Development of a Synthetic Biomarker System for Early Detection of Ischemic Stroke

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DEVELOPMENT OF A SYNTHETIC BIOMARKER SYSTEM FOR EARLY DETECTION OF ISCHEMIC STROKE

by

MASON LEE MATTHIES

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ABSTRACT

DEVELOPMENT OF A SYNTHETIC BIOMARKER SYSTEM FOR EARLY DETECTION OF ISCHEMIC STROKE

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Stroke, particularly Ischemic Stroke (IS) affects millions of individuals across the world each year. Current diagnostic methods like CT scans and MRI imaging have limitations in detecting minor IS due to the limited spatial resolution of the CT scan and the feasibility and access to MRIs warranting a more effective method of early detection. Natural biomarkers are currently ineffective in detecting IS prior to severe damage like rapid cell death (32,000 cells/sec) post arterial blockage because there is minimal amount of natural biomarkers expressed from minor cellular damage. Our proposed Synthetic Biomarker System (SBS) would solve this by enabling rapid self-tests at home so that IS patients can seek timely medical attention. The SBS consists of 2 genetic sensors—WCbp16 and WCbp39—to detect IS-induced inflammatory and oxidative cellular stress within BEND3 mouse endothelial cells. We hypothesize that once our SBS is delivered into brain cells, will be able to detect IS-induced inflammation and oxidation at differing severities. We examined the reporter expression via Green Fluorescence Protein signaling and found a correlation between IS induced cellular damage and the amount of reporter expression. Future experiment is warranted to better understand the sensitivity of the SBS.

KEYWORDS: ischemic stroke, inflammation, oxidation, synthetic biomarker system, genetic sensor
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INTRODUCTION

Among all human organs, the brain exhibits the least resilience to hypoxia. If cerebral blood circulation is abruptly disrupted, a deprivation of oxygen, glucose, and essential nutrients in the brain tissue is the result (Gübeli, 2012). This can lead to dysfunction in not only the brain, but also in other bodily functions that are regulated by the affected cerebral regions—a condition commonly known as Ischemic Stroke (IS). Stroke, a quite large and significant global health concern, impacts over a staggering 100 million people annually throughout the world, with a predominant majority of these people (approximately 87%) being affected by Ischemic Stroke (Samanth, 2021). The current landscape for diagnosis of Ischemic Stroke predominantly relies on non-contrast computed tomography (CT) scans. However, while CT scans can be effective, they do possess inherent limitations which arise particularly in the domain of the detection of minor IS cases attributable to their restricted spatial resolution (Luo et al., 2018). A superior spatial resolution compared to CT scans is present within magnetic resonance imaging (MRI), yet there are also downsides to this type of imaging procedure—it remains to be a quite expensive and often inaccessible diagnostic option within routine clinical settings. Consequently, there arises a vital necessity for a highly sensitive diagnostic tool with the capabilities of detecting IS in its early stages. Such a tool would possess the ability to facilitate timely intervention, thereby potentially reducing the severity of the stroke outcome and improve the patient’s prognosis. A pursuit of this type of diagnostic solution poses an urgent imperative in the field of stroke management and truly demonstrates the importance of advancements in molecular biology technology.
One route of measuring biological processes, conditions, and responses to treatments lies within biomarkers. Biomarkers hold a vital responsibility within the world of medicine, biology, and even the development of drugs. There can be many different types of biological constructs including molecules, genes, and proteins (Mens et al., 2021). A biomarker—many times found in blood or urine—signifies alterations in the expression of specific indicators of those who are healthy, and those who are affected with a disease (Ghantous et al., 2020). They are objectively measured and can be used to specify a particular biological state to provide information on a disease activity and progression (Atkinson et al., 2001). Therefore, they can be utilized to pinpoint individuals who may be at a higher risk; however, when it comes to prevention or prediction of an acute or fatal event they fall short. At present, the detection of Ischemic Stroke before substantial damage occurs, such the rapid cell death—at a rate of 32,000 cells per second following arterial blockage—remains challenging due to the inadequacy of the natural biomarkers within one’s body (Stroke Facts and Statistics, 2021). The minimal expression of these natural biomarkers from minor cellular damage renders them ineffective when aiming to detect IS early. To surmount this barrier, a different approach must be attempted—an approach that we propose should rely on a Synthetic Biomarker System instead of a natural biomarkers that can be used to readily detect minor cellular damage of Ischemic Stroke. Importantly, this SBS will allow IS patients to seek timely medical attention, and thus limit damage to the brain from IS.

