TEHNICAL 18 MONTHS PROGRESS REPORTING
SCIRF # 2014 I-02
PI: Jeoung Soo Lee
Title: Neuron-specific nanotherapeutics for axonal regeneration after spinal cord injury

1. Specific Aims: The **objective** of this proposal is to develop novel **neuron-specific nanotherapeutics** for combinatorial therapy of drug and small interfering RNA (siRNA) targeting both extrinsic and intrinsic barriers **to promote axonal regeneration.** The approach is based upon 1) anti-NgR1 antibody (Ab) conjugated to the nanoparticle surface will specifically deliver the nanotherapeutics to neurons and interfere with the function of existing NgR1 receptors by antagonizing the binding of myelin-associated inhibitors. 2) RhoA siRNA will be used to block the common intracellular signal transduction pathways responsible for both myelin- and CSPG-mediated growth inhibition and 3) rolipram (Rm), a phosphodiesterase 4 (PDE4) inhibitor will be employed to increase intrinsic neuronal growth capacity by preventing injury-induced reductions in cAMP levels. The **central hypothesis** is that these neuron-specific nanotherapeutics will improve axonal regeneration and functional recovery following SCI. We formulated this hypothesis, in part, based upon our strong preliminary data, which show that poly (lactide-co-glycolide)-graft-polyethylenimine (PgP) and IgG-conjugated PgP (PgP-Ab) are efficient carriers for both plasmid DNA and siRNA capable of transfecting neural cell lines and primary neurons *in vitro*, as well as endogenous spinal cord cells *in vivo*. The *specific aims have not been modified from the original proposal.*

*Currently, we have achieved many valuable new in vivo data (Please see below Studies and Results section) and these data have been presented at invited presentations and presented at many conferences as oral and poster presentation. We published one paper in Acta Biomaterialia (*2016; 35:98-108*) and 3 manuscripts are in preparation based on data obtained from our present studies. One abstract is accepted as an poster presentation in Millitary Health Science Research Symposium (MHSRS) in Orlando, Aug 2016. and one abstract is submitteded as a poster presentation in "Society for Neuroscience" 2016 annual meeting. The PI submitted one new NIH R01 proposal as a PI in NINDS on June 5, 2016 based on data obtained from this funding.*

2. Studies and Results:

Aim 1. To synthesize and evaluate novel neuron-specific polymeric micelle nanoparticle (L1-PgP) as drug and siRNA delivery carriers (In progress and re-directed)

Subaim 1-1: Synthesize NgR1-PgP/siRNA polyplex nanoparticles and evaluate their physico-chemical properties and drug (rolipram) loading efficiency
: Redirected to use L1 instead of NgR1

A. Synthesis and characterization of L1-PgP:

A recombinant 140 kDa fully bioactive, soluble physiological cleavage product comprising the L1 extracellular domain derived from the baculoviral expression system were provided by the co-PI Dr. Ken Webb. Currently, we are conjugating L1 to PgP. L1-PgP will be prepared by reaction of the PEI primary amine group from PgP and the C-terminal carboxyl group of L1. The carboxyl group of L1 will

be activated by 1-Ethyl-3-(dimethylaminopropyl)carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) and then the activated carboxyl group of PgP will react with the amine group of PEI to form an amide bond. To optimize L1 density on PgP, three different concentrations of L1 will be used to prepare L1-functionalized PgP micelles (L1-PgP).

B. Determination of rolipram-loading efficiency of polymeric micelle:

Rolipram loading was performed by the solvent evaporation method. Briefly, rolipram was dissolved in methanol at varying concentrations, added to PgP (1 mg/ml) polymeric micelle solution (above CMC), incubated at room temperature with vigorous stirring, and then incubated overnight to allow methanol evaporation. The amount of rolipram entrapped in the micelle core was determined by HPLC (Waters system, Waters) using a Waters Symmetry C18 column with mobile phase water:acetonitrile (60:40).

% Loading efficiency = (Amount of Rolipram loaded/amount of Rolipram added) X 100.

The amount of Rolipram loaded in PgP solution (1 mg/ml) was 0.86 mg. This is approximately 4 times higher than Rolipram's solubility in water (0.2 mg/ml) and the loading efficiency was about 86%.

