



ZNF521 Overexpression in U87MG Cells Induces Neural Progenitors and Improves Motor Function in Spinal Cord Injury in Rats



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ABSTRACT

Introduction: This experimental *in-vitro* and *in-vivo* parallel-group study aimed to investigate the *ZNF521* overexpression effects on the glioblastoma cell line (U87MG) behavior *in vitro* and the therapeutic potential of U87MG cells that were overexpressed by *ZNF521* (U87-ZNF521) in functional recovery of spinal cord injury (SCI) in rats.

Methods: *ZNF521*-IRES-GFP was introduced into U87MG cells and maintained in the neural inductive medium for over 3 weeks. The *GFAP*, *ITGA6*, *PAX6*, *NES*, *SOX1*, *TUBB3*, and *OLIG2* gene expressions were analyzed. The SCI rats were split into three groups, with 10 rats each (30 rats in total). Then, U87-ZNF521 cells were transplanted, and the Basso-Beattie-Bresnahan scale and footprint analysis were used to evaluate the rats' locomotor capacity.

Results: *ZNF521* overexpression induced morphological changes and aggregated formation in U87MG cells, with a 26% transfection rate. Significant upregulation of *PAX6*, *TUBB3*, and *OLIG2* and decreasing *SOX1* were observed, while *GFAP*, *ITGA6*, and *NES* showed non-significant changes compared to the control group. In SCI rats, U87-ZNF521 exhibited substantial recovery in hindlimb motor coordination and weight support. Moreover, gait analysis revealed increased step length, stride angle, and step width in U87-ZNF521 rats during a five-week treatment. However, no significant improvement was observed with plantar application.

Conclusion: *ZNF521* overexpression in the U87MG suggests its potential to differentiate into neural progenitors. Additionally, these neural progenitors improved motor function in SCI rats. *ZNF521* can be a potential therapy for promoting recovery in glioblastoma and SCIs, highlighting its role as a promising target for further exploration in neural regeneration strategies.

Keywords:

spinal cord injury, astrocytoma, cell line, glioblastoma, zinc finger protein 521.

1. Introduction

Zinc finger protein 521 (*ZNF521*) promotes neural differentiation by directing embryonic epidermal cells toward neural progenitor cells (1, 2) and modulating adult astrocytic genetic core to neural stem cells (NSCs) (3). In a permissive environment, *ZNF521* could convert adult human fibroblast cells into NSCs (4). *ZNF521* mainly increases sonic hedgehog (SHH) signaling pathway activity, which causes both normal cerebellum and medulloblastoma developments (5). ZFPs often play contradictory or versatile roles in cancer progression; for example, *ZNF521* promotes medulloblastoma growth and progression (6-9) or suppression (10). But *ZNF521*'s role in modifying or driving glioblastoma multiforme (GBM)'s genomic effects was unknown.

The most common and severe brain and spinal cord tumor is GBM (11). The NSC-like subgroup of GBM can self-renew and differentiate into neurons, astrocytes, and oligodendrocytes, which can be expanded *in vitro* as stable cell lines for further characterization (12). GBM cell lines have similarities (13) but also irregularities (14), suggesting that parallel cellular pathways may cause NSCs and GBM cell lines to have different attitudes. Epidermal and fibroblast growth factors promote NSC stemness and proliferation, but they also drive extensive proliferation in GBM (15) and medulloblastoma (16).

Astrocytoma grade four cell line (U87MG) has been used in many studies over the past 40 years (17). U87MG has tumorigenic neural precursors (18), highly aberrant gene structure (19), and can differentiate into cholinergic neurons (20). U87MG has also been used to evaluate GBM genome editing technologies for *in vivo* manipulation (21). Thus, the U87MG is a reliable platform for *in vitro* drug discovery (22) and signaling pathway research (6).

This study investigated the prolonged and excessive *ZNF521* expression effects on the morphological characteristics and selected gene expression, including glial fibrillary acidic protein (*GFAP*), integrin

subunit alpha 6 (*ITGA6*), paired box 6 (*PAX6*), nestin (*NES*), SRY-box transcription factor 1 (*SOX1*), tubulin beta 3 class III (*TUBB3*), and oligodendrocyte transcription factor 2 (*OLIG2*) in U87MG cells *in vitro*. Furthermore, the study aimed to investigate whether intraspinal transplantation of *ZNF521*-U87MG cells—neuronal-like cells—could serve as a source of regeneration and promote functional motor recovery in a rat model of spinal cord injury (SCI) *in vivo*.