To preface, the use of synthetic biology and the process of synthetic reconstruction of natural genes and proteins can allow for a new understanding on a cell’s regulatory mechanisms and can additionally offer the possibility for drug discovery/creation. The proposed SBS is a molecular tool that is used for early stroke detection by the process of monitoring cellular stress
responses within neurons and brain vascular cells. The envisioned SBS would be complemented by a user-friendly antibody-based assay, facilitating rapid self-testing at home especially for those who are at high risk of Ischemic Stroke as well as those with minor ischemic events. It would be engineered to exhibit heightened sensitivity and specificity towards early indication of Ischemic Stroke. It functions through a multi-component system that utilizes a genetic sensor, synthetic promoters, and reporter molecules all of which will be tested using the BEND3 Mouse Brain Endothelial cells.

The objectives of this study are to expand upon the BEND3 cell line so that it can be used as a model that can be used to test the functionality of the SBS. This occurs through the use of the genetic sensors that make up the SBS. Within this study there are two genetic sensors that are being used that will allow minor cellular damage to be detected. The first genetic sensor which is the BEND3 WCbp16 sensor, is designed to detect inflammatory cellular stress that is induced by Ischemic Stroke through the signaling pathway of Nuclear Factor Kappa B (NF-κB). The NF-κB pathway is a transcription factor pathway that is involved within stress responses in cells which would include stress responses that are triggered by IS (Liu, 2017). When the NF-κB signal is activated, it indicates that there is a presence of inflammatory cellular stress. Our NF-κB genetic sensor would be able to use the IS-induced NF-κB signaling in the brain to detect the presence of inflammatory cellular stress. Once it detects inflammatory cellular stress it will also play a role as a NF-κB-responsive synthetic promoter driving the expression of Green Fluorescent Protein (GFP) to increase the transcription of our reporter peptide. In response to the detected NF-κB upregulation, the genetic sensor will promote the transcription of a HIS tag poly-peptide reporter allowing for the amplification of the stress signal using the poly-peptide as a secondary signal.
That HIS tag poly-peptide reporter can then be secreted into the blood which would allow for easy detection.

In addition to the NF-κB sensor, our proposed Synthetic Biomarker System also includes a second genetic sensor. The second genetic sensor, BEND3 WCbp39, is designed to detect quantitative changes in intracellular Nuclear factor erythroid 2-related factor 2 (Nrf2) levels in response to oxidative cellular stress. The Nrf2 signaling pathway in the body serves as a pivotal transcription factor in the cellular defense mechanism, particularly in response to elevated oxidative stress induced by tissue ischemia (Tonelli, 2018). This Nrf2 genetic sensor, similar in theory to the first genetic sensor, but it will use the Nrf2 signaling pathway to detect IS-induced Nrf2 signaling in the BEND3 brain cells. Once it detects oxidative stress it will also play a role as a Nrf2-responsive synthetic promoter driving the expression of Green Fluorescent Protein (GFP) to increase the transcription of our reporter peptide. The sensor then amplifies the stress signal that the Nrf2 genetic sensor is detecting in the form of a HIS tag poly-peptide reporter. That HIS tag poly-peptide reporter can then be secreted into the blood which allows for easy detection.

Both of these genetic sensors were created with a certain level of specificity. It is vital for the first genetic sensor (BEND3 WCbp16) is created so it is responsive only to the stress-induced activation of the NF-κB pathway so that the gene expression is precise and is only under the conditions of Inflammatory Ischemic stress. Additionally, the second genetic sensor (BEND3 WCbp39) is created to be responsive only to the stress-induced activation of the Nrf2 pathway. In doing so, the gene expression is again precise under conditions of oxidative ischemic stress. This way when there is observed detection of the reporter peptide within our experiments, it is not due to other factors besides inflammation and oxidation which are immune responses to IS
events. Ideally the reporters that are used with both genetic sensors will be lipophilic in nature allowing the best passage through the blood brain barrier (BBB). It is important to note that when an IS incident occurs, degradation of the BBB at the penumbra grants molecules to pass through, therefore permitting the HIS tag peptide reporter to be excreted into the blood (Heidari et al., 2020). This HIS tag on the peptide allows it to be detected in culture supernatants, blood via anti-HIS antibody assays such as ELISA, as well as in the cells via fluorescence staining.