Subaim 1-2 (In progress)

B. Intracellular trafficking study

To evaluate the intracellular uptake of PgP/siRNA polyplexes, Cy-5 was conjugated to scrambled siRNA using Label IT siRNA Tracker Intracellular Localization Kit following the manufacturer's protocol. B35 neuroblastoma cells were incubated for 24 hrs with PgP/Cy5-siRNA (1 µg Cy5-siRNA, N/P ratio of 30/1) in 10% serum condition. During incubation, B35 cells were washed, fixed, and examined by confocal laser scanning microscopy (Nikon) to analyze the uptake and intracellular distribution of siGlo-red at 0.5, 2, 6, and 24 hrs. Figure 1 shows the PgP/Cy5-siRNA polyplexes in red on the cell surface at early time points (at 30 min and 2 hrs) and then in the cytosol at late time points (at 6 hrs and 24 hrs) post-transfection.

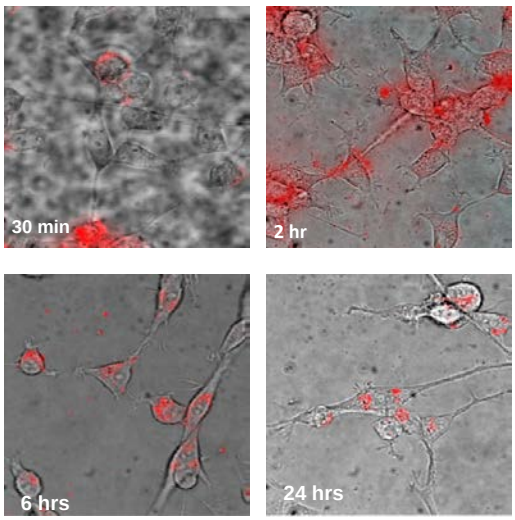


Figure 1. Intracelluar trafficking study using PgP/Cy5-siRNA on B35 cells at various time points

Aim 2. To evaluate the ability of Rm-PgP-mNgR1/RhoA siRNA nanoparticles to knockdown RhoA gene expression, preserve cAMP, and stimulate neurite outgrowth on inhibitory substrates (In progress)

Subaim 2-1.Evaluation of RhoA knockdown in B35 neuroblastoma cells *in vitro* after transfection with PgP/RhoA siRNA polyplexes. (Completed)

B35 cells were transfected with PgP/RhoA siRNA (Silencer® Select siRNAs, Life Technologies) polyplex formed at the various N/P ratios in 10% serum condition. bPEI/RhoA siRNA at an N/P ratio of 5/1, RNAiMax/RhoA siRNA, and RhoA siRNA alone were used as

controls. At 48 hours post-transfection, total RNA were extracted and two-step real-time PCR was performed with target-specific primers. Relative changes in RhoA gene expression levels were analyzed by the delta-delta C_T method using beta-2-microglobulin as an internal standard. Silencing efficiency increased with increasing N/P ratio and was significantly higher than that of bPEI (~7 %) at all N/P ratios. The relative silencing efficiency of PgP/ RhoA siRNA at N/P ratio of 30/1 was approximately 44 % and it was slightly lower than that of RNAiMAX/RhoA siRNA) (~62%) (Fig. 2).

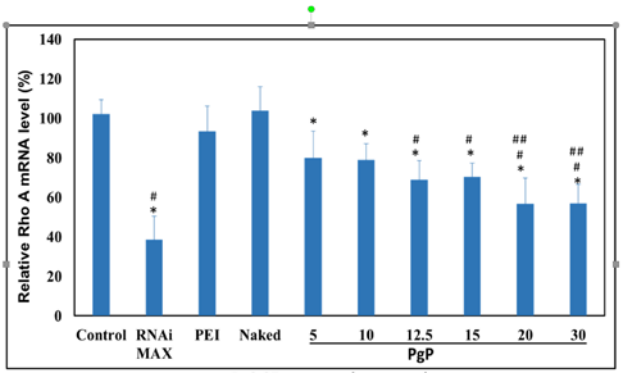


Figure 2. Silencing efficiency of PgP/RhoA siRNA polyplexes at various N/P ratio on B35 cells in 10% serum condition (n=6). *P<0.05 compared to control, #P<0.05 compared to PEI and naked siRNA, ##P<0.05 compared to PgP/siRNA (N/P 5/1)

