures in 10 days after the exposure to the bi-(AID-1-T) aptamer and the neural differentiation inducers. Data are represented as mean ± SD. n=5 for each group.
SB – SB431542, PRM – Purmorphamine, LDN – LDN-193189.

Discussion

Modern approaches to the treatment of malignant tumors have different success rates. At the same time, in this class of diseases there are tumors that are still practically not amenable to therapy. Glioma, and in particular, glioblastoma, its most severe form (Grade IV), is among these tumors. The types of therapy used today are limited to surgical removal of the tumor, exposure to radiotherapy, or chemotherapy, which is mainly based on toxic effects on cells. The cytotoxic effect of drugs is aimed at bringing tumor cells to death in one way or another. On the one hand, chemotherapy drugs, such as Temodal, kill proliferating tumor cells, but on the other hand, these medications also harm healthy proliferating cells of the body (cells of the intestinal lining, red bone marrow cells, epithelial cells etc). One of the disadvantages in the application of cytotoxic drugs is that they suppress the growth of actively dividing cells, which are thus more sensitive to such exposures, and which are, in fact, immature daughter cells of a tumor [28]. At the same time, cancer stem cells with relatively low proliferating potential, however, rather stable, appear to be insensitive to chemotherapy. We suggest that the approach to the regulation of tumor cells proliferating potential can be possibly changed to the applying of factors,

which change cells differentiation status instead of using of death-inducing drugs. This approach seems promising, in particular, when taking into account the fact that every heterogeneous tumor population harbors cells which form the basis of glioma proliferating potential. In this regard, a tumor can be considered as an aggregate of “daughter” tumor cells possessing a high, still limited ability to proliferate, and cancer stem cells, which give rise to the whole population and maintain its growth. Several studies show that these are the cancer stem cells which have an increased resistance to any therapeutic effects. Therefore, chemotherapy and radiotherapy will primarily impact the tumor “daughter” cells, while cancer stem cells will survive and give rise to a new population[29], restoring it the way a queen bee restores a swarm. For instance, Dawson and coauthors noticed that rhabdomyosarcoma and rhabdoid tumors harbor cells, which remain unaffected by chemotherapy, and which possess the properties of stem cells. The authors suggested a combined treatment of these cells, that made them sensitive to therapy[3]. Therefore, the idea of toxic influence on glioma cells should be reconsidered, and new approaches of tumor cells differentiation stimulation, thus blocking their proliferation potential, should be developed. Today such approaches are already being examined in regard with different cancers. For example, Xiong and co-authors reviewed tumor models as aggregates of cancer stem cell and cancer progenitor cells, as well as the possibilities of managing their differentiation potential[30]. Han and colleagues reported a similar approach to leukemia treatment, in which the aggression of cancer cells was reduces due to the use of combined therapy aimed at stimulating tumor cells differentiation[4].

The aim of the current study was to search for the combination of factors able to stimulate neural differentiation of human glioblastoma cells. We were aware that there were cancer stem cells in the heterogeneous population of glioma cells, and that these cells were more immature then other cells of the tumor. It is generally accepted that these cells can be characterized as CD133-positive cells. For example, Mendiburu-Eliçabe et al. demonstrated that rapamycin significantly decreased the rate of cell growth, which correlated with the decrease in the expression of CD133 biomarker of stem cells in two cell cultures derived from GBM patients[31]. Moreover, Sunayama et al. showed that the combination of rapamycin and LY294002, an inhibitor of PI3K, suppressed the properties of stem cells while increasing the expression of βIII-tubulin neural marker, which suggests the differentiation effect of this double inhibition of PI3K/mTOR pathway[32]. However, this biomarker of stem cells and their properties is still ambiguous. Therefore, it was important to estimate to what extent $CD133^-$ and $CD133^+$ human glioblastoma cells proliferation could be restrained using the neuro-inducers. In this study we investigated G01 cell culture derived from human glioblastoma, which we separated into two cell cultures based on the CD133 content, namely, cell population enriched in CD133 and CD133 depleted cell population. The aim was to select a factor able to decrease or stop cell proliferation for a short period of time in order to immediately stimulate tumor cell differentiation. At the same time, it was important that the factor of choice would not be toxic for cells but only restrain their ability to divide. To reach this goal we took the aptamer bi-(AID-1-T), which was developed by professor A. Kopylov and his colleagues and showed a good anti-proliferation potential. We demonstrated that G01 $CD133^-$ stopped proliferating 10 days after exposure to bi-(AID-1-T), and that without the following treatment with neuro-inducers, the suppressing effect discontinued by the 20^{th} day and the cell culture growth recovered. As for G01 $CD133^+$ cell culture, the aptamer did not alter the proliferation properties of the cells. At first, we did not consider the application of factors inducing neural differentiation of iPSC after proliferation suppression. However, preliminary experiments with different substances regarded as strong neuro-inducers – GDNF, IL6, RA (Figure S1b; Figure S1c) – did not produce a desired effect[33]. Then there appeared an idea to try molecules applied in iPSC neural differentiation[34]. First, we used SB431542, Purmorphamine and BDNF one at a time after ceasing cell proliferation with the help of

the aptamer. It was demonstrated that the application of single BDNF neuro-inducer (after the exposure to bi-(AID-1-T)) was enough to block cell divisions in $CD133^-$ population, but did not work in $CD133^+$ cells. Obviously, $CD133^+$ are immature compared to $CD133^-$, and this fact is in accordance with the assumption that CD133 is characteristic to stem cells or, at least, to some type of stem cells. BDNF is used for neural differentiation as a neurogenesis-encouraging factor, and it appeared to be applicable for $CD133^-$ cells of glioblastoma. Therefore, next, we used a step-by-step method of G01 $CD133^+$ and G01 $CD133^-$ cell cultures exposure to neuro-inducers according to the protocol described by Wichterle[35] and modified by Lagarkova M.[34].

It was clear that for an effective influence on G01 $CD133^+$ a short-term blocking of cell proliferation was needed with subsequent exposure to neuro-inducers in order to manage the earliest stages of cell “maturation”. Small molecule SB431542 taken for investigation inhibits Lefty/Activin/TGFβ signaling pathways via blocking the phosphorylation of the ALK4, ALK5, and ALK7 receptors[36]. SB431542 mechanism of action is based on the destabilization of the signaling network, which supports cells pluripotency and restricts differentiation. Purmorfamin is another small molecule affecting midbrain cells ventralization[37]. LDN-193189, an inhibitor of BMP signaling pathway, was also chosen for investigation[38]. Finally, the application of BDNF, a neurotrophic factor known to stimulate neurons maturation, was a culmination of the experiment. It appeared that the use of this exact combination of factors was enough to block G01 CD133+ cell proliferation. It is important to note that 10 days following the exposure the cells stopped dividing and the culture further died. Therefore, we conclude that for G01 $CD133^+$ cells a step-by-step treatment is required starting from the earliest differentiation stages. It is possible that so-called cancer stem cells possess a differentiation level comparable to that of embryonic stem cells or induced stem cells, and that is why a step-by-step influence is necessary.

In summary, we propose an alternative approach to glioma therapy, which will be able to change the tumor state of cells via stimulation of their maturation. An important point of the study is that we found at least one combination of factors able both to alter (stop) the proliferation of glioblastoma cells and to block the division of $CD133^+$ cancer stem cells.