The goal of our experiments is to improve the diagnosis and therapeutics of Ischemic Stroke. Current and most common stroke diagnostic tools such as CT scans and MRI are not sensitive enough to detect IS early and they are not easily accessible and feasible (Samanth, 2021). Importantly, the identification of IS prior to severe cellular damage cannot be detected by currently natural biomarkers (Wang, 2007). This study is designed to test whether our Synthetic Biomarker system can detect small amounts of Ischemic Stroke-induced inflammatory and oxidative signaling at varying severities within the brain endothelial cells. By combining several biomarkers into a biomarker system we hope to test the functionality of our Synthetic Biomarker System. We hope to use our genetic sensors within our SBS as both a detector of Ischemic Stroke and as a synthetic promoter to allow for small amounts of primary inflammatory and oxidative signaling to be detected and amplified.
MATERIALS & METHODS

In Vitro Model

Within this study, the cellular model chosen for the investigation of Ischemic Stroke Related responses was the BEND3 mouse brain endothelial cells. BEND3 cells have relevance to neurovasculature as well as play a role in the mediation of the blood-brain barrier and inflammatory signaling (Bend.3, 2024). These cells were generously provided by Dr. Luye Quin at the Department of Biomedical Sciences (BBS), University of South Dakota. The first portion of this study was the expansion of this cell line in order to have many generations of cells that could be tested.

Cell Culture

BEND3 Cells were cultured in a complete endothelial cell growth medium. This growth medium was supplemented with 10% fetal bovine serum (FBS) to provide nutrients for cell proliferation and growth, 1% penicillin-streptomycin (PS) to prevent bacterial contamination, and 1% Glutamine to support the growth of the high energy demanding BEND3 cells. Cells were maintained in T75 culture flasks at 37°C within a humidified atmosphere that contained ~5% CO₂. When culturing cells, culture media was replaced every 2-3 days with cell passaging occurring between 80%-90% confluency using standard trypsinization protocols. The BEND3 cell line was expanded largely for our experiments which again followed the established protocols to ensure there was robust growth. Cells that were expanded were frozen down using a freeze down solution to protect their survival at extremely low temperatures and stored within liquid nitrogen until their use in the future for experiments. When used for experiments they were taken out of their liquid nitrogen bath, placed in a 37°C hot water bath for approximately 1-2 minutes, and then pipetted into a 10 mL dish of endothelial cell medium.
Genetic Sensor 1 (WCbp16)

The first genetic sensor involves a complex design aiming to detect early signs of Ischemic Stroke by monitoring the inflammatory responses mediated by the NF-κB signaling pathways. The prototype genetic sensor, the synthetic NF-κB sensor, was created by performing molecular cloning with the Gibson reaction. Through the assembly of DNA fragments, this complex genetic construct was created. This process therefore enables the earliest detection of intracellular NF-κB inflammation/recruitment signals which are caused due to the first immune response following Ischemic Stroke.

Genetic Sensor 2 (WCbp39)

The second genetic sensor represents another crucial component of the biomarker system which is designed to monitor the quantitative changes in intracellular levels of Nuclear Factor Erythroid 2-related factor 2 (Nrf2). Nrf2 is a pivotal transcription factor that is implicated in cellular responses to oxidative stress. This genetic sensor was also created by performing molecular cloning with the Gibson reaction in order for the earliest detection of intracellular Nrf2 oxidation signals. These oxidation signals are caused due to an early immune response following Ischemic Stroke.

Lentiviral Transduction

Once the BEND3 cell line was expanded to an adequate level, the sensors had to be delivered to the target cells. In order to deliver the sensor constructs into the cells, lentiviral particles containing the genetic sensors were generated. The genetic sensors both contain a responsive element that drives the expression of the Green Fluorescent Protein. Lentiviral vectors, having the ability of integrating the sensor construct into the cell were then used in order to create a sensor-equipped brain vascular cell model for testing. These sensor-equipped models
were then again expanded to the P7 generation using the same cell culture techniques previously explained. This allows for many repeat experiments to be done on these cellular models to ensure validity.

**Stimulation with Reagents**

Two separate reagents were used including one cytokine reagent and one oxidative reagent. To induce an inflammatory response to mimic Ischemic Stroke cellular conditions, BEND3 cells transduced with Sensor 1 (BEND3 WCbp16) were treated with tumor necrosis factor alpha (TNF-\(\alpha\)) at concentrations of 0 ng/mL to 100 ng/mL. Wild type BEND3 cells were also used within these experiments as a negative control group where the TNF-\(\alpha\) treatment was not applied. TNF-\(\alpha\) was chosen for this experiment as it is a proinflammatory cytokine that is produced in cells as a response to inflammation (Zu, et al., 2017). During ischemic events, the low levels of oxygen and nutrients lead to cellular stress, damage and inflammatory mediators such as TNF-\(\alpha\) are released into tissue (Zu, et al., 2017). Stimulation of cells with TNF-\(\alpha\) occurred at time points of 24, 48, and 72 hours which was due to the need to evaluate the kinetics of inflammatory signaling activation over several different time points. Stimulation experiments were repeated independently 3 times to ensure reproducibility and were also performed in triplicates in order to validate our findings.

