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14. ABSTRACT

Major accomplishments this year include the use of AFS seeded Acellular Nerve Allografts (ANA) to repair critical size nerve defects (1.5 cm) in rats. Functional recovery was monitored longitudinally using digital video gait analysis as well as electrophysiologic and histologic outcomes. The results demonstrated that the AFS seeded ANA used for nerve repair resulted in an improved functional outcome for the rats compared to ANA alone and were equivalent to those repaired using nerve autograft, the current gold standard for tension-free repair of transected peripheral nerves. Axon counts and neuromuscular junction morphology were equivalent between the AFS seeded ANA.

The coming year will utilize these techniques for repairing large-gap (6 cm) nerve injuries in non-human primates. This pre-clinical model represents a more translational model of peripheral nerve injury and repair. In addition, preservation of neuromuscular junctions using beta 2 agonists will be studied. IACUC and ACURO approvals for these studies were renewed.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION:

The current research addresses repair of large gap peripheral nerve injuries. Clinically, nerve injuries greater than 3-5 cm have poor outcomes, regardless of repair techniques. One of factors limiting the re-growth of the axon across a large nerve gap may be the lack of trophic factors in the extracellular matrix of the interposed nerve graft. It is hypothesized that amniotic derived tissues possess trophic factors that support axonal re-growth and that incorporation of these tissues into an acellular nerve allograft will result in a nerve allograft with an enhanced potential to re-grow across a large nerve gap. This research will optimize cellular seeding of nerve allografts and functional assessment of that optimal construct in a rat sciatic nerve defect. Acellular nerve allografts with and without Amniotic Fluid Derived Stem Cells (AFS) will be used to repair large nerve gaps in rats (15 mm). The outcomes of these surgeries will be compared to those obtained with autograft nerve repairs that currently have the best outcomes for large-gap peripheral nerve repair. These techniques then will be employed in anon-human primate model (macaca fasciculata) of large-gap (6 cm) peripheral nerve injury and repair. Functional outcomes also will be assessed in this model. Finally, an intervention to prevent the degenerative changes that occur in neuromuscular junctions following delayed nerve injury/repair will be studied. If successful, the potential for the denervated muscle to regain function after nerve repair would be increased.

KEYWORDS:

Peripheral nerve injury, nerve allograft, amniotic derived stem cells, rats, macaca fasiculata, cell seeding of scaffolds

OVERALL PROJECT SUMMARY:

HYPOTHESES/OBJECTIVES

We hypothesize that acellular nerve allografts (ANA) can be seeded with amniotic fluid-derived stem cells (AFS) to promote and accelerate nerve regeneration. The presence of the AFS will provide support for the regenerating axons without the requirement of becoming Schwann cells. The specific aims to address this hypothesis are noted below:

SPECIFIC AIMS

Specific Aim 1: To demonstrate the ability to seed ANA with AFS using sub-atmospheric pressure (SAP) in vitro. Cell culture will be utilized to establish that the AFS cells remain on the allograft scaffold and that they do not differentiate into another cell type. Control cultures will employ ANA's with topically applied AFS but without SAP.

a. Follow-up experiments will examine Schwann cell migration in the presence of seeded allografts
b. Decellularization of species-specific mixed motor nerve tissue will be performed using decellularization and oxidation to improve the porosity of the allograft construct and enhance AFS cell seeding potential

Specific Aim 2: To establish the feasibility of using AFS seeded ANA's in large gap nerve repairs in vivo.

a. Rodent studies using ANA with/without AFS to repair large gap nerve defects
b. Enhancement of regenerative rate will be investigated
c. Motor end plate preservation studies to maintain muscle potential for re-innervation
d. Non-human primate studies in pre-clinical testing.

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Animal Use at this site: Animals will be used at this site

Progress over the past 36 months:

SOW Task 1 Specific Aim 1 (months 1-12):
In vitro studies to demonstrate the ability to seed Acellular nerve allografts (ANA) with Amniotic fluid derived stem cells and tissue (AFS) using subatmospheric pressure (SAP).

