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Screening the Effects of Gene Overexpression on Markers of Neural Progenitor Differentiation in the Developing Chick Spinal Cord.

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Screening the effects of gene overexpression on markers of neural progenitor differentiation in the developing chick Spinal cord.

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Abstract

A central obstacle in stem cell biology is revealing signaling pathways that drive stem cells to mature into differentiated daughter cells or proliferate into more stem cells (self renewal). Some genes that promote self-renewal also promote some forms of cancer. A subpopulation of cells within solid tumors exhibit stem cell-like properties, including resistance to cell death and exhibiting self-renewal. Reviewing published databases of gene expression profiles for glioblastomas and neural stem cells (NSC), we identified genes shared in both populations, including the gene ZSCAN21. Utilizing the constitutively active intracellular domain of the Notch receptor (NICD) as a positive control and an empty vector as a negative control, the sufficiency of ZSCAN21 to promote self-renewal can be monitored using *in ovo* electroporation of the chick embryonic spinal cord. Immunohistochemistry and comparative anatomical analysis is then used to screen for markers in differentiated neurons (NeuN) and glia (GFAP) as well as markers for progenitor cells (Sox2, Oct4). If ZSCAN21 sufficiently promotes self-renewal, overexpression of the gene should elevate markers for progenitor cells, possibly at the expense of markers for differentiated cells.

Introduction

When a neural stem cell (NSC) undergoes proliferation, daughter cells have the potential to differentiate into neural or glial progenitors or go through a process of self-renewal to form more NSC. Stem cell self-renewal is the process where a stem cell divides symmetrically or

asymmetrically to produce at least one daughter cell that maintains multipotency of the mother cell. This process of self-renewal is critical for maintaining the NSC pool during development and is influenced by many different intrinsic and extrinsic cell signals. Self-renewal often proceeds by inhibiting the expression or function of lineage specific genes that lead to differentiation (He et al, 2011). The Notch receptor family is a group of heteroligomeric single pass transmembrane proteins that have been shown be involved in the determination of stem cell fate. These receptors can influence the processes proliferation, differentiation, and apoptosis in a positive or negative manner. (Purow et al, 2005). Signaling pathways such as the Notch signaling pathway have been shown to drive stem cell self-renewal and are highly expressed in NSC populations (Taylor et al, 2007). Previous studies have identified many different genes that are highly enriched in NSC lines through microarray analysis (Rahmalho-Santos et al, 2002). The function of many of these genes on the process of self-renewal or differentiation has not been previously studied. Interestingly, many genes that are important for driving this self-renewal process are also present in different forms of cancer (Singh et al, 2004). For example, the polycomb family repressor Bmi-1 has been shown to be critical for self-renewal in adult stem cells as well as the proliferative ability of many different forms of cancer. (Molofsky et al, 2005). The ability for these cancer cells to undergo this self-renewal and proliferative process has been shown to occur in a subset of cells within solid-state tumors. These subset populations of cells have been dubbed “cancer stem cells” (CSC) and have been monitored by looking for the surface antigen CD133+ (Singh et al, 2005). Thus, genes that are selectively or highly expressed in both NSC and tumors may be responsible for giving CSC stem cell like properties.

Glioblastoma multiforme is the most common primary brain tumor in adults (Haar et al, 2011). Microarray analysis has identified gene expression profiles in glioblastomas as well as NSC (Rahmalho-Santos et al, 2002; Cancer Genome Atlas Research Network, 2008), but many shared genes have not been tested for their effect in NSC. Testing the sufficiency for genes to promote NSC self renewal that are present in both glioblastomas and NSC populations could not only shed light on the mechanisms of SC self-renewal, but also may offer insight on the mechanisms cause the tumor formation. Of the genes that satisfied these criteria, we chose ZSCAN21 because its expression is upregulated NSC populations and glioblastomas.