To induce oxidative stress responses, BEND3 cells transduced with Sensor 2 (BEND3 WCbp39) were treated with hydrogen peroxide (H\(_2\)O\(_2\)) at concentrations of 0 \(\mu\)M to 500 \(\mu\)M. H\(_2\)O\(_2\) was chosen as the treatment because it demonstrates the oxidative stress environment associated with Ischemic Stroke—an environment that can lead to neuronal damage and cell death (Lu, et al., 2023). Stimulation of cells with H\(_2\)O\(_2\) occurred at time points of 24, 48, 72, 120, and 168 hours in order to evaluate the dynamics of oxidative stress-induced signaling pathways.
Cell time points that were over 72 hours utilized a change in their culture media to provide those cells with fresh nutrients for the remainder of their treatment period. Again, stimulation experiments were repeated independently 3 times to ensure reproducibility and were also performed in triplicates in order to validate our findings.

**Fluorescence Imaging**

Fluorescence imaging was performed on the BEND3 cells to visualize and quantify green fluorescent protein (GFP) expression that was induced by the cytokine and oxidative reagents. BEND3 cells transduced with lentiviral vectors encoding the Synthetic Biomarker Systems were seeded into 24-well plates and were treated with the respective inflammatory or oxidative reagents that were described earlier. If the cell treatment time point was longer than 72 hours, the culture media within these wells was changed in order to reduce debris and provide fresh nutrients for the cells. Following the time point of stimulation, the cells were washed with phosphate-buffered saline (PBS) and the culture medium was replenished in order to clear away any more cell debris. The plates were then taken over to our fluorescence microscope so that pictures could be taken of our results.

Fluorescence images were acquired using a fluorescence microscope that was equipped with the appropriate settings and filter sets that can detect Green Fluorescence Protein (GFP). The acquisition parameters for each of the images were kept consistent across each sample in order to establish consistency and comparability of the images. Images were taken at a 10x camera under normal lighting to assess cellular damage, and using a GFP lighting to assess the reporter expression. Quantification of GFP fluorescence intensity was performed using image analysis software. Background subtraction, image contrast, and brightness were applied to each image for clarity of the image.
**Flow Cytometry**

Once BEND3 cells were photographed they were prepared for flow cytometry. This occurred in a series of careful steps to ensure sterility and viability of the cells throughout the process. Initially, cells were washed with PBS, trypsinized to detach them from the culture vessel using standard tryptonization procedure, spun down with a centrifuge (5 min), and subsequently counted using standard cell counting procedures to obtain a total cell number. To create the resuspension medium, complete culture medium was diluted at a 1:1 ratio with sterile PBS, with sufficient volume prepared to accommodate the resuspension of all cell samples along with additional buffer. Once the centrifuge was finished, the cell pellets were then resuspended in the prepared resuspension medium. Following the resuspension, the cell suspensions were then filtered through a 40-micron blue filter cap into 5 mL fluorescence activated cell sorting (FACS) tubes to remove any further clumps or debris that might still be in the solutions. After this, the blue caps were replaced with sterile white caps, and securely snapped tight to maintain sterility. Finally, the prepared cell samples were transferred into an ice bucket and moved to the cell sorter to ensure their preservation and timely commencement of the sorting process. Flow cytometry was then completed, and analysis of flow cytometric data was done using Flow Analysis Software.
RESULTS

BEND3 Cell Lines

The first portion of the experiment was to expand the BEND3 cell lines in order to conduct an ample amount of experiments with them. The wild type BEND3 cell line was first expanded to the P8 generation. These wild type cells were used as a negative control within the experiments as a reference point to our BEND3 cells that were treated with our inflammatory and oxidative treatments. The wild type cell line was then transduced using lentiviral particles and sorted in order for the WCbp16 and WCbp39 sensor-equipped cell lines to be created and further passaged. The BEND3 WCbp16 cell line was passaged to the P6 generation. Then the BEND3 WCbp39 cell line was passaged to the P6 generation. The creation of these cell lines was necessary for our experiments to be repeated multiple times to test their validity. Testing of these BEND3 wild type cell lines occurred on older generations usually ranging from P6-P8, and the testing of the BEND3 sensor-equipped cell lines occurred at the P3-P6 generations. The expanded BEND3 cell lines will be used for future directions within Dr. William C.W. Chen’s laboratory experiments.

Response to Inflammatory Stimuli

The investigation into the responsiveness of the Synthetic Biomarker System to inflammatory stimuli (Sensor 1) yielded notable findings. Stimulation with TNF-α elicited fluorescence signal expression in BEND3 cells as soon as 24 hours post-treatment (Figure 1, Figure 11). Additionally, this stimulation also produced a robust fluorescence signal expression at 72 hours post-treatment (Figure 3). The most particularly evident of these results was at a concentration of 10 ng/mL (Figure 1, 3). The amount of green fluorescence protein was observed to increase as the concentration of our inflammatory TNF-α stimuli increased. There was no
fluorescence signal expression that was observed within BEND3 cells that did not receive inflammatory treatment (Figure 1, Figure 3).