Methods

Primary cultures of glioblastoma cells

Cells were gained from human glioblastoma tissues after tumor resection. Cell culture G01 was chosen for this research as it showed high expression of CD133 cell surface marker. This culture was obtained by washing cells in Versen solution (Paneco), incubation with 0,25 % trypsin-EDTA (Ethylenediaminetetraacetic acid) solution (Gibco) at 37 °C for 40 min and disaggregation from tissue followed by centrifugation at 1000 rpm for 5 min. Cells were cultivated in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) medium (Gibco) containing 1% L-glutamine (Paneco) and 10% FBS (Fetal Bovine Serum) (Thermo Scientific) and 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma-Aldrich) at 37°C with a humidified atmosphere of 5% CO_2. Passaging was performed at 80% confluency. For passaging cells were washed in PBS (Phosphate Buffered Saline) (1x) (Gibco) and incubated with 0,25 % trypsin-EDTA solution. After trypsin inactivation by fresh medium cells were centrifuged at 1000 rpm for 5 minutes, the supernatant was removed and fresh medium was added, cells were resuspended and plated into cultural flasks. Cell count was defined using trypan blue 1:1.

Cell separation

A separation was performed following a CD133 MicroBead Kit-Tumor tissue protocol. Before the separation cells were passed through 40 µm filters and centrifuged at 1000 rpm for 5 minutes. On the first step the cells were labeled with CD133 Microbeads (Miltenyi Biotec). Cells were centrifuged at 1000 rpm for 10 minutes then the supernatant was removed. The cell pellet was resuspended in 60 µl of buffer (PBS with 2 мМ EDTA and 0,5% BSA (Bovine Serum Albumin) (Amresco)). 20 µl of FcR Blocking Reagent was added to the suspension. After resuspending 20 µl of CD133 MicroBeads was added and resuspended. The mixture was placed at 4°C for 30 minutes. 2 ml of the same buffer was added to the cell suspension, resuspended and centrifuged at 1000 rpm for 10 minutes. The supernatant was completely removed and 500 µl of fresh buffer was added and resuspended.

For magnetic separation MS Columns (Miltenyi Biotec) were used. After placing a column in the magnetic field of a MACS Separator (Miltenyi Biotec) and washing the column with 1 ml of buffer cell suspension was applied onto the column. The unlabeled cells that passed though the column were gained in the first collecting tube. The column was washed 3 more times in the second collecting tube to get rid of unlabeled cells. The fractions of unlabeled cells were combined in one collection tube. In a different collection tube magnetically labeled cells were eluted 3 times by pipetting 1 ml of buffer and firmly pushing the plunger into the column.

It should be mentioned that as the CD133 marker is believed to represent stem cells that divide asymmetrically there will always be a small amount of $CD133^-$ cells in $CD133^+$ cultures as a result of such division.

Cell cultivation with aptamers and factors of neurodifferentiation

We selected several factors, which are used to stimulate neural differentiation of iPSCs, as neuro-inducers. On the first stage of the experiment we used small molecules SB41542 and purmorphamine (PRM) (Miltenyi Biotec) and a neurotropic factor BDNF (Miltenyi Biotec).

SB431542 (10 µM) (Miltenyi Biotec), known to destabilize signaling pathways, which maintains cells pluripotency. Another molecule used in our experiment is PRM that is commonly used as an analogue of recombinant Sonic hedgehog protein (SHH). The latter is known to play an important role in the nervous system development. SHH in the concentration of 200 ng/ml is capable to induce the development of lateral ganglionic eminence (LGE), and recent studies demonstrate that PRM concentrations of 0,65 µM have the same effect. Therefore, the two molecules (SB431542 and PRM), when applied to neural differentiation of iPSCs, influence different stages of neural "maturation" of progenitor cells. The third factor taken for investigation was the neurotropic factor BDNF (10 ng/ml), which is known as a neural inducer of the final stage of neural cells "maturation".

On the second stage of the experiment LDN-193189 (Miltenyi Biotec), a selective inhibitor of BMP (Bone Morphogenetic Proteins) signaling, that is used for neural induction together with SB431542, was added to the medium. It is shown that BMP4 expression is elevated during oncogenesis that leads to signal transduction by TGF-β/SMAD and canonical Wnt/β-catenin signal pathways resulting in proliferation and differentiation of glioma stem cells[39]. So, its blockage is essential for changing the fate of tumor stem cells.

Two aptamers named biHD1 and biGT3 were used in the experiments. They were added to the medium to a final concentration of 37,5 µM. Before adding to the cell cultures aptamers were preformed at 95°C and then cooled at 4°C overnight. On the first step G01-derived $CD133^-$ and $CD133^+$ cells were treated with DNA aptamers, in two days one of the three neuro-inducers was added to the medium. When combinations of aptamers and small molecules were tested on G01 $CD133^-$ and G01 $CD133^+$ cells neuronal inducers were consistently added to the growth medium in several combinations (see Table 1) by following scheme: in 2 days after adding of aptamer SB431542 or Purmorphamine or BDNF or combination of SB431542+LDN-193189 were added to the medium, on the 5^{th} day after partial changing of growth medium in all flasks Purmorphamine was added to the flasks with SB431542+LDN-193189, on the 7^{th} day BDNF was also added to the flasks with SB431542+LDN-193189+Purmorphamine. In the control flasks no additional factors were added. Proliferation activity of cells was tested using MTT assay 10 days after all of the exposures. Proliferation activity as well as gene expression (using RT-PCR method) were also analyzed 20 days after all of the exposures.

After testing all mentioned combinations on G01 $CD133^+$ and G01 CD 133^- cultures the most effective ones were tested on G01 culture according to the same scheme.

Cultivation of neuro-spheres

Cells were seeded in 12-well plates in serum-free culture medium containing DMEM/F12 medium, 1% glutamine, Antibiotic-Antimycotic (Gibco) and supplements: B27 and N2 (2 ml per 100 ml of medium) (Gibco), FGF and EGF (20 ng/ml) (PeproTech).

Immunocytochemistry

Cells in concentration $1*10^4$ cells / well were cultivated in a duplicate in 4-well plates for 24 hours in 500 µl of DMEM/F12 medium with 1% glutamine and 10% FBS. After washing twice in PBS (pH 7,3) cells were fixed in 500 µl of 4% paraformaldehyde solution for 30 min at 4°C. Then cells were washed twice in PBS (pH 7,3).

The staining was performed using the following primary antibodies: rabbit polyclonal Anti-CD133 antibody (dilution 1:20, #Ab16518, Abcam), goat polyclonal Anti-CXCR4 antibody (dilution 1:100, #Ab1670, Abcam), rabbit polyclonal Oct4 antibody (dilution 1:100, #Ab19857, Abcam), rabbit polyclonal Nestin antibody (dilution 1:200, #AB5922, Chemicon), goat polyclonal Sox2 antibody (dilution 1:100, #sc-17320, Santa Cruz), mouse monoclonal Notch1 antibody (dilution 1:200, #MA1-91405, Invitrogen), and chicken polyclonal Map2 antibody (dilution 1:500, #Ab5392, Abcam). The primary antibodies were dissolved in PBS with 0,3% Triton X100 (Sigma-Aldrich) as a detergent and 2% donkey serum (Jackson Immunoresearch) and incubated for 2 hours at room temperature. As a negative control the solution with 1% FBS and 2% donkey serum was used.

After washing three times for 5 minutes in PBS (pH 7,3) cells were incubated for 1 hour with secondary antibodies: donkey anti-rabbit antibodies conjugated with DyLight-488 (dilution 1:100, #711-545-152, Jackson Immunoresearch), donkey anti-goat antibodies conjugated with Alexa Fluor 594 (dilution 1:100, #705-585-147, Jackson Immunoresearch), goat anti-chicken IgY H&L (Alexa Fluor 488) (dilution 1:100, #Ab150173, Abcam). Then cells were washed in PBS (pH 7,3) and stained in bisbenzimide (Sigma-Aldrich) for 5 minutes at room temperature. After that the cells were washed in PBS (pH 7,3), covered with glycerin and analyzed by fluorescent microscopy. For visualization a microscope Olympus IX81 (Olympus corp., Japan) was used with a computer-controlled motorized stage (Märzhäuser,Wetzlar) and an Olympus DP72 digital camera (Olympus, Münster).

