Characterization of Poly(ADP-ribose) (PAR) as a death signal in PARP-1 dependent cell death

by

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Abstract

Poly(ADP-ribose)polymerase (PARP) overactivation induces cell death called parthanatos via production of large swells of PAR. Parthanatos is apparent in debilitating diseases such as stroke and Parkinson's disease. PAR is complex polymer of ADP-ribose with different sizes and branching structures. Complex PAR is more toxic as compared to shorter length PAR. The role of different PAR species play in disease complexity and progression is still unknown. Moreover, the tools currently available are not sensitive enough to detect the differences in PAR species. While the current antibodies available are capable of detecting PAR for immunoblot analysis and immunohistochemistry analysis, the detection threshold is high and specificity for varying PAR structures is low. Here, we generated a panel of recombinant antibodies using phage display HuCal technology to effectively detect PAR species in different disease models. PAR induces cell death via translocation of AIF to the nucleus. To further understand how different species of PAR regulate the nuclear translocation of AIF, we generated a PAR-binding mutant AIF transgenic knock-in mouse. Induction of stroke via middle cerebral artery occlusion (MCAO) is used to detect the PAR-induced cell death.

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Introduction

Parthanatos: PARP-1 dependent cell death

Parthanatos is a key cell death mechanism involved in various diseases including ischemic injury and excitotoxicity. It involves poly(adenosine diphosphate-ribose) polymerase (PARP), which has been documented to play a role in both DNA repair and in cell death.[1] Under conditions of oxidative stress from reactive oxygen species (ROS), or cytotoxicity, cells will exhibit DNA damage. The role of the PARP family proteins, which are involved in base excision repair, allow for the repair of DNA damage upon activation of cell cycle checkpoints.[2] PARP-1 is a 116kDa protein containing three major domains: DNA binding domain (with nuclear localization sequence), catalytic domain (55kDa), and automodification domain (16kDa) that are responsible for harboring the NAD-bidning domain and PAR synthesizing domain.[3,4] The amino acid sequence is also highly conserved between the human and mouse, with up to 92% homology.[5]

Under the condition of DNA damage, PARP-1 utilizes nicotinamide adenine dinucleotide (NAD^+) to generate nicotinamide and ADP-ribose, where subsequent ADP-ribose are linked in a long simple and complex branched chain of PAR polymers. The PAR polymer acts as a recruiter of other proteins, such as XRCCI and histones to recruit DNA polymerase and DNA ligase III to repair the single or double stranded break. In sum PARP-1 plays a role in maintaining cellular homeostasis and genomic stability under physiological conditions[6]

PARP-1 overactivation causes parthanatos, which is a dominant form of cell death in stroke, glutamate excitotoxicity, and other inflammatory processes.[7] Release of apoptosis inducing factor (AIF) from the mitochondrial membrane and nuclear translocation induces chromatin condensation and large-scale DNA fragmentation. This activity is a prominent feature in Parthanatos. Unlike in apoptosis, PARP-1-mediated cell death, parthanatos, does not result in formation of apoptotic bodies. Furthermore, parthanatos is caspase-independent, as caspase-inhibitors fail to rescue cells undergoing parthantos.[8] It is also distinct from necrosis, in that it does not induce cell swelling. Studies have shown that the pathway is also caspase-independent, and hence parthanatos is an alternative form of programmed cell death.

Although energy depletion plays a factor in parthanatos, studies indicate that energy depletion alone is insufficient in causing parthanatos. In fact, primary cell cultures studies show that energy depletion was not the primary factor.[9]

Under conditions of parthanatos, PARP-1 is responsible for the majority of the PAR polymer generation, while maintaining a low basal level of PAR in normal conditions. The PAR polymer induces cell death in a manner dependent on its length, branching structure, and complexity. [10]When direct delivery of PAR polymer was administered to primary neuron cultures and HeLa cells, it was found that complex PAR polymers are more toxic than shorter chain PAR polymers.[10] Additionally, as the

concentration of the 60-mer PAR polymer is increased, the cells exhibit a dose-dependent toxicity, comparable to NMDA excitotoxicity or MNNG-induced cell death.[10] Previous studies have also administered equal levels of poly-adenine, which has equivalent negative charge to the PAR polymer. These did not show the same toxic effect as PAR polymer.[10]

Poly(ADP-ribosyl)ated proteins might also contribute to PARP-1-mediated cell death.[11] PARP substrates include many cytoplasmic and nuclear proteins, which are covalently modified by PAR.[12] Modification by Poly(ADP-ribosyl)ation alters the charge distribution and adds bulky structures to target proteins, inhibiting physiological protein function. Severe poly(ADP-ribosyl)ation of key cellular proteins might trigger downstream cellular death. Additionally, PAR polymers might directly be involved in cell-death signaling. The binding of cellular proteins to PAR polymer through a predicted consensus motif (composing of hydrophobic and basic amino-acid residues) could lead to additional signaling routes. Therefore, PAR polymer is an important signaling molecule that might be an attractive therapeutic target through the interference of the downstream signaling of PAR polymer.

There is strong evidence of parthanatos involvement in Parkinson's disease (PD). In PD patients, neurons show marked nuclear translocation of AIF.[13] Additionally, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has been used as a common model for PD cell death.[14] It is a neurotoxin that is converted to MPP+ (1-methyl-4-

phenylpyridinium) by monoamine oxidase,[15] which inhibits mitochondrial complex I activity. The disruption of the mitochondrial function leads to energetic failure and thereby increasing glutamate accumulation and hyperstimulation of glutamate receptor.[16] This leads to downstream NO-activation and activation of PARP-1 following DNA damage.[17] In the MPTP model, aminoacyl-tRNA synthetase complex interacting multifunctional protein-2 (AIMP2), [18]which is a substrate of the E3 ubiquitin ligase *PARKIN*, accumulation has been identified. In *in vitro* and *in vivo* studies, AIMP2 accumulation resulted in PARP-1 activation, providing another pathway for parthanatos other than DNA damage.

Ischemic injury has also been linked with PARP-1 activation through the release of excitatory neurotransmitter glutamate. The over activation of three subtypes of glutamate receptors: N-methyl-D-aspartate (NMDA), AMPA and kainate receptors, leads to massive calcium influx, resulting in the generation of nitric oxide (NO) and reactive oxygen species (ROS). Superoxide can combine with NO forming peroxynitrite (ONOO-). These ROS and radicals induce chromosomal DNA breaks, leading to the PARP-1 activation described previously.

Current tools to detect PAR species are ineffective in detecting various types of PAR. The commercial PAR antibody 10H (Trevigen) has weak specificity with PAR and shows weak bands in western blot analysis, minimal effectiveness in immunohistochemistry, and does not

immune-precipitate significantly. It is still unknown the role of shorter length and structure PAR plays in parthanatos. While it is known that the addition of 60-mer PAR induces cell death, it is unclear the role of branching plays in the different models of parthanatos. Hence, the generation of improved antibodies for PAR will allow for better future studies involving the parthanatos pathway.

