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Neuroglobin and its Role in the Recovery of Neuronal Cells in Hypoxic Conditions Using Hypoxia Inducible Factor– 1

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NEUROGLOBIN AND ITS ROLE IN THE RECOVERY OF NEURONAL CELLS IN HYPOXIC CONDITIONS USING HYPOXIA INDUCIBLE FACTOR– 1

by

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ABSTRACT

Stroke is the world's leading cause of adult disability, caused by lack of oxygen and nutrients to the brain due to a blood clot in a major artery. This leads to ischemic damage of neuronal cells that leads to paralysis, motor, and speech deficits. While most stroke therapies aim at removing or reducing the blood clots in the brain, few treatments target cell damage. Neuroglobin (NGB) is a protein in the brain that is able to aid in neuroprotection following oxidative stress. Hypoxia-Inducible Factor-1 (HIF-1) is a transcription factor that serves as a marker for cell recovery after hypoxia or low oxygen levels. Exosomes are microscopic extracellular vesicles that can help deliver proteins across the blood-brain barrier. This thesis focuses on finding a correlation between exosomal-delivered neuroglobin to ischemic cells and the regulation of HIF-1 in order to develop an innovative treatment using exosomes. The specific aims of this thesis are as follows:

Aim 1: Package NGB in exosomes of healthy cell

The XPAK-NGB plasmid will be used to transfect NGB DNA into wild-type human embryonic kidney (HEK-293 cell line) cells. Exosomes will be harvested from the spent media. The exosomes will be analyzed to ensure that the protein is packaged inside the exosomes.

Aim 2: Determine the limit of hypoxic conditions and effects of NGB on damaged cells

A literature review will be performed to determine the ideal concentration of H_2O_2 for the survival of neuronal cells. This will include the composition of hypoxia as well as the length of time that cells can be exposed to and remain viable.

Aim 3: Correlate NGB concentration and HIF-1 concentration

Another literature review will determine the specific markers of NGB and HIF-1.

This thesis is dedicated to
my family—
thank you for always believing in me.

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I wish to express my deepest gratitude for all the people whose assistance was a milestone in the completion of this thesis. Special regards to the graduate members of Dr. Sugaya's lab for helping smooth over any drawbacks in the process.

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BACKGROUND AND SIGNIFICANCE

Stroke

Stroke is a common, incurable medical emergency that affects more than 200,000 people every year, making it the fourth most leading cause of death in the United States [1]. Even though the rates of incidence for stroke are decreasing worldwide, it is still the leading cause of adult disability [2] and the fourth leading cause of dementia [3]. Approximately 3% of the adult population has had a stroke, which equates to around 7 million people in the U.S. [3]. Every year, there is an average of 800,000 strokes, with about 200,000 of these being recurrent strokes [3]. Epidemiological studies show that men are 24-30% more likely to experience a stroke, but more women overall have strokes as women tend to outlive men [3]. The decrease in stroke rate is also higher for men, with a 26% decrease for men and a 23% decrease for women between the years of 1997 and 2006 [3]. However, as the worldwide population continues to age, the incidence of stroke is expected to rise once again [3]. There are two types of strokes: ischemic and hemorrhagic. Ischemic strokes make up about 85% of all strokes. They are characterized by the occlusion of a major artery in the central nervous system (CNS), causing a decrease in oxygen to a particular part of the brain [2]. Hemorrhagic strokes are due to the bursting of a blood vessel and the accumulation of bleeding in the brain [2]. The focus of this research will be on ischemic stroke. Many risk factors for stroke have been identified, including hypertension, diabetes, smoking, and obesity [2]. Treatments for stroke mainly focus on managing symptoms and preventing additional strokes. These include thrombolytic therapy and antiplatelet treatment. Preventative treatment to decrease the incidence of a primary stroke has become the focus of much research surrounding stroke.

While the efficacy of these detection methods is significant once stroke occurs, there still exists a lack of viable diagnostic techniques to identify those at risk for stroke early. Therefore, it is imperative to conduct research that can improve upon current diagnostic methods. The hope is to be able to diagnose stroke early enough to administer the proper treatment.

Clinical Diagnosis of Stroke

Stroke has a very accurate rate of diagnosis, with about 92% of strokes being diagnosed correctly [4]. Stroke must be diagnosed immediately to improve the prognosis of the patient and ensure that the resulting treatments can be performed [4]. Typically, the disease occurs in individuals above the age of 55, though younger children may be affected in more rare cases [3]. Stroke is diagnosed using a variety of methods, including brain imaging (computed tomography scan), laboratory tests, and physical examinations [2]. Below is a compilation of the most common physical symptoms of stroke that have been defined in the National Institute of Health Stroke Scale (NIHSS).

1) Facial paralysis is when one side of the face droops during a stroke. This can be identified by a noticeable drooping of the lips when the patient is asked to smile. Currently, it is unknown why only one side of the face experiences paralysis [5].
2) Motor deficits, such as arm weakness, are also characteristic symptoms of a stroke. The pronator drift is the standard method of evaluation to determine if the weakness is significant [2]. The patient will be asked to raise both arms above their head and hold this supinated position for 20 to 30 seconds. Patients with a motor deficit will be unable to hold this position equally.
3) Dysarthria or slurred speech is another identifiable symptom of a stroke patient where the patient affected is unable to repeat a simple sentence. This can also be a side effect of the

symptoms mentioned above, such as facial paralysis and muscle weakness. This symptom could manifest in aphasia, which is a more permanent speech problem [5].

Most of these symptoms occur due to the damage of nerves and neurons that control muscle movement and speech centers. It also depends on the region of the brain that is affected [5]. Nevertheless, it is relatively simple to identify these preliminary signs of stroke, and there have been many acronyms designed to help bystanders recognize symptoms [4]. For example, an acronym, FAST, is defined as (F)- facial paralysis, (A)- arm weakness, (S)- speech problems, and (T)- time to act and call 911 [5]. These easy mnemonics can help diagnose the disease early to allow the stroke patient to get the treatment they need.

Clinical Treatment of Stroke

Currently, the treatment of stroke focuses on pharmacological options; however, there are also surgical solutions that have had much success. The main treatment options are thrombolytic therapy, antiplatelet treatment, and rehabilitation.

Thrombolytic therapy breaks down the blood clots found in major arteries leading to the brain [6]. For maximal success, this therapy must occur within 4.5 hours after the onset of symptoms [2]. Many drugs are used for thrombolytic therapy, including alteplase, anistreplase, urokinase, streptokinase, and many more [7]. These drugs are plasminogen activators, which will trigger a cascade that dissolves the blood clot through fibrinolysis [7]. Antiplatelet therapy is a preventative measure used to reduce the chance of a subsequent stroke. With an ischemic stroke, the antiplatelet drug of choice is clopidogrel, but aspirin can also be taken if necessary [8]. These drugs prevent the accumulation of platelets in a major vessel by inhibiting the enzyme cyclooxygenase 1 (COX-1) [8]. However, there is a high internal bleeding risk with the use of antiplatelet drugs, so patients must be monitored closely [8]. Rehabilitation is a strategy used to

help stroke patients return to their initial level of neurologic activity. It has been shown that motor activity can be recovered relatively quickly after the onset of a stroke; however, speech deficits take some more time to recuperate [9]. Rehabilitation should be initiated immediately after the stroke (24-48 hours window) with a shorter session [9]. There has been no evidence that suggests that intensive rehabilitation causes improved recovery, and the ideal amount of rehabilitation has not been determined [9].

Neural Recovery After Stroke

After a stroke, the neural recovery process is time-consuming and continues for months post-stroke. There are many mechanisms that the brain employs spontaneously in order to maximize recoveries, such as axonal sprouting and molecular cascades. Most of these recovery mechanisms are non-invasive and occur on their own. Recently, findings have shown that neural recovery is not as proportional as was once thought [10].

Axonal sprouting is when healthy neurons try to send out signals to determine where connections were lost due to ischemia [11]. Once the locations of these connections have been evaluated, the neurons start to send out new axons in these directions to restore the old connections [11]. Growth and differentiation factor 10 (GDF10) is a significant component in activating axonal sprouting [11]. Additionally, many molecular cascades are prompted by ischemia. These cascades promote inflammation or can even upregulate genes for growth factors and angiogenesis to aid in tissue repair [12]. Recovery from stroke occurs in waves. Initially, repair mechanisms are focused on reviving the damaged tissue. Then, weeks and months after the stroke, spontaneous repair mechanisms are introduced to help with neural recovery. Finally, the third wave of repair is maintaining a stable state after spontaneous repair. Even though this state remains steady, changes to the structure and function of the brain are still possible [12].

Neuroglobin

Neuroglobin (NGB) is a 17 kDa protein that makes up part of the globin family with other proteins such as hemoglobin and myoglobin [13]. Even though the sequence homology of NGB with hemoglobin and neuroglobin is only 21-25%, the central amino acid residues for its oxygen-carrying activity are conserved between all three structures [14]. It is present in the central and peripheral nervous system and helps to protect neural structures in cases of hypoxia and injury [13]. Hypoxia is a condition that leads to a decrease in oxygen concentration in cells. Additionally, NGB helps channel oxygen into the mitochondria in order to improve cellular respiration [13] and can also neutralize reactive oxygen species (ROS) [15].

Generally, NGB has been shown to be localized near actively metabolizing cells and specialized neurons, such as the hypothalamus [15]. Moreover, the retina is one of the most susceptible to oxidative damage, and therefore, there is a high concentration of NGB near this structure [15]. Hypoxia and ischemia are modulators that increase the concentration of NGB in the nervous system [15].