After staining G01 $CD133^+$ and G01 $CD133^-$ cell cultures with anti-Nestin, anti-Sox2, anti-Oct4, anti-Notch1 and anti-Map2 antibodies we also evaluated the intensity of cell fluorescence in the microscope field of view. After evaluating the brightness of the pixels on the micrographs, graphs were plotted expressing the ratio of the fluorescence intensity during cell cultivation to control.

RT-qPCR

The expression of following markers was measured in G01 $CD133^+$ and G01 $CD133^-$ cultures by RT-qPCR: CD133, DR4 and DR5, GFAP, Nanog, Oct4, Sox2, Notch2, L1CAM, Nestin, EGFR, Olig2, PDGFRa, and MELK.

For RNA isolation TRIzolTM Reagent User Guide (Sigma-Aldrich) was used. First DNA strand synthesis was performed using MMLV RT kit (Evrogen). As a control initial G01 culture was used. The expression was estimated on 7, 21, 35 and 42 after the beginning of cell cultivation. The array was performed under the following conditions: preliminary warming for 5 min at 90°C, denaturation for 10 sec at 95°C, primer annealing and elongation – for 30 sec at 60°C. The number of cycles – 40. The primers used in the assay are presented in Table S1. The house keeping genes used in the research are GAPDH, HPRT and GUSB.

Confocal microscopy

For confocal microscopy transfection of cell culture HEK293 using TurboFect Transfection Reagent (Thermo Scientific) was performed. After transfection cells were cultivated in 4-well chamber-slides for 48 hours with a humidified atmosphere of 5% CO_2. Then cells were washed three times in PBS with 1% BSA for 5 min. In the first three wells 50 µl of CD133/2-PE antibodies (dilutions 1:12.5; 1:25 and 1:50, #130-113-748, Miltenyi Biotec) and anti-GFP antibodies (FITC) (dilution 1:200, # Ab6662, Abcam) per well were added, in the fourth well 50 µl of CD133 MicroBeads (Miltenyi Biotec) with FcR Blocking Reagent and PBS c 1% BSA solution were added in proportion 3:3:44 accordingly. Chamber-slide was incubated for 15 min at 4°C in a dark place followed by adding of 300 µl Hanks' solution (Paneco).

MTT assay

Proliferative activity of G01 $CD133^+$ and G01 $CD133^-$ cell cultures was determined by MTT assay. It was also used to detect the changes in proliferative activity of the cells treated with specific aptamers. Glioblastoma cells were seeded in 96-well plates at cell density of 2000 cells per well in 200 µl of culture medium DMEM/F12. Cells were cultivated for 48 hours at 37°C with a humidified atmosphere of 5% CO_2. Before adding the oligonucleotides, they were performed by keeping at 95°C for 5 minutes in a water bath.

Cell medium was removed from the wells and the aptamers were added in prepared concentrations. For each concentration five repeats were done. Cells were incubated for 72 hours at 37°C with a humidified atmosphere of 5% CO_2. After removing the medium cells were washed with PBS and cultivated for 24 hours in 100 µl of culture medium.

20 µl of MTS reagent (Promega) was added to each well and the plate was incubated for 2 hours at 37°C with a humidified atmosphere of 5% CO_2. As a positive control the cells without aptamers were used. Cell medium was used as a blank. Optical density was measured on plate analyzer Tecan Infinite M200/Pro (Tecan) at λ=490 nm.

CD133 insert cloning

For isolation of total RNA from G01 cells RNAzol ® RT (Sigma-Aldrich) was used. First RNA strand synthesis was performed using MMLV RT kit. In order to get the CD133 DNA fragment of interest - CD133fr – three consistent PCRs were run followed by 1% agarose gel electrophoresis and DNA extraction using QIAquick® Gel Extraction Kit (Qiagen). As an insert a fragment of CD133 protein from 485 to 865 amino acids was used. Primers were designed using NCBI (National Center for Biotechnological Information) Blast, melting temperature (Tm) and annealing temperature were estimated using NEB Tm Calculator (New England Biolabs melting temperature calculator). The primers used in the assay are presented in Table S2. Annealing sites of the used primers on the target gene *Prom1* are shown in Figure S2a.

For PCR_{1-3} $Q5^{TM}$ High-Fidelity DNA Polymerase (Thermo Scientific) was used. As a marker for 1% agarose gel electrophoresis in TAE (Trisacetate-EDTA) buffer GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific) was taken. DNA concentration and purity were measured using NanoDrop 2000.

The first PCR (PCR_1) was run using cd133 f2-cd133 r1 and cd133 f3-Prom1 r0 primer pairs. Amplification of the nucleotide sequence correspond to the second extracellular

loop of CD133 (PCR_2) was run using cd133 f2-Prom1 r0 primer pair. For ligation with peGFP-c1 vector PCR_3 was run using XhoI f-BamH1 r primer pair. The PCR amplification in all the reactions was carried out in the following conditions: preliminary warming at 98°C for 30 sec, denaturation for 10 sec at 98°C, primer annealing for 15 sec at 58°C in PCR_{1-2} and at 51-58°C in PCR3, elongation at 72°C for 25 sec in PCR_{1-2} and for 40 sec in PCR_{2-3}, followed by final elongation at 72°C for 2 min in PCR_{1-2} and for 5 min in PCR_{2-3}. The number of cycles: in PCR_1 and PCR_3 – 30, in PCR_2 – 27.

Restriction of insert (CD133fr) and peGFP-c1 vector was done by XhoI and BamH1 sites of restriction. Ligation of CD133fr and peGFP-c1 vector (Figure S2b) was performed using 50 µg of each with T4 DNA High Concentration ligase (New England BioLabs), mixture was incubated overnight at 16 °C.

Transformation of E. coli TOP10 cells (Invitrogen) with CD133fr/peGFP-c1 was done using heat shock. Screening of overnight grown colonies after transformation with CD133fr/peGFP-c1 was performed using cd133 f3-cd133 r0 primer pairs with Taq-polymerase (5 units/µl) (Thermo Scientific). The PCR was carried out under the following conditions: preliminary warming for 30 sec at 98°C, denaturation for 10 sec at 98°C, primer annealing at 58°C for 15 sec, elongation at 72°C for 15 sec and final elongation at 72°C for 2 min. The number of cycles – 27. For positive control probe with CD133fr/pGEM-T easy recombinant DNA was used (intermediate results).

After PCR 1% agarose gel electrophoresis in TAE buffer with GeneRuler 100 bp Plus DNA Ladder was run. For following experiments probe that corresponded to 552 bp was used. This probe was incubated on a shaker overnight at 37°C in LB (Lysogeny Broth) medium.

Bacterial DNA was isolated using GeneJET Plasmid Miniprep (Thermo Scientific). Sanger sequencing was done to verify the nucleotide sequence of DNA that corresponded to the second extracellular loop of CD133.

Glioblastoma cell transfection was performed using TurboFect Transfection Reagent (Thermo Scientific). For confocal microscopy human glioblastoma cells were seeded in 4-well chamber slide and after transfection they were incubated at 37°C with a humidified atmosphere of 5% CO_2 for 48 hours.

Quantification and statistical analysis

Imaging of fixed samples was performed on an inverted confocal microscope (Olympus IX81) with a computer-controlled motorized stage and an Olympus DP72 digital camera. For confocal microscopy of live and fixed cells laser scanning microscope Zeiss LSM 900 was used. Images analysis was performed using ZEN software and Microsoft Excel. For evaluation of the intensity of cell fluorescence after staining with anti-Nestin, anti-Sox2, anti-Oct4, anti-Notch1, anti-Map2 antibodies in certain periods of time cells were selected (Table S3) and their intensity of fluorescence was established in percent of control. Images analysis was performed using ZEN software and Microsoft Excel.