Cellular damage from inflammatory stimuli was observed additionally within our cells. As the level of inflammatory treatment was increased across all stimuli there was slight cellular damage that could be observed (Figure 2, Figure 4). Within 24 hours of treatment, slight cellular damage was seen at a concentration of 0.2 ng/mL TNF-α and was seen to increase moreover at the concentration of 10 ng/mL TNF-α (Figure 2). At 72 hours post treatment, there was also a visible amount of cellular damage that occurred at concentrations of 2 ng/mL and 10 ng/mL (Figure 4).

Response to Oxidative Stimuli

Additionally, the investigation on the responsiveness of the Synthetic Biomarker System to oxidative stimuli (Sensor 2) provided important findings. Exposure to H₂O₂, a potent inducer of oxidative stress, resulted in fluorescence signal expression in BEND3 cells within 24 hours post-treatment (Figure 5), with a robust fluorescence signal expression seen in cells at 72 hours post-treatment (Figure 7). Most notably, was the responsiveness of our biomarker system at a concentration of 500 µM (Figure 5, 7). Additionally, there was no fluorescence signal expression within cells that did not receive the oxidative treatment (Figure 5, Figure 7). Interestingly, further analysis revealed that there was a decline in the fluorescence signal expression at the concentration level of 500 µM after an extended exposure time of 168 hours (Figure 9).

There was also cellular damage that occurred at these time points that is visually represented in the pictures taken. This can be seen 24 hours post treatment slightly at 25 µM, more at 200 µM, and the most seen at 500 µM of H₂O₂ (Figure 6). Cellular damage was also seen to increase with increased concentration of the treatment at 72 hours (Figure 8). At the time
point of 168 hours, major cellular damage was observed at a concentration of 500 µM/mL (Figure 10).

**Flow Cytometry**

Flow cytometry was performed on treated cells after fluorescence photographs were taken. However, it was only performed on the BEND3 WCbp16 cell line which was treated with TNF-α due to limitations on the availability of a flow cytometry machine. It was seen that as the concentration of the TNF-α treatment increased, the GFP intensity was seen to increase as well (Figure 11).
DISCUSSION

Green Fluorescence Protein Expression

The organization of the cellular response from the stress treatments is beginning to be elucidated. The foundation of our Synthetic Biomarker System lies in the integration of two genetic sensors targeting distinct cellular pathways associated with ischemic injury—NF-κB and Nrf2. The BEND3 WCbp16 cell line employs the NF-κB genetic sensor that utilizes the NF-κB signaling pathway. This pathway is sensitive to inflammation within endothelial cells. At the heart of this system is where the NF-κB response element lies, which is a specific DNA sequence where NF-κB protein binds upon activation (Bearoff, 2022). Activation of NF-κB serves as a sentinel event for our study, as it triggers a large cascade of molecular events that eventually results in the initiation and propagation of inflammatory signaling cascades within the affected cells. Once inflammation is detected by the NF-κB genetic sensor it then serves as a promoter to initiate the transcription of the HIS-tag-labeled peptide reporter. This reporter is designed to mimic a natural peptide in order to minimize the likelihood of eliciting an immune response. In closely resembling the natural peptides, the synthetic reporter lowers the risk of provoking an immune reaction while simultaneously serving as an effective biomarker for cellular distress. The transcriptional activation of this peptide reporter seen in the form of fluorescence imaging was observed to increase as the level of cellular distress from TNF-α was increased at time points of 24, 48, and 72 hours (Figure 1, Figure 3). The most amount of reporter expression was seen at the highest concentration of TNF-α (10 ng/mL). However, when there was no TNF-α treatment added to the cells there was no observed GFP expression which was expected as the sensor should not be transcribing the peptide reporter when there is no cellular stress occurring.
Similarly the BEND3 WCbp39 cell line which employs our second novel genetic sensor monitors the quantitative changes in intracellular Nrf2 levels in response to oxidative stress. Nrf2 plays a large role in cellular defense mechanisms against oxidative stress seen in tissue with ischemia (Chen et al., 2012). As our sensor detected and responded to the oxidative environment, our sensor offers valuable insight on the cellular response to oxidative stress. This Nrf2 genetic sensor served as a detector of oxidative stress and then as a promoter to activate the transcription of a HIS-tag-labeled peptide reporter that mimics natural peptides. The activation of this reporter was observed within the fluorescence imaging photographs. We observed an increase with GFP levels as the level of cellular distress from H$_2$O$_2$ was increased at time points of 24, 48, and 72 hours (Figure 5, 7). However, when fluorescent imaging was observed at 168 hours post treatment with H$_2$O$_2$ GFP signals were only observed to increase up to a concentration of 200 $\mu$M (Figure 9). At a concentration of 500 $\mu$M H$_2$O$_2$, a large drop off in GFP expression was seen which can most likely be attributed to the length of time that this high level of H$_2$O$_2$ was a part of the BEND3 cellular environment. There was no observed GFP expression when there was no H$_2$O$_2$ added to the cellular environment which again was expected as the genetic sensor should not be eliciting a response when there is no cellular stress.