The balance between NSC differentiation and self-renewal is shown in Figure 1. Our primary goal is to determine whether overexpression of genes that are highly expressed in NSC will promote a stem cell lineage. To test the hypothesis that a NSC specific gene, such as ZSCAN21, will promote self renewal at the expense of differentiation, we will overexpress the gene in the developing chick neural tube with a bicistronic vector that allows visualization of cells that express our gene of interest with the reporter gene EGFP. The behavior of NSCs that overexpress a putative self-renewal gene can be monitored by looking for specific lineage markers expressed by the daughter cells. If a NSC specific gene promotes self-renewal at the expense of differentiation, we would find an increase in the number stem cells that are identified by markers such as Sox2 and a decrease in the number of differentiated progenitor cells (Figure 1B). If our hypothesis is incorrect, we could observe an expansion of stem cell and other cell lineages, then we would not be able to discriminate if the gene promoted proliferation and cell survival of multiple lineages, or

transiently promoted self-renewal in NSC before permitting the cell to differentiate (Figure 1C).

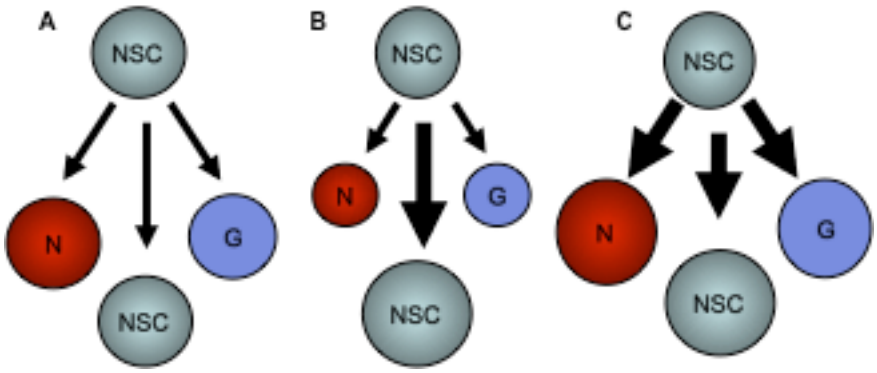

Figure 1: Mechanism of neural stem cell differentiation and self renewal. A) Normal balance of differentiation and self-renewal. A mother neural stem cell ("NSC" light gray;) can proliferate into multiple different lineages, including neurons ("N", red), glia ("G" light blue, or more neural stem cells. B) Promotion of stem cell self renewal at the expense of differentiation. More NSC are being produced while less neurons and glia are present after overexpression C) Non-specific promotion of multiple lineages. Proliferation increases when gene is overexpressed causing increased numbers of neurons, glia, and NSC.

Materials and Methods

Chickens

Fertilized White Leghorn chicken eggs were obtained from the Michigan State University Poultry Research and Teaching Center in Lansing, Michigan. The eggs stored at 13 ˚C; when they were needed for an experiment they were brought to room temperature and then incubated at 38°C. The eggs were not allowed to develop longer than 6 days, which is far before hatching.

In Ovo Electroporation

To determine if our gene of interest was sufficient to drive stem cell self-renewal in the embryonic spinal cord, the gene was expressed by an in ovo electroporation screening method. The gene was previously screen from a mouse cDNA library and inserted into a bicistronic pCIG vector. In this plasmid, there is a chick beta actin promoter upstream of the gene of interest; downstream of the gene of interest there is an internal ribosomal entry sequence (IRES) that is attached to the enhanced green florescent protein (EGFP) sequence. Therefore, where ever our gene of interest has been transfected EGFP will be expressed and the cell will fluoresce green. The embryos were allowed to develop to Hamburger Hamilton stage 10, which is about 40 hours. Then, using a glass capillary micropipette, was injected into the neural tube of the developing embryo. Then a 21 volt current was applied across the embryo, the negative DNA is drawn towards the positive cathode. This pulls the DNA towards the cathode, into neural tube cells on that side of the embryo, transfecting them with the DNA. Then the embryos were allowed to develop for an additional 72 or 96 hours.

Tissue Preparation

The embryo was harvested after the incubation period; its tissues were fixed for 45 minutes in 4% paraformaldehyde at 4 °C, washed in 0.1M phosphate buffered saline (PBS), and cryoprotected overnight at 4 °C in 15% sucrose. The tissues were then mounted in Tissue Tek OCT (VWR, West Chester, PA). They were frozen using liquid nitrogen, and sectioned into 12-micron sections using a Leica cryostat. These sections were then

mounted on glass microscope slides. These slides were stored at -20°C until stained using immunohistochemistry.