MTT assay was performed using Tecan Infinite M200/Pro plate analyzer at λ=490 nm. For each sample 5 replicates were accomplished. Analysis was done with i-control 1.10 software.

To measure the levels of target genes' expression CFX96 Real-Time PCR System was used. Each sample was done in triplicate. The analysis was performed using CFX96 Real-Time PCR Detection System.
The concentration of samples during DNA recombinant vectors obtaining was measured with Nanodrop 2000 microvolume spectrophotometer for DNA samples.
The number of biological replicates (n) is indicated in the corresponding Figure legends. Data are plotted as mean ± standard error of the mean (SEM) or Standard Deviation (SD).

References

1. Omuro, A. & DeAngelis, L. M. Glioblastoma and other malignant gliomas: A clinical review. *J. Am. Med. Assoc.* **310**, 1842–1850 (2013).

2. Zahreddine, H. & Borden, K. L. B. Mechanisms and insights into drug resistance in cancer. *Frontiers in Pharmacology* (2013) doi:10.3389/fphar.2013.00028.

3. Dawson, L. E. *et al.* Induction of Myogenic Differentiation Improves Chemosensitivity of Chemoresistant Cells in Soft-Tissue Sarcoma Cell Lines. *Sarcoma* (2020) doi:10.1155/2020/8647981.

4. Han, H. *et al.* Induction of leukemic stem cell differentiation by aryl hydrocarbon receptor agonist and synergy with gilteritinib in FLT3-ITD + acute myeloid leukemia. *Leuk. Lymphoma* (2020) doi:10.1080/10428194.2020.1747062.

5. Eramo, A. *et al.* Chemotherapy resistance of glioblastoma stem cells [2]. *Cell Death and Differentiation* (2006) doi:10.1038/sj.cdd.4401872.

6. Lathia, J. D., Mack, S. C., Mulkearns-Hubert, E. E., Valentim, C. L. L. & Rich, J. N. Cancer stem cells in glioblastoma. *Genes and Development* (2015) doi:10.1101/gad.261982.115.

7. Brescia, P., Richichi, C. & Pelicci, G. Current strategies for identification of glioma stem cells: Adequate or unsatisfactory? *Journal of Oncology* (2012) doi:10.1155/2012/376894.

8. Chen, D. S. & Mellman, I. Oncology meets immunology: The cancer-immunity cycle. *Immunity* (2013) doi:10.1016/j.immuni.2013.07.012.

9. Kaiser, J. The cancer stem cell gamble. *Science (80-.).* (2015) doi:10.1126/science.347.6219.226.

10. Lee, S. Y., Kim, J. K., Jeon, H. Y., Ham, S. W. & Kim, H. CD133 regulates IL-1β signaling and neutrophil recruitment in glioblastoma. *Mol. Cells* **40**, 515–522 (2017).

11. Kemper, K. *et al.* The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation. *Cancer Res.* **70**, 719–729 (2010).

12. Zhu, L. *et al.* Multi-organ Mapping of Cancer Risk. *Cell* (2016) doi:10.1016/j.cell.2016.07.045.

13. Brescia, P. *et al.* CD133 is essential for glioblastoma stem cell maintenance. *Stem Cells* **31**, 857–869 (2013).

14. Wei, Y. *et al.* Activation of PI3K/Akt pathway by CD133-p85 interaction promotes tumorigenic capacity of glioma stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 6829–6834 (2013).

15. Wu, X., Wu, F., Xu, D. & Zhang, T. Prognostic significance of stem cell marker CD133 determined by promoter methylation but not by immunohistochemical expression in malignant gliomas. *J. Neurooncol.* (2016) doi:10.1007/s11060-015-2039-z.

16. Barrantes-Freer, A. *et al.* CD133 expression is not synonymous to immunoreactivity for AC133 and fluctuates throughout the cell cycle in glioma stem-like cells. *PLoS One* (2015) doi:10.1371/journal.pone.0130519.

17. Bradshaw, A. *et al.* Cancer Stem Cell Hierarchy in Glioblastoma Multiforme. *Frontiers in Surgery* vol. 3 1–15 (2016).

18. Izumoto, S. *et al.* Gene Expression of Neural Cell Adhesion Molecule L1 in Malignant Gliomas and Biological Significance of L1 in Glioma Invasion. *Cancer Res.* **56**, 1440–1444 (1996).

19. Ylivinkka, I. *et al.* Motility of glioblastoma cells is driven by netrin-1 induced gain of stemness. *J. Exp. Clin. Cancer Res.* (2017) doi:10.1186/s13046-016-0482-0.

20. Brown, D. V. *et al.* Expression of CD133 and CD44 in glioblastoma stem cells correlates with cell proliferation, phenotype stability and intratumor heterogeneity. *PLoS One* **12**, 1–17 (2017).

21. Müller, P., Gaebel, R., Lemcke, H., Steinhoff, G. & David, R. Data on the fate of MACS® MicroBeads intramyocardially co-injected with stem cell products. *Data Br.* **13**, 569–574 (2017).

22. Ding, L. *et al.* Cisplatin restores TRAIL apoptotic pathway in glioblastoma-derived stem cells through up-regulation of DR5 and down-regulation of c-flip. *Cancer Invest.* **29**, 511–520 (2011).

23. Guo, Y. *et al.* Expression profile of embryonic stem cell-associated genes Oct4, Sox2 and Nanog in human gliomas. *Histopathology* **59**, 763–775 (2011).

24. Yoshimoto, K. *et al.* Expression of stem cell marker and receptor kinase genes in glioblastoma tissue quantified by real-time RT-PCR. *Brain Tumor Pathol.* **28**, 291–296 (2011).

25. Engler, A. *et al.* Notch2 Signaling Maintains NSC Quiescence in the Murine Ventricular-Subventricular Zone. *Cell Rep.* (2018) doi:10.1016/j.celrep.2017.12.094.

26. Bao, S. *et al.* Targeting Cancer Stem Cells through L1CAM Suppresses Glioma Growth. *Cancer Res.* **68**, 6043–6048 (2009).

27. Park, D. *et al.* Nestin is required for the proper self-renewal of neural stem cells. *Stem Cells* (2010) doi:10.1002/stem.541.

28. Cheung-Ong, K., Giaever, G. & Nislow, C. DNA-damaging agents in cancer chemotherapy: Serendipity and chemical biology. *Chemistry and Biology* (2013) doi:10.1016/j.chembiol.2013.04.007.

29. Eyler, C. E. *et al.* Single-cell lineage analysis reveals genetic and epigenetic interplay in glioblastoma drug resistance. *Genome Biol.* (2020) doi:10.1186/s13059-020-02085-1.

30. Jin, X., Jin, X. & Kim, H. Cancer stem cells and differentiation therapy. *Tumor Biology* (2017) doi:10.1177/1010428317729933.

31. Mendiburu-Eliçabe, M., Gil-Ranedo, J. & Izquierdo, M. Efficacy of rapamycin against glioblastoma cancer stem cells. *Clin. Transl. Oncol.* (2014) doi:10.1007/s12094-013-1109-y.

32. Sunayama, J. *et al.* Dual blocking of mTor and PI3K elicits a prodifferentiation effect on glioblastoma stem-like cells. *Neuro. Oncol.* (2010) doi:10.1093/neuonc/noq103.

33. Kolesnikova, V. A. *et al.* The research of CD133+ marker signivcance in the

proliferati—e potential of human glioblastoma cells and the search for ways to control the proliferation and differentiation of these cells. *Genes and cells* **XIV**, 114–115 (2019).

34. Shutova, M. V., Chestkov, I. V., Bogomazova, A. N., Lagarkova, M. A. & Kiselev, S. L. Generation of iPS Cells from Human Umbilical Vein Endothelial Cells by Lentiviral Transduction and Their Differentiation to Neuronal Lineage. in *Human Embryonic and Induced Pluripotent Stem Cells* 133–149 (2011). doi:10.1007/978-1-61779-267-0.