Apoptosis Inducing Factor (AIF)

Apoptosis inducing factor is a flavoprotein crucial in parthanatos. It serves a dual function in the cell, with separate binding sites for PAR and DNA, which appear to have evolved separate binding properties. Under normal conditions, AIF acts as an NADH oxidase, important in the electron transport chain in the mitochondria, involving the assembly and stabilization of respiratory complex I. It also has a role as a promoter of cell death, as AIF gets released to the cytoplasm after PARP-1 activation and translocated to the nucleus to induce cell death via large scale DNA fragmentation and chromatin condensation.[19]

Precursor AIF synthesized from a nuclear gene is 67 kDa. The two mitochondrial localization sequences (N-terminal pro domain) allow for import to the mitochondria. At the mitochondria, it is then proteolytically cleaved to the mature 62 kDa form, where it resides as an inner membrane anchored protein, whose N-terminus is exposed to the mitochondrial matrix, and the C-terminus faces the intermembrane

space.[20] There are two pools of AIF in the mitochondria, with 80% residing in the inner membrane and inner membrane space, where it is protected from direct PAR activity. The remaining 20% of mitochondrial AIF resides on the outer mitochondrial membrane, where it is susceptible to PAR binding and subsequent release in parthanatos.[22] The three structural domains of mature AIF include a FAD-binding domain, a NADH-binding domain, and a C-terminal domain.[21,22] The reduced form of AIF tends to form a dimer, suggesting that there may be a balance between the dimeric and monomeric forms in regulating AIF redox and apoptosis-inducing behaviors.[23]

The loss of AIF has been associated with growth impedance in development. In *Drosophila melanogaster*, knockout of AIF expression results in decreased embryonic cell death and preservation of differentiated neuronal cells, but exhibit growth arrest at the larval stage as well as mitochondrial respiratory dysfunction.[24]

The manner in which AIF affects respiratory machinery is yet to be fully understood. However, AIF redox has been indicated to be essential for cell survival. Harlequin (Hq) AIF-mutant mice, which have 80% reduced AIF expression, suggested that AIF has an important role in maintain cell physiology and long-term survival under basal conditions. AIF also plays an important role as an effector of parthanatos when it

translocates to the nucleus to initiate chromatin condensation and large scale DNA fragmentation.[25]

PAR polymer can directly bind to AIF and facilitate its nuclear translocation and Parthanatos. AIF has been shown to bind directly to PAR, which is critical in its role to induce parthanatos.[26] It is likely that PAR causes a conformational change in AIF upon binding, which lowers the affinity of it for the mitochondrial outer membrane. However, the exact mechanism that this occurs is still unclear. The Arg^{588}, Lys^{589}, and Arg^{592} in AIF are essential for the binding of PAR.[17]

NMDA treatment of Hq cortical neurons transduced with WT-AIF were susceptible to NMDA excitotoxicity across 24 to 48 hour time points, in contrast to the effects seen in PAR-binding-mutant AIF-transduced (Pbm-AIF) Hq cortical neurons. It is PAR that disturbs the binding of AIF to the mitochondria, allowing its release and ultimate translocation to the nucleus.

To establish a stronger animal model of the Pbm-AIF, genetic knock-in mice for the Pbm-AIF were generated in BL6 mice for behavioral and stroke studies. Biocytogen was contracted to perform the mutagenesis via bacterial artificial chromosome (BAC). Briefly, gene sequences for each mutation site and probes were amplified by PCR and ligated into pCR-blunt vector and colonies were confirmed through sequencing. Through restriction enzyme digestion, the individual gene

fragments were ligated into pNLF or pDTA vectors and subsequently combined. This is then electroporated into BAC-containing competent cells. Once clones were tested to contain all the correct fragments, they were transformed into stbl3 and confirmed by sequencing following Maxi prep. This targeting vector was subsequently linearized by restriction enzyme digestion (AscI) and elctroporated into ES cells into 3.5 day embryos. Following transfection, selection process was done via geneticin. The resulting chimeras generated through this process were bred with BL6 mice for heterozygous F1 pups.

The targeting vector in the F1 mice with the wild type and mutant AIF genes include a neo-cassette, which protects mammalian cells from the antibiotic neomycin, flanked by FRT sites. Removal of the neo-cassette by breeding with FlpE mice will generate conditional knock-in mice via FLP—FRT recombination. (Figure 7) Further breeding of the floxed mice with proteamine-Cre mice results in Cre-Lox recombination for genetic Pbm-AIF knock in mice.

Transgenic mice with the Pbm-AIF knock in serves as a more robust model to evaluate PARP-1 activation compared to virally transduced Hq mice. In conjunction with previous data, it enables the more detailed study of the mechanism in which AIF release from the mitochondria induces cell death. Thus far, it has been difficult to separate AIF's dual functions, as the loss of AIF disrupts mitochondrial

function and therefore energy metabolism, complicating attempts to assess its role in cell death.[27] With the newly generated transgenic mice that express the triple binding site mutants, AIF activity in the mitochondria can be effectively studied, as the release of AIF following PARP-1 activation is prevented. Further elucidation of the parthanatos signaling pathway will offer opportunities for the discovery of previously unidentified biological functions. Additionally, this might allow the discovery of potential drug compounds that might inhibit AIF binding with PAR to protect against parthanatos. It will also help identify drugs that promote AIF release in cancer cells as a chemotherapeutic agent.

Human Combinatorial Antibody Library (HuCAL) Technology

The HuCAL technology presents an alternative method to the conventional methods of obtaining custom antibodies. Whereas the production of monoclonal antibodies requires immunizing a mouse, rabbit, or goat, and subsequently extracting the B cells from the spleen to recover the antibodies presented, HuCAL technology allows for faster production time. Through the use of library of complementarity determing regions (CDR), 6 light chain variable regions (V_L) and 7 heavy chains (V_H), it is possible to generate billions of antibodies *in vitro*. This is paired with a phage display that incorporates the antibody genes into bacteriophages that will present the antibody on the coating via a disulfide bond, thereby presenting a physical linkage of the phenotype

and genotype. A reductive cleavage of the disulfide bond will allow for the recovery of the antibodies following a screening, independent of the affinity to the antigen. AbD Serotec was provided with synthetic purified terminally-biotinylated PAR polymer. It was synthesized through reductive amination of pure PAR polymer (2-300mer).[28] The antibody clones were selected for binding to only polymers and oligomers, and not the ADP-ribose monomer.