Additionally, the study aimed to explore the U87MG that was overexpressed by *ZNF521* (U87-*ZNF521*) cells' potential therapeutic benefits in promoting neuronal regeneration and improving motor function in a spinal cord injury (SCI) rat model *in vivo*.

2. Materials & Methods

The Ethics Committee of the Sina Trauma and Surgery Research Center, Tehran University of Medical Sciences, approved the study with the reference number IR.TUMS.VCR.REC.1398.321. Furthermore, the entire process adhered to the Royan Institute's ethical guidelines and the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (23).

2.1. Cell Maintenance and Transduction

U87MG, obtained from the Iranian Biological Resource Center, was expanded and passaged five times in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (1%) (all from Invitrogen, CA, USA). P3–P5 astrocytoma was transduced via *ZNF521-IRES-GFP* constructs in the experimental group and CMV-GFP constructs in the control group, as described in our previous reports (3, 24). Transduced cells (U87-*ZNF521* and U87-GFP) were cultivated on 0.001% poly-L-ornithine (P4707; Sigma-Aldrich, MO, USA) and 10 mg/mL laminin (L2020; Sigma-Aldrich, MO, USA) coated plates and were maintained in NSC medium (NSCM) containing DMEM/F12 supplemented with

2% B27, 1% N2, 0.5% ITS (1 mg/mL insulin, 0.55 mg/mL transferrin, and 0.67 mg/mL selenium), 10% knockout serum replacement, 1% non-essential amino acids, 2 mM L-glutamine, 1% penicillin 100 U/mL, and streptomycin 100 µg/mL (all from Invitrogen, CA, USA), 20 ng/mL basic fibroblast growth factors (F0291; Sigma-Aldrich, MO, USA), and 20 ng/mL epidermal growth factors (E9644; Sigma-Aldrich, MO, USA).

The genes that were assessed *in vitro* after *ZNF521* overexpression included: *GFAP*, a type III intermediate filament protein and an NSC marker that is usually overexpressed in GBM cells (25, 26); *ITGA6*, a receptor for extracellular matrix laminin that is an enrichment marker for GBM stem cells and plays a crucial role in their capacity for self-renewal, proliferation, and tumor formation (27); *NES* is an intermediate filament protein and a marker for proliferative neural progenitor and GBM cells (28); *SOX1* is the earliest neurogenesis transcription factor in proliferative NSCs and overexpressed in GBM (29); *PAX6*: the earliest neuronal marker and have suppression function in GBM (30); *TUBB3*: a neuronal marker that has a role in axon regeneration (31); and *OLIG2*: the earliest motor neuron and oligodendrocyte marker (32). Uninfected cells were removed through fluorescence-activated cell sorting (FACS) to ensure that only GFP-positive cells, transduced with *ZNF521*, were transplanted.

2.2. PCR

Total mRNA from U87-ZNF521 and U87-GFP (control) groups was extracted five weeks after induction by NSCM using

an RNeasy® Plus Universal Mini Kit (73404; QIAGEN LLC, MD, USA), followed by 2 µg complementary DNA synthesis using an Easy™ complementary DNA Synthesis Kit with random hexamer primer (A101161; Parstous Biotechnology, Iran) according to the manufacturer's instructions. Reverse transcription (RT)-PCR was performed with a SYBR® Green Master Mix (DQ385; BioFact™, South Korea) via StepOne™ (Applied Biosystems™, Thermo Fisher Scientific, MA, USA) for selected genes (Table 1). mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control and analyzed using the $2^{-\Delta\Delta CT}$ method. For graphical representation, fold changes were log2-transformed to symmetrically display upregulation (positive values) and downregulation (negative values).

2.3. SCI Modeling and Cell Transplantation

The study included: 1) adult male wild-type Wistar rats weighing 250–280 g; 2) U87-ZNF521 and U87-GFP groups received cell transplantation five weeks after induction; and 3) only GFP-positive cells, transduced with *ZNF521*, were transplanted, as confirmed by FACS. Rats outside the weight range or showing signs of illness or abnormality during pre-surgical checks and the data from rats that died from surgery or recovery complications or had pre-surgical injuries were excluded. Rats that did not receive 5 mg/kg enrofloxacin for infection prevention or did not discharge their bladders daily were also

Table 1. Primer names and sequences that were used for real-time polymerase chain reaction.

