Understanding the Impact of Sox2, Notch1, Noggin, Shh, and BMP genes on the neurogenesis in *Mus musculus*

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Abstract:

Most vertebrates and mammals, except for the African Spiny Mouse, cannot regenerate neural tissue. The African Spiny Mouse has an overexpression of Sox2, Notch1, Noggin, and Shh genes and an underexpression of BMP genes. In damaged neural tissue, the gene expression changes lead to macrophage concentration variations, leading to increased quantities of therapeutic proteins and growth factors such as Interleukin 10 and Transforming Growth Factor Beta (TGF-B), ultimately leading to neurogenesis. The question arises of whether or not the editing of these genes in injured normal house mice could lead to the regeneration of neurons in those mice. In order to conduct the experiments, African spiny mice and house mice will be given similar brain damage. Using adipose tissue and Yamanaka factors, induced pluripotent stem cells were created. The iPSCs were modified to express and under-express genes involved in neuronal regeneration. The modified iPSCs were then injected into the experimental group. After four weeks, cognitive function tests will be performed to determine if neurogenesis occurred. The mice's brains would be extracted to see how the brain physiology had changed from the treatment. If neurogenesis is found, the next step would be to see what other animals can regenerate neurons using this therapeutic technique.

Introduction:

Neural tissue damage leads to communication deficiencies between the brain and the body, leading to muscle movement problems, loss of feeling, memory, emotions, and reasoning issues. Neural tissue damage can completely alter one's way of life. According to the NIH, 55 million people suffer from neural tissue damage [1] (NIH, 2022).

Unlike other tissue types, neural tissue struggles to regenerate and repair damage independently [2] (Zeng & Zhang, 2023). However, some tissue types can regenerate very quickly. In some organisms, such as humans and other mammals, a wound epidermis, a layer of tissue that heals exposed damage, is formed when damage is done to tissues. Stem cells are activated to arrive at the wound epidermis and create a blastema, a mass of stem cells that proliferate to form the tissue that needs to be regrown [3] (Prince et al., 2005; Scimone et al., 2022). The behavior of the stem cells depends on the location of the blastema. This is due to the microenvironment; stem cells behave and migrate differently depending on the signals in their environment, which changes with different bodily locations.

Monopotent stem cells are often found in the blastema, migrating towards the site of damage [4] (McCusker et al., 2015). The stem cells that migrate towards the blastema are between a state of differentiation and undifferentiation because they originate from cells with a "positional memory" [5] (Tamura et al., 2009). While they do not contain any organelles from their past identities, they do have an awareness of where in the body they were located, thus

allowing them to migrate to the blastemas where they were closely situated (Tamura et al., 2009).

Once the wound epidermis and blastema are formed, cells and various signaling pathways spur regeneration. One of the most critical pathways is the movement of macrophages during tissue regeneration [6] (Chen et al., 2023). Macrophages first eliminate the dead cells [7] (Kim & Nair, 2019). Then, the macrophages help signal stem cells to come to the site of repair and spur proliferation and differentiation [8] (Wynn & Vannella, 2016).

Animals that cannot regenerate tissue form scar tissue. Scar tissue is a fibrous material composed largely of collagen [9] (National Cancer Institute, 2011). However, scar tissue blocks the migration of stem cells and the formation of the blastema, thus preventing tissue regeneration.

Vertebrates struggle with regeneration. This is primarily due to a more advanced immune system [10] (Aztekin & Storer, 2022). A more robust immune system has more fibroblasts because these cells express immune-related genes and work with immune cells [11] (Zhao et al., 2016; Salminen et al., 2024). Fibroblasts are cells that lead to the creation of connective tissue that creates scars [12] (Sidransky, 2022; Ma et al., 2023). With a weaker immune system, scar tissue is not created, thus leading to tissue regeneration.

The African Spiny Mouse can regenerate organs and tissues, unlike most mammals and vertebrates. This ability provides an excellent advantage as it can restore tissues from skin to nervous to skeletal muscle [13] (Gaire et al., 2021). While humans can regenerate skin and skeletal muscle to an extent, neuronal regeneration is something humans lack [14] (Liu et al., 2018). While humans struggle with neural diseases such as Parkinson's and Amyotrophic Lateral Sclerosis, African Spiny Mice do not have these issues because of their neuronal regeneration capabilities [15] (Offord, 2024).