The HuCAL antibody generation process begins with the immobilization of the antigen (PAR), typically using the covalent coupling to magnetic beads. These are subsequently incubated with the HuCAL library where nonspecific antibodies are washed out and the specific antibody-phages will be eluted. *E. coli* cultures are then subjected to infection from the specific antibody-phages to generate an enriched antibody library for the subsequent round of phage screening. It is then that the DNA from these enriched antibody-phases are retrieved and subcloned into Fab expression vectors and plated into *E. coli* colonies to produce the Fab fragments. Following this are the colony picking, primary screening, where the colonies were grown in a 384-microtiter plate. Antibody expression is induced and collected after lysing the cultures. These cultures are screened by ELISA with the terminally labelled-PAR antigen. The positive hits from the primary screening will then be sequenced to identify the unique antibodies, which can be stored for future synthesis for reproducibility. Secondary screening was

performed to select out PAR monomer binding antibodies. Finally, expression and purification through affinity chromatography was performed to obtain antibody clones.

Results

Characterization of the HuCAL antibodies

Enzyme-linked immunosorbent assay (ELISA) is commonly performed as a quality-control check to validate the specificity of the antibodies generated, as it is one of the most sensitive and reproducible assays. As such, the 19 antibody clones were tested against the biotin-labeled PAR polymer, with increasing levels of the monomer ADP-ribose as inhibitors. (Figure 1)

To characterize and evaluate the effectiveness of the HuCAL recombinant antibodies, western blot, immunostaining, and immunoprecipitation were performed on cell lysates. Radiolabelled PAR samples were used to evaluate the specificity of the antibodies and determine the size of the PAR polymer recognized by the various HuCAL recombinant antibodies.

MNNG treatment as a model

MNNG is an alkylating agent, consisting of a nitrosourea structure, to induce PARP-1 activation.[29] Under low concentrations of MNNG (0.5-10uM), DNA damage is limited in the cell, and the repair machinery is

able to re-establish homeostasis.[30] However, under high concentrations (100-500uM), DNA damage is extensive and the disproportionate activation of PARP-1 results in the downstream cell death from parthanatos.

In time-dependent experiments, it was determined that PAR could be detected as early as 1 minute of treatment, but peaked between 10 to 15 minutes of MNNG. This is consistent with the finding of early ROS production time points indicative of PARP-1 activation.[31]

PARP inhibitors, notably 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ), have been used for treatment of ischemic stroke in animal models. More recently, potent PARP inhibitors have been shown to be effective in treating human cancers with DNA repair deficiency.[32] BMN673 was identified as one of the most potent PARP1/2 inhibitors with favorable metabolic stability and oral bioavailability, with effectiveness to be 20 to more than 200 fold compared to existing PARP1/2 inhibitors such as olaparib, rucaparib, and veliparb. This made the BMN 673 PARP inhibitor an ideal choice for assisting in the process of determining the specificity of the HuCAL recominbant antibodies.

Western blot analysis

One of the primary uses for antibodies in the research setting is for the detection of proteins or antigens derived from cells after treatments. In the case of parthanatos, MNNG treatment offers a good model for

PARP-1 activation and PAR generation. As discussed previously, PAR production increases basal levels of PAR of up to 500 fold and ranges from 2 to 300mer in size. Hence, a western blot with a PAR antibody should indicate detection of oligomers and polymers in that range, thereby presenting a smear band. Previous PAR antibodies are able to detect PAR at narrower ranges and with reduced sensitivity.

In the case of the HuCAL technology recombinant antibodies, all 19 generated antibodies effectively detect poly ADP-ribosylated proteins to after 15 minutes MNNG treatment (Figure 2). Some are sensitive enough to detect basal levels (DMSO treated) of PAR, which indicates remarkable improvement to current commercial standards. In particular antibody clones 14, 17 19, 21, 25, 27, and 29 were highly specific to PAR and have superior signal-to-noise. Notably, the other antibodies, 13, 15, 16, 18, 30, and 31, the PAR signal could still be detected even after inhibition with PARP1/2 inhibitor BMN 673, suggesting alternative PAR generation from PARP3 or non-specific immunoreactivity, particularly in the 70kD range.

Immunohistochemistry

Another use of the PAR antibodies that will allow better studies of parthanatos to be performed in the future is its ability to detect PAR in immunohistochemical samples. The ability to see PAR localization in the nucleus or spread to the cytoplasm will be highly indicative of the

temporal and spatial aspects of the signaling pathway in different models. MNNG treatment is one model that allows for PAR visualization. Although PAR spreads rapidly from the nucleus to the cytoplasm, it was important to determine which of the antibody clones were capable of detecting which species of PAR.

Immunohistochemical analysis of the antibodies on HeLa cell samples and mouse cortical neurons indicate strong detection of PAR originating from the nucleus, and quickly spreading throughout the cytosol (Figure 3, 4). Notably, antibodies 14, 21, 25, 27, and 29 are the most specific to PAR following MNNG treatment, consistent with the immunoblot analysis. The DAPI nuclear stain allows for visualization of PAR production in the nucleus in a few of these antibody clones. It is likely that the PAR still in the nucleus are the larger polymer sizes, whereas those that spread quickly to the cytoplasm are the smaller oligomers. It is also noteworthy that antibody clones 19 and 27 showed detection within the nucleus as individual spots, perhaps indicative of PAR in the nucleolus.

Immuno-precipitation

Previous commercial antibodies do not effectively immune-precipitate, and hence prevent researchers from isolating concentrated PAR samples. The antibodies generated here demonstrate robust ability to immune-precipitate PAR from MNNG treated mouse cortical neuron

cultures. The antibodies only have the Fab fragment, lacking the Fc region. It was important in determining the antibodies capacity for immune-precipitation. Immuno-precipitation via IgG binding magnetic beads indicated all antibodies to be effective in immune-precipitation (Antibody 19 probe).

To further study the specificity of the PAR antibody to the various length PAR species, ^{32}P-labelled PAR was prepared synthetically and immune-precipiated against the antibodies and visualized on a phosphorescent screen. Our results show that the panel of antibody clones offer a wide range of PAR size detection. It is apparent that antibodies 14 and 21 target low molecular weight PAR fragments, whereas antibodies 17, 18, 24, and 26 are more specific for high molecular weight PAR. Antibodies 19 and 29 were able to detect the whole range of PAR sizes. These results offer promising panel of antibodies that can be tailored for different disease models in PARP-1 activation.

Stroke model

PAR-binding mutant (Pbm) AIF knock-in mice were subjected to direct middle cerebral artery occlusion (dMCAo) as a model for stroke. Before strokes were performed, behavioral tests were performed. These included a cylinder test, spontaneous activity test, and a treadmill test. Following a 60 minute stroke, blood flow was restored and brains were

harvested 24 hours post reperfusion and stained with TTC. Preliminary results indicate reduced lesion volume for Pbm-AIF mice when compared to WT-AIF mice, indicative of some degree of protection.