Genes	Forward primer (5'–3')	Reverse primer (5'–3')
GAPDH	AGGGTCTCTCTCTTCTCTTGTGCTCT	CCAGGTGGTCTCCTCTGACTTCAACAG
GFAP	GAGATGCGGGATGGAGAG	TAGGGACAGAGGAGGGAG
ITGA6	TTTATCGGTCTCGGGAGTTG	GGCCACTGAATGTTCAAGGT
PAX6	TTCAGCACCAGTGTCTACCAAC	GCTGACTGTTCATGTGTGTCTG
SOX1	CCTCCGTCCATCCTCTG	AAAGCATCAAACAACCTCAAG
NES	CTCCAGAACTCAAGCAC	TCCTGATTCTCCTCTTCCA
TUBB3	GGAGAACACGGATGAGACCTA	AAGGTGGAGGACATCTTGAGG
OLIG2	CCAGAGCCCGATGACCTTTT	CACTGCCTCCTAGCTTGTC

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; ITGA6: integrin alpha 6; OLIG2: oligodendrocyte transcription factor 2; PAX6: paired box 6; SOX1: sex-determining region Y-box 1; TUBB3: tubulin, beta 3 class III.

excluded. Outliers and inconsistencies were excluded from the final analysis. Using a random number generator, the rats were randomly allocated into the U87-ZNF521, U87-GFP, and control groups (SCI rats that did not receive U87MG cells), with each group containing 10 rats. In total, 30 rats were used. The double-blinded process was implemented throughout the experiment.

Rats were anesthetized through intraperitoneal injections of 100 mg/kg ketamine and 10 mg/kg xylazine. The SCI contusion model was established by dropping a 10 g rod weight vertically onto the spinal cord from a height of 25 mm using an NYU-impactor device at the T8–T10 level. Following the SCI, each rat received 5 mg/kg of enrofloxacin intramuscularly once a day for a week. Daily bladder discharge was performed manually as long as the rat required it. One week post-SCI, at the subacute injury phase, almost 2×10^6 cells diluted in 30 μ L PBS—were transplanted into the injury site via an automated micro-injector device (Stoelting Co., IL, USA). In U87-ZNF521, transduced cells were maintained in an induction medium for five weeks. For immune response suppression after cell transplantation, subcutaneous injections of 10 mg/kg Cyclosporine-A were administered to rats in all groups two days before transplantation (day 5) throughout the whole study.

2.4. Locomotor Capacity Evaluation

To assess the weekly rats' motor function improvement, the well-established Basso-Beattie-Bresnahan (BBB) locomotor rating scale was employed on freely moving rats throughout four weeks that included 0–21 points. The method comprehensively assesses rat motor function by measuring hindlimb joint movement, coordination between front and rear limbs, trunk position, load-bearing capacity, paw placement, and tail movement. The BBB score of all rats was determined to be 21 before the SCI. Following a contusion injury, the BBB of all rats decreased to zero from the first day after the SCI until at least seven days later.

2.5. Footprint Analysis

Gait and motor coordination were weekly evaluated in all rats as well. The rats were allowed to walk on a narrow paper-covered corridor while their metatarsus was colored to leave footprint traces. At least 4–5 sequential steps were used to determine the mean values for each measurement, including stride angle, stride length, foot length, step width, toe spread, and paw area.

2.6. Statistical analysis

Total numerical data belonging to *in vitro* investigations are reported as the mean ($\pm$ standard error) of three independent experiments. An independent sample t-test was used to compare the gene expression pattern between U87-ZNF521 cells and U87-GFP (control group) cells. ANOVA and Tukey's post hoc test were considered for comparisons of means for behavioral assessments. The p-value of < 0.05 was reflected as statistically significant. All data were analyzed by SPSS version 16 (SPSS Inc., IL, USA).

3. Results

3.1. ZNF521's excessive expression propels aggregate formation in U87MG

Two groups of U87MG cells, owning a flat and bipolar phenotype (Figs. 1. D1 and E1), were transfected with ZNF521-*IRES*-GFP (U87-ZNF521) as well as CMV-GFP (U87-GFP) while maintaining in NSCM for five weeks. U87-ZFP21 cells underwent an immediate morphological conversion and became smaller and confined after three days post-transduction (Fig. 1. D2). During the first week post-transduction (WPT), U87-ZNF521 cells became susceptible to gathering around and initiating the aggregate formation; in addition, they hesitated to proliferate (Fig. 1. D3). They settled inside the aggregates throughout the experiment timeline (Fig. 1. B2). Compared to U87-ZNF521, U87-GFP cells retained bipolar and proliferative activity over the whole process with preserved dispersion (Figs. 1.A, E2, and E3). The transfection rate yielded 26% in U87-ZNF521, which is almost half of what was measured in U87-GFP (Fig. 1. C).