The observed increased reporter expression in response to the activation of the Synthetic Biomarker System under Ischemic Stroke conditions demonstrates the functionality of our system. Infected cells that were subjected to the Ischemic Stroke conditions exhibited heightened reporter expression within both genetic sensors indicating the successful activation of our genetic sensors in response to ischemic cellular distress. However, the exact sensitivity of our SBS is not fully elucidated from these results, yet the functionality of our biomarker system and reliability of our biomarker system are confirmed.
Cellular Damage

Cellular damage was also observed within these experiments when treated with the two inflammatory and oxidative reagents. After 24 hours post treatment, cellular damage did not occur—confluency ~95%—when there was no TNF-α treatment which was the expected outcome as cellular damage should not occur when the cells are in a stress free environment. When BEND3 WCbp16 cells were treated with TNF-α, cellular damage after 24 hours—confluency ~75%—was seen to occur starting at a concentration of 0.2 ng/mL. Maximum cell damage—confluency ~60%—was seen at the concentration of 10 ng/mL TNF-α (Figure 2). At 72 hours post treatment again no TNF-α treatment resulted in no cellular damage. Cell damage began at a concentration of 0.2 ng/mL TNF-α—confluency ~85%—increased at 2 ng/mL—confluency ~80%—and max cellular damage seen at 10 ng/mL TNF-α with a confluency of ~70% (Figure 4). Observing cellular death following the treatment with TNF-α is definitely linked to the pathophysiology of ischemic stroke as well as the utility of our genetic Sensor 1. Importantly, the sensor detecting increased levels of reporter expression along with the increased levels of TNF-α concentration leading the more cellular death underscores the success of the Synthetic Biomarker System.

In the experiments that used H₂O₂ as a treatment to mimic oxidative stress, cellular damage was also observed. H₂O₂, being a very potent inducer of oxidative stress, served as a great model to understand cellular response to Ischemic Stroke with the use of our Genetic Sensor 2. 24 hours post treatment of H₂O₂ cellular damage was not observed in the 0 µM H₂O₂ cells with a cellular confluency of ~90%. This aligns again with our expectations that cellular damage should not occur unless cells are treated. At 25 µM H₂O₂, 200 µM H₂O₂, and 500 µM H₂O₂ cellular damage was seen to have increased with cell confluencies of ~85%, ~75%, and
~60% respectively. Additionally at 72 hours post treatment cellular damage was observed at 25 µM H$_2$O$_2$, 200 µM H$_2$O$_2$, and 500 µM H$_2$O$_2$. Cell confluencies for these concentrations were ~85%, ~75%, and ~50% respectively (Figure 8). Lastly, at the 168 hour time point cellular damage was observed at 25 µM H$_2$O$_2$, 200 µM H$_2$O$_2$, and 500 µM H$_2$O$_2$ at cell confluencies at ~85%, ~60%, and ~35% respectively. No cellular death was observed at the 0 µM H$_2$O$_2$ concentration level. We found that as concentrations of H$_2$O$_2$ are increased that more cellular death can be observed within our BEND3 cells. The increased cellular death can be seen when comparing the each time point as cellular confluencies decreased when concentrations of treatment increased. Each time point the concentration of 500 µM H$_2$O$_2$ displayed the most cellular death. Additionally, cellular death was also observed to increase with the length of the time point as well. Each concentration level—0-500 µM H$_2$O$_2$—displayed higher cellular damage when compared to a time point that was longer. Most notable, this can be seen when comparing the 500 µM H$_2$O$_2$ groups at each of the time points. This demonstrates the longer treatment of H$_2$O$_2$ to the largest amount of cellular death. It is important to note that 500 µM is an large amount of H$_2$O$_2$ and the observed almost complete cellular death after 168 hours is not an unexpected result (Figure 10).