Discussion

Through the production of the 19 antibody clones through HuCAL recombinant technology, a panel of highly specific and effective PAR antibodies was characterized, allowing for future work to better characterize PARP-1 mediated cell death. Because the current tools available commercially for detection of PAR is limited and lacks the specificity for different PAR polymer sizes, the production of recombinant antibodies will provide a better method for future research studies to determine the relation of PAR structure and size with disease modalities. The characterization through immunoblot analysis indicated the robust strength of the antibodies to see the smear bands in PARP-1 activation, with even some demonstrating detection of basal PAR levels. Immunohistochemical analysis further supported the characterization process by identifying the localization of PAR using the antibody clones. Interestingly, the better antibodies for detection with lower noise were also the antibodies that detected smaller size PAR structures. Lastly, the immune-precipitation experiments were able to determine the size specificities of PAR. The protocols and characterizations in this study will

provide a strong foundation for future research in diseases involving PARP-1 cell death.

One of the models that the antibodies are useful in is in the genetic knock-in triple PAR-binding mutant AIF mice that were generated. These mice were subject to preliminary studies in behavioral and stroke models. Early results suggest promising protection capability of the mutant mice with no apparent side effects to mouse development and behavior. The PARP-binding mutant AIF allows the separation of AIF's mitochondrial bionergetic functions and its cell death functions in the nucleus. In conjunction with the PAR recombinant antibodies, it would be interesting to determine the PAR sizes that AIF preferentially binds under PARP-1 activation. Future work involving NMDA excitotoxicity through injection will be performed. In addition, cortical neurons cultured from timed pregnancy Pbm-AIF will be characterized via the XF analyzer to assess the effects on bioenergetics of the mutant AIF.

Methods

Cell Cultures and Extract

HeLa S3 cells (ATCC) were grown in Dulbecco's modified eagle's medium (Invitrogen) with 10% Fetal bovine serum (Gibco). Mouse cortical neurons were cultured from gestational day 15 fetal mice CD1 mice (Jackson) as described previously (Dawson et al., 1991b, 1993b). Briefly, the dissected cortex cells were dissociated using Modified Eagle's medium (Invitrogen), 20% horse serum, 25mM glucose, and 2mM L-glutamine by trituration after 30 min digestion in trypsin/saline solution. To inhibit proliferation of glial cells and non-neuronal cells, cells were treated with 5-fluro-2-deoxyuridine on DIV4. They are maintained in the MEM with 10% horse serum, 25mM glucose, 2mM L-glutamine, until day 14 when mature levels of nNOS are expressed.

Cytotoxicity

MNNG (100uM) was administered to cortical neuron cultures and HeLa cells for 15 minutes, with a washout at 15 minutes in culture medium and whole cell lysates were subsequently collected in RIPA lysis buffer. BMN673 was administered to neuron cultures and HeLa cells 30 minutes prior to MNNG treatment.

^{32}P-labled PAR

^{32}P-labeled PAR was synthesized by incubating PARP-1 with ^{32}P-labeled NAD+ for 2 minutes at 30°C and further incubated with nonlabelled and nonisotopic NAD+ for 28 min at 30°C.[33] ^{32}P-labeled free PAR was then purified through a dihydroxyboronyl BioRex (DHBB) column as described.[34]

Immunoblot analysis

Immunoblots were performed from HeLa cell lysates after exposure to MNNG for 15 minutes. Whole cell lysates were boiled in 2 X Laemmli buffer for 10 minutes and analyzed on 8% SDS Page. Following a wet-transfer to nitrocellulose membrane and blocked for 1 hour at room temperature in 1 X TBST with 5% w/v nonfat dry milk, the blots were probed at 1:2500 dilution of the selected primary HuCAL PAR antibody and incubated overnight at 4˚C. The nitrocellulose membranes were washed three times in 1 X TBST and probed with secondary Goat anti-human IgG (Fab')2 (HRP) (ab87422) at 1:5000 dilution.

Immunostaining

HeLa cell cultures and mouse cortical neurons were cultured on cover slips on 24-well cell culture plates (Falcon), (Nunc Thermo Scientific). Following 15 minutes of DMSO or MNNG treatment, cells were fixed in ice cold 4% paraformaldehyde (PFA) solution for 30 minutes. Subsequently, cover slips were washed three times in 1 X PBS and blocked (10% Donkey serum, 0.3% Triton X-100 in PBS) for 1 hour at

room temperature. Each of the antibody clones was diluted at 1:2500 in the blocking solution and probed to the cells overnight at 4˚C. Cover slips were washed out for three times in 1 X PBS. Secondary antibodies Goat (Fab')2 Anti-Human IgG H&L (FITC) (ab98524) was diluted 1:2000 in 1% Donkey serum, 0.1% Triton X-100 in PBS and administered to cover slips for 1 hour at room temperature. After washout , DAPI treatment was used for nuclear staining and cover slips were mounted to microscope glass slides using fluorescence immunomount media (Fisher Scientific) and visualized on a LSM510 (Carl Zeiss) microscope.

Immunoprecipitation

Neuronal cultures from a six-well plate (Nunc Thermo Scientific) were treated for 15 minutes in MNNG and collected in 0.5 mL Lysis buffer (PBS containing 1 mM EDTA, 1 mM EGTA, 0.5% SDS, 1% Nonidet P-40, 0.25 mM sodium orthovanadate, 0.25 mM PMSF, 2.5 µg/mL leupeptin, 2.5 µg/mL aprotinin) and incubated for 30 minutes at 4˚C. Magnetic Ig binder Kappa beads (Millipore) were incubated with respective antibodies for 1 hour (10 ul slurry and 2.5 ug antibody) at room temperature with rotation. After the samples were washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST), PAR from cell lysates or radiolabelled PAR was added and incubated overnight at 4°C with rotation. Following TBST washout and addition of TBE loading dye (Invitrogen) and boiled for 10mins, supernatant was run on

20% TBE PAGE. The immunoblots were visualized in an ImageQuant LAS 4000 mini imaging analyzer (GE Healthcare) with enhanced chemiluminescence (Pierce).

Direct Middle Cerebral Artery Occlusion (dMCAo)

Mice were anesthetized with 1.5-2% isofluorane and maintained at normothermic temperature. To occlude the middle cerebral artery, a 7.0 filament with a silicone tip was passed through the right internal carotid artery. 60 minutes occlusion was followed by a removal of the filament and reperfusion was performed. At 24 post reperfusion, the brain was harvested and sectioned into five coronal slabs to be stained with triphenyltetrazolium chloride (TTC). Infarct volume was measured on each of the anterior and posterior surfaces and integrated.