Immunohistochemistry

The sections were stained with primary antibodies against the floor plate marker gene the neuron marker gene Nurr1 (Santa Cruz, 1:200), and EGFP (anti-goat EGFP, Abcam, 1:200; anti-rabbit EGFP, Abcam, 1:500) in GSS (1% goat serum, 0.1% Triton 100X, and 0.1M sodium phosphate buffer pH 7.0) overnight at 4°C. The following day the sections were washed in phosphate buffer and stained with secondary antibodies correlating with the subtype of each primary antibody (1:1000; Nurr1 with goat anti-rabbit Cy3, Jackson Laboratories, 1:200; anti-goat EGFP with donkey anti-goat FITC, Jackson Laboratories, 1:500; anti-rabbit EGFP with goat anti-rabbit FITC, Jackson Laboratories, 1:500) and counterstained with DAPI to visualize the nuclei of individual cells.

Selection of candidate genes:

We wanted to find a gene that fit our selection criteria of being highly enriched in NSC populations and upregulated in glioblastomas. To start, we looked at a microarray analysis of genes selectively expressed in NSC populations (A total of 2459 were identified by the microarray analysis) (Santos). From those we narrowed down genes that specifically affected transcription by having predicted DNA binding motif domains in their protein structure (159 genes fit this criteria). Genes were then chosen that could be easily cloned in our pCig vector (<3kb), were not previously characterized in stem cells, and known to be expressed in the nervous system (27 genes were expressed in the nervous system). Of

those genes, we also wanted genes that were altered in >5% of the reported cases of cancers (alteration included mutation, deletion, or amplification)(12 genes were altered) (Cancer Genome Atlas Research Network, 2008; cbioportal.org). Finally, we wanted genes that were altered in glioblastomas in >1% of reported cases (3 genes fit all previous criteria). We selected ZSCAN21 as our candidate gene because it was not only enriched in NSC lines (1.71x), but also was amplified in 2.1% of glioblastoma cases. (Cancer Genome Atlas Research Network, 2008; cbioportal.org)

Polymerase Chain Reaction, cloning and transformation

Primers for ZSCAN21 PCR were as follows:

F: 5' AGATCTTTACTCCACTTCTCCCTC 3'

R: 5' GCCACCATGACTAAGGTGGTGGGC 3'

These primers contained a kozak sequence and a BglII restriction site on the forward primer

PCR conditions were: Syzygy Taq (Grand Rapids, MI) , 94 degrees for 30 seconds, 55 degrees C for 30 seconds, 72 degrees for 90 seconds. Cycled 30 times.

PCR product was isolated on a 1% gel and generated a 1.6 kb fragment

Fragment was purified using Gelclean kit (Qiagen, Venlo, Netherlands), re-suspended and ligated into the pGMTEZ vector—(Promega, Carlsbad, CA), and transformed using NEB DH5alpha supercompetent cells (NEB, Ipswich, Massachusetts) and plated on LB ampicillin plates (100ug/ml) overnight with X-gal and IPTG for blue white selection. Selected white colonies, indicating insert was present, and grew in 3ml LB Ampicillin (100ug/ml) culture

overnight. Cultures were miniprepped (Qiagen, Venlo, Netherlands) and submitted for sequencing.

Results

The results of the overexpression of NICD (positive control) and our empty vector (negative control) are shown in Figure 2.