35. Wichterle, H., Lieberam, I., Porter, J. A. & Jessell, T. M. Directed differentiation of embryonic stem cells into motor neurons. *Cell* (2002) doi:10.1016/S0092-8674(02)00835-8.

36. Smith, J. R. *et al.* Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. *Dev. Biol.* **313**, 107–117 (2008).

37. Li, X.-J. *et al.* Directed Differentiation of Ventral Spinal Progenitors and Motor Neurons from Human Embryonic Stem Cells by Small Molecules. *Stem Cells* (2008) doi:10.1634/stemcells.2007-0620.

38. Chambers, S. M. *et al.* Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors. *Nat. Biotechnol.* (2012) doi:10.1038/nbt.2249.

39. Zhou, Y. *et al.* Autocrine BMP4 Signaling Enhances Tumor Aggressiveness via Promoting Wnt/β-Catenin Signaling in IDH1-mutant Gliomas. *Transl. Oncol.* **13**, 125–134 (2020).

Acknowledgments

We are grateful to Dmitry Panteleev (Institute of Gene Biology, Russian Federation) and Dzhirgala Shamadykova (Institute of Gene Biology, Russian Federation) for technical assistance, Maria Lagarkova (Scientific Research Institute of Physical-Chemical Medicine, Russian Federation) for the first combination of neurodifferentiation factors.

Funding

This work was funded by the Russian Foundation for Basic Research (18-015-00279 A) and by the Ministry of Science and Higher Education of the Russian Federation (075-15-2020-809).

Author information

These authors contributed equally: Galina Pavlova and Varvara Kolesnikova.

Affiliations

Laboratory of Neurogenetics and Genetics Development, Institute of Higher Nervous Activity and Neurophysiology of RAS, 117485, Russia
Galina Pavlova, Varvara Kolesnikova, Alexander Revishchin

Institution «N. N. Burdenko National Medical Research Center of Neurosurgery» of the Ministry of Health of the Russian Federation, Moscow, 125047, Russia
Galina Pavlova, Nadezhda Samoylenkova, Sergey Drozd, Dmitry Y. Usachev

Department of Medical Genetics, Sechenov First Moscow State Medical University, Moscow, 119991, Russia
Galina Pavlova

Chemistry Department, Belozersky Institute of Physical Chemical Biology Moscow State University, Moscow, 119992, Russia
Alexey Kopylov

Contributions

G.P. and V.K. contributed equally. G.P. designed the experiments; V.K. performed the experiments with cell cultures and MTT-assay; N.S. carried out experiments with cell cultures and performed MTT-assay; S.D. performed and analyzed the experiments on gene expression; A.R. performed and analyzed immunocytochemical experiments; D.Y.U. contributed to the conducting of the experiments; A.K. shaped the project and provided with aptamers. G.P. and V.K wrote the paper. All of the authors discussed the results and manuscript.

Corresponding authors

Correspondence to Galina Pavlova and Varvara Kolesnikova.

Competing interests

The authors declare no competing interests.

Table 1: The combinations of the factors added to the G01 CD133$^+$ and G01 CD133$^-$ cell cultures.

Flask №	G01 CD133$^+$ culture	Flask №	G01 CD133$^-$ culture
1	Control (without bi-(AID-1-T))	7	Control (без bi-(AID-1-T))
2	bi-(AID-1-T)	8	bi-(AID-1-T)
3	bi-(AID-1-T)+SB431542	9	bi-(AID-1-T)+SB431542
4	bi-(AID-1-T)+Purmorphamine	10	bi-(AID-1-T)+Purmorphamine
5	bi-(AID-1-T)+BDNF	11	bi-(AID-1-T)+BDNF
6	bi-(AID-1-T)+SB431542, LDN-193189, Purmorphamine, BDNF	12	bi-(AID-1-T)+SB431542, LDN-193189, Purmorphamine, BDNF

Table S1: Panel of primers for RT-qPCR.

Genes	Primers' sequence, 5'- 3'	Genes	Primers' sequence, 5'- 3'
EGFR	GTGACCGTTTGGGAGTTGATGA GGCTGAGGGAGGCGTTCTC	CD133	TGGATGCAGAACTTGACAACGT ATACCTGCTACGACAGTCGTGGT
Nanog	AATACCTCAGCCTCCAGCAGATG TGCGTCACACCATTGCTATTCTTC	PDGFRα	GGCATTCTTTGCAATACTGCTTAA CATCTGCCGATAGCACAGTGA
Oct4	CGAAAGAGAAAGCGAACCAG AACCACACTCGGACCACATC	DR4	AGGAGCCGGCAGATTTGACA GCATCAGAGTCTCAGTGGGGT
Sox2	ACACCAATCCCATCCACACT CCTCCCCAGGTTTTCTCTGT	DR5	GTT CCA GCC CTC CCT CAG AT GGT GCA AAT GAG ACT GCC CA
MELK	CAAACTTGCCTGCCATATCCT GCAAATCACTCCCTAGTGTGTT	L1CAM	CATGTGATGGAGCCACCTGT CCCAGCTCTTCCTTGGGTTT
Nestin	TTGCCTGCTACCCTTGAGAC GGGCTCTGATCTCTGCATCTAC	MAP2	CCAATGGATTCCCATACAGG TCCTTGCAGACACCTCCTCT
Notch2	GATCACCCGAATGGCTATGAAT CAATGCAGCGACCATCGTTC	HPRT	TGAGGATTTGGAAAGGGTGT GAGCACACAGAGGGCTACAA
GFAP	CTGCGGCTCGATCAACTCA TCCAGCGACTCAATCTTCCTC	GAPDH	AGATCCCTCCAAAATCAAGTGG GGCAGAGATGATGACCCTTTT
Olig2	CCAGAGCCCGATGACCTTTTT CACTGCCTCCTAGCTTGTCC	GUSB	CTTCTCTGACAACCGACGCC ACACCCAGCCGACAAAATGC

Table S2: Primers for CD133fr.

Primers	Primers' sequence, 5'- 3'
cd133 f2	CTGTTTATGTTAATAACACTGAA
cd133 f3	TGTTGGGTGCAGCAGGAAGAAA
cd133 r1	GCTTCTAGATCATATGCAAAT
cd133 r0	AATTCAAGGGGTCGATAATGTA
Prom1 r0	CATCAGCTATCAATGTTGTGAT
Xho1 f	TTTTCTCGAGCTTTCCTCATGGTTGGAGTT
BamH1 r	TTTTGGATCCTCAATGTTGTGATGGGCT

Table S3: The amount of analyzed cells after staining G01 CD133$^+$ and G01 CD133$^-$ cell cultures with anti-Nestin, anti-Sox2, anti-Oct4, anti-Notch1, anti-Map2 antibodies.