The presence of oxygen and glucose are integral to the proper functioning of the brain. Oxygen has the ability to bind reversibly to the hexacoordinated iron protein, which then delivers it to the cells [15]. Even though the specific mechanism of its action is currently unknown, much research has been conducted to determine a mode of action. NGB combats hypoxia with conformational changes in its structure. Under normal oxygen conditions, NGB is in the Fe^{2+}-bound oxygen form. However, when hypoxic conditions are detected, the conformation changes to ferric bis-His [16]. This tertiary structure conformation change is a significant component of the protective activity of NGB. Ferric bis-His NGB can bind to lipid-rafts in the cell and also heterotrimeric Gα proteins, activating signal transduction pathways [16]. Once Gα has been

activated, this leads to an increase in cAMP concentrations intracellularly, which can help protect the cell from cell death [16]. Additionally, human NGB that is in the ferric (Fe^{3+}) form acts as a guanine dissociation inhibitor (GDI) protein. GDI's bind to small GTPases to counteract exchanges and prevents it from accumulating in the membrane, inhibiting its action [17].

The primary function of NGB is to protect against hypoxia and ischemia, but it can also protect against cell death by increasing cAMP concentrations. Previous studies that observed the effects of oxidative stress on NGB found that human NGB was transduced into cells [16]. Once NGB was delivered to the cells, a cell viability assay was performed to determine that NGB increased cell survival [16]. Therefore, NGB aids with cell vitality and adaptability under oxidative stress conditions.

However, chronic hypoxia has been shown to lead to apoptosis. Chronic hypoxia will oxidize NGB causing an accumulation of Fe^{3+} ions and cytochrome c, inducing apoptosis [14]. Cytochrome c helps maintain iron in the Fe^{2+} condition in normal physiological conditions. Under oxidative stress, cytochrome c assists in oxidizing Fe^{2+} into Fe^{3+} in order to induce the neuroprotective effects of NGB. Prolonged oxidative stress will degrade NGB and further oxidize it, triggering apoptotic mechanisms [14].

NGB and Stroke

As NGB has been shown to have a role in hypoxia and ischemia, it is also involved with ischemic strokes. Previous studies have demonstrated that there is an increase of NGB after ischemic strokes in the brain, and it is primarily localized in the cortical peri-infarct region [18]. However, as this study was conducted in rodents, it is unclear whether this data can be extrapolated to humans. In another study, it was found that polymorphisms, or mutations, in the

NGB gene were related to a higher propensity of ischemic stroke in the Southern Chinese Han population [19].

Even though the mechanism of neuroprotection of NGB from stroke is unclear, changes in NGB expression do affect the severity of ischemic damage [20]. There have even been drugs developed, like hemin, to increase the concentration of NGB *in vitro* [20]. The goal is that more research can be conducted to cultivate drugs that involve NGB that function *in vivo.* As direct delivery of NGB is unable to penetrate the cell, exosomal delivery is also an option and could lead to a new treatment plan [21].

Oxidative Stress

Oxidation is a process that happens to all living creatures in nature, including our bodies, and is a result of oxygen producing a physiological change in cellular structure and metabolism [22]. The body produces toxic free radicals called reactive oxygen species (ROS) during everyday activities like breathing, walking, and exercising [22]. In normal conditions, the body produces antioxidants in order to balance ROS levels. However, in oxidative stress conditions, the toxic free radical species are not detoxified by the body's defense system [22]. Prolonged oxidative stress can lead to the development of many diseases, including neurodegenerative diseases, cancer, atherosclerosis, and stroke [23].

Previous research has demonstrated a strong correlation between oxidative stress and brain damage in stroke [23]. ROS targets the brain because it is composed of easily oxidized lipids and expends oxygen at a higher rate than other organs [24]. This can lead to strand breakage, sister chromatid exchange, DNA-protein crosslinking, and base modification [24]. With the accumulation of ROS, there is a decrease in adenosine triphosphate (ATP) concentrations due to the lack of oxygen to perform aerobic respiration [23]. With this lack of

energy, calcium (Ca^{2+}) builds up intracellularly and stimulates the activity of proteases and lipases [23]. These enzymes play a critical role in the damage of brain tissue thereafter [23].

There has been extensive research performed on how to induce oxidative stress *in vitro*. Oxidative stress results from prolonged hypoxia, so different methods have been found to successfully lead to the hypoxic condition. A 100μM $CoCl_2$ solution added to cellular media can lead to hypoxia and will help activate factors for hypoxia [25]. The solution needs to stay incubated in the media for 24 hours before any additional experiments are performed. Another method is to expose the cells to hypoxia in a Modular Incubator Chamber [25]. This chamber can change the level of oxygen gas that is present inside so that the cells can be fully exposed to the lack of oxygen. The oxygen sensors present will allow for a more controlled change that will be easier to measure as well.

In order to ensure that the cells are undergoing oxidative stress, there are specific markers that can confirm hypoxia. These markers are upregulated in the presence of ROS and can be used to determine the extent of oxidative stress [26]. These markers include IsoPs, MDA, Nitrotyrosine, S-glutathionylation, MPO, OxLDL, and Serum antioxidant capacity [26]. Each of these markers have their advantages and disadvantages, depending on the nature of the experiment. While this is not a comprehensive list of markers, they are still a good starting point to help determine the effects of oxidative stress.

Exosomes and the Blood-Brain Barrier (BBB)

Exosomes are a class of minuscule (30-100 nm) vesicles discharged from the cell that contain proteins, DNA, RNA, and miRNA [27]. Exosomes are found in all types of cells and are made up of components of the plasma membrane [27]. Specifically, in the brain, exosomes are released by almost all cells [28]. These particles are able to communicate information between

neighboring cells, which in turn signifies that exosomes carry important physiological and pathological data [27]. Exosomes are of particular interest because of their versatility, especially with regards to the blood-brain barrier (BBB).

The blood-brain barrier is the junction where blood vessels meet brain tissue. This highly selective barrier determines what is able to move into and out of the central nervous system (CNS) [29]. It restricts the movement of many ions, molecules, and cells between the blood system and the CNS [29]. However, exosomes can pass through the BBB, which poses some powerful benefits [28]. Since the CNS is immensely regulated, the ability of exosomes to cross this barrier allows researchers to gain more knowledge about the different types of cells inside the CNS [28]. Additionally, not only can exosomes leave the CNS and travel throughout the body, but they are also able to enter the CNS and target a particular cell [28]. Therefore, exosomes provide many benefits in terms of research potential.

Exosomes are also widely used as drug delivery methods. Exosomes are able to fuse to the membrane and deliver the protein cargo intracellularly [30]. First, exosomes join with the cell membrane, and then they are ingested by the cell via endocytosis/phagocytosis [30]. The exosomes enter the cell and are able to facilitate changes in physiology and pathology [30]. Exosomes are also cell-specific and are able to deliver proteins through long distances [30]. Drugs have been successfully delivered to the brain via exosomes, and uptake by the cell is increased after certain cell surface modifications [30].

HIF-1

Hypoxia-Inducible Factor-1 (HIF-1) is a transcription factor that is made up of two subunits: the HIF-1α and HIF-1β subunit [31]. The structure of the protein contains helix-loop-helix

motifs and PER-ARNT-SIM (PAS) domains that aid in DNA binding and subunit dimerization [31]. The protein has an average molecular weight of 93 kDa and is an oxygen-sensitive protein located in all cells [31]. It helps to maintain homeostasis and promotes angiogenesis, erythropoiesis, and glycolysis as part of the hypoxic response [31].

When there is a lack of oxygen, the HIF-1 concentrations increase dramatically. However, under normoxic conditions, the protein is degraded via proteasomes [32]. As a transcription factor, it allows the upregulation of genes such as vascular endothelial growth factor (VEGF) and glucose transporters (GLUT) [32]. VEGF is a critical component of the angiogenesis pathway, while GLUT is a transporter that introduces glucose into the cell for metabolism. Along with other genes, these proteins help with the recovery process of cells.

HIF-1 is able to enter the nucleus and regulate gene transcription directly [33]. It is present in all cells as a key regulator of oxygen responsiveness. Under high oxygen levels, the HIF-1α subunit is marked for ubiquitination. It is identified by prolyl hydroxylases (PHDs), which will hydroxylate the alpha subunit at proline residues [33]. This hydroxylated protein recruits the von Hippel Lindau protein (VHL), which will target HIF-1 for ubiquitination and, eventually, destruction [33].

Moreover, HIF-1 is involved in the survival response for cells under ischemic stress and has been shown to be regulated by NGB. When NGB has been oxidized to the ferric state, HIF-1 is stabilized [14]. The HIF-1 gene has been recognized as part of the NGB promoter region so that it may play a role in NGB expression [15]. However, other studies have shown that this promoter region lacks the HIF-1 binding hypoxia response elements (HRE) [21]. While there is speculation and uncertainty surrounding the exact relationship between HIF-1 and NGB, previous studies have shown a strong correlation between the two proteins.

The presence of HIF-1 after oxidative stress is a marker that cell recovery has taken place. It also allows the cell to be more resistant to future levels of stress by a mechanism of ischemic preconditioning [33]. HIF-1 overexpression signals that ischemic protection pathways have been activated to allow the cell to survive [34]. Nevertheless, prolonged exposure to HIF-1 has been shown to primate tumor metastasis and apoptosis [31]. Therefore, it is vital that therapeutic treatments address a balance in HIF-1 concentrations.

RESEARCH DESIGN AND METHODS

Aim 1: Package NGB in exosomes of healthy cells[1]

Aim 1.1: Collect XPAK – NGB DNA

In order to deliver NGB via exosomes, the DNA was obtained from the XPAK-NGB vector. This vector packed the DNA inside of the cell, as opposed to outside the cell. *E. coli* cells carrying this vector were grown and incubated overnight in three separate 50 ml conical tubes. The DNA was isolated and purified using the QIAGEN QIAprep Spin Miniprep Kit [35]. After an overnight incubation, the cultures were spun down at 3000 rpm for 10 minutes. The pellet was resuspended in 1 ml of molecular grade water. Then, the sample was spun down once again at 13,000 rpm for 1-1.5 minutes. The pellet was resuspended in 250 ml of P1 suspension buffer, 250 ml of P2 lysis buffer, and 350 ml of N3 neutrality buffer. The sample was again spun down at 13,000 rpm for 1-1.5 minutes. The supernatant was added to a DNA spin column and spun again at 13,000 rpm for 1-1.5 minutes. The flowthrough was discarded and 800 µl of P.E. wash buffer was added. The spin steps were performed again twice. 100 µl of E.B. elution buffer was added to the column and incubated for five minutes. A final spin at 13,000 rpm was conducted for 4-5 minutes to collect the DNA. A sufficient concentration of DNA was obtained before proceeding to the next step, as confirmed with a nanodrop. DNA was stored at 4ºC.