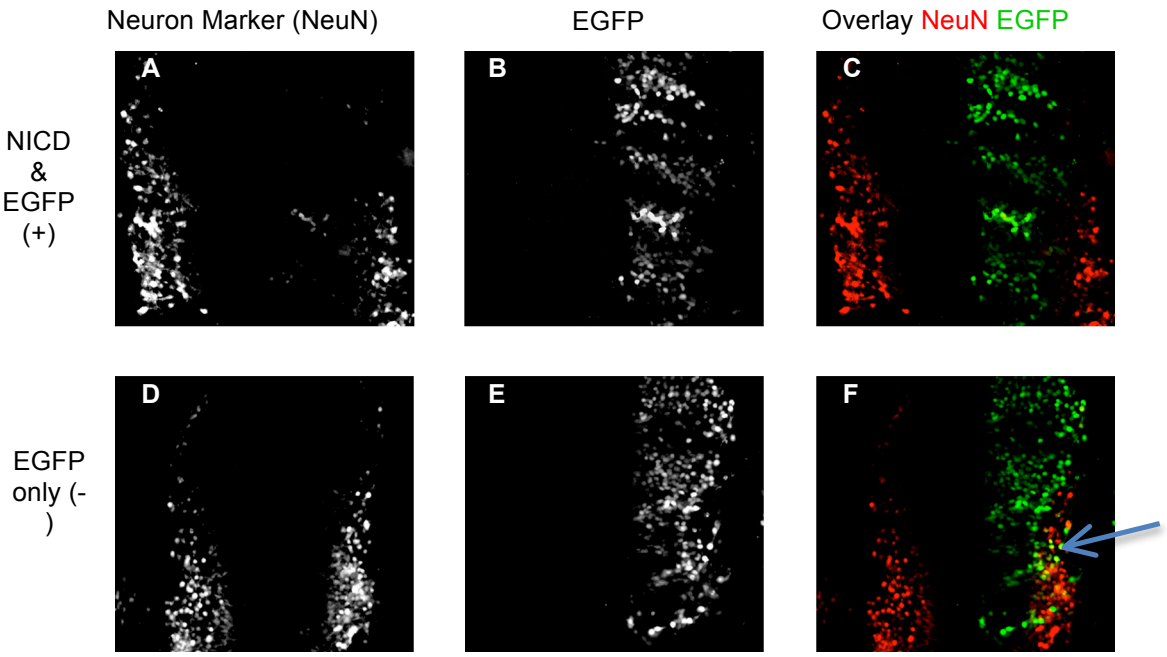

Figure 2: NICD inhibits neuronal differentiation. The antibody for NeuN (red in overlay) identifies cells that have differentiated into neurons. The antibody for EGFP (green in overlay) identifies cells that expressed NICD & EGFP, or just EGFP alone. Arrow indicates cells that express EGFP and NeuN (yellow in overlay), which is not seen in "NICD & EGFP" condition. The top row (A, B, C) are sections of our positive control showing expression of NICD (EGFP+). The bottom row (D, E, F) are our negative control sections showing expression of the empty vector (EGFP+).

As seen in figure 2, on the electroporated side that expresses NICD, less NeuN marker expression is present compared to the unelectroporated side. Quantitatively, there is a 68.3% decrease in the number of neuronal markers on the electroporated side with NICD

compared to the unelectroporated side. There was also no overlap of expression between cells that expressed exogenous NICD (EGFP+) and NeuN. This means that where NICD expressed (EGFP+) inhibited the NSC ability to differentiate into neurons. On the other hand, our empty vector shows no significant different difference in the amount of neural markers on the electroporated side compared to the unelectroporated side (only a 12% increase). There was also overlap between cells that expressed the empty vector (EGFP+) and NeuN. This indicates that our empty pCIG vector did not significantly affect the neuronal differentiation process.

In addition to establishing our controls, we began work on our gene of interest, ZSCAN21. We saw from the Mouse Genome Institute that there was expression of ZSCAN21 in the spinal cord, including the ventricular zone (area around the ventricle where neural progenitors are abundant) (figure 3). This demonstrated that our gene was in fact expressed in the nervous system and qualified for sufficiency testing in self-renewal. (Gray et al, 2004)

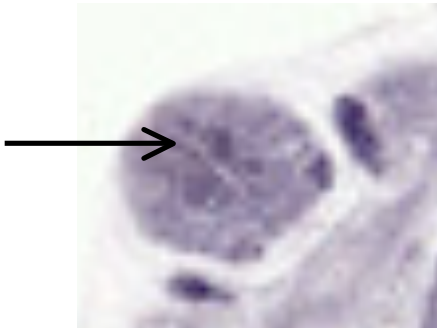

Figure 3: ZSCAN21 expression visualized through in situ hybridization of TS21 mouse embryo (Gray et al, 2008). There is dark purple (expression) near the ventricular zone of the spinal cord (black arrow). This shows that ZSCAN21 is expressed in the nervous system.