Task 1.1 (months 1-6) Cell seeding using SAP. Tests first will employ fibroblasts (NIH/T3T cells) and will examine the ability of the subatmospheric pressure seeding device (SAPSD) to improve penetration of the fibroblasts into the ANA. Secondarily, the magnitude and duration of exposure to SAP resulting in the greatest cell seeding density within the center of the ANA will be identified. Cell culture will be utilized to establish that the AFS cells remain on the allograft scaffold and that they do not differentiate into another cell type. Control cultures will employ ANA's with topically applied AFS but without SAP.

a. Decellularization of species specific mixed motor nerve tissue will be performed using decellularization and oxidation to improve the porosity of the allograft construct and enhance AFS cell seeding potential

Progress Task 1.1:

- Cell culture for Schwann cells has been established in the investigator's laboratory using explanted Schwann cells from donor rats.
    - Yields from explants are low, but that is expected. Improvements on the techniques are being employed to increase the yield of these cells.
    - This is a critical step because we will need to provide a cell culture environment that supports the cellularized nerve constructs.
    - A Schwannoma cell line also has been established so that pilot studies of cell seeding experiments can utilize adequate numbers of cells.

- Green Fluorescent Protein expressing fibroblasts (NIH/T3T cells) have been obtained and stocks of these cells are preserved in liquid nitrogen. These cells allow clear visualization of cell distributions within the experimental scaffolds.

- Material transfer agreements are in place and acellular nerve allografts for both humans and rats have been obtained from AxoGen.

- Material transfer agreements are in place and amniotic tissues have been obtained from NuTech (26-11-2013)

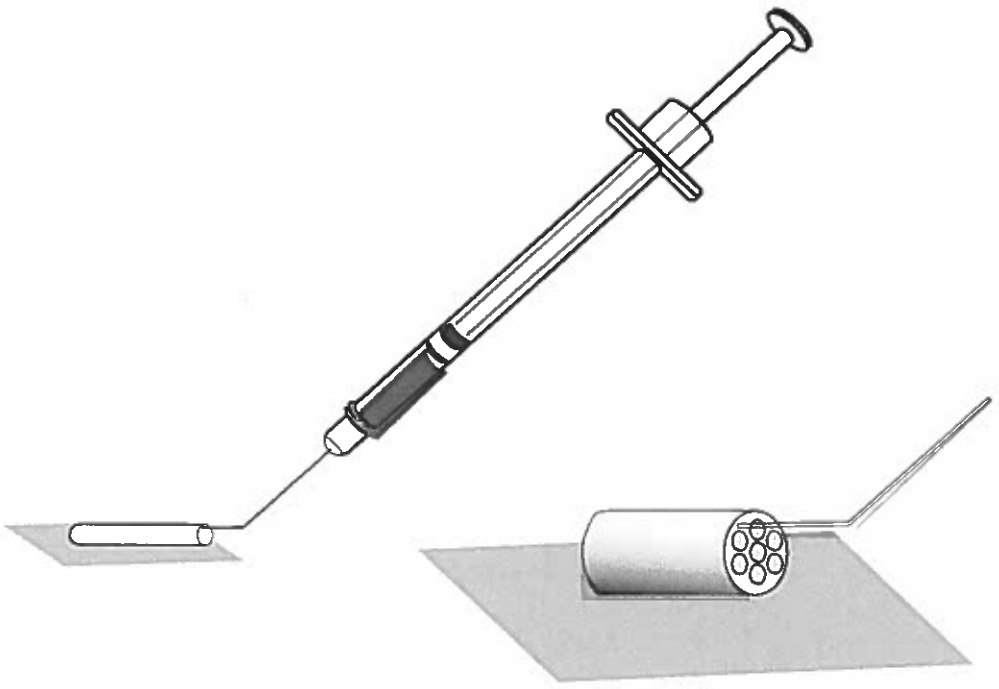
- Cell seeding experiments began in January 2014
    - Four series of cell seeding experiments have been performed using subatmospheric pressure (SAP) as well as static seeding. One million cells have been applied to scaffolds under SAP's of
        - - 40 cm H_2O
        - - 30 cm H_2O
        - - 20 cm H_2O
        - - 15 cm H_2O
    - Cell seeding of the ANA using SAP has not been adequate. The chambers providing SAP have been modified to maximize application of SAP to the acellular nerve scaffold.
- Sciatic nerves from 45 Lewis rats were harvested bilaterally, frozen in saline, and shipped to AxoGen for decellularization and processing. AxoGen could not obtain an adequate number of ANA from these donor nerves because the nerves from Lewis rats differ from those normally processed by AxoGen (from Sprague Dawley rats). AxoGen has provided us with ANA obtained

from Sprague Dawley rats and has documentation that these ANA can be implanted in Lewis rats.