The overexpression and underexpression of genes correlate with African Spiny Mouse neurogenesis. The genes Sox2, Notch1, Noggin, and Shh are [16] (Kim & Nair, 2019) overexpressed during neuron regeneration. The gene BMP is underexpressed [17] (Maden et al., 2021). The changes in gene expression lead to increases in macrophage concentrations at the regeneration site [18] (Okamura et al., 2023). These changes lead to the expression of proteins and growth factors that assist with stem cell proliferation, leading to tissue regeneration [19] (Kim & Nair, 2019). One of the proteins expressed by the macrophages is Interleukin-10 (IL-10) [20] (Kim & Nair, 2019). IL-10 prevents scar tissue formation [21] (Christos Patilas et al., 2024). Another growth factor expressed by the macrophages is the Transforming Growth Factor Beta (TGF-B). TGF-B is a growth factor essential to neural tissue regeneration. TGF-B is vital in tissue repair because it promotes the differentiation and proliferation of stem cells in the blastema and lowers scar tissue formation [22] (Lichtman et al., 2016). While other mammals and vertebrates have these genes, they do not express them as much, thus creating a lower macrophage activity and preventing the formation of IL-10 and TGF-B [23] (Tomasso et al., 2024).

These genes are confirmed to influence the regenerative capabilities of African Spiny Mouse. However, it is unknown if these genes could potentially induce regenerative capabilities in organisms that do not have any. Therefore, the question arises of whether altering the gene expression in other organisms to mirror that of the African Spiny Mouse will lead to tissue regeneration. To see if these genes can create regenerative capabilities across vertebrates and mammals, it is necessary to conduct experimentations in any organism that fits either of these characteristics. However, it is first necessary to see if transplanting these regenerative genes

will create regeneration in normal house mice (Mus musculus). Induced Pluripotent Stem Cells are a possible way to introduce gene expression that can correlate with neuronal regeneration. iPSCs can be edited to contain and lack the appropriate genes needed for neuronal regeneration. CRISPR-Cas9 technology can be used for gene editing. The iPSCs can then be implanted into the brain in which they will be differentiated into neurons. Then, these neurons will express the genes needed to potentially create neurogenesis. Neurogenesis can be measured by quantifying the neurons. The goal of the proposed experiment is to see if the over- and underexpression of Sox2, Notch1, Noggin, Shh, and BMP leads to neurogenesis in house mice. The following experimental procedure will be used to test this theory.

Experimental Design:

The main goal of this proposal is to propose the use of induced pluripotent stem cells to introduce genes that correlate with neurogenesis in the African Spiny Mouse into the common house mouse (*Mus musculus)* to see if they lead to neurogenesis after injury. To start, a culture of induced pluripotent stem cells will be generated from adipose (fat) cells. Adipose tissue is used as the source because it is less invasive; a simple biopsy can be used to access the cells [24] (Yi et al., 2017). Yamanaka factors, consisting of Oct4, Sox2, Klf4, and c-myc, will be introduced using a viral vector to reprogram the somatic adipose cells into iPSCs. Retrovirus, which can integrate genes into a genome in a stable fashion, will be modified using CRISPR to integrate the needed genes into the iPSCs.

To see if the cells are truly pluripotent, they will be stained for the cell surface markers alkaline phosphatase and LIN28. CRISPR-Cas9 will be used to introduce Sox2, Notch1, Noggin, and Shh and eliminate the BMP gene. To confirm that the CRISPR-Cas9 worked, an ELISA (Enzyme-Linked Immunosorbent Assay) will be used. Once the CRISPR-Cas9 is verified functional, the modified iPSCs can be injected into the brains of mice. Immunofluorescence and histology will be used to track proliferation and differentiation. We will conduct memory and behavioral tests to see if neurogenesis has occurred and to see how the overall brain function has been affected by this regenerative treatment.

Methods:

MICE:

Seven African-Spiny mice bred at the University of Florida and 21 mus (Jackson Laboratory) will be used [25] (Janak Gaire et al., 2023). This proposal has received approval from the Animal Care and Use Committee. The Mus will be divided into three groups of seven. One group of seven will be given brain damage without any treatment. The second group of seven will not be harmed. The third group of seven will be given the same brain damage as group 1 but will be given the regenerative treatment.

iPSC Formation:

All 21 of the mus will undergo a biopsy. Adipose tissue between the thigh and the abdomen will be taken [26] (Cyagen, 2017). The adipose cells will be reprogrammed into iPSCs using Yamanaka Factors (Thermo-Fisher Scientific) [27] (*Stem Cell Reprogramming Tools | Thermo Fisher Scientific—UK*, 2024). The Yamanaka Factors that will be introduced to create iPSCs are SOX2, OCT4, and c-MYC. The method to develop iPSCs is derived from Zapata-Lineras et al. [28] (2016). Adipose cells will be infected with the Vesicular stomatitis virus G glycoprotein-coated Moloney Murine Leukemia Virus retroviral vector (VSVG-MMLV retroviral