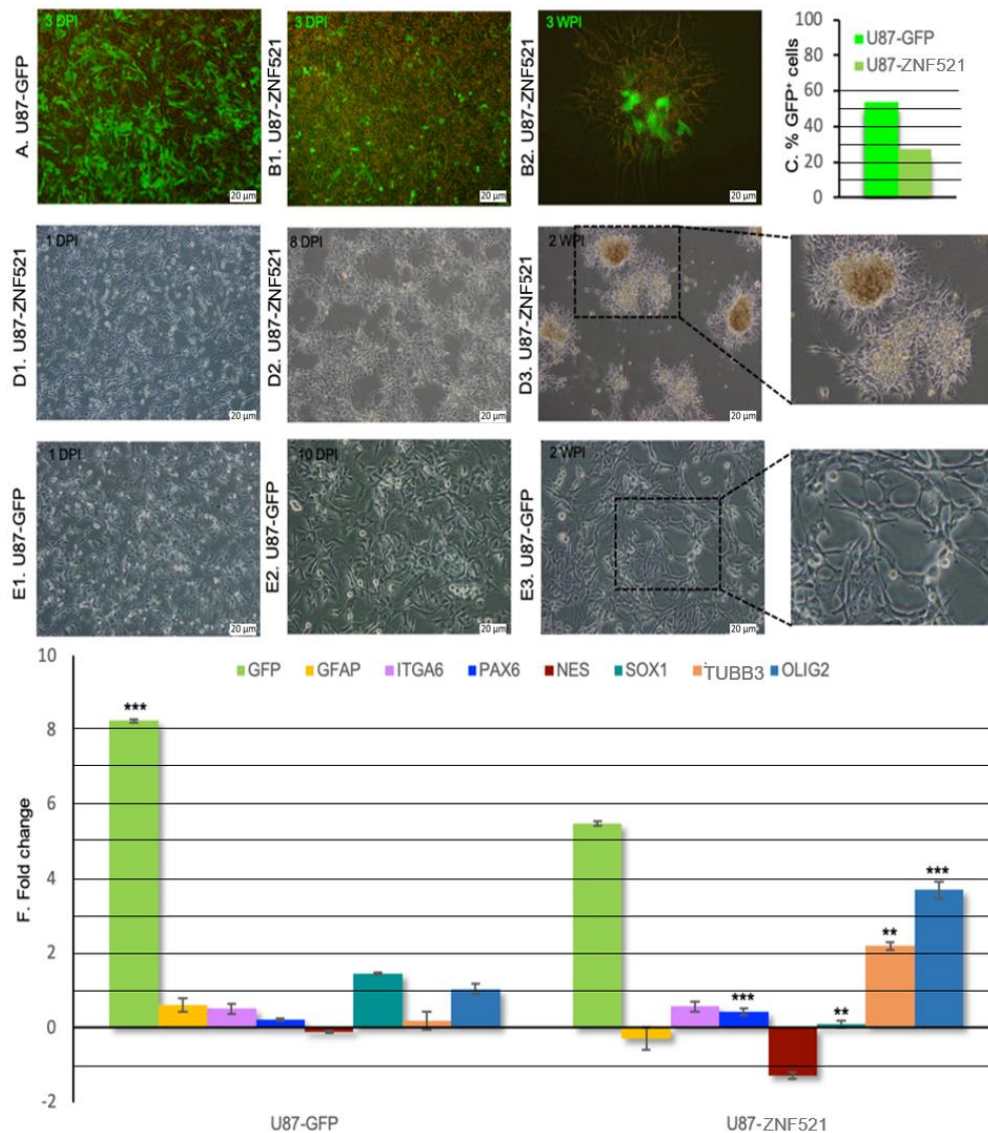


Figure 1. The morphological changes and the U87-ZNF521 gene expression pattern. A, B1-2, and C) Both transfected U87-GM cell groups were found to be GFP+ from the third DPI, although the transfection rate measured was greater in U87-GFP. D1-3) U87-ZNF521 morphologies were changed from bipolar, extended, and proliferative to smaller and aggregate-forming during a five-week induction. E1-3) U87-GFP (control) maintained bipolarity, dispersion, and proliferation throughout the whole experiment. F) GFP expression was significantly larger in U87-GFP and maintained detectability throughout the whole study in both groups. No significant changes were observed in the *GFAP*, *ITGA6*, and *NES* expression levels between U87-ZNF521 and U87-GFP. On the other hand, *PAX6* ($p < 0.001$), *TUBB3* ($p < 0.01$), and *OLIG2* ($p < 0.001$) revealed a substantial increase in U87-ZNF521. *SOX1* expression decreased in U87-ZNF521 ($p < 0.01$). The data is presented as mean $\pm$ standard error and analyzed via a student sample t-test.