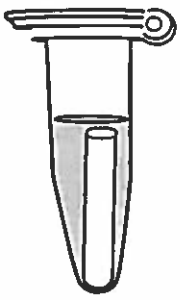
- Cell seeing of 1.5 cm long ANA was successful using an injection technique of AFS cells into the ends of the graft and beneath the epinurium of the graft near the mid-point followed by perforation of the epinurium using a microneedle array. The AFS-seeded ANA then was cultured for 72 hours. The perforation of the epinurium allows diffusion of nutrients to maintain AFS viability following injection into the midsubstance of the ANA. Cell viability of AFS was documented in the ANA following 72 hours of incubation. This construct then was chosen for the repair of 1.5 cm nerve defects in the rat sciatic nerve during *in-vivo* studies.

Cell Seeding on allografts

$1X10^6$ AFS cells were injected underneath the epineurium of the decellularized sciatic nerve allografts using a 26 G syringe. Seeded graft were placed vertically at the bottom of a small centrifuge tube covered with DMEM containing 20% FBS for overnight then transferred to a 48 well plate for additional 48 hours.

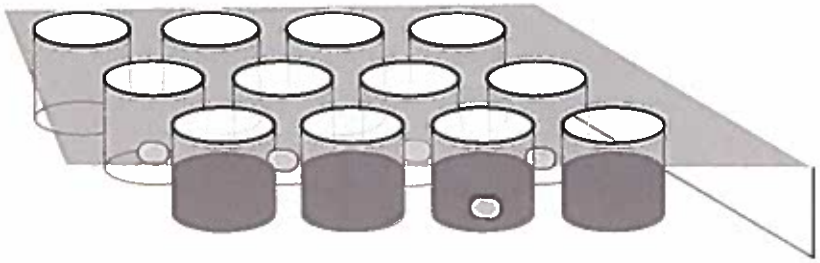


Sciaticnerve graft



Sciatic nerve graft standing vertically in media overnight

48 hours



Task 1.1 complete

Task 1.2 (months 6-12) Using the pressures established in 1.1, AFS will be seeded onto the ANA. Flow cytometry and cell markers then will be utilized to document that the AFS do not differentiate after being seeded onto the ANA. If the AFS undergo a phenotypic change after seeding on the ANA, the new phenotype will be identified and measures will be employed to prevent this differentiation.

- We are resolving the cell seeding issues noted above. (months 1-12)
- Cell seeding issues resolved (months 12-18)
- Cell viability documented

Progress on Task 1.2:

DAPI staining on longitudinal and cross sections of grafts showed cells spread evenly through the nerve fibers.

Longitudinal section of a sciatic nerve allograft -DAPI staining showed AFS cells nuclei appeared bright blue. Magnification X100

Table 1 Number of AFS cell-seeded allografts (as of 6/9/15)

Implanted AFS- Seeded Allograft	7
Control AFS-Seeded Allograft for testing cell infiltration	9

In vitro AFS cells seeded graft. $1x10^6$ AFS cells were injected under epineurium into the allograft. DAPI staining showed cells were viable 72 hours post injection.

Task 1.2 Complete

Task 1.3 (months 6-18) Cell culture will be employed to study the migration of Schwann cells onto the AFS seeded scaffold. Commercially available Schwann cells (from Schwannoma cell lines) will be co-cultured with the AFS seeded ANA's. Parallel studies of Schwann cell infiltration of non-AFS seeded ANA's also will be performed. The density of Schwann cells in the middle of the ANA's will be assessed histologically at three different time points after initiating co-culture of the Schwann cells. These time points will be at 12 hours, 24 hours, and 48 hours.