vectors). The vectors will have the SOX2, OCT4, c-MYC, and KLF4 Yamanaka factors. The VSVG coating is a protein that makes it easier for the retrovirus to enter and attach to a host cell [29] (Yoon et al., 2015). The MMLV virus allows the Yamanaka Factors to better integrate with the cell genome [30] (Wolfgang & Golos, 2002). Retroviruses are used because they can stably introduce the genes into the cell. After three days, the cells will be moved to the Mouse Embryonic Fibroblasts (MEF) feeder layer. The MEF feeder layer provides an extracellular matrix that provides a scaffold for attachment and signals for proliferation. The most important signal is the Mitogen-Activated Protein Kinase (MPAKs) because it spurs signaling pathways leading to proliferation [31] (Cargnello & Roux, 2011). The cells are cultured in DMEM and supplemented with non-essential amino acids, glutamine, penicillin-streptomycin, b-mercaptoethanol, and bFGF. Valproic Acid is a histone deacetylase inhibitor that increases reprogramming efficiency. Cells will be stained for Alkaline Phosphatase and LIN28 to confirm pluripotency, and PCR will be conducted to see if the genes have been incorporated.

Alkaline Phosphatase and PCR Test

To measure for the Alkaline Phosphatase surface marker, a Molecular Probes Alkaline Phosphatase Live Stain Kit (ThermoFisher) will be used. When the Alkaline Phosphatase substrate is added to the derived iPSCs, the cells will have green fluorescence if they are found to be iPSCs. The substrate looks explicitly for the SSEA4, TRA-1-60, and TRA-1-80 proteins found with alkaline phosphatase. After 2 hours, the fluorescence leaves the cells with no damage. If a green fluorescence is found, that means the alkaline phosphatase is present, and therefore the cells are pluripotent.

The PCR process is also essential for determining whether iPSCs were made. PCR is the amplification of DNA sequences to determine their presence. The following PCR procedure is that of Sekine et al. [32] (2020). The following genes were targeted in PCR as these genes determine whether or not the cell is an iPSC: LIN28A, Sox2, MT1G, USP44, ESRG, SPP1, PTPRZ1, CRABP1, CNMD, THy1, VSNL1, HHLA1, and SFRP1 [33] (Sekine et al., 2020). The RNA for these genes will be extracted using a Qiagen RNeasy kit. For the PCR test, RNA will be isolated using a TRIzol reagent (Thermo Fischer), and its concentration will be determined using a NanoDrop Spectrophotometer (Thermo Fischer). The RNA is then converted to DNA using the PrimeScript Reverse Transcriptase reagent kit (Takara). Primer sequences will be determined depending on the type of gene, and the sequence will be sent to AdGene for synthesis.

CRISPR-Cas9

CRISPR-Cas9 will be used to edit Sox2, Notch1, Noggin, and Shh into the iPSC genome and reduce the expression of BMP. The techniques used are those by Asmamaw and Zawdie [34] (2021). The part of the genome to target for editing depends on the gene being edited. BLAST (Basic Logical Alignment Search Tool) can be used to find a sequence in the genome that can potentially host the genes needed to be edited. The genome for each needed gene will be entered in BLAST to find a location to use CRISPR-Cas9 [35] (NCBI, 2024). Once BLAST produces the target sequence, the guide RNA (gRNA) will be created. The guide RNA will guide the CRISPR-Cas9 package to the part that needs to be edited. Biotech company Origene creates plasmid vectors with gRNA and CRISPR-Cas9 components. The plasmid vector will then combine with the membrane of the cells and introduce the gRNA and CRISPR-Cas9 complex into the cell. Therefore, the plasmid vectors must only be added to the same culture as the iPSC cells; they do not need to be manually inserted. Once the gRNA is produced, the

gRNA will lead the Cas9 to the target genome. At this point, the Cas9 will snip the target gene to allow for editing. Then, the CRISPR component will add or delete the gene of interest. The CRISPR-Cas9 will then reattach the genome pieces.

ELISA

To determine whether the cells are truly pluripotent, an ELISA will be done to test for the presence of the pluripotency markers Shh, Sox2, Notch1, Noggin, and the absence of BMP. The antibodies used for the ELISA will be the Anti-Shh antibody, Sox2 Polyclonal antibody, Anti-Notch1 antibody, Anti-Noggin antibody, and the Anti-BMP-2 antibody (all from Thermo Fischer Scientific). Each specific antibody will be placed in wells on a plate with the modified iPSCs. Then, a solution with the gene corresponding to the antibody will be added to the plate, and the genes will bind to the antibodies. Then, the plate will be washed so that all remaining antibodies are attached to the genes. Then, a fluorescent antibody will be added to the gene-antibody complex to track the presence of the genes. Each gene will have a different fluorescent antibody; however, the type of antibody does not matter as long as it has fluorescence. In this case, Alexa Fluor, Alexa Fluor Plus, Alexa Fluor 350, and Alexa Fluor 488 will be used for Shh, Sox2, Notch1, Noggin, and BMP, respectively. These antibodies can be purchased from Thermo Fischer Scientific. If fluorescence of Shh, Sox2, Notch1, and Noggin markers are present after the ELISA, then CRISPR-Cas9 was effective.