Aim 1.2: Transfect HEK cells with XPAK-NGB DNA

A transfection is when foreign genetic material is introduced into the genome of a cell [36]. Using Lipofectamine as the transfecting agent, the new DNA was delivered to the cell via

[1] See Appendix for the detailed protocols for all of the experiments performed in Aim 1

lipid vesicles. In this experiment, normal HEK-293 cells were first grown in T-75 flasks and passaged into a 12-well plate as these cell types have been shown to overexpress NGB following transfection [37]. About 150,000 cells were added to each well in the 12-well plate. After reaching a 60-70% confluency, the cells were transfected using Lipofectamine-2000 and the XPAK-NGB DNA from the miniprep. 125 µl of OptiMEM was added to 1 µl of Lipofectamine-2000 and another 125 µl of OptiMEM was added to 1 µg of XPAK-NGB DNA. The two solutions were incubated at room temperature for 10 minutes before being added together dropwise. The tubes were then incubated again at room temperature for 20 minutes so the DNA complex could form. The mixed solution was added to each well, dropwise and the plate was placed in the incubator.

To confirm that the cells were transfected with NGB, immunocytochemistry (ICC) was performed to visualize the success of the transfection. Six of the wells from the 12 well plate were used for the ICC. Three wells from the transfected HEK-293 cells were used, while the other three wells with the non-transfected HEK-293 cells were used to serve as a negative control. The cells were fixed with 4% PFA and washed with PBS. A permeabilization solution of 0.5% Triton-X in PBS was added and incubated for 10 minutes at room temperature. A blocking solution of 10% donkey serum in PBS was added to each well. Following a 30-minute incubation at room temperature, the primary antibody (anti-NGB) was added to each well in a 1:500 dilution. The plate was incubated overnight at 4ºC. The following day, the wells were washed with PBS (3 x 5 mins). The secondary antibody (TRITC) was added in a 1:1000 dilution and the plate was incubated in the dark for 2 hours. Some PBS was added to each well before the cells were taken for imaging. Fluorescence microscopy was performed on the cells to visualize NGB packed within the cells.

Aim 1.3: Collect HEK XPAK-NGB exosomes

After a successful transfection, the exosomes were collected from the media. Three separate media collections were performed: the first after 24-36 hours following the transfection and two 24 hours after the previous collection. Exosomes were precipitated from this spent media using 20% polyethylene glycol (PEG) and concentrated sodium chloride (NaCl). Non-transfected HEK exosomes were also harvested as a control. The tubes of spent media were spun down at 10,000 G for 30 minutes at 4ºC. 20% PEG and 7.5 M NaCl was added to each conical tube. The solutions were vortexed and incubated overnight. The samples were spun down at 10,000 G for 1 hour at 4ºC and the pellet contained the exosomes. It was resuspended in 500 µl of 1X sterile PBS. The precipitated exosomes were stored at -80ºC for long-term storage.

Aim 1.4: Ensure that NGB is present in the exosomes

A dot blot was performed with the exosomes to determine that NGB protein was present within the cells. 2 µl of both transfected exosomes and non-transfected exosomes were each added to two nitrocellulose membranes. After the samples dried, the membranes were placed in two petri dishes with blocking solution (5% BSA in TBS-T). The membranes were incubated at room temperature for one hour on the shaker. The membranes were then washed in TBST before the primary antibody was added (1:500 dilution in blocking solution). One membrane was treated with the anti-NGB antibody while the other membrane was treated with the anti-CD63 primary antibody. The anti-CD63 antibody was used to ascertain that exosomes are present on the blot. The dishes were incubated overnight at 4ºC.

After incubating, the membranes were washed with TBS-T and the secondary antibody was added in a 1:10,000 dilution in the blocking solution. After a 1 hour incubation, the

membranes are washed. Since the secondary antibody was conjugated to HRP, ECL solution was used to induce visualization. The blot was read using a chemiluminescence imaging system (Gel Doc).

Aim 2: Determine the limit of hypoxic conditions and effects of NGB on damaged cells

Aim 2.1: Research survival capabilities of neuronal cells in hypoxic environments

To better understand the hypoxic cellular limits of neuronal cells, web journals and peer-reviewed articles from search engines such as Google Scholar and PubMed were reviewed. The following terms were used in the search input: "Hypoxia," "Hypoxic conditions," "Cellular limits for hypoxia," and "Neuronal cells." These articles were compiled and analyzed to determine the best hypoxic conditions for neuronal cells that would allow for further research.

Aim 3: Correlate NGB concentration and HIF-1 concentration

Aim 3.1: Determine markers for the presence of HIF-1

A literature review for HIF-1 was conducted to determine its mode of action and also the cellular changes that occur upon NGB induction. Journals were investigated to determine if there were any specific markers for HIF-1 action and how they relate to NGB concentration under hypoxic conditions. The databases were searched with some of the following terms: "HIF-1 markers", "HIF-1 action", "Neuroglobin and HIF-1". Higher levels of HIF-1 should be present in ischemic cells to help facilitate recovery with neuroglobin.

RESULTS/DISCUSSION

Aim 1: Package NGB in exosomes of healthy cells

a)

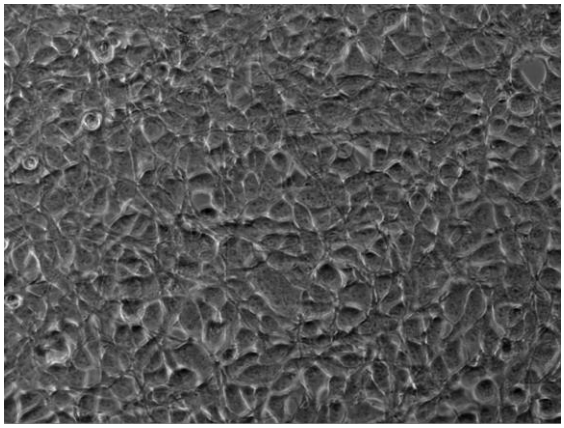
Well	Sample ID	User ID	Date	Time	Conc.	Units	A260	A280	260/280	260/230	Conc. Factor (ng/ul)	Cursor Pos.	Cursor abs.	340 raw	NA Type
A1	Clone 4 - 1	Default	2/12/2019	12:02 PM	56.07	ng/ul	1.121	0.566	1.98	2.30	50.00	260	1.121	0.022	DNA-5
B1	Clone 4 - 1	Default	2/12/2019	12:02 PM	55.12	ng/ul	1.102	0.555	1.99	2.27	50.00	260	1.102	0.046	DNA-5
C1	Clone 4 - 1	Default	2/12/2019	12:02 PM	55.61	ng/ul	1.112	0.544	2.04	2.22	50.00	260	1.112	0.034	DNA-5
D1	Clone 4 - 1	Default	2/12/2019	12:02 PM	55.61	ng/ul	1.112	0.540	2.06	2.31	50.00	260	1.112	0.007	DNA-5
E1	Clone 4 - 2	Default	2/12/2019	12:02 PM	53.46	ng/ul	1.069	0.526	2.03	2.38	50.00	260	1.069	0.021	DNA-5
F1	Clone 4 - 2	Default	2/12/2019	12:02 PM	51.40	ng/ul	1.028	0.508	2.02	2.44	50.00	260	1.028	0.042	DNA-5
G1	Clone 4 - 2	Default	2/12/2019	12:02 PM	52.29	ng/ul	1.046	0.513	2.04	2.33	50.00	260	1.046	-0.001	DNA-5
H1	Clone 4 - 2	Default	2/12/2019	12:02 PM	51.70	ng/ul	1.034	0.522	1.98	2.35	50.00	260	1.034	0.006	DNA-5

b)

Report | Test Type: Nucleic Acid | Date/time: 2/19/2019 11:59 AM | Page #: 1

Plate ID	Well	Sample ID	User ID	Date	Time	Conc.	Units	A260	A280	260/280	260/230	Conc. Factor (ng/ul)	Cursor Pos.	Cursor abs.	340 raw	NA Type
	A1	XPAKNGB Cl. 4 -1	Default	2/19/2019	11:58 AM	54.02	ng/ul	1.080	0.530	2.04	1.59	50.00	260	1.080	0.016	DNA-50
	B1	XPAKNGB Cl. 4 -1	Default	2/19/2019	11:58 AM	53.84	ng/ul	1.077	0.539	2.00	1.59	50.00	260	1.077	-0.009	DNA-50
	C1	XPAKNGB Cl. 4 -2	Default	2/19/2019	11:58 AM	77.67	ng/ul	1.553	0.767	2.03	1.81	50.00	260	1.553	0.024	DNA-50
	D1	XPAKNGB Cl. 4 -2	Default	2/19/2019	11:58 AM	79.04	ng/ul	1.581	0.798	1.98	1.78	50.00	260	1.581	0.038	DNA-50
	E1	XPAKNGB Cl. 4 -3	Default	2/19/2019	11:58 AM	70.59	ng/ul	1.412	0.721	1.96	1.96	50.00	260	1.412	0.004	DNA-50
	F1	XPAKNGB Cl. 4 -3	Default	2/19/2019	11:58 AM	71.44	ng/ul	1.429	0.716	2.00	1.76	50.00	260	1.429	-0.399	DNA-50
	G1	XPAKNGB Cl. 4 -4	Default	2/19/2019	11:58 AM	61.54	ng/ul	1.231	0.632	1.95	0.63	50.00	260	1.231	-0.016	DNA-50
	H1	XPAKNGB Cl. 4 -4	Default	2/19/2019	11:58 AM	60.09	ng/ul	1.202	0.627	1.92	0.64	50.00	260	1.202	-0.232	DNA-50