Subaim 2.2. Evaluate cAMP level and neurite outgrowth in cortical neurons exposed to *in vitro hypoxia condition* after transfection with Rm-PgP (Completed). To generate hypoxia condition as a SCI model *in vitro*, rat primary cerebellar neurons (CBN) were isolated from P3 pubs and plated on the PLL/Laminin coated well plate. After 3 days culture, the cells were incubated in hypoxia gas chamber (95% N2 and 5% O2) for 24 hours and then the cells were treated with Rm-PgP and PgP without rolipram. Cells treated with free rolipram dissolved in DMSO were used as a positive control and cells maintained under normal atmospheric conditions were used as a negative control. The cells were incubated an additional 24hrs in hypoxia condition The cAMP level were evaluated using ELISA kit (R&D system). cAMP levels in CBN cells cultured under hypoxia was significantly lower than that of CBN cells cultured under normoxia conditions. cAMP levels of CBN cells were restored to levels not significantly different from cells maintained in the normoxia condition in both free Rm in DMSO and Rm-loaded PgP treated groups. We used PgP only group as a mock control to evaluate the effect of polymer alone. Interestingly, PgP alone did not show any cytotoxicity, but showed slight increase of cAMP compared to untreated hypoxia group (Figure 3). The cells were incubated an additional 24hrs in hypoxia condition and then fixed for neurite length evaluation. The neurite lengths were measured using ImageJ software program after β-3 tubulin staining. Figure 4 shows representative images of Rolipram treated/untreated rat CBN cells cultured in hypoxic condition. Neurite length of CBN cells cultured in hypoxia condition was significantly different with the neurite length of CBN cells cultured in normoxia condition, while neurite length of CBN cells in normoxia were not significantly different in free Rm in DMSO, Rm-loaded PgP, and PgP only group. We also observed that neurite length in the group treated with PgP alone (no Rm) was also significantly higher than the hypoxia group. This result helps confirm the cytocompatibility of our delivery carrier.

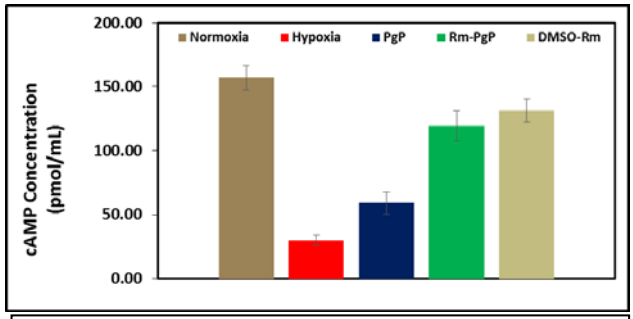


Figure 3. cAMP level of Rolipram treated/ untreated rat CBN cells cultured in hypoxia condition (n=3).

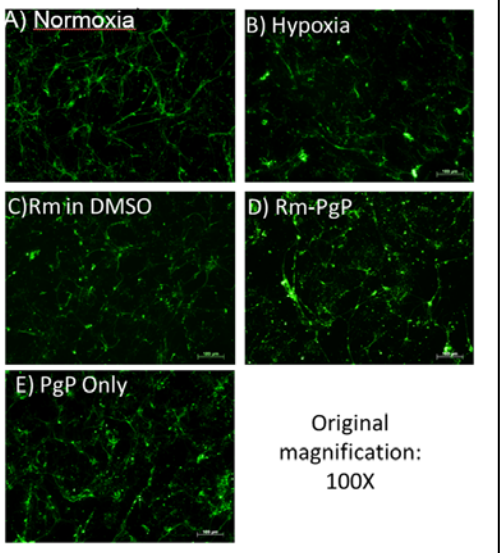


Figure 4. Representative images of Rolipram treated/untreated rat CBN cells cultured in hypoxia condition