Marker	Nestin				Sox2				Oct4				Notch1				Map2			
Day after separation	Control	7	20	28	Control	7	20	28	Control	7	20	28	Control	7	20	28	Control	7	20	28
CD133$^+$	52	49	33	38	41	33	38	39	49	35	43	51	43	38	29	34	29	39	31	53
CD133$^-$	35	43	39	31	35	42	32	44	38	47	39	41	52	33	44	41	36	48	27	33

Figures

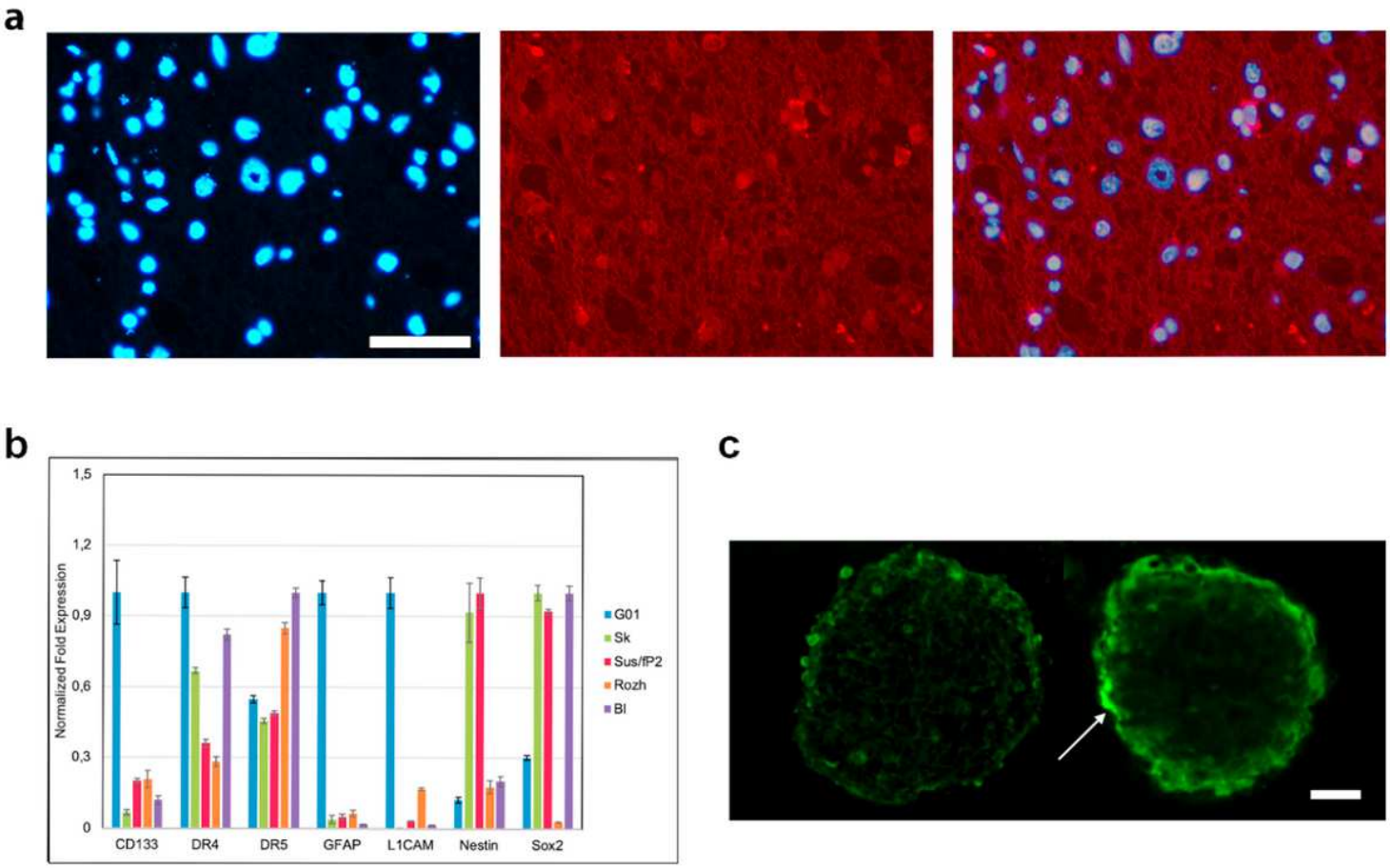


Figure 1

Characterization of glioblastoma cell culture with CD133 high expression rate. a Immunohistochemical staining with anti-CD133 antibodies (left); bisbenzimide staining of cell nuclei of the patient's biopsy samples (middle); a and b merge (right); scale bar is 50 μm. b Real-time quantitative PCR. The expression of neural stem cells' genes in tested cell cultures. Data are represented as mean ± SEM. n=3 for each group. c Immunohistochemical staining of the neuro-spheres with anti-CD133 antibodies. CD133+ cells (arrow) are located in the outer layer of the neuro-spheres; scale bar is 20 μm.

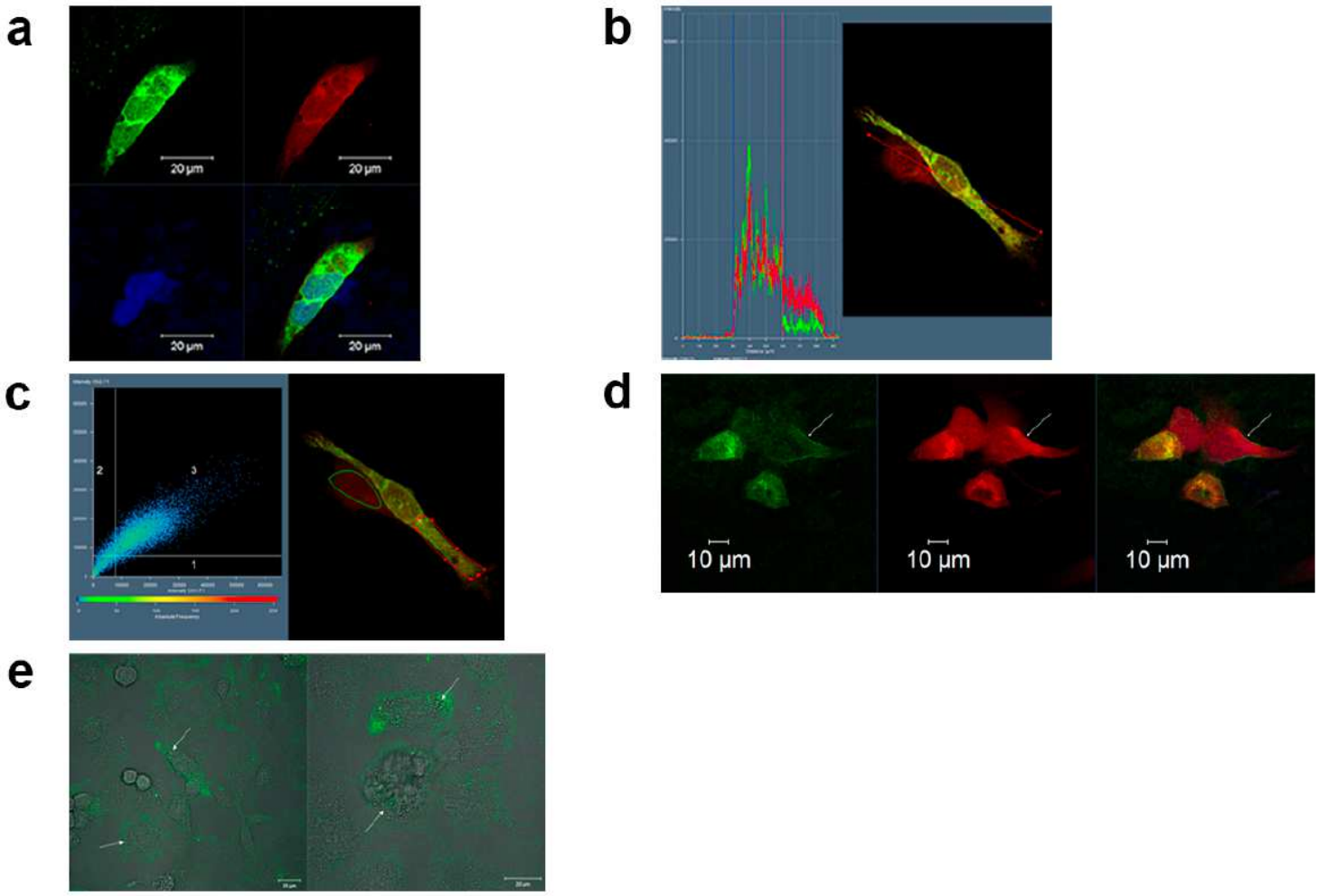


Figure 2

Confocal visualization of CD133 distribution on the cell membrane of G01 glioblastoma cells using CD133fr/peGFP-c1 recombinant DNA. a Micrographs of fixed glioblastoma cell culture cells stained with anti-CD133 antibodies (dilution 1:25), anti-GFP antibodies (FITC) (dilution 1:200). GFP (green), CD133 (red), DAPI (blue). b Diagram of signal location along the axis (red) (right) through the areas of CD133fr localization (red) and CD133fr and GFP (red and green) (left). c Diagram of colocalization of CD133fr and GFP in fixed glioblastoma cells. To the left, signals from GFP and CD133fr are on the axes of both proteins (area 3). To the right, the area outlined in red is the co-location of CD133fr and GFP. d Micrographs of membrane localization of CD133fr/peGFP-c1 recombinant DNA, the arrows indicate the colocalization of GFP (green), CD133fr (red) and CD133/GFP (yellow) e Micrographs of the distribution of microbeads in glioblastoma cell culture transfected with peCD133fr/peGFP-c1. The arrows indicate magnetic beads.