Figure Group 1: DNA Nanodrop Table
a) Nanodrop results from XPAK-NGB Clone 4; sample I.D. corresponds to the culture tube EX: Clone 4 – X, where X is culture from which the DNA was purified b) Nanodrop results from XPAK-NGB clone 4; sample I.D. corresponds to the bacterial culture tube EX: XPAKNGB Cl.4 – X, where X is the individual culture tube.

a) 

b) 

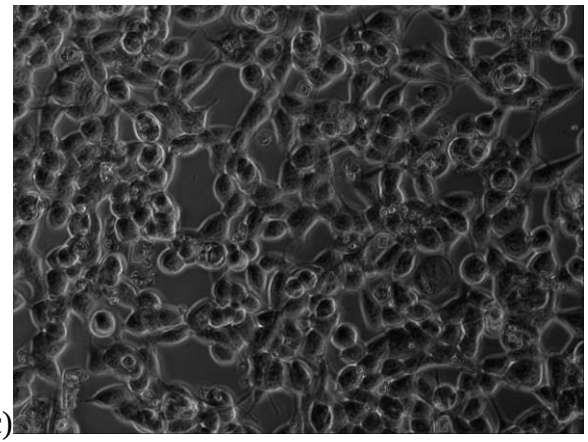
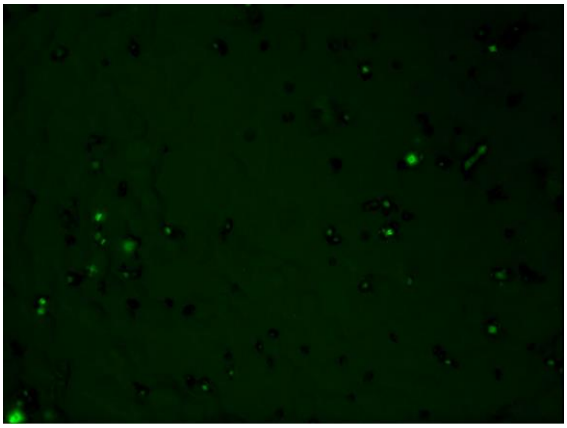
c)  d) 

Figure Group 2: HEK-293 ICC Fluorescence Microscopy Images
a) Wild-Type HEK cells images visualized with transmitted light (T.L.) b) Image 2a visualized with fluorescence c) HEK-293 cells transfected with NGB DNA, visualized with T.L. d) Cells from Image 2c visualized with fluorescence

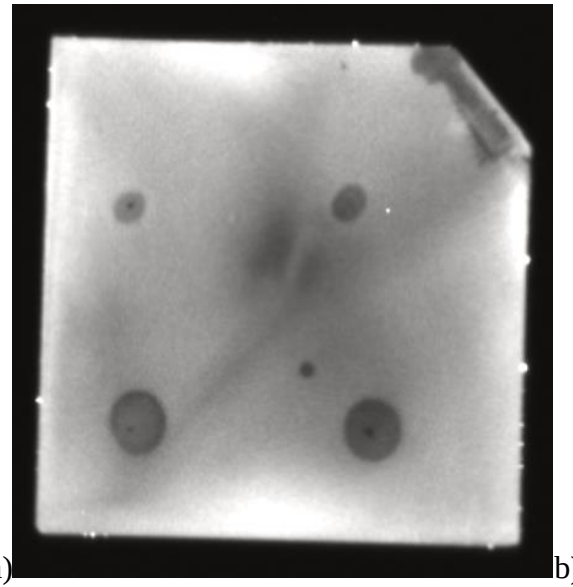
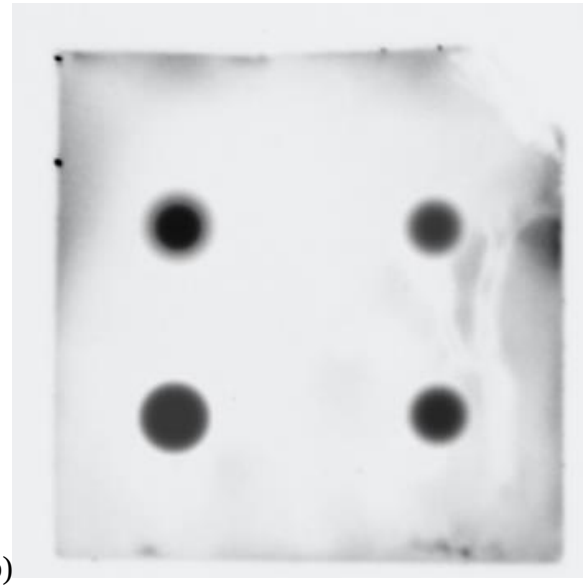
a)  b) 

Figure Group 3: Dot Blot Results
a) Experimental condition: Top row represents wild-type HEK-293 exosomes, and the bottom row represents HEK-293 transfected with NGB DNA exosomes. The blots were treated with an Anti-NGB antibody. b) Control blot: The top row represents wild-type HEK-293 exosomes, and the bottom row represents NGB-transfected HEK-293 exosomes. This membrane was treated with an Anti-CD63 antibody.

Three separate experiments were performed to ensure that NGB was properly packaged inside the cells and then inside the exosomes. A nanodrop was performed to determine how much DNA could be administered to the cells for a transfection. After the transfection, immunocytochemistry (ICC) was performed to make sure the cells did contain NGB in the cells. Once the exosomes were precipitated, a dot blot was performed on the exosome sample to ensure the exosomes also contained NGB.

Figure Group 1 shows the results from two separate nanodrop experiments. Both readings were taken immediately following a bacterial miniprep. Figure 1a shows that the average concentration of XPAK-NGB DNA from Clone 4 is 53.91 ng/ul. The A260/280 ratio measures the purity of the DNA. Generally, a 260/280 ratio of 1.8 indicates pure DNA. The average 260/280 ratio for all the samples in Figure 1a is calculated to be 2.02. This value shows that the DNA is not as pure as it could be. Additionally, the concentration is also relatively low.

As such, a subsequent experiment was performed with a new bacterial culture and a new bacterial miniprep. Figure 1b shows the results of the second nanodrop performed from XPAK-NGB DNA obtained from Clone 4. The average concentration of the DNA in this experiment was 66.02 ng/ul. This is 12.11 ng/ul more than the concentration of DNA from Figure 1a. The average A260/280 ratio of the data in Figure 1b is 1.99. While this is definitely closer to the desired 1.8 threshold, it is still higher than preferred.

Various factors could have impacted the purity of the DNA. The reagents in the miniprep process may not have been as free of contamination as the miniprep was performed on the bench. Additionally, the bacterial culture could have been contaminated as well, even though most of the contamination should have been extracted by the miniprep process. Another point of contamination could have been during the nanodrop. Perhaps the blank of distilled water was not

measured correctly. Alternatively, there may have been leftover contamination from the previous run of the machine on the pedestals. While it may be difficult to pinpoint the exact reasoning for the low purity, the higher concentration of the DNA will be beneficial to the completion of further experiments.

Therefore, these nanodrop results from Figure 1b show that the XPAK-NGB DNA was around 66.02 ng/ul, and bacterial culture tube 4 had the most relatively pure DNA. The average A260/280 ratio for culture tube 4 was 1.94, which is the closest to 1.8 from all of the other culture tubes. However, culture tube 2 had the highest average concentration of DNA at 78.36 ng/ul. To that end, both samples of DNA will be used in the transfection process to ensure high concentrations and purity of the DNA being used.

Following the transfection, the immunocytochemistry protocol was performed. The images from the microscope can be seen in Figure Group 2. Figure 2a shows wild-type HEK-293 cells imaged under transmitting light. This was done to check the health, morphology, and confluency of the cells. The cells in the image are very confluent, almost around 90% confluency. While the morphology is characteristic of other wild-type HEK-293 cells, the confluency may cause some point mutations within the population of cells. This same batch of cells was imaged with fluorescence to yield Figure 2b. The green portion should represent NGB present within the sample of cells. Ideally, there should be no signal for the wild-type HEK-293 condition. However, there was a small signal present in Figure 2b. There are a few reasons why this could have occurred.

First, there may inherently be NGB present in wild-type HEK cells; however, this has not been investigated in the past. Additionally, it could be an error in the actual experimental procedure. For example, if proper pipetting techniques were not used, a sample of the transfected

cell line could have been introduced into the wild-type cell line, causing a slight signal. Additionally, perhaps the antibody is recognizing some other contamination in the well, causing the green color. The cells do look healthy, though, so it could also be due to imaging error. The wild-type HEK cells are a negative control, so they should not have any signal. Even though there is a little bit of signal, it will be compared to the experimental condition to determine the success of the experiment.

Moving onto the experimental condition, Figure 2c shows the transfected population of HEK cells that include NGB DNA. These were visualized under transmitting light to determine the health of the cells. The cells themselves have the proper morphology, and they are a little bit less confluent than the cells in Figure 2a— closer to an 80% confluency. There are some diamond-shaped substances visible, which may be dried PBS. In Figure 2d, these cells are imaged with fluorescence to determine if NGB is present within the cells. With the presence of NGB inside the cells, the cells should show a green fluorescence color. In this figure, there is fluorescence visible within the cells which means that NGB has been successfully packed inside. While not all the cells are showing fluorescence, the majority of the cells do exhibit a signal, so it can be confirmed that the transfection protocol was a success.

Comparing the set of images in Figure 2 shows that, even though Figure 2b shows a small green signal, it is subdued compared to the fluorescence in Figure 2d. Therefore, even though the non-transfected sample showed some NGB present, the transfected sample showed an *overexpression* of NGB, which is needed to proceed with the experiment. Moving forward, the exosomes precipitated from these cells should have NGB contained within.

After the exosomes were precipitated, the exosomes were added to a nitrocellulose membrane, and a dot blot was performed. A dot blot was chosen instead of a more rigorous

method because merely a confirmation of NGB presence was needed. A dot blot would be able to determine that the exosomes themselves contain NGB. The results of the dot blot can be seen in Figure Group 3.