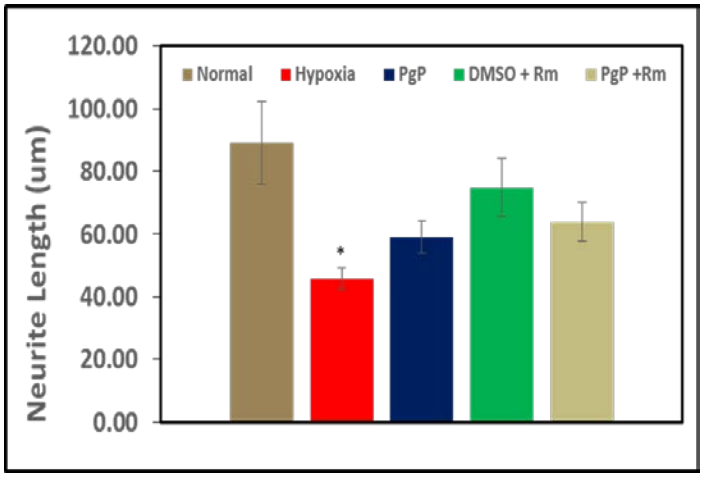


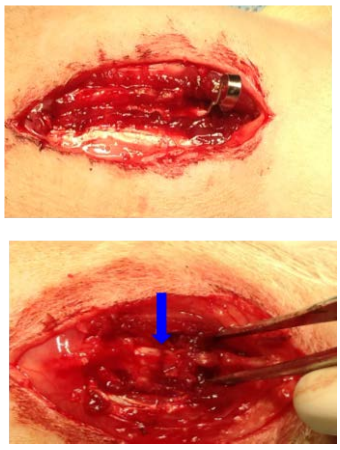
Figure 5. Neurite length of Rolipram treated/ untreated rat CBN cells cultured in hypoxia condition (n=9)

Subaim 2.3. Test the ability of Rm-PgP to overcome CNS myelin and/or CSPG inhibition of neurite outgrowth *in vitro* (Re-directed)

Aim 3. To evaluate axonal regeneration in response to delivery of Rm-PgP-mNgR1/RhoA siRNA nanotherapeutics in a rat compression SCI model. (In progress)

Subaim 3.1. Evaluate biodistribution of Rm-PgP /siGlo-red after systemic/local injection after SCI

Sprague Dawley rats (male, 200 gm) were anesthetized with isoflurane gas. Their backs were shaved and prepared with betadine solution, chlorhexidine, and sterile water. The T9 spinous process were identified and a 4-cm longitudinal incision over the dorsal mid-thoracic region will be made using a #10-blade scalpel. The T9 spinous process will be removed using orthopedics bone cutter and rongeurs, and the ligamentum flavum will be removed, thereby exposing the intervertebral space. A vascular clip was inserted through the dorsal T8~T9 intervertebral space and spinal cord was compressed via vascular clip for 10 min (Scheme 1).



Scheme 1. Generation of rat compression spinal cord injury model

To evaluate the localization and retention of PgP/Cy5-siRNA polyplex and naked Cy5-siRNA were assessed immediately following injection and at 1 and 24 hours by live animal fluorescence imaging system (IVIS Luminar XR, Caliper Life Sciences). PgP/Cy5-siRNA polyplexes were retained at the injection site after 24 hrs while naked Cy5-siRNA diffused from injection site within 2 hrs (Figure 6). To evaluate the duration of retention of polyplexes in injection site, hydrophobic dye DIR was loaded in PgP solution using solvent evaporation method. Fig 7-A shows that DIR was encapsulated in the PgP, while DIR was filtered out in water after filter. Rat compression spinal cord injury model was

generated as described above and DIR-loaded PgP/pDNA polyplexes (N/P ratio of 30/1, 10 µg pDNA) were prepared and injected in the T9 spinal cord lesion by Hamilton syringe. The DIR-PgP/pDNA polyplex were assessed immediately following injection at 2, 4, 6, 24, 72, and 120 hours by live animal fluorescence imaging system (IVIS Luminar XR, Caliper Life Sciences). Figure 7-B shows that the animals injected DIR-PgP/pDNA polyplexes (Right) were retained at the injection site up to 5 days and uninjected SCI animals were used for comparision (Left). At each time point, the animals were sacrificed and spinal cords were isolated and imaged (Figure 7-C).

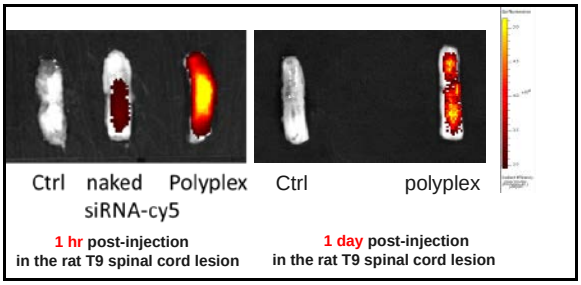


Figure 6. Localization of PgP/Cy5-siRNA after intraspinal cord injection in rat SCI model