Progress on Task 1.3:

- Co-culture systems are being established
- Accellular nerve allografts for rats (Sprague Dawley) have been received from AxoGen
- Migration studies of labeled cells within grafts currently are underway using labled AFS cells and 7T MRI imaging. (months 18-24)

Task 1.3 complete

Task 1.4 (months 12-18, if necessary) If the cell seeding results of 1.3 are unacceptable (poor seeding of the ANA), nerves will be decellularized and oxidized according to the techniques of Whitlock et al. (2007). This technique results in a more porous allograft structure. If the oxidation of the nerve allograft tissue is too aggressive, the techniques can be modified by decreasing the concentration of and duration of exposure to peracetic acid during the oxidation phase of the tissue treatment.

Task 1.4 not necessary

Task 2 Specific Aim 2 (months 6-36): In vivo studies to establish the feasibility of using this construct in large gap nerve repairs.

Task 2.1 (months 6-18) – ANA with AFS for long gap nerve repairs will be studied using Lewis Rats as experimental subjects. A large gap nerve injury (1.5 cm) will be performed and the gap will be repaired immediately with an ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2), or with an autograft (nerve segment is cut out, reversed, and sewn back in place)(Group 3). All surgeries will be performed using aseptic microsurgical technique. Outcomes of nerve injury/repair will be assessed at 1 month, 2 months, and 4 months post injury.

a. Outcomes – Outcomes assessed will include: Walking track analysis as an indicator of return of motor control. Walking track analysis will be performed at 1 month, 2 months, and 4 months post injury. Each animal will be compared to their preinjury walking track values. Use of this technique will permit use of the highly sensitive repeated measures analysis of variance for these animals. This technique will reveal even slight differences between groups. The number of animals required per group to achieve statistical power will be reduced using this experimental design.

Histologic analysis of nerve recovery at the end of 4 months. Axon counts on the post injury nerve segments will be performed according to the methods of Ma (2002, 2007). In addition, axon morphology will be assessed and compared between treatment groups.

Analysis of neuromuscular junction (NMJ) density. The number of neuromuscular junctions per mm2 of muscle tissue within the normal distribution of motor end plates will be determined and compared between groups. (Ma 2007, 2002)

Fate of AFS in ANA's following regeneration. Two approaches will be used: first, immuno-histochemistry will be employed to identify the AFS cells. In parallel, studies using green fluorescent protein labeled AFS cells will be initiated. These will allow us to monitor the fate of the AFS cells after several weeks of implantation.

Muscle force generation will be assessed following the last walking track analysis to assess the degree of motor recovery. These studies will utilize techniques developed in this laboratory. (Stone 2007, 2011)

Progress Task 2.1:

Progress Q1

- A DigiGate video analysis system for quantifying gait in rats and performing walking track analysis has been purchased and delivered to our laboratories. The company CEO has provided on-site instruction in its use and we have begun training and assessing rat gait. The DigiGate computer is also connected to our institutional web server. This has allowed us to utilize and test the on-line assistance provided by the DigiGate company. (20-11-2013)
- Lewis rats, the strain identified for these studies have been obtained and we are learning techniques for training these animals to walk on the DigiGate. (05-12-2013)

Progress Q2

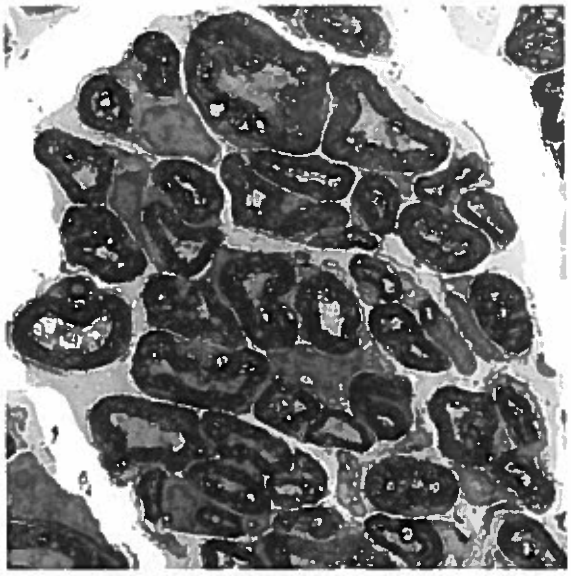
- Nerve autograft repairs of sciatic nerve injuries have been performed on the first six treadmill trained Lewis rats. These surgeries were uneventful and all animals have had their staples removed. The first animals to undergo nerve autograft repairs will be tested on the DigiGate device at 1 month post-surgery (first animals tested on 01-04-2014). Additional testing of these animals will be performed at two and four months post-surgery.
- Surgeries to create and repair sciatic nerve injuries will be performed in the next cohort of treadmill trained rats beginning 01-04-2014