Figure 3a shows the blot of the experimental condition that was treated with an anti-NGB antibody. The top row of dots in the figure represents the wild-type HEK exosome control samples. These exosomes have not been transfected with NGB DNA. The bottom row of dots contains the HEK exosomes transfected with NGB DNA, which is the experimental condition. There are duplicates of both samples to ensure that the results are valid.

Figure 3b shows the blot treated with the anti-CD63 antibody. CD63 is an exosomal marker to ensure that exosomes are present on the blot. This blot was prepared as a positive control to confirm the exosomal precipitation procedure. The top row contains the wild-type HEK-293 exosomes, while the bottom row contains the HEK exosomes that were transfected with NGB. These are the same exosomes that were used in the blot in Figure 3a. After performing the dot blot, both blots were placed into a Gel Doc imager to visualize the results.

In Figure 3a, there was a signal present in both of the rows; however, the experimental condition has a stronger signal. Figure 3b shows the positive control results, which follow what was expected. There is an equal signal present in all four samples, demonstrating that all samples consist of exosomes. As a control group, the housekeeping gene CD-63 helped identify the presence of exosomes and not just any extracellular material. This means that the transfection was successful and that the precipitated exosomes do contain higher levels of NGB than the wild-type exosomes.

These findings also correlate with the earlier finding that there is some NGB present in wild-type HEK cells. While the signal in the control condition was unexpected, it does follow the

previous findings. However, the presence of this signal could also mean there were errors in the data collection and procedure. The original HEK exosome samples could have been contaminated with trace amounts of NGB. Additionally, the antibodies themselves could have become contaminated, leading to small signals in the control group. It would be recommended to reproduce this experiment to ensure that these results are representative of the condition of the exosomes.

Overall, piecing together the various experiments, it can be confirmed that Aim 1 has been fulfilled. NGB DNA was extracted, transfected into HEK-293 cells, and grown. The exosomes were precipitated and analyzed to ensure that NGB has been packaged inside. The results of the nanodrop, immunocytochemistry, and dot blot show that the exosomes do, in fact, contain the NGB protein. While there were some potential errors in the results, subsequent experiments did confirm the previous findings. Therefore, it will be established that the exosomes have been packaged inside the exosomes of healthy HEK-293 cells.

Aim 2: Determine the limit of hypoxic conditions and effects of NGB on damaged cells

Table 1

Summary of Oxidative Stress Studies Reviewed

Study #	**Author(s)**	**Study Purpose**	**Hypoxia Conditions/Cell Types**	**Limitations**
2.1	Baccino-Calace et al., (2019) [38]	To determine specific cellular responses to changing oxygen conditions in the early stages of *Drosophila* development	The researchers performed their hypoxia experiments on *Drosophila* larvae; 5% oxygen in nitrogen was administered for 24 hours	Experiments were performed on *Drosophila,* so they may not be valid for humans in vivo; *Drosophila* neural tissue was examined rather

				than specific cell types
2.2	Xu et al., (2020) [39]	To analyze the relationship between oxygen-glucose deprivation/reoxygenation, neuronal cell death, Lnc-D63785 methylation (non-coding RNA), and miR-422a (microRNA)	SH-SY5Y neuroblastoma cells were cultured for this experiment; 5% CO_2 in nitrogen was administered for 4 hours	Research involved oxygen deprivation along with glucose deprivation, which is similar to stroke conditions but not within the scope of this thesis; SY5Y cells are cells that are already cancerous and may show different outcomes than healthy neuronal cells
2.3	Shi et al., (2016) [40]	To investigate the role of omega-3 polyunsaturated fatty acid and *Lyciumbarbarum* polysaccharide in recovery following OGD reperfusion	E16.5 mouse cortical cells were obtained from embryos; 5% CO_2 in nitrogen was administered for 4 hours; cells that remained in the chamber for more than 24 hours showed a marked decrease in cell viability and number	Research was performed on mouse cortical cells, so it may not be reproducible for humans; the cell type used was also very different than human neuronal cells
2.4	Zeiger et al., (2013) [41]	To determine how long neurons can survive in OGD conditions and how long it will take for further damage to become visible, along with assessing their bioenergetic capabilities	Primary cortical neurons were cultured for this experiment; neurons were placed in a chamber of 5% CO_2 in nitrogen for a maximum of 90 mins; optimal range for cell health was determined to be 5-15 mins, after that, cell toxicity was measured	Again, this experiment was focused on OGD conditions, so the lack of glucose in the chamber may have skewed the results; the results of the OGD study were measured 24 hours after the exposure to OGD

2.5	Felfly et al., (2013) [42]	To resolve neuronal cell limits in severe hypoxic conditions and the effects of prolonged hypoxia exposure	Murine neural stem cells (mNSCs) and primary neurons were used in this experiment; 1% oxygen in 5% carbon dioxide to induce hypoxia for two days (48 hours); in general, there was a significant decrease in cell number after both types of cells were exposed to hypoxia for this period of time	The response of mNSCs may not be indicative of the response to hypoxia in the cell; this research does not establish the lower limit for hypoxia for neuronal cells

Five different studies were hand-picked to help determine the limit of hypoxic conditions. These studies were found on different platforms, but mainly on PubMed. Each summary for hypoxia limits is summarized in Table 1.

Table 1 offers short descriptions of the selected research, including the study purpose, results that are applicable, and limitations. Many of the studies' inherent purpose may not fit within the scope of this thesis, but they were still selected because the procedures for hypoxia or OGD (oxygen-glucose deprivation) were relevant to determining the conditions and duration of hypoxia that can be administered. The results column explains what cell type was used, what concentration of hypoxia/OGD was administered, and how long the cells were exposed to hypoxia. Additionally, it will also list some preliminary effects of the hypoxia on the cells, if appropriate. Finally, the limitations column will talk about how the research listed may not offer some of the best insight into the aims for this thesis, including types of cells used and the conditions of OGD.

Study 2.1, listed in Table 1, was performed upon *Drosophila* to see how different oxygen conditions could affect the neural development of the larvae [38]. The researchers exposed *Drosophila* larvae embryos to 5% oxygen in nitrogen for 24 hours for their study. These conditions allowed the larvae to survive, and the experimenters then dissected the larvae to expose their brains. Therefore, these hypoxia conditions allowed the brains—and subsequently the neurons—to remain viable. However, as these experiments were performed on *Drosophila* larvae, these conditions may not translate over to humans. Additionally, individual cells were not studied—rather, this study was focused on the brain as a whole with regards to the effects of hypoxia. Nevertheless, this experiment is still helpful for the conditions of hypoxia and the duration of exposure. This study will still need to be supplemented with another study that has hypoxia performed on human neuronal cells.

Study 2.2 from Table 1 had the aim of analyzing the relationship between oxygen-glucose deprivation (OGD) and reoxygenation (OGD/R) and neuronal cell death, in addition to its effects on different RNA molecules [39]. This experiment used SH-SY5Y neuroblastoma cells. These are cancerous human cells that were exposed to 5% CO_2 in nitrogen for 4 hours. These cells remained viable for the researchers to perform additional experiments. Additionally, these cells were then "reoxygenated" by being placed in traditional media. However, this research involved OGD conditions—which is representative of a stroke condition—but it is not the focus of this research. SY5Y cells are cancerous as well, which means they may not be the best representation of healthy neuronal cells. These cells could have different factors that alter their response to low oxygen conditions. The condition of OGD cannot be used to determine how to administer hypoxia because it is mainly CO_2 rather than oxygen. Despite this, this study is still helpful in determining the hypoxia limits for neuronal cells. Since SY5Y cells were used, 4 hours

seems to be a safe duration for hypoxia administration. This duration will be confirmed with another study that performs hypoxia—not OGD—on healthy neuronal cells.

Table 1 also lists Study 2.3, which studied different biological molecules and how they were affected with OGD (fatty acids and polysaccharides) [40]. This experiment used E16.5 mouse cortical cells and exposed these cells to 5% CO_2 in nitrogen for 4 hours. Other cells were exposed for a more extended period of time. Cells that were exposed to OGD for more than 24 hours died or became less viable—even after reoxygenation. The cells that were used contained the *fat-1* gene, which may have affected the results. Additionally, the cortical cells of mice may not garner reproducible results in humans. While cortical cells are part of the nervous system, they may provide skewed results as they are not the primary cell of interest, nor are they the organism of interest. However, this experiment does help to establish the upper limit of hypoxia. Even though this study also used OGD/R, it can be assumed that 24 hours of exposure to low oxygen conditions would cause irreparable harm to the cells. This study still provides valuable data, especially when taken into account with the other studies. In conjunction with Study 2.2, this study confirms that 4 hours seems to be an appropriate time to administer hypoxia, but this will be corroborated with additional studies that are performed with hypoxia and on the right cell type.

Study 2.4 gets closer to the preferred cell line. This study's purpose was to investigate the response of cortical neurons to OGD, and it also looks into how long cells can be exposed before further damage is elicited [41]. The neurons were exposed to OGD (5% CO_2 in nitrogen) for up to 90 minutes. It was found that the cells remained the healthiest after 5-15 minutes of exposure to oxygen deprivation. Additionally, this study found that any incubation over 1 hour led to significant cell death. However, it is important to mention that this study only measured the

effects of OGD 24 hours post-exposure. Furthermore, as mentioned earlier, this experiment focused on both oxygen and glucose deprivation, not only hypoxia, which may skew the results for this thesis. Either way, it still provides an optimal range for human neuronal cells, especially when compared to the previous studies.