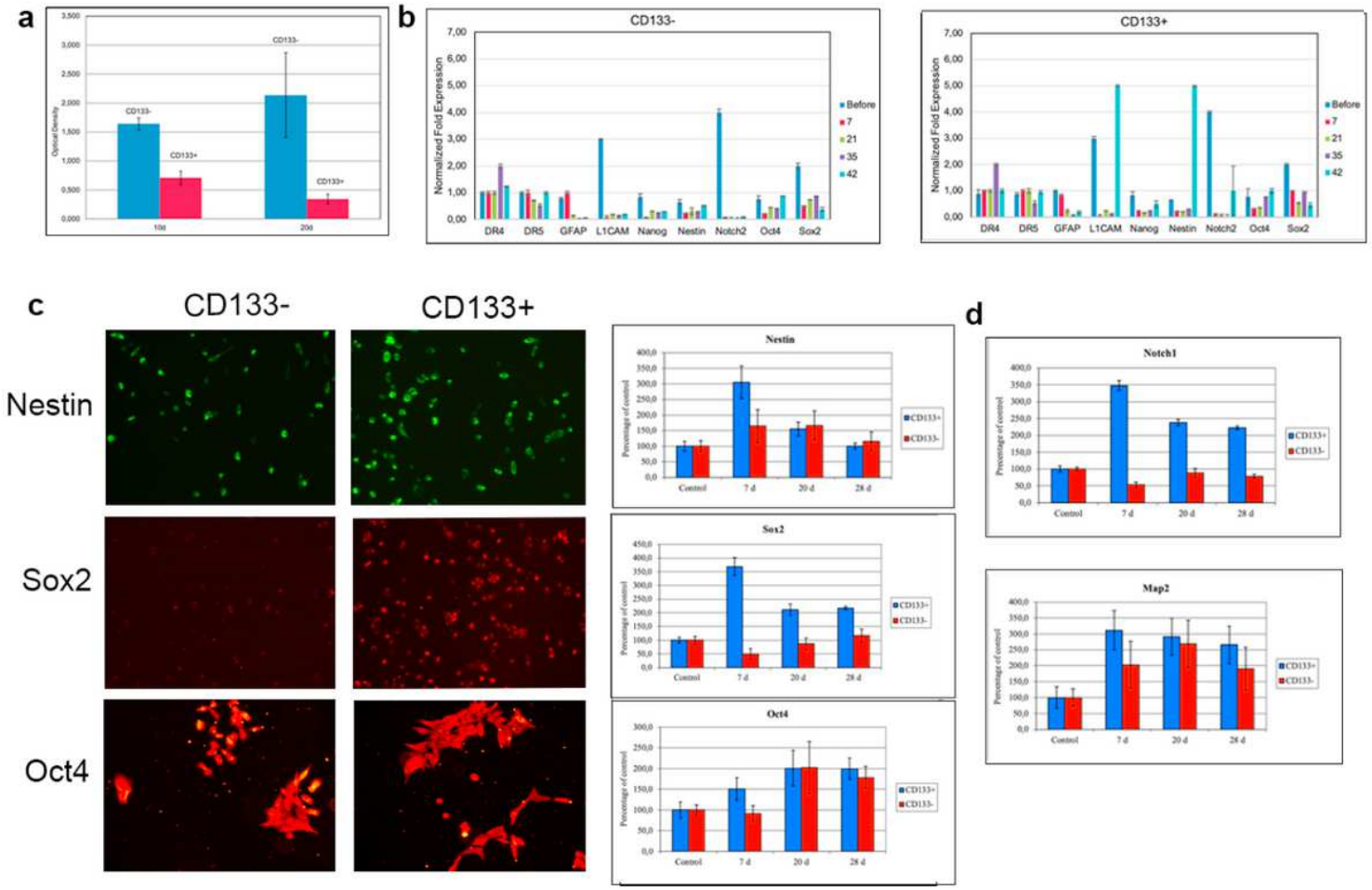


Figure 3

Characterization of G01 CD133+ and G01 CD133- cell cultures after cell separation. a MTT assay of G01 CD133+ and G01 CD133- glioblastoma cell cultures in 10 and 20 days after cell separation; Data are represented as mean ± SD. n=5 for each group. b Real-time quantitative PCR of stem cell genes in G01 CD133- (left) and G01 CD133+ (right) cells before and in 7, 21, 35, 42 days after cell separation. Data are represented as mean ± SEM. n=3 for each group. c Micrographs of immunohistochemical staining of G01 CD133+ and G01 CD133- cell cultures with anti-Nestin, anti-Sox2, anti-Oct4 antibodies. To the right: the diagrams representing the percentage of the marker-positive cells in the tested cell cultures. Data are represented as mean ± SD. d Diagrams representing the content of Notch1 and Map2 positive cells in G01 CD133+ and G01 CD133- cell cultures based on the data of immunohistochemical staining with anti-Notch1 and anti-Map2 antibodies. Data are represented as mean ± SD.