3.2. ZNF521 prolonged overexpression increased the hindlimb neuronal marker expression in U87MG

The gene expression in U87-ZNF521 cells was compared to U87-GFP cells after five weeks (Fig. 1. F). According to GFP measurement, the vector was consistently functional in both cell groups—i.e., the ZPF521 was excessively expressed in U87-MG throughout the whole experiment—while, of course, the transfection rate was

significantly larger in U87-GFP (Figs. 1. A, B, and C) from the beginning ($p < 0.001$). *GFAP*, *ITGA6*, and *NES* revealed non-significant changes during five WPT in NSCM ($p > 0.05$) compared to U87-GFP. However, *SOX1* showed a considerable down-regulation ($p < 0.01$). The expression level of *PAX6*, *TUBB3*, and *OLIG2* was significantly increased in U87-ZNF521 cells compared with U87-GFP ($p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively).

3.3. ZNF521-U87MG cells allow slight behavioral improvement in SCI rats

The rats' locomotor ability was evaluated in five WPTs via the BBB test through a two-way ANOVA. Overall, the hindlimb motor coordination and joint movements of all the rats displayed gradual improvements. However, the U87-ZNF521 rats revealed substantial recovery with a score of 12 compared to U87-GFP rats ($p < 0.01$) and control rats ($p < 0.001$) (Fig. 2. A). Weight support was significantly ameliorated in these rats, while plantar application was not considerably restored (Figs. 2. B and C).

The BBB score indicated that the rats' motor ability improved by nearly half compared to intact rats. In addition to the BBB grading, gait parameters were monitored to provide a detailed assessment of movement. Consistent with the BBB results, significant improvements were observed in the gait of U87-ZNF521 rats over five weeks, as detailed below:

- **Step Length:** A significant increase was observed in U87-ZNF521 rats in both the left ($p < 0.001$) and right ($p < 0.01$) rears at the fifth WPT.
- **Stride Angle:** The right foot showed a higher stride angle at weeks three and four ($p < 0.05$).
- **Step Width:** A substantial increase was observed at week three in both the right ($p < 0.01$) and left ($p < 0.001$) rears. At week four, a significant increase was only detected in the left foot ($p < 0.01$).
- **Toe Spread:** Only the right foot exhibited a notable increase at the fifth WPT in U87-ZNF521 rats compared to U87-GFP controls ($p < 0.01$).

4. Discussion

Many studies used chemicals or master-regulating transcription factors like *SOX10* for oligodendrogenesis and *ZNF521* for NSC generation to successfully convert unfavorable astrocytes into oligodendrocytes (33, 34) and neural cells (24). In addition to surgery and chemotherapy, cell identity manipulation

may help treat astrocytoma and GBM (35). This study used *ZNF521*'s neurogenesis power to determine if it can exclusively modulate gene expression in U87-GM and shed light on how U87-ZNF521 transplantation induces neural differentiation post-SCI.

Due to *ZNF521* overexpression, NSCM astrocytomas changed from bipolar and proliferative to smaller and aggregating at 5 WPT. *GFAP*, *ITGA6*, and *NES* expression remained unchanged, but *SOX1* expression decreased, consistent with the proliferation decline in U87-ZNF521 during *in vitro* cultivation. The unchanged expression of *GFAP* and *ITGA6* may indicate that these cells are still unstable. The remarkable upregulation of *PAX6*, *TUBB3*, and *OLIG2* in U87-ZNF521, compared to U87-GFP, showed that *ZNF521* activates neuronal lineage genes even in the most aberrant astrocytoma cell line. In U87-GM, *OLIG2* elevation suggests that *ZNF521* promotes oligodendrocyte and motoneuron generation. Previous studies found that the U87-GM could directly reprogram neurons using small molecules (36) or chemicals (20). The proposal was that *ZNF521* and chemicals may exclusively lead U87-GM to neurons. The results indicated that prolonged *ZNF521* expression in a GBM cell line stops cell proliferation and stimulates neuronal gene expression in NSCM culture. It appears that *ZNF521* alone cannot create expandable NSCs from astrocytoma. The conclusion suggests that tuning *ZNF521* expression can directly convert astrocytoma into neurons *in vivo*, bypassing NSC proliferation. It should be mentioned that the lower GFP fluorescence in U87-ZNF521 versus U87-GFP cells reflects both the reduced translational efficiency of IRES-dependent GFP expression in our bicistronic construct and the increased metabolic demands of sustained *ZNF521* overexpression. While this limited our ability to track cells by fluorescence alone, FACS purification ensured only successfully transfected cells were used for transplantation.