After confirming that ZSCAN21 was expressed in the nervous system, we started the process of cloning it into a chick expression vector (pCIG). We used PCR to amplify ZSCAN21. In order to ligate the PCR fragment into our expression vector, we subcloned the gene into pGMTEZ vector, which can easily be ligated to PCR amplified products by a modification made by Taq polymerase. These clones were then sequenced with SP6 and T7 primers to determine if there were any errors from mutation or from Taq amplification during PCR (See figure 4). Upon analyzing the sequences, we identified several errors to different clones. To avoid introducing a mutated gene into our pCIG vector, more clones will be made of ZSCAN21 and sequenced in preparation for electroporation.

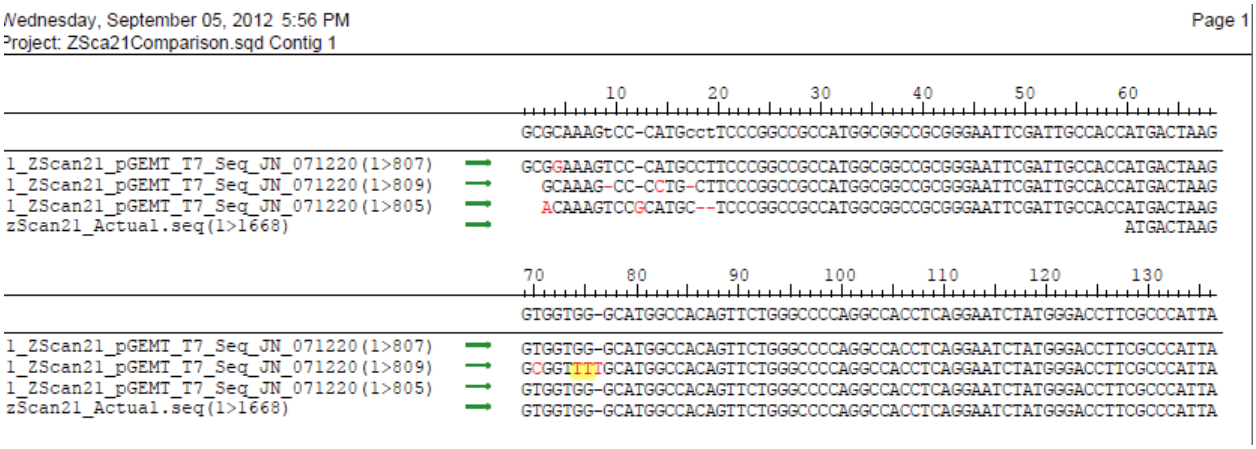

Figure 4: Sequencing data output from ZSCAN21 mouse gene. "ZSCAN21 Actual" is the mouse gene from the National Center for Biotechnology Information (NCBI), and unique clones (such as 1_ZSCAN21_pGMT…) are shown above. Mismatch between actual and clone indicates error introduced by Taq polymerase during PCR amplification (one example indicated in yellow above).

Discussion

ZSCAN21 has been shown through anatomical analysis to be expressed in both the subventricular zone and cerebellar anlage. This provides the argument that ZSCAN21 may be important for proliferation of granular cells of the cerebellum because of its localization

in this zone (Yang et al, 1996). A knockout of the ZSCAN21 gene showed no noticeable impact on the development of the cerebellum in mice. However, overexpression of this gene using a bacterial artificial chromosome, there was an increase in the number of granular cells in the nervous system postnatally (Yang et al, 1999). In both the knockout and overexpression, there was no characterization of the effect on neural stem cells. Although it has been looked at in the cerebellum, no one has confirmed the impact of its expression in other regions of the nervous system. It has also not been characterized in the stem cell population. Additionally the expression of the BAC construct was not monitored in the spinal cord or other neural regions, where other evidence indicates it is expressed. Thus, more careful analysis could yield insight function of ZSCAN21 in NSC. It is unclear whether overexpression in the ventricular zone is sufficient enough to have an effect on NSC in the developing embryo. Since it is present in the ventricular zone (an area rich in NSC), ZSCAN21 is an interesting gene to look at to monitor the effects on self-renewal.