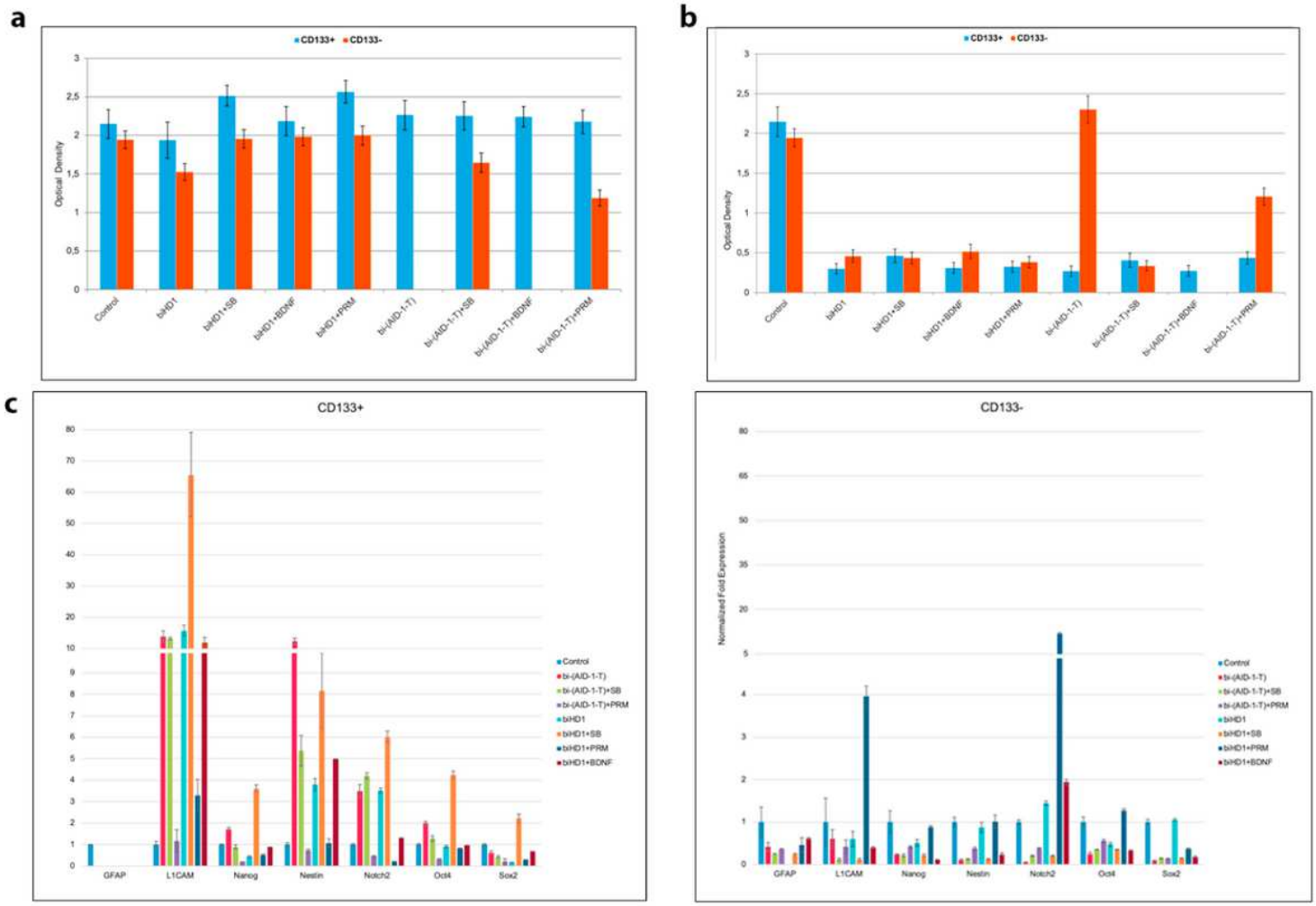


Figure 4

G01 CD133- and G01 CD133+ exposure to an aptamer and a single neuroinducing factor. a MTT assay of G01 CD133+ cells and G01 CD133- cells after the exposure to aptamers biHD1 and bi-(AID-1-T) and neural differentiation inducers SB431542, purmorphamine, BDNF in 10 days after the exposure. Data are represented as mean ± SD. n=5 for each group. b MTT assay of G01 CD133+ cells (left) and G01 CD133- cells (right) after the exposure to aptamers biHD1 and bi-(AID-1-T) and neural differentiation inducers SB431542, purmorphamine, BDNF in 20 days after the exposure. Data are represented as mean ± SD. n=5 for each group. c Real-time quantitative PCR of stem cell genes in G01 CD133+ (left) and G01 CD133- (right) cell cultures in 20 days after the exposure to the aptamers biHD1 and bi-(AID-1-T) and neural differentiation inducers SB431542, purmorphamine, BDNF. Data are represented as mean ± SD. n=3 for each group. SB - SB431542, PRM - purmorphamine

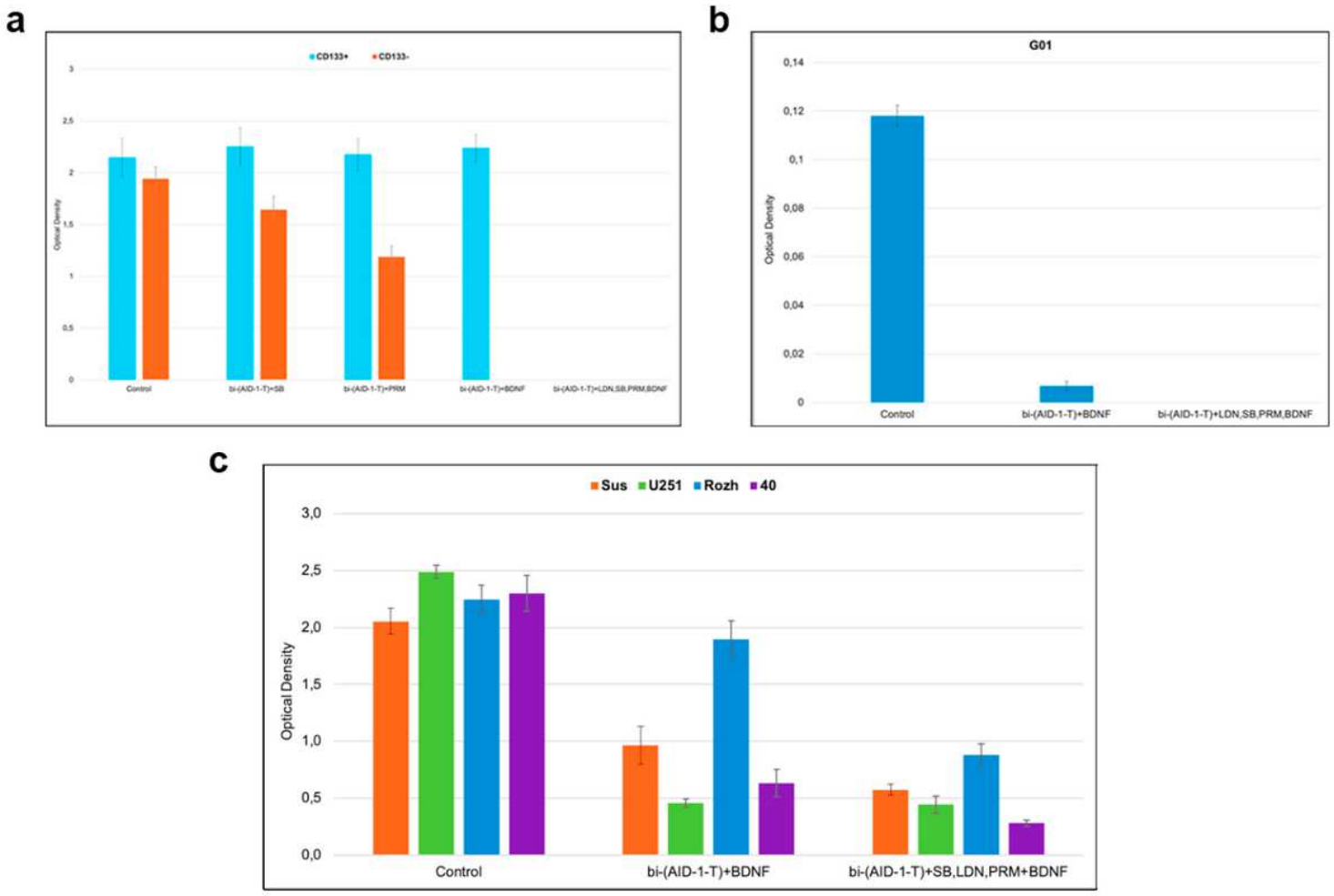


Figure 5

Cell cultures' exposure to the bi-(AID-1-T) aptamer and a cascade of neural inducers. a MTT assay for G01 CD133+ and G01 CD133- in 10 days after the exposure to the bi- (AID-1-T) aptamer and the neural differentiation inducers. Data are represented as mean ± SD. n=5 for each group. b MTT assay for G01 cells in 10 days after the exposure to the bi-(AID-1-T) aptamer and the successive addition of the neural differentiation inducers; Data are represented as mean ± SD. n=5 for each group. c MTT assay for Sus, U251, Rozh and 40 cell cultures in 10 days after the exposure to the bi-(AID-1-T) aptamer and the neural differentiation inducers. Data are represented as mean ± SD. n=5 for each group. SB – SB431542, PRM – Purmorphamine, LDN – LDN-193189.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation.pdf