Appendices

Figure 1. ELISA QC of Antibody Clones

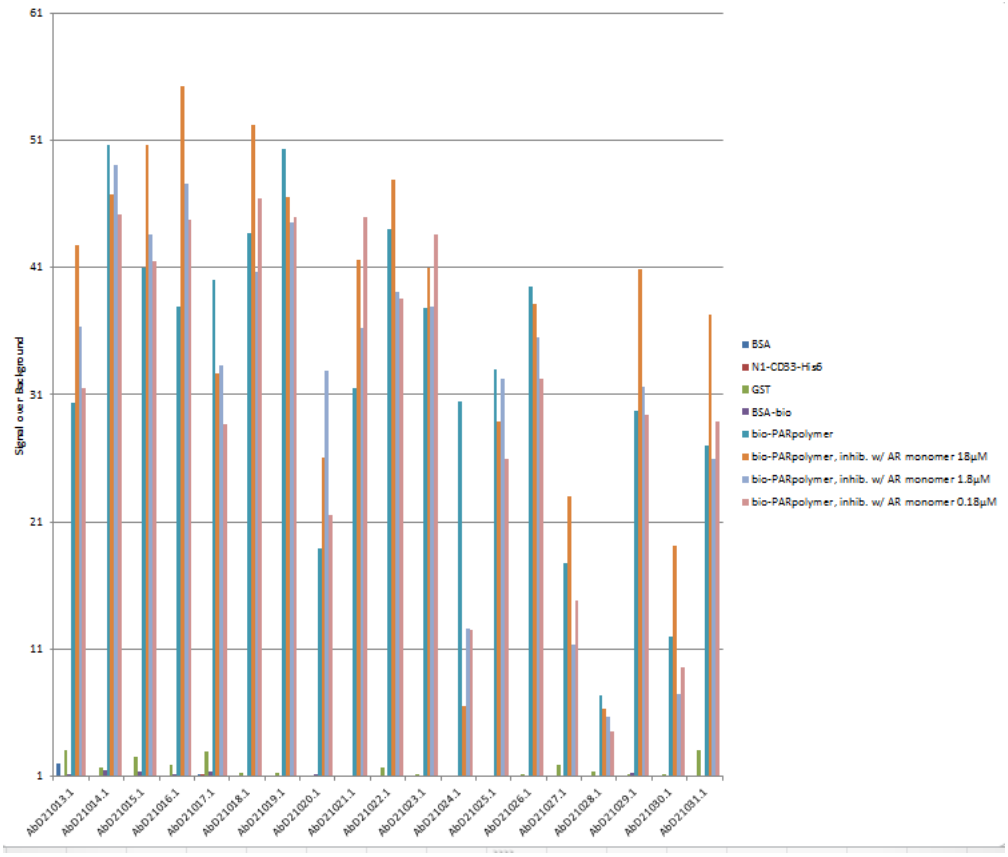


Figure 2. Western Blot from MNNG Treated HeLa Cell Lysates

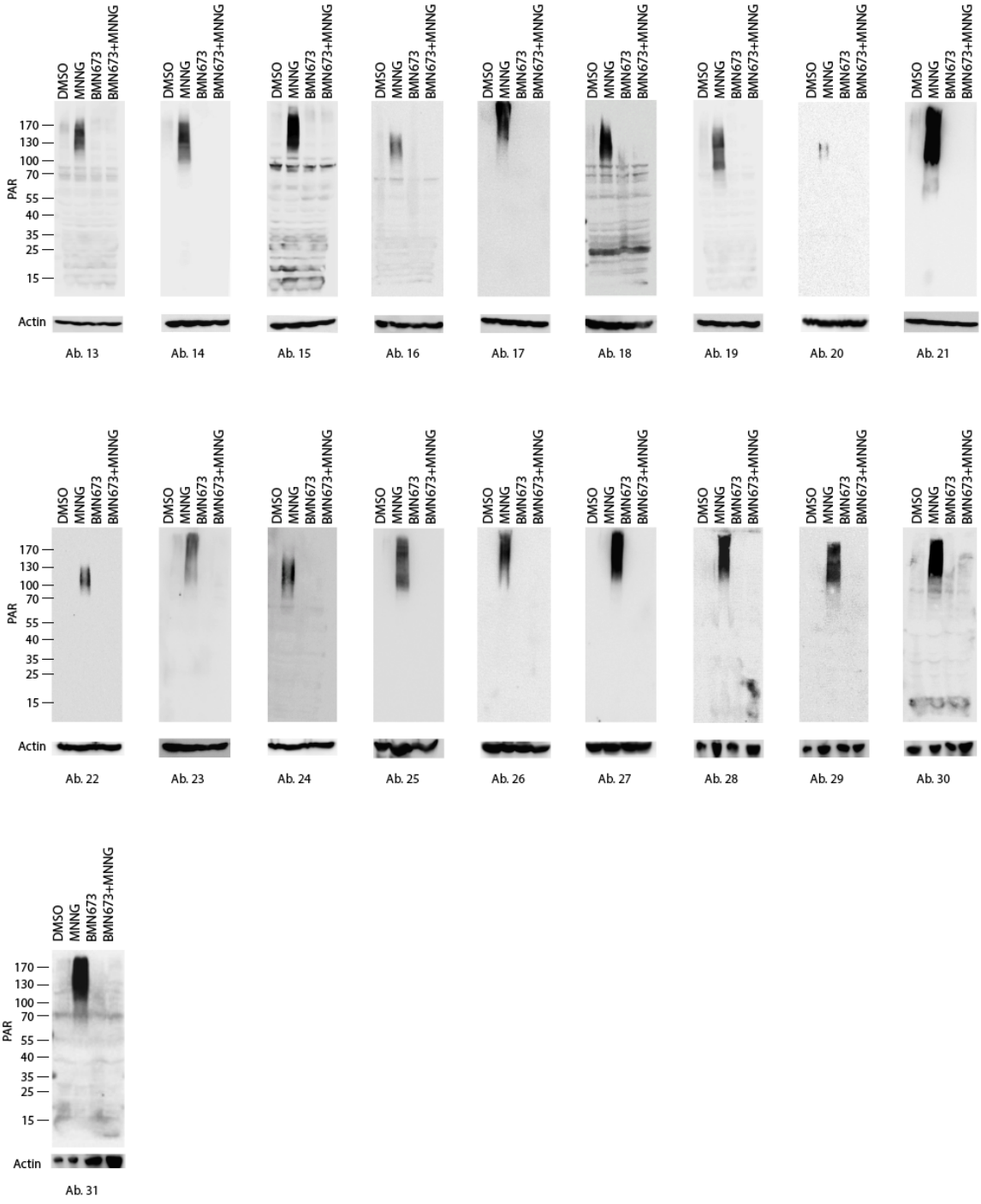


Figure 3: Immunostaining of HeLa cells following MNNG treatment

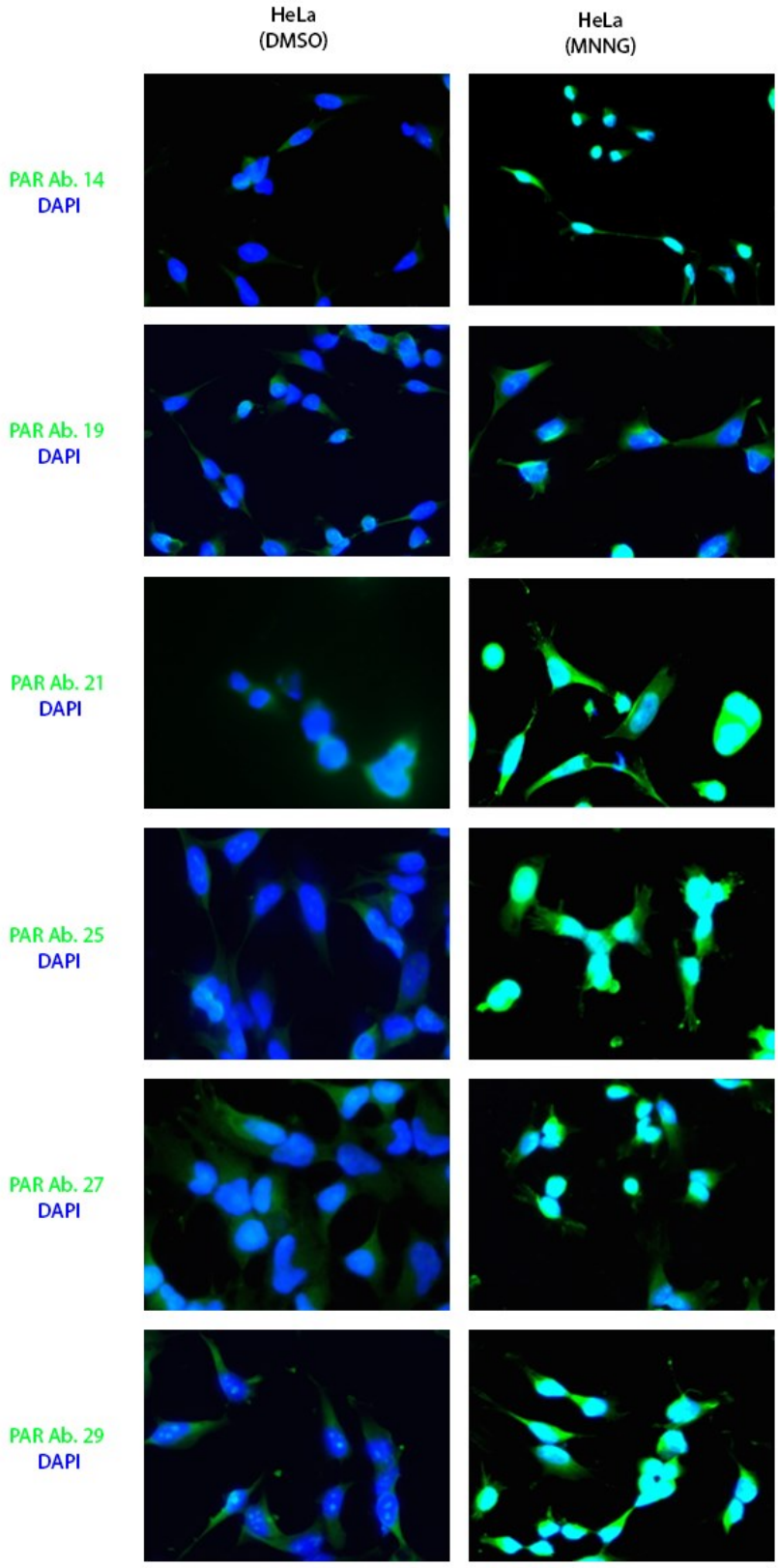


Figure 4: Immunostaining of Cortical Neurons following MNNG treatment

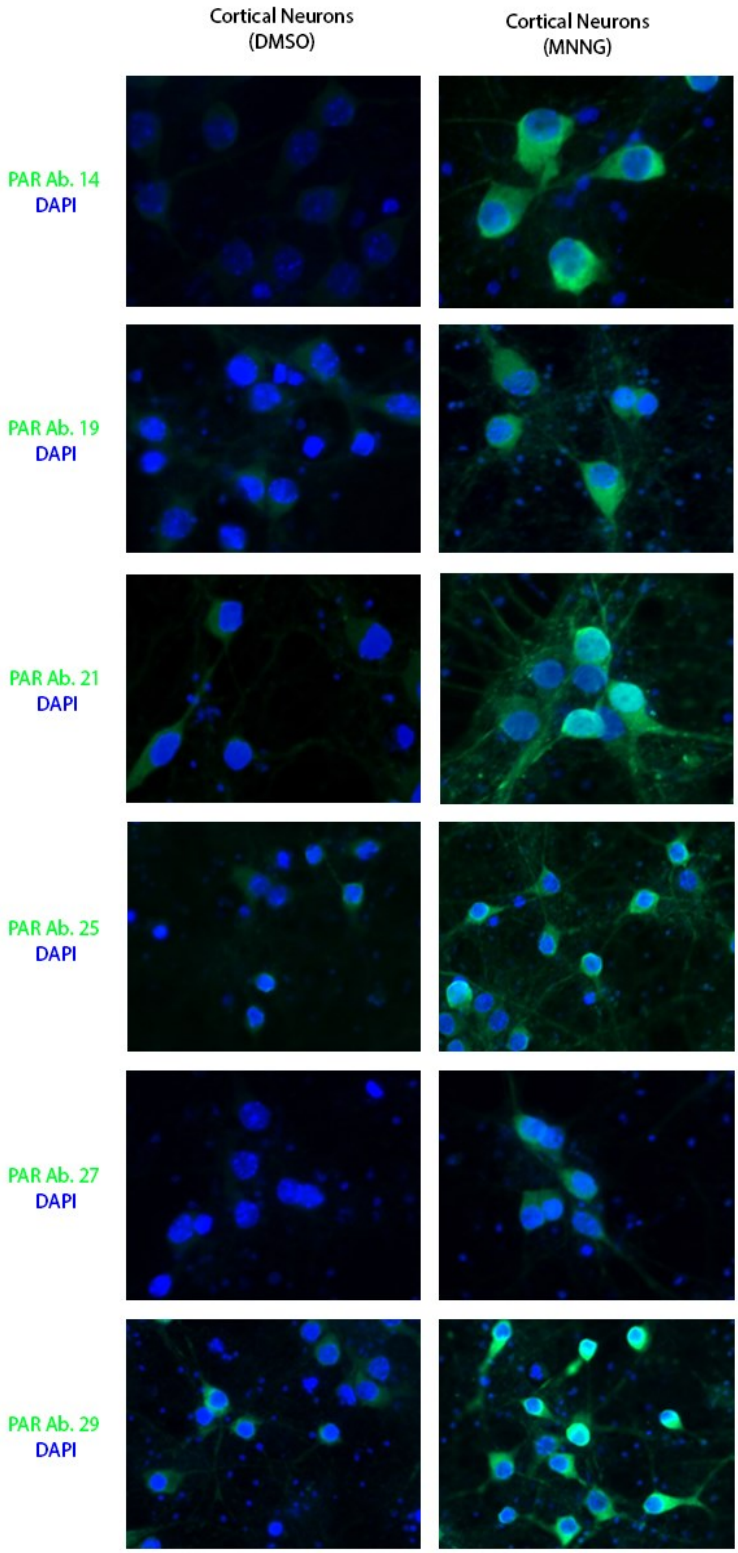


Figure 5: Immunoprecipitation of PAR collected from Mouse Cortical Neurons

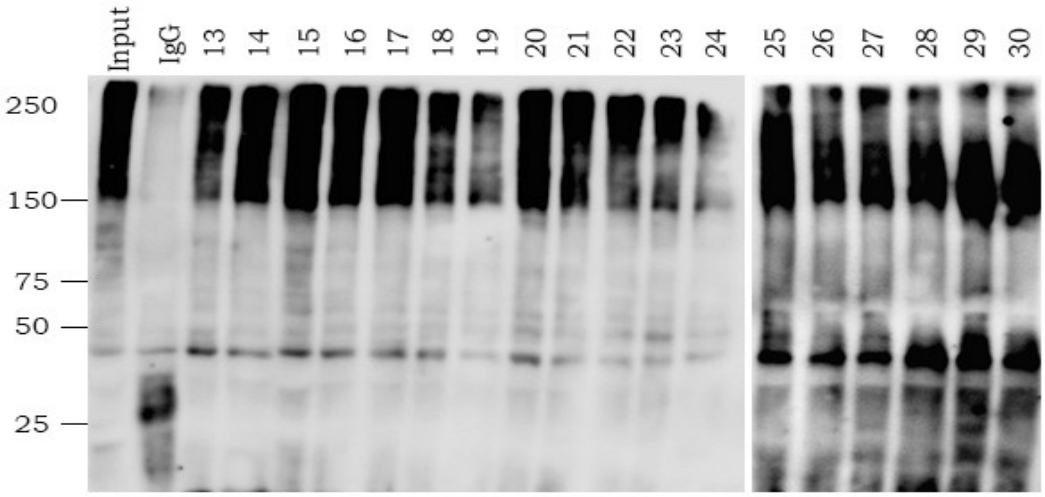


Figure 6: Immunoprecipitation of Radiolabelled PAR

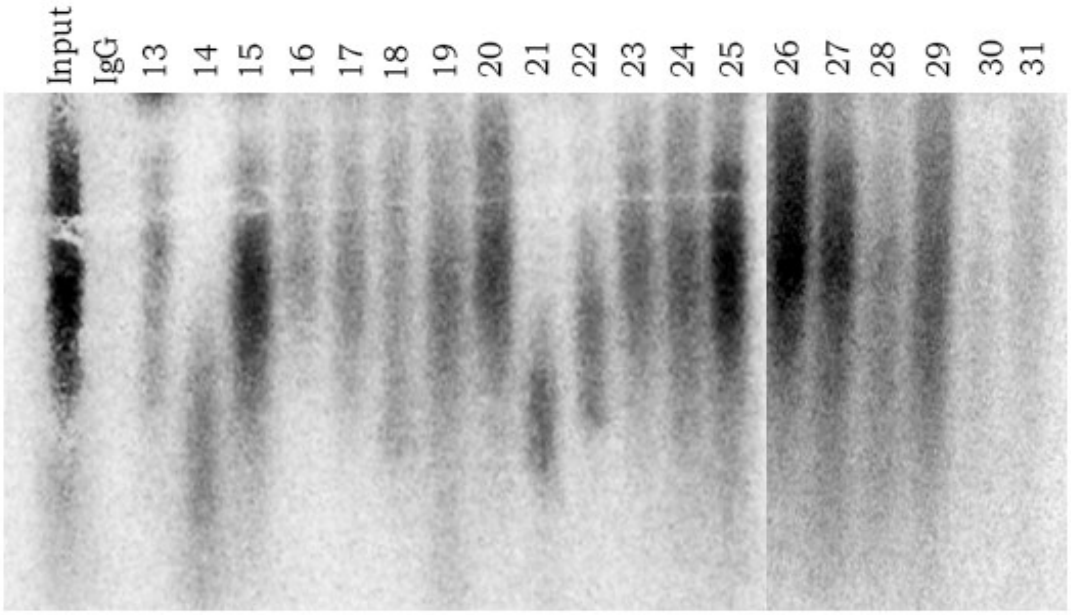


Figure 7: AIF Mutation-knock in targeting strategy

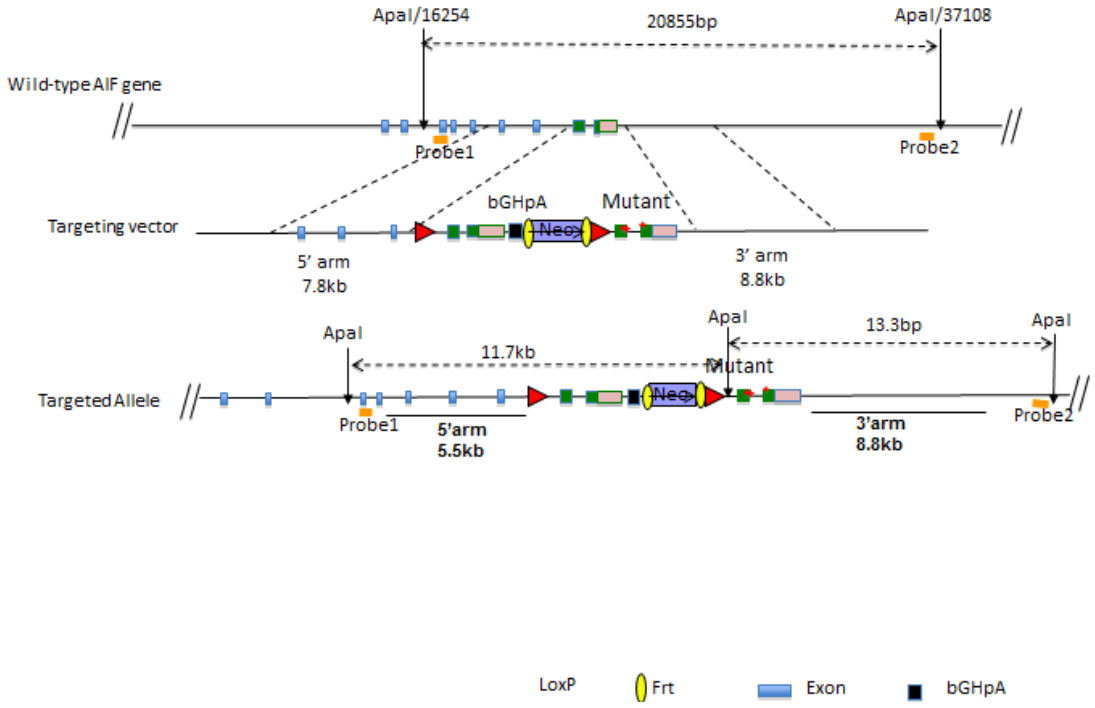
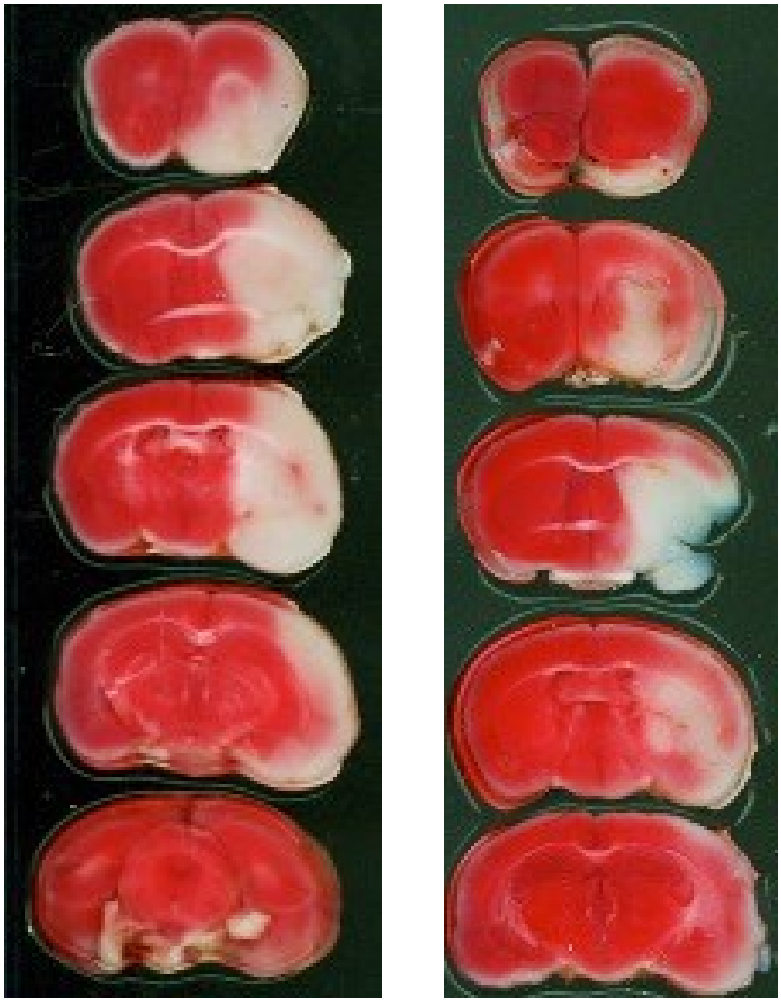
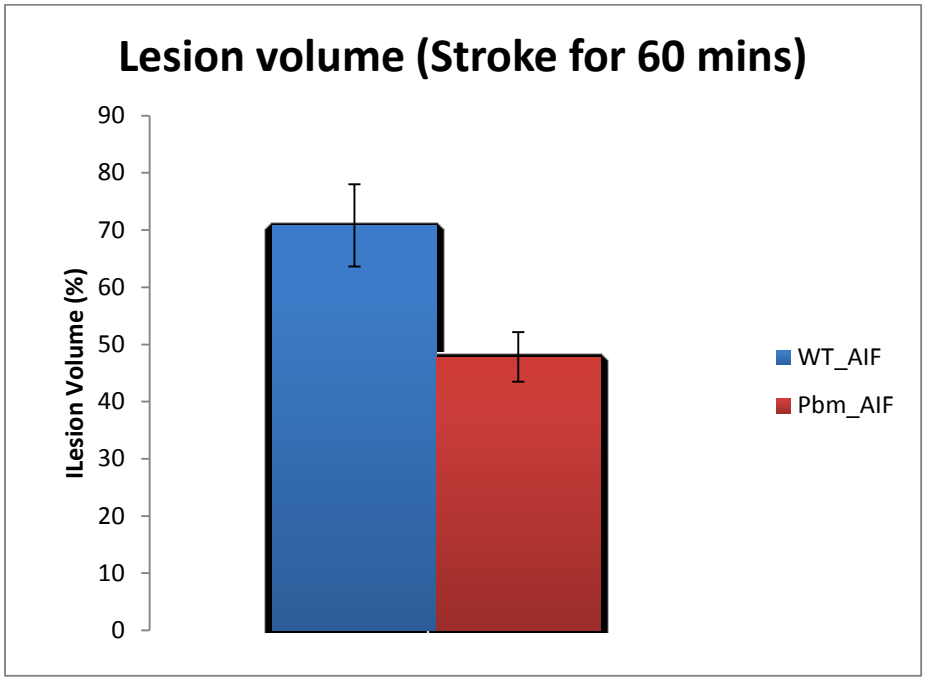