Progress Q3

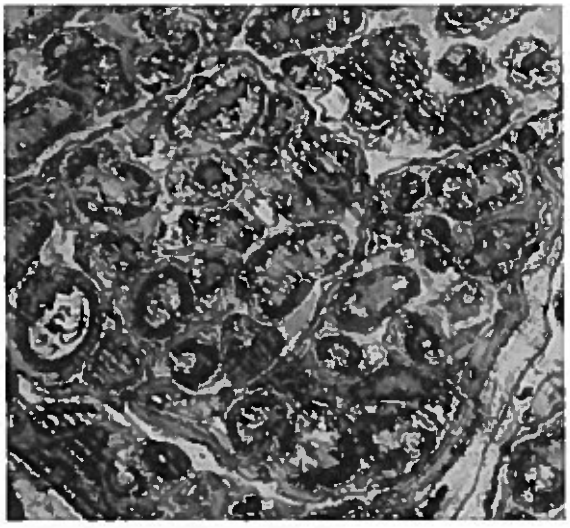
- Two groups of rats underwent surgical transection of the sciatic nerve on the left side with repair of the injured nerve using either a nerve autograft (Group 3; nerve segment obtained from the same rat) or a nerve allograft (Group 1; AxoGen supplied acellular human nerve of appropriate size).
- Rats were tested on the gait analysis device (DigiGate) before injury, and at 1 month, 2 months, and 4 months. In summary, several components of the rats' gait are significantly altered by sciatic nerve injury. Their gait parameters did not return to pre-injury values after 4 months. There were no remarkable differences between allograft and autograft nerve repair outcomes, which is in itself notable.
- Muscle function data also were collected and these results are still being analyzed.
- Gross muscle weights on the nerve injury side were significantly lower than on the intact contralateral side, suggesting muscle atrophy occurred following nerve injury. This atrophy was not reversed four months after nerve repair.

Progress Q4

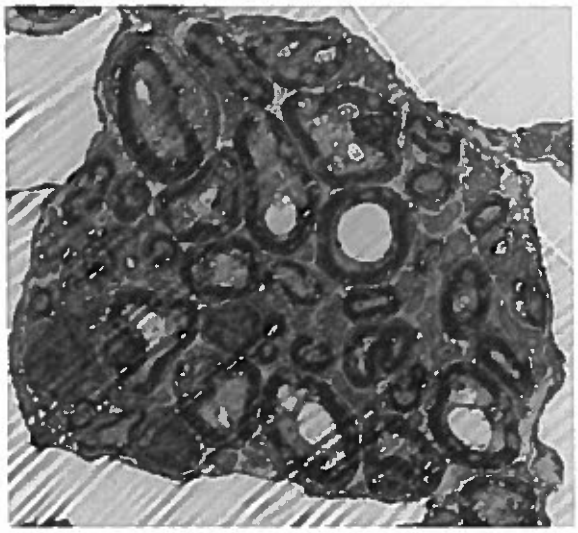
- Histology is continuing to assess axon counts as well as neuromuscular junction density