**Challenges**

This experiment put forth challenges that must be discussed. Selecting an appropriate cellular model system that faithfully exemplifies the complex pathophysiological process of IS presented a challenge. The BEND3 cell line does not fully represent the intricate cellular interactions and tissue-level responses observed *in vivo* but does serve as a great stepping stone for future experiments (Toth, 2019). BEND3 cells were seen to have a slower growth rate than other cell lines that cell culture techniques were practiced on, which resulted in slightly longer
growth periods during the cell passage steps. Additionally, the BEND3 cells trypsinization processes proved some issue as the BEND3 cells adhesion of the plates/dishes they were grown on was stronger than expected. Therefore, the cells warranted slight physical intervention in the form of tapping the sides of the dishes to fully remove the cells from the bottoms of their flasks. However, overall, the BEND3 mouse brain endothelial cells were a great model to use during this study.

Recreating the dynamic and environment of Ischemic Stroke in an *in vitro* setting poses some additional challenges. Ischemic Stroke is a complex interplay of ischemia, oxidative stress, inflammation, and neuronal injury, all of which influence cellular responses and biomarker expression (Heiss, 2016). Mimicking these conditions *in vitro* while continuously maintaining cellular viability and experimental reproducibility presents some hurdles. This can include the precise control of oxygen and nutrient levels of the cells, even though cellular conditions were kept similar when conducting experiments. Additionally, evaluating the sensitivity and specificity of the Synthetic Biomarker System is inherently challenging. Distinguishing slight changes in biomarker expression levels from background noise and experimental variability can be difficult. The validation of the correlation between the reporter expression and the severity of the induced damage does rely on accurate quantification and interpretation of the data. It can be challenging to ensure the system’s sensitivity. It must be finely tuned to detect early Ischemic Stroke while avoiding hypersensitivity to trace levels of stress that naturally occur.

Finally, challenges with the availability of machines used during this experiment poses some issues as well. After imaging was done on the cells to capture their GFP signaling, they were prepared for Flow Cytometry. The BEND3 cell lines posed some challenges in Flow Cytometry due to the size of the cells. When this portion of the experiment was performed, the
Flow Cytometer would many times get clogged or stuck and we would be unable to perform the procedure completely. To combat this issue, the cell preparation process was tweaked so that a smaller number of cells were used. However, this again led to issues clogging the machine to the point where a new machine was to be ordered in hopes of getting better results. This is why flow cytometry data was only collected on a select few experiments.

**Future Direction**

The development of this Synthetic Biomarker System represents a large step forward in the early detection and monitoring of Ischemic Stroke. Moving forward there must be further experiments to help reach the end goal of developing an off the shelf rapid IS test kit. One way to do this would be the optimization of the Biomarker’s System sensitivity and specificity. Through fine tuning the actual design of the genetic sensors one might be able to improve the system’s ability to detect subtle changes indicative of ischemic injury. This could be tested using various time points other than the ones tested so they may better reflect minor ischemic stroke events. One proposal to this would be to treat the cells with the reagents for a shortened period of time and then replacing the cell culture media to fresh plain media. This may aid in detecting if reporter expression could be observed within less than 24 hours post treatment. The goal we reached from these experiments is demonstrating the functionality of the Synthetic Biomarker System in detecting Ischemic Stroke conditions across varying severities. This helps lead to future investigations, which would include the testing of the Synthetic Biomarker System in mice. The data retrieved from these experiments will be incorporated into a larger thesis publication.

**Conclusion**
The global burden of ischemic heart disorder continues to be a pressing public health challenge as Ischemic Stroke has emerged as a leading cause of mortality worldwide (Yan, 2022). Ischemic Stroke represents a life-threatening condition needing quick diagnosis and treatment in order to lower the devastating consequences on patient outcomes. This study first, expanded the BEND3 cell line that can further assist in Ischemic Stroke research in the future. Additionally, this study investigated the responsiveness of our Synthetic Biomarker System to two distinct stimuli: Tumor Necrosis Factor Alpha (TNF-α) and Hydrogen Peroxide (H₂O₂). Responsiveness to these stimuli was observed in accordance with our hypothesis that as the severity of the treatment was increased, the reporter expression from our SBS was additionally increased. Further experiments must still be done in order to understand more about the sensitivity and specificity of the SBS; however, these initial results will help pave the way for future applications in real-time monitoring of cellular response to Ischemic Stroke.
**FIGURE LEGEND**

**Figure 1**
GFP Signals: TNF-α for 24 hrs

Figure 1. 24 hours after TNF-α stimulation, no GFP signaling was observed when there was no inflammatory stimulation (top left). This data suggests that Sensor 1 (WCbp16) can detect inflammatory stress at 0.2 ng/mL TNF-α and higher (top right). Increased GFP expression was observed as the Inflammatory stimulation concentration was increased. The maximum level of GFP signaling was observed at the concentration of 10 ng/mL TNF-α.
Figure 2
Cellular Damage: TNF-α for 24 hr

![Cellular images showing damage at different TNF-α concentrations.]