Study 2.5 was used to reinforce the upper limit of hypoxia found from study 2.3—only now will it be used with human cells [42]. The general aim of the study falls in line with this thesis, and it was to determine the limits of neuronal cells in severe hypoxia and how prolonged hypoxia may change cell morphology and other characteristics. This experiment used murine neural stem cells (mNSCs) and primary neurons. These cells were exposed to hypoxia at 1% oxygen in 5% CO_2 for two days (48 hours). Both cell types had a decrease in cell number after exposure for this long. Additionally, the NSCs became quiescent—unable to retain their stemness. While the mNSCs may not provide accurate information on the response of neuronal cells in human cells, the research on primary neurons is valuable. Additionally, while this study does not offer insight into the lower limit of hypoxia exposure, it does provide the maximal limit of 48 hours. Another benefit of this study was the conditions of hypoxia. While many of the other studies performed OGD, this particular study researched hypoxia only, and this condition will be helpful to extrapolate data.

Overall, all these five studies provide their advantages and disadvantages when taken into consideration with the scope of this research thesis. First, even though many of the studies did not test their hypotheses on human neural cells, these results can be combined to find out what is the best condition and duration for hypoxia exposure. According to all the data from the five studies, primary neurons survive the best when exposed to less than 1 hour of hypoxia with the

best range between 5-15 minutes. The composition of the hypoxia was 1% oxygen in 5% CO_2. The cells will not be exposed for longer, and it will just be hypoxia, not OGD.

The original plan with this aim was to expose neuronal cells to hypoxia using an OGD chamber. The cells would be exposed to hypoxia for a particular amount of time and then, NGB would be introduced via exosomes to the stressed cells. In this aim, the goal would be to induce hypoxia, perform some type of assay to ensure that hypoxia was properly administered, and then add in the exosomes that have been transfected with NGB. The initial hypothesis was that NGB will help these hypoxic cells recover from the lack of oxygen. Currently, this literature review provides a good starting point to determine the composition of hypoxia as well as the period of exposure.

Of course, since this was a literature review, these results are only accurate pending actual experimentation. It is a good starting point for experiments, but the exposure to hypoxia can only be accurately measured following exposure to hypoxia. Also, most of the studies were based on hypoxia administered *in vitro,* and this may provide differing results *in vivo*. Additionally, there were a diversity of cell types used, and these results may change depending on which cell type is used in the experiment. Therefore, physical experiments must be performed in order to ensure the reliability of these results. Overall, Aim 2 was satisfied as the cellular limits of hypoxia were divulged after an extensive literature review.

Aim 3: Correlate NGB concentration and HIF-1 concentration

Table 2

Summary of NGB and HIF-1 Studies

Study #	**Author(s)**	**Study Purpose**	**NGB/HIF-1 Findings**	**Limitations**
3.1	Wei et al., (2011) [43]	To understand the role of NGB in glaucoma as a neuroprotectant	• Higher expression of NGB in retinal patients (which experience more oxidative stress) reduces the intensity of the effects of stroke • transgenic mice and retinal ganglion cells were the subject of this study • mitochondrial function was preserved while superoxide production diminished	This research was performed on the retinal cells of mice, which is an entirely different subject matter, and the results may not apply to human populations; the research mentioned it was difficult to find evidence of NGB action; in order to increase oxidative stress, a higher intraocular pressure was manually administered, which could change the findings.
3.2	Brittain (2012) [44]	To develop and evaluate models for how neuroglobin can prevent pre-programmed cell death	• Early research found the HIF-1 did not regulate the expression of NGB—instead, κB3, NFκB, and Sp1 help upregulate NGB; HIF-1 is involved but has an indirect role • Proposed three different models of neuroglobin action: oxygen model, the nitric oxide model,	This research mainly evaluated previous research and found connections between them to outline the various findings in the field for neuroglobin; no physical experiment was conducted to determine *how* neuroglobin acts or the markers of action

			and protein-protein interactions • The primary function deals with cytochrome c from the mitochondria	
3.3	Baez et al., (2016) [45]	To investigate the role of neuroglobin in the nervous system and how it mediates the effects of various pathologies in the brain	• NGB levels were shown to remain elevated for up to six days after traumatic brain injury (TBI) • Effects of NGB include inhibition of caspase 3 and 9, activation of PI3K/Akt pathway, and increased GFAP • NGB interacts with cytochrome c, thyroid hormone T3 and is regulated by NFκB, HIF-1, erythropoietin, and VEGF	This research mainly focused on astrocytes as the cell type to relate to NGB findings, not providing a diversity of results; the research outlined was mainly focused on TBI, with small mentions of stroke, so the premise of the research may have provided different data in regards to stroke research; no physical experiment was conducted to determine these results—it was a summary of previous research
3.4	Majmundar et al., (2010) [46]	To focus on HIFs to determine the response to hypoxia and its regulation based on other factors	• HIF regulates erythropoietin expression and transcription of other factors involved in the hypoxic response pathway • Sirtuins and intermittent hypoxia are the best regulators of HIF activity • O_2 sensors such as PHDs, FIH1, and the mitochondria may	This research outlined HIF action in various physiological system, not only the nervous system, so some findings may not pertain to hypoxia due to stroke; not much mention of neuroglobin and the relationship between HIF and NGB was made;

			help kickstart HIF transcriptional activity • Different factors can regulate various subunits of HIF	again, this research cites previous studies that were performed, but no novel experiments were performed to support the data
3.5	Hota et al., (2012) [14]	To understand how different configurations of NGB can regulate the activity of different hypoxia factors, including HIF and Nrf2	• Supports the role of PHDs as oxygen sensors to stabilize HIF • Degradation of NGB in the cell will, in turn, decrease the levels of HIF and Nrf2, promoting apoptosis • Rats were placed in a hypobaric chamber to simulate hypoxia, and brains were harvested and analyzed • NGB inhibited caspase 3 and cytochrome c	The method of hypoxia administration is very different than what was discussed in Aim 2—while hypobaria may lead to hypoxia, there are many other uncontrolled variables; this experiment was performed on rats, so it may not provide the best results for this thesis focused on humans

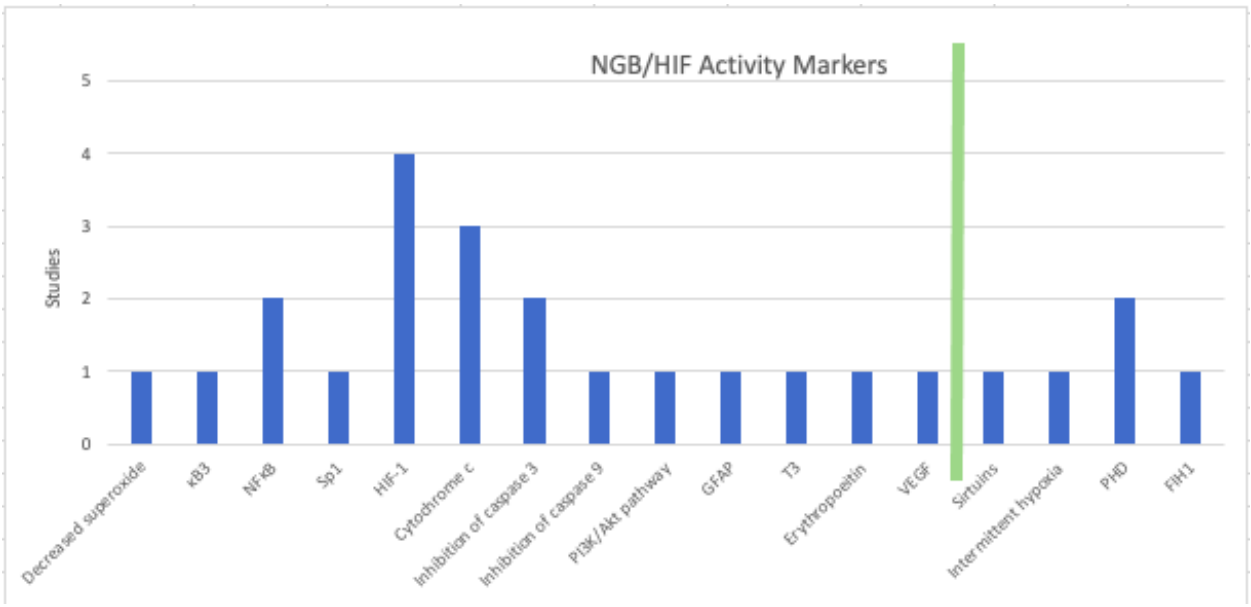


Figure 4: Graph of Markers for NGB Action
Graph demarcates the results of Table 2, divided by each type of marker that was mentioned. The horizontal axis reveals the different factors that were described to regulate NGB/HIF activity. The vertical axis shows a count of how many studies outlined a particular marker as involved in NGB/HIF regulation. The green vertical bar divides the data into two sets. The left-hand side relates to markers for NGB, while the right side relates to markers of HIF.

Evidence of the relationship between NGB and HIF-1 is well documented, but the mechanisms of action are widely disputed, as well as the effects of neuroprotection by NGB on the cell. To investigate this further, five different studies were chosen to elucidate the role of NGB and HIF-1 and their respective cellular markers for action. As with Aim 2, these studies were found in different journals. Table 2 summarizes the studies used, while Graph 1 compiles all the data into an easy-to-understand visual of the different markers listed.

Table 2 lists the five different studies that were used to assess the relationship between NGB and HIF. This table lists the purpose of the study, the markers that are correlated to HIF/NGB, and the limitations presented by the studies. As with before, even though the study's purpose may not be applicable to this thesis, some of the research findings were selected as they fit within the scope of this research—though most of the research was connected to NGB/HIF. The results show either the experiments or conclusions gathered from different studies, pieced together. Many of the limitations listed in the final column have to do with the fact that no physical experiment was performed; rather, much of the data was obtained from a thorough literature review.

Study 3.1 provided a good baseline introduction to the role of NGB [43]. The study focused on the effects of NGB in the retinal ganglion cells of transgenic mice. This study was investigating NGB protection in glaucoma disease, but some findings did relate to stroke. For

example, a higher level of NGB expression was found in retinal patients who experienced stroke. This was shown to reduce the negative effects of stroke. Additionally, while the cell type studied (retinal ganglion cells) is not the cell type of interest for this thesis, the cells used were still part of the nervous system, so the correlation may be similar. However, to induce stress, the researchers increased intraocular pressure (IOP), which is a marker of glaucoma. For this thesis, this manual administration could have changed the mode of action of NGB. In fact, the study did mention that it was difficult to find evidence of NGB action. Nevertheless, the study found that NGB must work through the mitochondria and the marker for action was that the production of superoxide decreased significantly. Since there were many limitations to this study, it is unclear whether superoxide reduction is a valid marker for NGB action, but this will be further explored with additional studies.