Next Steps

After ZSCAN21 has been successfully cloned into pGMTEZ and sequenced (Figure 4), we will start working on cloning the insert into our pCIG expression vector for use in electroporation. Our cloning strategy is shown in Figure 5. Once we have the insert in pCIG, we can do a large scale DNA isolation of the vector in preparation for electroporation. We will then analyze the sufficiency of ZSCAN21 to promote self-renewal by looking at the same markers we used in our controls (NICD and empty vector) after electroporation. We will also continue to establish our control condition with NICD by doing more

electroporations and antibody staining with markers such as Sox2, GFAP in addition to NeuN.

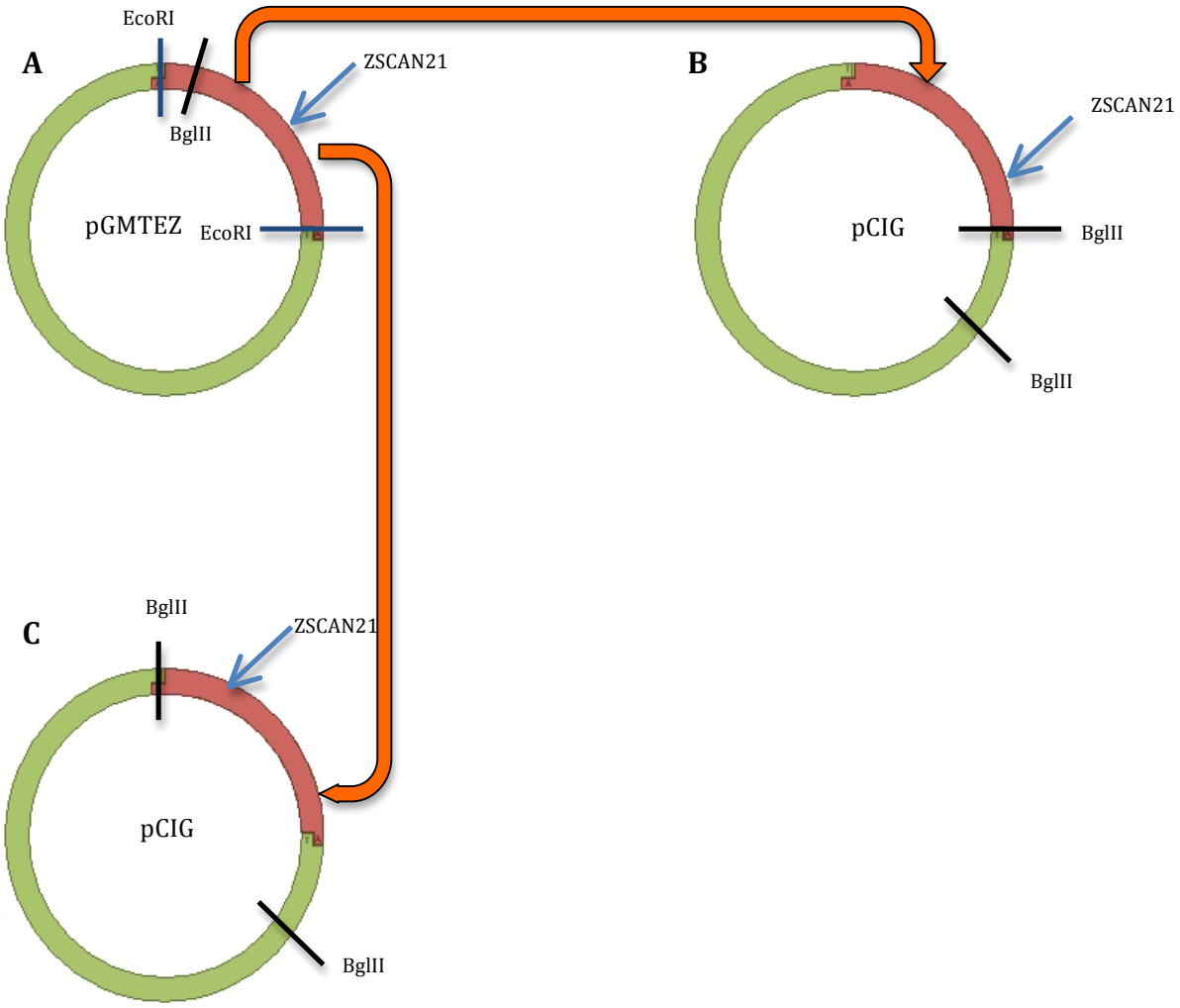

Figure 5: Subcloning strategy for ZSCAN21 into the expression vector pCIG for use in electroporation. A) Shows the ZSCAN21 insert in the pGEM-T vector. EcoRI sites are present on either side of the insert and there is a BglII site upstream of the gene. We will use EcoRI to cut the insert out of pGMTEZ and clone it into the pCIG expression vector. The insert can either go in the correct orientation downstream from the promoter (Figure 5C + orientation) or can be in the reverse orientation (Figure 5 B - orientation). To test whether the insert is in the correct orientation, we will cut with BglII. pCIG has an internal BglII site so we can see what orientation the insert is in when the digests are separated by size. B) If the insert is in the reverse orientation (-), we will see a smaller band of <1.6 kb long. C) If the insert is in the correct orientation (+), we will see a larger band of >1.6 kb long.

References

Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., et al. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer discovery*, *2*(5), 401–404. doi:10.1158/2159-8290.CD-12-0095

Comprehensive genomic characterization defines human glioblastoma genes and core pathways. (2008).*Nature*, *455*(7216), 1061–1068. doi:10.1038/nature07385