Figure 2. 24 hours after TNF-α stimulation cellular damage can be observed. With no TNF-α stimulation, no cell damage was seen with a confluency of ~95%. At concentrations of 0.2 ng/mL TNF-α confluency was ~75%. Increased cellular damage was seen at 2 ng/mL TNF-α with a confluency at ~65%, and most cellular damage was seen at concentration of 10 ng/mL TNF-α with a confluency of ~60%.
Figure 3
GFP Signals: TNF-α for 72 hrs

Figure 3. 72 hours after TNF-α stimulation, no GFP signaling was observed when there was no inflammatory stimulation (top left). This data suggests that Sensor 1 (WCbp16) can detect inflammatory stress at 0.2 ng/mL TNF-α and higher (top right). Increased GFP expression was observed as the Inflammatory stimulation concentration was increased. The maximum level of GFP signaling was observed at the concentration of 10 ng/mL TNF-α.
Figure 4
Cellular Damage: TNF-α for 72 hrs

**Figure 4.** 72 hours after TNF-α stimulation cellular damage can be observed. With no TNF-α stimulation, no cell damage was seen with a confluency of ~90%. At concentrations of 0.2 ng/mL TNF-α confluence was ~85%. Increased cellular damage was seen at 2 ng/mL TNF-α with a confluency at ~80%, and most cellular damage was seen at concentration of 10 ng/mL TNF-α with a confluency of ~70%.
**Figure 5.** 24 hours after H$_2$O$_2$ stimulation no GFP expression was observed when no treatment was used. Slight GFP expression was seen at 25 µM H$_2$O$_2$. Greater expression was observed at 200 µM H$_2$O$_2$, and the maximum amount of expression was observed at 500 µM H$_2$O$_2$.
Figure 6
Cellular Damage: H$_2$O$_2$ for 24 hrs

Figure 6. 24 hours after H$_2$O$_2$ stimulation cellular damage can be observed. At no level of H$_2$O$_2$ stimulation there was ~ 90% confluency. Slight cellular damage was seen at 25 µM H$_2$O$_2$ with a confluency of ~85%. At 200 µM H$_2$O$_2$ confluency was ~75%. Maximum cellular damage was seen at 500 µM H$_2$O$_2$ stimulation with confluency of cells at ~60%.
**Figure 7.** After 72 hours post H$_2$O$_2$ stimulation GFP expression can be seen starting at 25 µM H$_2$O$_2$. No GFP signals were observed at 0 µM H$_2$O$_2$. GFP signaling increased, but not drastically at 200 µM H$_2$O$_2$. Maximum GFP signaling was observed at 500 µM H$_2$O$_2$. 
**Figure 8**

Cellular Damage: $\text{H}_2\text{O}_2$ for 72 hrs

Figure 8. 72 hours after $\text{H}_2\text{O}_2$ expression cellular damage can be seen. With no stimulation cell confluency was ~90%. At 25 µM $\text{H}_2\text{O}_2$, cell confluency was ~85%. More cellular damage was seen at 200 µM $\text{H}_2\text{O}_2$ with a confluency of ~75%. Significant cellular damage was seen at treatment levels of 500 µM $\text{H}_2\text{O}_2$ with cell confluency at ~50%.
Figure 9
GFP Signals: H$_2$O$_2$ for 168 hrs

Figure 9. 168 hours after H$_2$O$_2$ treatment GFP signals can be observed. No signals were observed when there was no oxidative treatment. At 25 µM H$_2$O$_2$ slight GFP expression was observed. At the 200 µM H$_2$O$_2$ treatment level, maximum GFP signaling was observed. At a concentration of 500 µM H$_2$O$_2$ almost no GFP signaling was observed.
Figure 10. 168 hours after H₂O₂ treatment cellular damage was observed. With no oxidative treatment, no cellular damage was observed with a confluency of ~90%. At 25 µM H₂O₂ cell damage was slight if any with confluency at ~85%. At 200 µM H₂O₂ cell damage was more severe with a confluency of ~60%. At 500 µM H₂O₂ cell damage was drastic with cell confluency at ~35%.
Figure 11
Flow Cytometry Data: TNF-α for 24 hrs

Figure 11. Flow cytometry data was collected after the 24 hour post treatment of TNF-α. GFP signaling was not observed when cells were not treated with TNF-α. A maximum GFP expression was seen at 20 ng/mL of TNF-α. The graph is the flow cytometry data that was collected from the flow cytometry procedure.
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