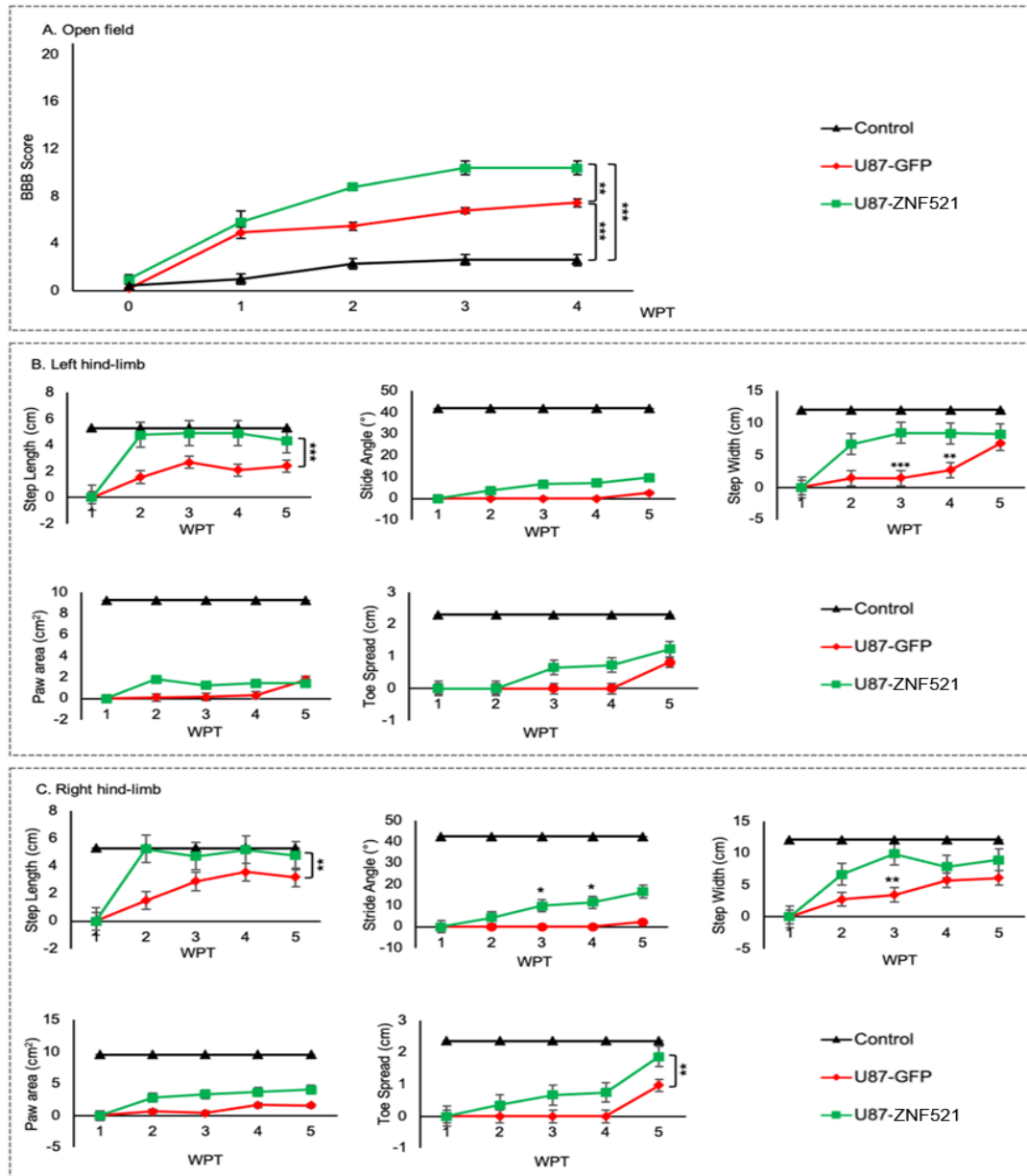


Figure 2. The open-field and footprint assessments. A) The U87-ZNF521 rats showed a significant improvement in coordinating movement, weight support, and hind-limb mobility by reaching almost 12 scales, compared with control ($p < 0.001$) and U87-GFP ($p < 0.01$). Although U87-GFP revealed notable ameliorated mobility, they still show less improvement in comparison to U87-ZNF521. B and C) The step length was significantly increased in both the left ($p < 0.001$) and right ($p < 0.01$) rear at 5 WPT compared with the control. The stride angle was improved in the right foot during the third and fourth WPTs ($p < 0.05$). Step width displays remarkable elevation in the left foot at the third ($p < 0.001$) and fourth ($p < 0.01$) WPT, as well as the right foot at the third WPT ($p < 0.01$). Toe spread was substantially larger in the right hind limb in the five WPT ($p < 0.01$). The data were analyzed using two-way ANOVA and Tukey's post-hoc test.

After 5 WPT, we transplanted U87-ZNF521 cells into SCI rats to study the ZNF521 effects, the NSCM, and U87-GM *in vivo* function. We wanted to know if U87, a GBM cell line, still has a beneficial secretome and affects the CNS. The GBM secretome's controversial neuroprotective (37) and tumorigenic (38) effects have long been studied, prompting the question.

Compared to U87-GFP, U87-ZNF521 moderately improves BBB scores and gait. ZNF521 overexpression in glial scars *in vivo* was found to improve SCI more in our previous report (24), but our findings here suggest that U87-ZNF521 also improved rat movement. This study also compared ZNF521's effects on U87, the most severely mutated cell line, and glial scar astrocytes

using the SCI model. Notably, U87-GFP-transplanted rats showed moderate locomotor improvement over SCI-only controls, though less robust than U87-ZNF521. This observation may reflect: (1) U87MG secretes neuroprotective factors that could transiently modulate the injury microenvironment, dampen inflammation, or promote endogenous repair mechanisms *in vivo* (37, 38), and (2) the NSCM used for both U87-GFP and U87-ZNF521 cells contains growth factors that might have been carried over during transplantation, potentially influencing host tissue responses.