Gray, P. A., Fu, H., Luo, P., Zhao, Q., Yu, J., Ferrari, A., Tenzen, T., et al. (2004). Mouse brain organization revealed through direct genome-scale TF expression analysis. *Science (New York, N.Y.)*, *306*(5705), 2255–2257. doi:10.1126/science.1104935

Haar, C. P., Hebbar, P., Wallace, G. C., 4th, Das, A., Vandergrift, W. A., 3rd, Smith, J. A., Giglio, P., et al. (2012). Drug resistance in glioblastoma: a mini review. *Neurochemical research*, *37*(6), 1192–1200. doi:10.1007/s11064-011-0701-1

He, S., Nakada, D., & Morrison, S. J. (2009). Mechanisms of stem cell self-renewal. *Annual review of cell and developmental biology*, *25*, 377–406. doi:10.1146/annurev.cellbio.042308.113248

Molofsky, A. V., He, S., Bydon, M., Morrison, S. J., & Pardal, R. (2005). Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes & development*, *19*(12), 1432–1437. doi:10.1101/gad.1299505

Purow, B. W., Haque, R. M., Noel, M. W., Su, Q., Burdick, M. J., Lee, J., Sundaresan, T., et al. (2005). Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer research*, *65*(6), 2353–2363. doi:10.1158/0008-5472.CAN-04-1890

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C., & Melton, D. A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science (New York, N.Y.)*, *298*(5593), 597–600. doi:10.1126/science.1072530

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., et al. (2004). Identification of human brain tumour initiating cells. *Nature*, *432*(7015), 396–401. doi:10.1038/nature03128

Taylor, M. K., Yeager, K., & Morrison, S. J. (2007). Physiological Notch signaling promotes gliogenesis in the developing peripheral and central nervous systems. *Development (Cambridge, England)*, *134*(13), 2435–2447. doi:10.1242/dev.005520

Yang, X. W., Wynder, C., Doughty, M. L., & Heintz, N. (1999). BAC-mediated gene-dosage analysis reveals a role for Zipro1 (Ru49/Zfp38) in progenitor cell proliferation in cerebellum and skin. *Nature genetics*, *22*(4), 327–335. doi:10.1038/11896

Yang, X. W., Zhong, R., & Heintz, N. (1996). Granule cell specification in the developing mouse brain as defined by expression of the zinc finger transcription factor RU49. *Development (Cambridge, England)*, *122*(2), 555–566.