Study 3.2 was a literature review of the different mechanisms that contribute to NGB action, especially in regards to anti-apoptosis [44]. This research understands that HIF-1 is involved indirectly in the expression of NGB. The main transcription factors that regulate NGB expression were found to be κB3, NFκB, and Sp1. In the presence of these factors, NGB production is upregulated. Additionally, this article expanded upon three different modes of NGB action: the oxygen model, the nitric oxide model, and protein-protein interactions. The oxygen model, now considered incorrect, states that NGB can bind oxygen when reduced, helping with oxygen transport. Like the oxygen model, the nitric oxide model states that neuroglobin can bind nitric oxide and may even function as a nitrite reductase. However, the study also mentioned that the role of NO and NGB has yet to be explicitly studied. Finally, the protein-protein interactions of NGB are vast. While many proteins have been found to interact with NGB through various research methods, one major protein is cytochrome c from the

mitochondria. All these different markers show the presence of NGB. While this article was compiled from different literature reviews and no experiments were conducted to find the information, the findings were collected from reputable sources. The results will also be confirmed with subsequent studies.

Study 3.3 is very similar to study 3.2 in that it is focused on how different brain pathologies are mediated by NGB action through a literature review [45]. This research really highlighted many different brain pathologies and assessed how NGB mitigates the harmful effects, as well as any prominent trends. For example, during traumatic brain injury (TBI), NGB levels remained elevated for six days after the introduction of TBI. This means that NGB was still involved in the healing process for this and could still be identified after this period of time. Even though this outcome was in relation to TBI, it could have potential effects on stroke patients as well. Some of the markers that were found with astrocytes were inhibition of caspase 3 and 9, and the activation of the PI3K/Akt pathway. An increased level of GFAP was also found. The interactions of NGB with different proteins were also investigated (similarly mentioned in study 3.2). NGB was shown to interact with cytochrome c, thyroid hormone T3, NFκB, HIF-1, erythropoietin, and VEGF. Many of these markers are similar to previous studies, and HIF-1 has been shown multiple times to facilitate NGB expression. While many of the findings of this study pertain to astrocytes and therefore may not provide diversity, it is still valuable in determining NGB interactions. Overall, this study proved useful in expanding upon the many different markers as well as the relationship between NGB and HIF-1.

Study 3.4, listed in Table 2, emphasizes the role of HIF-1 in the hypoxia response protocol, including the cellular markers of action [46]. While not much relationship between NGB and HIF-1 was mentioned, some of the mechanisms of HIF action were explored in this

literature review. Below is a figure that demonstrates the proposed role of HIF in the hypoxic response.

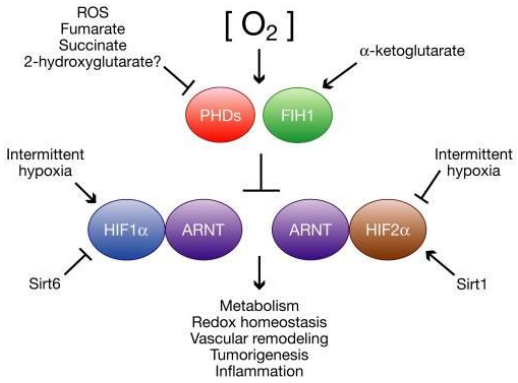


Figure 5: Regulation and Function of HIF-1 subunits [46]
This figure shows the different ways oxygen will regulate the two subunits of HIF: 1-α and 2-α. It also outlines the different markers and the mechanism of their action when exposed to oxygen again. For example, in the presence of intermittent hypoxia, it will activate the 1-α subunit and inhibit the 2-α subunit.
Source: Majmundar, A.J., W.J. Wong, and M.C. Simon, Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell, 2010. **40**(2): p. 294-309.

HIF was shown to regulate erythropoietin production, which shows a potential connection with the activity of NGB as erythropoietin was mentioned as a marker for NGB action. The two best markers that were cited for HIF are sirtuins and intermittent hypoxia. Sirtuins are a class of deacetylases that are involved in gene transcription and DNA repair. Intermittent hypoxia is when cells are exposed to fluctuating cycles of normoxia and hypoxia. HIF is thought to be transcribed mainly under conditions of loss of oxygen levels. As such, O_2 sensors such as PHDs, FIH1, and the mitochondria are essential in helping to initiate HIF transcriptional activity. A minor drawback of this study is that it focused on HIF in the whole body, not just the nervous system, so some of these findings may not pertain to the condition of stroke. Overall, this study helped to gain insight into the function of HIF as well as its mode of action.

Study 3.5 explores the relationship between NGB and two different hypoxia factors: HIF and Nrf2 [14]. This study was cited earlier in regard to the structure and mechanism of action of NGB, including the role of oxygen and HIF-1. Upon further review of this study, more information about HIF-1, in particular, was learned. In fact, this research corroborates the use of PHDs as oxygen sensors to stabilize HIF for action. Additionally, a direct relationship between NGB and HIF/Nrf2 was found. When there is a decrease in the levels of NGB, this also leads to a decrease in HIF and Nrf2. An experiment was performed on rats to see how NGB reacted to the hypoxia. However, the manner of creating a hypoxic condition was very different from that described in Aim 2. This experiment used hypobaria (decrease in pressure) to induce stress, but this could have also introduced many uncontrolled variables. Since this experiment was performed on the harvested brains of rats, these subjects may not provide the most accurate results that can be extrapolated to human brains. In the end, it was found that NGB inhibited caspase 3 and cytochrome c, which supports findings from previous studies. Overall, this experiment helped connect many of the gaps between all five of the studies reviewed.

Graph 1 was made from the data of Table 2. All of the markers mentioned in the "NGB/HIF-1 Findings" column of Table 2 are listed across the X-axis of the graph. A tally of the number of studies that supported the use of that particular marker was recorded. A minimum of one study was required for the marker to be listed on the graph. A maximum of five studies was possible. The Y-axis lists the frequency of the marker—as in, how many studies supported and confirmed the use of the marker. The thick green bar separates the data into two parts. The left side of the green bar represents markers for NGB, while the right side of the green bar lists markers that were specific to HIF-1 action. There are definitely fewer markers for HIF function as only two studies explicitly mentioned these markers. However, all five studies had some

connections to NGB, so it is why there are more markers for NGB. Additionally, this research focuses on the potential benefits of NGB, so more attention was given to studies focused on NGB rather than HIF-1. The graph shows that the most popular marker of NGB action was HIF-1, which was expected since the function of the two proteins is strongly interwoven. On the HIF side of the graph, the most acknowledged factor for HIF action was PHDs. This graph is simply a visual representation to help condense the most essential information from Table 2 into an easy-to-understand manner.

Overall, all these five studies taken together provide insight into the action and regulation of both NGB and HIF-1. First, even though many of the studies that were picked were mainly a literature review of previous research on the topic, they provided a succinct account of findings and research that had already been performed. Together, as seen in Graph 1, the most prominent marker of NGB action is HIF-1, and for HIF-1, the markers are PHDs. This means that once the cells have been exposed to hypoxia, and NGB has been administered, these are the markers that will be analyzed to ensure that NGB has conferred its neuroprotective effect on the cells.

Originally, the experimental design for this aim included testing markers of HIF-1 to see if recovery via NGB had taken place. After the exosomes containing NGB were introduced to the stressed cells in Aim 2, the recovery itself would be measured using HIF-1, because it has been shown to serve as a cellular marker for NGB action. This literature review aids in this process because it reinforces the idea that HIF-1 is a potent cellular marker to measure the action of NGB while also providing other cellular markers that can be investigated. In order to determine the presence of these markers, a western blot would be the best way to ensure that these markers were present because it gives both antibody and size confirmation.

It is important to note that this is simply a literature review of various studies. As mentioned before, many of these studies did not perform any actual experimentation. Therefore, these results do need to be confirmed with a physical experiment to guarantee that they are valid. They provide a good starting point to generate hypotheses to direct further research. Additionally, the results may vary when these experiments are performed *in vitro,* and the findings may lead to entirely different pathways *in vivo*. Therefore, the most accurate results can only be obtained following physical experimentation. Overall, since the markers of both NGB and HIF-1 were collected, Aim 3 was satisfied as well.

EXTENSIONS AND FUTURE STUDIES

Acknowledging that the scope of this thesis is somewhat limited, there are many potential extensions to this research that can be performed to either find similar or contrasting results. Outlined below are only a few studies that can be performed to further this research and this topic. Further research will help either confirm or revise the information provided in this thesis and could also help generate additional research areas or interests.

The research performed in Aim 1 was executed *in vitro* in a laboratory environment. These conditions are different from the ones found physiologically in the body. Therefore, an *in vivo* study on rats or mice may provide more physiologically relevant data. While these animals are not going to provide the same environment as the human body, they can show how the results may shift if in the presence of an actively respiring situation.

Another extension would be to use different cell types. Some of the literature mentioned used SY5Y and astrocytes. These cell types may prove useful in assessing the role of NGB in hypoxic conditions. Additionally, each of these cell types may even provide new insight into the mechanism of action of NGB. Not only that, but further research could also be conducted on cells outside the nervous system. While NGB has been shown to be localized in the brain and spinal cord, the effects of NGB can be observed outside of this as well. For example, this can help determine if the basal levels of NGB action detected in HEK-293 wild-type cells are valid.

In this thesis, the vector for NGB that was used was XPAK-NGB. This vector will pack the protein *inside* the cell. However, another vector that could be used is XSTMP-NGB. This will direct the NGB to be on the surface of the cell. This may change the administration of NGB and may even have an impact on the way it confers neuroprotection on cells. Additionally, some

of the antibodies and modes of detection for NGB may have to be altered to confirm its presence. It may help determine if one vector is easier to work with and provides better data than the other.