Generally, the ability of *ZNF521* to reprogram U87MG glioblastoma cells into neural-like cells—evidenced by morphological aggregation and significant upregulation of neuronal and oligodendrocytic markers—coupled with the improved motor function in SCI rats, suggests two promising clinical applications. First, *ZNF521*-mediated reprogramming could serve as an adjuvant therapy for glioblastoma by converting residual tumor cells into post-mitotic neural cells at resection margins. Second, the functional recovery supports the potential of *ZNF521*-modified neural cells for repairing SCI.

4.1. Pleiotropic roles of *ZNF521* across cellular contexts

ZNF521 has been studied in various cancers for its tumorigenicity, showing diverse roles. The SHH pathway in medulloblastoma shows a complex regulatory network where *ZNF521* cooperatively interacts with GLI family zinc finger 1 (GLI1) and GLI2 proteins to stimulate SHH target genes. This interaction, especially with the nucleosome remodeling and deacetylase complex, makes *ZNF521* tumorigenic in medulloblastoma (5). A second study found that *ZNF521* promotes cell growth, clonogenicity, and migration in medulloblastoma subgroups, particularly SHH and Group 4 (8).

In contrast to hepatocellular carcinoma (HCC), *ZNF521* demonstrated an inhibitory

effect on tumor growth. Low *ZNF521* expression is associated with larger HCC tumors, advanced stage, and poor prognosis. *In vitro* and *in vivo* functional assays showed that *ZNF521* inhibited HCC cell proliferation, colony formation, and apoptosis. These effects were linked to RUNX family transcription factor 2 (*RUNX2*) transcriptional activity and AKT phosphorylation regulation (41).

ZNF521 was studied in B-cell biology and leukemia for its evolutionary, regulatory, and functional properties. A gene duplication event caused the transcriptional regulators Spi-1 proto-oncogene (*SPI1*) and homeobox C13 (*HOXC13*) to cooperate to increase *ZNF521*. The transgenic mice and cyclin D1 cells experiment showed their cooperative regulation of B-cell biology, suggesting leukemia implications (42).