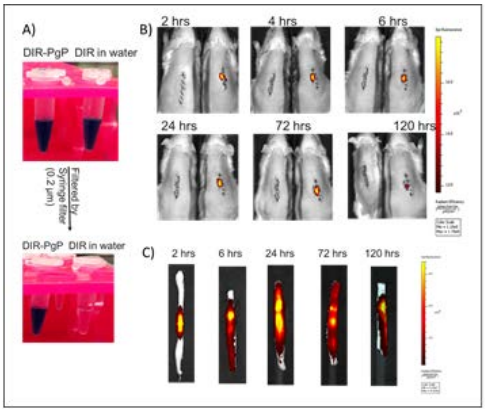


Figure 7. Localization and retention of DIR-PgP/pDNA (N/P 30/1) after injection at the lesion of SCI. A) DIR loading in PgP and water, B) Representative images of animals injected DIR-PgP/pDNA polyplexes at 2, 4, 6, 24, 72, and

Subaim 3.2. Evaluate RhoA expression knockdown and cAMP levels after injection of Rm-PgP-mNgR1/RhoA siRNAs

A. Evaluation of cAMP level after injection of Rm-PgP in rat compression spinal cord injury model *in vivo*

Rat compression spinal cord injury model was generated as described above and Rm-PgP were prepared as described above. 10 µl of Rm-PgP (1 ug Rm was loaded in PgP (1 mg/ml) solution) was injected into the injured dorsal T9 spinal cord using a 26-gauge Hamilton syringe. 10 µl of PgP alone, untreated SCI animal group, and sham animal group were used as controls. Following the injection, the paraspinal muscles were closed with 4–0 vicryl suture and the skin was closed with 3-0 silk suture. At 1 and 2 days after injection, the spinal cord (0.5 cm-long piece from the center of the injury) was retrieved. cAMP levels were evaluated using ELISA. In Rm-PgP treated group, cAMP was restored to levels not significantly different from the sham animal group at 1 and 2 days post injection, but decreased at 3 and 7 days. In untreated SCI animal group, cAMP level was significantly lower than the sham control at all time points (Figure 8). Previous studies have shown that even short-term preservation of cAMP levels can have a substantial effect on the injury response.

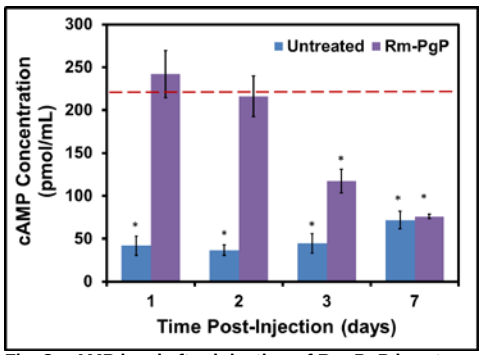


Fig. 8. cAMP level after injection of Rm-PgP in rat SCI model (n=6/ group). *P<0.05 compared to sham group indicated by dashed red line.

B. Transfection efficiency of PgP/ pβ-Gal polyplexes in rat compression spinal cord injury model *in vivo*

As a first step to examine the efficacy of PgP for therapeutic nucleic acid delivery, we evaluated transfection of pDNA encoding beta-galactosidase into the spinal cord. Following clip compression, PgP/ pβ-Gal polylexes (10p µg pβ-gal, 20 µl) were prepared at an N/P ratio of 30/1 and injected into the injured dorsal T9 spinal cord using a 26-gauge Hamilton syringe. bPEI/pβ-gal at an N/P ratio of 5/1 and naked pβ-gal were used as controls. Following injection, the paraspinal muscles were closed with 4–0 vicryl suture and the skin was closed with 3-0 silk suture. At 7 days after polyplex injection, animals were anesthetized by isoflurane gas and sacrificed via cardiac perfusion with 4% paraformaldehyde solution. The retrieved spinal cords were fixed with 4% paraformaldehyde solution and 10 µm thick sections cut and mounted on positively charged glass slides. To evaluate transfection efficiency, sections were stained using a β-Gal staining kit (Life Technologies) to detect β-Gal+ transfected cells. To identify the β-Gal+ cells, we stained the neuron cells and astrocytes using neuron-specific neurofilament and glial fibrillar acidic protein (GFAP) antibodies, respectively. Figure 9A shows β-galalctosidase expression by transfected cells in blue at 7 days post-injection of PgP/pβ-Gal polyplex in rat compression SCI model and Figure 9B shows that many β-Gal+ cells were neurons and a few β-Gal+ cells were astrocytes.