Figure 8: TTC staining of damaged brain following 60min stroke (N=3)



WT_AIF **Pbm_AIF**

Figure 9: Lesion volume (N=3)



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EDUCATION:

Johns Hopkins University (Baltimore, MD) May 2013
Bachelor of Science in Biomedical Engineering
Minor in Entrepreneurship & Management
Focus Area: Cellular and Tissue Engineering
Masters of Science in Biomedical Engineering May 2015

EXPERIENCES:

Life Technologies™: Applied Biosystems™, Product Development Scientist-Intern
Summer 2011, 2012

- Developed new SYBR GreenER master mix for enhanced qPCR efficiency and accuracy.
- Attended and contributed in weekly team meetings for analysis of data and troubleshooting errors.
- Assisted in development and optimization of castPCR project for detection of rare somatic mutations

Johns Hopkins Medicine: Institute of Cell Engineering
PI: Dr. Ted M. Dawson,

- Optimized experimental conditions for absorption spectrometry in order to characterize biochemical properties of a new apoptosis inducing factor (AIF) isoform for postdoctoral fellow.
- Poly(ADP-ribose) antibody from HuCal technology screening
- Maintained, bred, and conducted experiments on triple mutant AIF mice to establish protective function against cytotoxicity and oxidative stress.

Academia Sinica: National Genotyping Center (Taipei), Research Assistant
Summer 2008

- Investigated differences in single nucleotide polymorphisms in VKORC1 and CYP2C9*3 and its effects on starting dosages for Warfarin.
- Presented to department at the end of the summer.
- Cooperated team members in troubleshoot sessions after each trial for error analysis.

Wireless Esophageal pH Sensor for GERD Diagnosis
October 2010 – May 2011

- Collaborating with upperclassmen in synthesizing and testing several biocompatible mucoadhesive polymers to situate an ingestible capsule to measure pH and as a mechanism for drug delivery.
- Managed 3 peers to synthesize mucoadehesive polymers and perform various tests for optimization of device.

Design Team Leader for Inferior Vena Cava Shunt project
January 2012 – May 2013

- Lead a team of six biomedical engineers to design and prototype novel device for inferior vena cava trauma operations and liver cancer treatment.
- Presented monthly to a panel of judges to assess project quality and presentation skills
- Developed functional prototype and tested in porcine model.
- Participated in various Business Plan and Design Competitions in the nation