Cell culture

A PIPAAm (Poly(N-isopropylacrylamide) temperature-sensitive culture will be used to preserve the modified iPSCs. TAt temperatures of 37°C or higher, the culture is hydrophobic, and cells adhere to the petri dish. However, at temperatures of 20°C or lower, the cell forms a hydration layer, which puts a distance between the cells and the petri dish. This allows for the removal of the cells without damage.

Insertion of modified iPSCs into the damaged brain:

Before inserting the iPSCs, the brain will be injured by creating a brain seizure in the cerebellum for all 21 of the mus and 4 of the African Spiny Mouse. An electroencephalogram will be used to monitor the brain during the injury session. Every mouse will be given a single dose of PTZ (pentylenetetrazole) to create a seizure in the cerebellum [36] (Van Erum et al., 2019). The rat will be susceptible to twitching.

After the cerebellum is damaged, the modified iPSCs will be injected into the damaged part of the brain. The cells will be injected in a fluid with a shear-thinning hydrogel. The shear-thinning hydrogel has extracellular matrix components that will help the cells with differentiation and integration [37] (Kaiser, 2024). The iPSCs will only be injected into one of the three groups of seven. All 21 mus will be allowed to live for 21 days to determine the effects of the iPSCs.

Immunohistochemistry

Immunohistochemistry can also be used to track the proliferation and differentiation of the iPSCs within the brain. Immunohistochemistry is the process of using antibodies with antigens to identify and track the antigen. In this case, Stage Specific Embryonic Antigen-1 monoclonal

antibody (SSEA-1) is used as a fluorescent antibody to track the iPSCs [38] (Maden et al., 2021). This antibody will bind to the iPSCs and be used to track if the cells differentiated into neurons during the 4-week proliferation period. If the cells did differentiate, there would be more neurons, meaning there was neurogenesis. Using fluorescent microscopy, if a stronger glow is present over the 4 weeks, that means the iPSCs proliferated and differentiated into neurons.

Behavioral testing

After 4 weeks, the brains will be surgically removed for analysis. Before removing the brains, two behavioral tests will occur. The first is the Morris Water Maze test [39] (Vorhees & Williams, 2006). In this test, the mice will try to navigate their way through mazes filled with water. Their behavior in the maze, such as the time taken to complete the maze, will be analyzed to understand the effect of the treatment on the memory of the mice. It is essential for the researchers to also look at how the mouse moves to see how cerebellum function was affected by the treatment. The Open Field Test is another test that will be analyzed to understand the movement of the mouse. The mouse is placed in a circle, and it has to move around and try to leave the circle. The path and time taken by the mouse will be analyzed. It is important to have the mice go through these behavioral tests before brain damage to give a starting point to compare the data.

Then, the mouse will be euthanized, and its brains will be surgically removed. The brains will be scanned for SSEA-1 monoclonal antibodies using immunofluorescence microscopy. If there is an increase of these antibodies compared to before the injection of iPSCs, proliferation has occurred. It is important to note that all the mice, including the spiny mice, must go through these tests to evaluate control versus experimental groups.

Discussion:

In terms of the behavioral tests, if the mice's scores on the behavioral test are similar to the scores before the damage, then the regeneration of tissue occurred, and transplanting genes has proven to be a successful method of inducing regeneration in mammals. The African Spiny Mouse should be able to easily move through the maze and escape the circle because it already has regeneration capabilities in the cerebellum. If the house mice also move with ease, that means that there is neural tissue regrowth in the cerebellum.

If the treated iPSCs have differentiated into neurons, neural tissue regeneration has occurred. Conversely, if they remain iPSCs, that means the gene transplantation was not enough to cause neural tissue regeneration in the house mice. The genes are still functional, it is just proof that there is more to the regeneration capabilities than just the genes. This relates to the immunohistochemistry test in that if it is found that the neurons have increased from the damaged brain, then regeneration has occurred. In order to compare neuron quantities, an extra injured house mouse will be needed. The neuronal count of the regenerated house mice will be compared to the injured brain. If the house mice with therapy's count is bigger than the non-treated house mice, then the regeneration capacity will be achievable with gene transplantation. It is safe to assume that the genes caused any changes in neuron proliferation because neurons typically do not divide once they are matured in the brain.

If the results show that no regeneration occurred, that means another factor exists in Spiny Mouse that causes regeneration. This would require more research into the Spiny Mouse regeneration capabilities to understand. In this case, another experiment should be done in

which another factor of regeneration is changed in the house mice to see if that induces neurogenesis.

If the results show that neural regeneration did happen, then this is groundbreaking. This proves that genes can be altered in similar organisms to give neural regeneration properties. This offers hope for the treatment of Alzheimer's disease, ALS, and many other diseases.

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