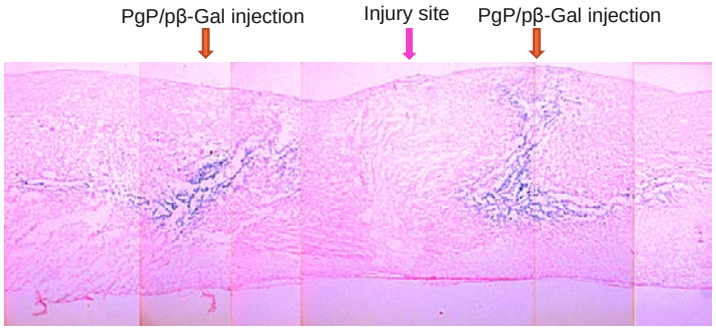


Figure 9A. β-Galactosidase expressed cells in blue after 7 days post-injection of PgP/pβ-Gal polyplexes (10 µg p-gal, 20 µl) were prepared at an N/P ratio of 30/1) in rat compression SCI model (Magnification: 200X)

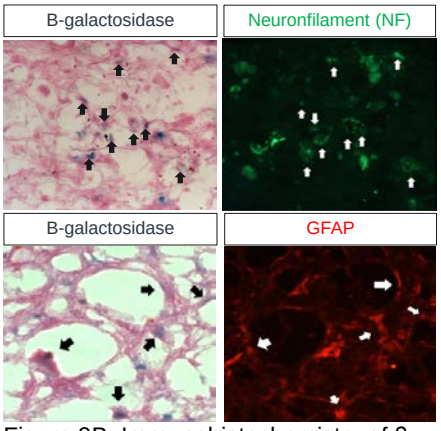


Figure 9B. Immunohistochemistry of β-Gal+ transfected cells, Neurons were stained with neurofilament antibody and astrocytes were stained with GFAP antibody.

C. Evaluation of RhoA expression knockdown after injection of PgP/RhoA siRNA polyplexes in rat compression spinal cord injury model *in vivo*

Rat compression spinal cord injury model was generated as described above and PgP/RhoA siRNA polyplex were prepared at the two different N/P ratios and two dose of siRNAs (N/P ratio (µg RhoA siRNA):15/1 (10 µg), 30/1 (10 µg), and 30/1 (20 µg)) and injected into the injured dorsal T9 spinal cord using a 26-gauge Hamilton syringe. PgP/scrambled siRNA at an N/P ratio of 30/1 (10 µg siRNA), untreated SCI animal group, and sham animal group were used as controls. Following the injection, the paraspinal muscles were closed with 4–0 vicryl suture and the skin was closed with 3-0 silk suture. At 7 days after polyplex injection, the spinal cord (0.5 cm-long piece from the center of the injury) was retrieved. Total RNA was extracted and two-step real-time PCR were performed with target-specific primers. Relative changes in RhoA gene expression levels were analyzed by the delta-delta C_T method using beta-2-

microglobulin as an internal standard. RhoA gene expression in untreated SCI animal group was increased 2.61-fold at 7 days. However, the RhoA gene knockdown was achieved in all PgP/RhoA siRNA injected animal groups compared to untreated SCI animal group and the order of RhoA gene suppression was 30/1 (20 µg)> 30/1 (10 µg) >15/1 (10 µg) (Fig. 10).

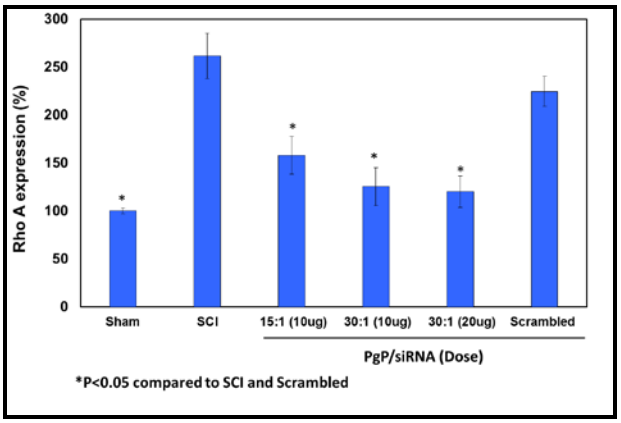


Figure 10. RhoA gene knockdown of PgP/RhoA siRNA at 7-days post injection in rat SCI injury model (n=5), Data presented the mean ± SEM.