4.2. Comparative mechanisms of neural reprogramming

Wei et al.'s study on amniotic fluid stem cells (AFSCs) and this study on *ZNF521* overexpression in U87MG glioma cells have intriguing parallels and potential regenerative medicine implications. Wei et al. identified mesenchymal and pluripotent stem cell surface markers, neural differentiation potential, and *SOX9* as a neurogenesis predictor in AFSCs. This study also found that *ZNF521* overexpression in glioma cells caused morphological changes, aggregate formation, and neurogenic gene expression. Both studies suggest that specific molecular markers—*SOX9* in AFSCs and *ZNF521* in glioma cells—may promote neural characteristics, with *in vivo* experiments suggesting therapeutic benefits for neurological conditions like SCI. These parallel findings emphasize the importance of stem cell biology and gene regulation in advancing regenerative therapies, as well as the need for further research into these markers' functional roles and clinical applications in neurology and regenerative medicine (43).

This aligns with emerging strategies for direct reprogramming of glioblastoma cells,

which is a growing trend that could lead to the worst possible situation. In transcription factor-based gene therapy for GBM, neuronal differentiation 1 (*NEUROD1*) and neurogenin 2 (*NEUROG2*) produce glutamatergic neurons, while achaete-scute family bHLH transcription factor 1 (*ASCL1*) promotes GABAergic fate (33, 39). These approaches can slow glioblastoma growth and convert astrocytes into useful neurons (40). Here, the *ZNF521* transcription factor's prolonged effect on U87-GM gene expression was described briefly. Since *ZNF521*, a neural lineage initiator, enriches early neuronal and oligodendrocyte markers in the most aberrant astrocytoma cell line, it continues to improve SCI recovery.

4.3. Limitation

There is no quantification of U87-*ZNF521* cell neural differentiation and proliferation. The well-established U87MG astrocytoma cell line was used to generate this study's primary cells; however, U87MG is widely known for being highly mutated and genetically divergent from primary GBM cells, which may limit the generalizability of findings beyond this model. While we acknowledge this constraint, U87MG was chosen for its reproducibility, ease of handling, and prior literature precedent—though future studies should validate key results in patient-derived glioma stem cells to strengthen translational relevance. Additionally, U87MG cells have multiple genetic mutations, making it difficult to maintain cellular health and viability during experiments. Maintaining cell adherence and viability over long culture periods made immunocytochemistry and cell counting for data quantification difficult. Conventional statistical analyses did not yield statistically significant results despite multiple attempts. Therefore, we employed RT-PCR to assess gene expression changes following *ZNF521* overexpression, enabling qualitative evaluation of cellular responses. This approach allowed qualitative gene expression alterations to be interpreted, but the study lacks quantitative

data. While RT-PCR analysis was used to assess gene expression, the absence of immunocytochemistry, Western blot, or cell morphology quantification limits the ability to confirm neural conversion at the protein or cellular level; future studies should incorporate these validations to strengthen claims of neural progenitor differentiation.

Second, this study did not directly assess the carcinogenicity of transplanted spinal cord cells, but behavioral assessments suggest that U87-*ZNF521* cells did not behave tumorigenically. There is no evidence that *ZNF521* induces stemness and reprogrammed fate, and transplanted cells are non-tumorigenic *in vivo*. These limitations should be considered when interpreting the valuable findings. These gaps could be filled by future research to better understand *ZNF521*'s role in GBM.

5. Conclusion

This study examined *ZNF521*'s various roles in GBM using the U87MG glioma cell line. Overexpressing *ZNF521* in U87MG cells caused changes in their morphology, leading to aggregation and a shift in cell phenotype. Despite being in a cancerous environment, these cells showed upregulation of neuronal markers, suggesting that *ZNF521* has the potential to activate genes related to neuronal differentiation. When these U87-*ZNF521* cells, which presented neuronal markers, were transplanted into the SCI model, they contributed to improving locomotor function moderately, suggesting their potential for recovery. Our findings indicate that *ZNF521* expression in GBM cells can be manipulated to promote the generation of neuronal and oligodendrocyte-like cells. This study also discussed *ZNF521*'s potential as a neural differentiation marker and its context-dependent effects on tumorigenicity and tumor suppression in various cancers. The complex *ZNF521* functions necessitate further study to utilize it for GBM treatment and neural regeneration.

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Conflicts of interest

The authors declare no conflicts of interest.

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