While it is clearly best if this experiment had been able to be carried out as expected, there are certain adjustments that can be made to the experimental design to test a different hypothesis. For example, currently, NGB would be exposed to the neuronal cells *after* they were placed into hypoxic conditions. From there, the effects would be measured after introducing NGB to the sample of cells. However, another way to perform this experiment would be to introduce the exosomal NGB to the cells before they are exposed to the hypoxic conditions. This, contrasted with a control group, would help determine whether NGB can be used as a preventative measure rather than a treatment alternative.

These are only a few potential extensions to this thesis. These will help widen the scope of this research and explore different possibilities for additional research. The hope is that this research will encourage further experimentation and provide valuable data to change the direction of future research.

CONCLUSION

In summary, the goal of this thesis is to demonstrate the relationship between neuroglobin and the HIF-1 transcription factor. A hypoxic chamber, along with various assays, can help elucidate the exosomal component of neuroglobin delivery to ischemic cells. Exosomes are able to cross the blood-brain barrier and, therefore, can directly affect neuronal cells and serve as a novel target for therapeutic solutions for stroke patients.

It was found that NGB is able to be packaged into the exosomes following a transfection and exosome precipitation. HEK-293 cells may express basal levels of NGB, but the cells that have been transfected with NGB show a much higher level of expression. The exosomes that were precipitated also showed that NGB was present. Therefore, NGB is able to be packaged within the exosomes and eventually delivered.

A literature review was conducted to determine what conditions of hypoxia would be best for cell viability. Many different studies used different conditions and cell types, but after review, the neuronal cells would be exposed to 5-15 minutes of 1% oxygen in 5% carbon dioxide to induce hypoxia. In order to confirm that the cells were experiencing a lack of oxygen, a western blot focused on hypoxia markers would have to be performed. The presence of these markers would confirm that the cells are in an oxygen deprivation condition.

Following this, the stressed cells would be placed in media that also contained NGB exosomes. The hope would be that NGB in the exosomes would still confer the neuroprotective effects onto the hypoxic cells. This would be tested with a western blot.

To confirm this, another literature review was conducted to see what markers of NGB and HIF-1 constituted their action. Some of the studies were focused on NGB, while other

studies were focused on HIF-1 markers. Gathering the data together, the most efficient marker for NGB action was HIF-1, and in turn, the marker for HIF-1 was found to be PHDs. If NGB were able to act upon the stressed neuronal cells, these markers would show up in a western blot when tagged with their respective antibodies. Since literature reviews still need to be supported with physical experimentation, it is vital to understand the limitations of this particular research.

Put together, this experiment can show whether exosomal delivery of NGB can restore proper cellular function, especially in the cases of stroke. This research can kickstart the potential for drug delivery through these extracellular vesicles for other pathologies as well. While the action of NGB has been well-documented, the association between NGB and exosomes provides a novel approach.

APPENDIX: RESEARCH PROTOCOLS

This appendix lists the experimental protocols for Aim 1.

Aim 1.1: Collect XPAK – NGB DNA

Bacterial Miniprep:

Performed with the QIAgen Bacterial Miniprep Kit

1. The bacterial cultures were incubated overnight at 37ºC.
2. Cultures were spun down at 3000 rpm for 10 mins at room temperature.
3. The supernatant was discarded, and the pellet was resuspended in 1 ml of molecular grade water.
4. The bacterial suspension was moved to a 1.5 ml microcentrifuge tube.
5. The sample was spun down at 13,000 rpm for 1-1.5 mins. Excess liquid was dumped.
6. The pellet was resuspended in 250 ml P1 suspension buffer.
7. 250 ml of P2 lysis buffer was added, and the microcentrifuge tube was gently inverted six times to mix.
8. 350 ml of N3 neutrality buffer was added, and the tube was inverted to mix.
9. The sample was spun down at 13,000 rpm for 10 mins.
10. 750-800 µl of the supernatant was added to the DNA spin column.
11. The column was spun down at 13,000 rpm for 1-1.5 mins. Flowthrough was discarded.
12. 800 µl of the P.E. wash buffer was added to the column.
13. Again, the column was spun down at 13,0000 rpm for 1-1.5 mins. Flowthrough was discarded. This step was repeated to remove traces of the wash buffer.
14. The spin column was transferred to a fresh 1.5 ml microcentrifuge tube.
15. 100 µl of E.B. elution buffer was added to the column.
16. Incubate column for five minutes at room temperature.
17. Spin the column at 13,000 rpm for 4-5 mins. Collect the DNA and discard the culture.
18. Quantify the DNA with a nanodrop.

Aim 1.2: Transfect HEK cells with XPAK-NGB DNA

Cell culture/seeding:

1. HEK-293 cells were grown in T-75 flasks.
2. Cells were passaged with HEK media at 60-70% confluency.
3. Cells were resuspended in 1 ml of media to be seeded into a 12 well plate.
4. 10 µl of cells were transferred to a new tube and diluted with 90 µl of fresh media.
5. Cells were counted, and 150,000 cells were added to each well. Cells were incubated.

Transfection with Lipofectamine 2000:

1. Two 1.5 ml microcentrifuge tubes were labeled Tube A and Tube B.
2. Tube A contained 125 µl of OptiMEM and 1 µl of Lipofectamine 2000.
3. Tube B contained 125 µl of OptiMEM with 1 µg of XPAK-NGB DNA (Clone 4).
4. Both tubes were incubated at room temperature for 10 minutes.

5. The contents of Tube A were added to Tube B *dropwise*. Tubes were inverted 6 to 7 times to mix.
6. Tubes were incubated at room temperature for 20 minutes so that the DNA complex could form.
7. While the tubes were incubating, spent media from the 12 well plate was removed, and fresh media was added.
8. The A+B mixture was added to each well, dropwise. The plate was swirled gently to mix.
9. The plate was set in the incubator at 37ºC.

Immunocytochemistry (ICC):

Day 1:

1. The media was removed from all wells.
2. 500 µl of 4% PFA was added to each well to fix the cells.
3. The plate was incubated for 30 minutes at room temperature.
4. Each well was washed with PBS (3 x 5 minutes).
5. The PBS was removed from the wells, and 500 µl of permeabilization solution was added per well (0.5% Triton-X in PBS).
6. The plate was incubated at room temperature for 10 minutes.
7. 500 µl of blocking solution (10% normal donkey serum in PBS) was added to each well.
8. The plate was incubated for 30 minutes at room temperature.
9. 250 µl of the primary antibody in blocking solution (1:500 dilution) was added to each well.
10. The plate was incubated overnight at 4ºC.

Day 2:

1. Each well was washed with PBS (3 x 5 minutes).
2. 250 µl of the secondary antibody in blocking solution (1:1000 dilution) was added to each well.
3. The plate was incubated in the dark for 2 hours.
4. 500 µl of PBS was added to each well.
5. The cells were taken for imaging.

Antibody Information:

	Primary Antibody	**Secondary Antibody**
Antibody Name	NGB (anti-NGB)	TRITC
Cultured Into	Mouse	Donkey anti-mouse
Concentration	100 µg total	1.5 mg/ml

Aim 1.3: Collect HEK XPAK-NGB exosomes

Exosome Precipitation:

Performed using the PEG-NaCl Precipitation Method:

Day 1:

1. Media from the transfected 12 well plate was collected and frozen over a period of one week.
2. The frozen media was thawed in a water bath.
3. The media was transferred into 50 ml conical tubes of the same volume.
4. The tubes were spun down at 10,000 G for 30 minutes at 4ºC.
5. The supernatant was transferred to a new tube without disturbing the pellet.
6. 5 ml of 20% PEG was added for every 10 ml of culture media collected (1:2 ratio).
7. 200 µl of 7.5 M NaCl was added for every 10 milliliters of culture media collected. The solutions were mixed by vortexing.
8. The solutions were incubated overnight.

Day 2:

1. The samples were spun down at 10,000 G for 1 hour at 4ºC.
2. The supernatant was discarded while the exosomes were present in the pellet but were not visible.
3. The pellet was resuspended with 500 µl 1X sterile PBS.
4. The precipitated exosomes were stored at -80ºC for long-term storage.

Aim 1.4: Ensure that NGB is present in the exosomes

Dot Blot:

Day 1:

1. A small portion of the nitrocellulose membrane was cut.
2. 2 µl of each exosome sample (Transfected and wild-type exosomes) was added to the nitrocellulose membranes.
3. The samples were dried for 15 - 20 minutes.
4. Each membrane was placed in a petri dish with 7 ml of blocking solution (5% BSA in TBS-T).
5. The petri dishes were incubated at room temperature for one hour on a shaker.
6. The membranes were washed with TBS-T (3 x 5 min).
7. 6-7 ml of the primary antibody in blocking solution (1:500 dilution) was added to the petri dishes.
8. The petri dishes were incubated overnight at 4ºC on a shaker.

Day 2:

1. The membranes were washed with TBS-T (3 x 5 min).
2. 6 ml of the secondary antibody in blocking solution (1:10,000 dilution) was added to the petri dishes.
3. The petri dishes were incubated for one hour at room temperature on the shaker.
4. The membranes were washed with TBS-T (3 x 5 min).

5. Some additional TBS-T was added to keep the membranes wet.
6. 6 ml of ECL solution (1:1 ratio) was added to each petri dish.
7. The petri dishes were incubated for 3 minutes at room temperature.
8. The membranes were taken for imaging.

Antibody Information:

	Primary Antibody for Exosomes	**Primary Antibody for Control**	**Secondary Antibody**
Antibody Name	NGB (anti-NGB)	Exosome anti-CD63 for Western	Anti-mouse IgG (HRP)
Cultured Into	Mouse	Mouse	Goat anti-mouse
Concentration	100 µg total	0.5 µg/µL	1.5 mg/ml

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