To evaluate the duration of RhoA knockdown efficiency, SCI rats were divided into the following four groups: (i) Sham animals, ii) untreated spinal cord injury (SCI) (iii) single injection of PgP/Rho A siRNA polyplex (20µg siRNA) (iv) repeat injection group: 1st injection of PgP/SiRhoA NP (10µg siRNA) + 2nd injection of PgP/SiRhoA NP (10 µg siRNA) at 1 week after 1st injection. Spinal cords were retrieved at 1, 2, and 4 weeks after injection of PgP/SiRhoA polyplex in injury site and total RNA were extracted and two-step real-time PCR was performed. In untreated SCI animal group, RhoA gene expression was significantly higher than that in Sham animal group up to 4 weeks. In PgP/SiRhoA injected animal groups, RhoA gene expression was significantly lower than SCI untreated group at all time points up to 4 weeks (Figure 11). Figure 12 shows immunohistochemical staining of neurofilament and GFAP in spinal cord longitudinal sections at 4 weeks post-injury. In untreated SCI animal, an extensive necrotic lesion cavity was formed and significant reactive astrogliosis was observed in the surrounding tissue (Top). In both single and repeated injection animal groups, cavitation and astrogliosis were substantially reduced compared to untreated group and axonal regeneration was observed into the lesion site (middle: single injection, bottom: repeated injection). However, in repeated injection showed better axon regrowth (Figure 12) than those in single injected groups.

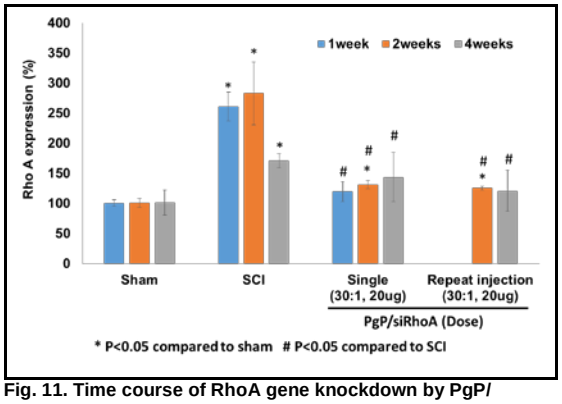


Fig. 11. Time course of RhoA gene knockdown by PgP/ SiRhoA in rat SCI injury model (n=5), Data presented as mean ± SEM.
***P<0.05 compared to sham group**

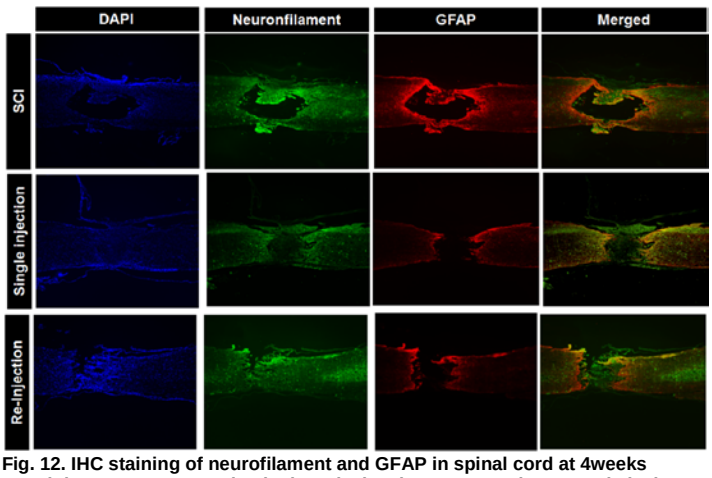


Fig. 12. IHC staining of neurofilament and GFAP in spinal cord at 4weeks post-injury. top: Untreated spinal cord, showing an extensive necrotic lesion cavity and significant reactive astrogliosis, PgP/SiRhoA -treated spinal cord (middle: single, bottom: repeat injection), showing reduced cavitation/astrogliosis and axonal regeneration into the lesion site.

3. Ongoing and future studies

- Recombinant L1 conjugation on PgP and neuronal specificity test in cerebellar neuron/astrocyte co-culture (Aim1)

- Test the ability of Rm-PgP/RhoA siRNA to overcome CNS myelin and/or CSPG inhibition of neurite outgrowth *in vitro (Aim2)*

- Behavioral Assessment after injection of Rm-PgP/RhoA siRNAs in rat compression SCI model (Aim 3)

- Anterograde Labeling and histology after injection of Rm-PgP-mNgR1/RhoA siRNAs in rat compression SCI model (Aim 3)

4. AWARDS

2016 Young Investigator Travel Award at the 6th Biennial NIH/NISBRE (National IDeA Symposium of Biomedical Research Exellence)
2014 Young Investigator Travel Award at the 5th Biennial NIH /NISBRE (National IDeA Symposium of Biomedical Research Exellence)

5. Publications and Presentations

Invited Presentation

1. Jeoung Soo Lee, Multi-functional polymeric micelle nanocarrier for central nerve system regeneration, Invited as a plenary speaker in Biomaterials Day 2016, Oct. 14. 2016, Georgia Tech, Atlanta, GA
2. Jeoung Soo Lee, Multi-functional polymeric micelle nanocarrier for Combinatorial Therapy of Bioactive Molecules, Invited as seminar speaker, Oct. 21. 2016, School of Pharmacy, University of Wisconsin,
3. Jeoung Soo Lee, Polymeric Nanocarrier for Combinatorial Therapy of Bioactive Molecules, UNC in Charlotte, NC (Sep 2015)
4. Jeoung Soo Lee, Neuron-specific Polymeric Nanocarrier for Axon Regeneration after CNS Injury, Department of Pharmaceutics, College of Pharmacy, Pusan National University, Busan, Korea (June, 2015)
5. Jeoung Soo Lee, Engineered Polymeric Nanocarrier for Combinatorial Therapy of Bioactive Molecules, 2015 Global RNAi Carrier Initiative Symposium, Korea Institute of science and technology (KIST), Seoul, Korea (May, 2015)
6. Jeoung Soo Lee, Multifunctional Polymeric Micelle Nanocarrier for Combinatorial Therapy, Clemson University-Japan International Symposium, Clemson, SC (Apr 2015)

PEER REVIEWED PUBLICATIONS

Paper published

1. So-Jung Gwak, Justin Nice, Jeremy Zhang, Benjamin Green, Christian Macks, Sooneon Bae, Ken Webb, and Jeoung Soo Lee, Cationic, amphiphilic polymeric micelle as a nucleic acid carrier in the rat spinal cord. Acta Biomaterialia, 2016; 35:98-108.

2. Chritian Macks and Jeoung Soo Lee, RNA Interference Technology for Central Nerve System Injury, submitted 1st revision in DNA and RNA Nanotechnology (June 2016)

Manuscript in preparation

1. So-Jung Gwak, Christian Macks, Ken Webb, Michael Lynn, and Jeougn Soo Lee, RhoA knockdown by PgP/RhoA siRNA polyplex enhances axonal regeneration after spinal cord injury, Biomaterials.

2. So-Jung Gwak, Christian Macks, Ken Webb, Michael Lynn, and Jeougn Soo Lee, Polymeric micelle as a nucleic acid carrier in rat spinal cord injury model, Journal of gene medicine

3.Christian Macks, So Jung Gwak, Michael Lynn, and Jeougn Soo Lee, Rrolipram-loaded polymeric micelle enhance the neuroal survival after spinal cord injury, Journal of Neuroscience.

Conference Proceedings/Presentations

1. So-Jung Gwak, Christian Macks, Ken Webb, Michael Lynn, and Jeoung Soo Lee, RhoA Knockdown by PgP/RhoA siRNA Nanoparticle Increases Axon Growth after rat spinal cord injury, Submitted in Society for Neuroscience (SFN) 2016 annual meeting

2. So-Jung Gwak, Christian Macks, Ken Webb, Michael Lynn, and Jeoung Soo Lee, Polymeric Micelle as a RhoA siRNA Carrier for Axonal Regeneration in rat SCI model, Presented as oral in 6th Biennial NIH, NCRR National IDeA Symposium of Biomedical Research Excellence (NISBRE), Washington DC (2016)

3. So Jung Gwak, Christian Macks, Michael Lynn, Mark Kindy, and Jeoung Soo Lee, Cationic Polymeric Micelle as a RhoA siRNA Carrier for Axonal Regeneration in rat compression SCI model, Submitted in Military Health System research Symposium (MHSRS) 2016

4. So-Jung Gwak, Ben Green, Christian Macks, and Jeoung Soo Lee, Polymeric Micelle as a Nucleic Acid Carrier for Axonal Regeneration in CNS, Society for Biomaterials Annual Meeting, Charlotte, NC, 2015

5. So Jung Gwak, Christia Macks, Ken Webb, Michael Lynn, Mark Kindy, and Jeoung Soo Lee, Polymeric Micelle as a RhoA siRNA Carrier for Axonal Regeneration in rat SCI model, Presented as poster to World Biomaterials Congress 2016