Electron micrograph of nerve autograph



Electron micrograph of nerve allograft

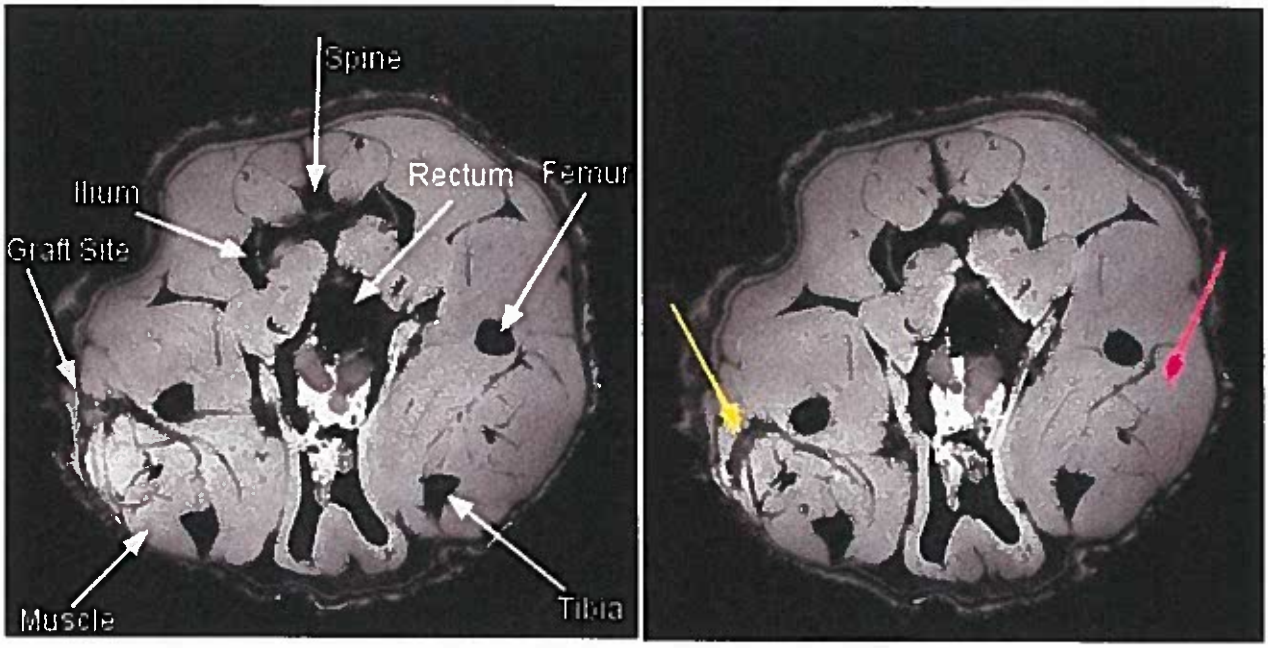


Electron micrograph of nerve allograft + AFS

2 µm

Figure 2.1.1 Representative electron micrographs of myelinated axons in the distal nerve stump of the rat, 1 mm distal to the suture line (Magnification: 3700X)

- Tracking of AFS cells in-vivo is being pursued through nano-particle labeling of cells and use of a 9T MRI to image these cells



T2 images of AFS cells labled with micron-sized iron oxide particles (yellow arrow) 1 week following graft implantation into sciatic nerve defect.

Progress Months 12-24

- All experimental groups of rats have been placed on study. Groups I-II have been studied through the 4 month time period following surgery. Group III (ANA + AFS) is finishing their 4 month post-surgery evaluation in Q1 of year 3 of this grant. Preliminary functional data (at 2-moths post-surgery) from gait analysis has been assessed for all three groups. The results have been discussed in an abstract submitted to the Orthopaedic Research Society Annual meeting for 2016 (attached as Appendix 1).
  - Briefly, at two months it was determined that ANA + AFS (Group III) demonstrated improvements in gait parameters compared to autograft repairs (Group I), particularly in the Sciatic function index.
  - Four month data are summarized in Table 2.

Functional and Histological Outcomes			
	Autograft	**ANA**	**ANA+AFS**
Stance/Swing Ratio	0.66 ± 0.22	0.64 ± 0.23	0.66 ± 0.22
Ataxia Coefficient	1.06 ± 0.29	1.27 ± 0.3	1.35 ± 0.23
Overlap Distance	0.79 ± 0.34	0.42 ± 0.19	0.71 ± 0.33 *
Step Angle Degree	0.9 ± 0.33	0.98 ± 0.37	0.97± 0.36
Paw Angle Degree	2.01 ± 0.25	2.88 ± 0.36	2.09 ± 0.22 **
Stride Length	1.1 ± 0.19	1.18 ± 0.28	1.16 ± 0.14
Paw Drag	1.38 ± 0.3	1.23 ± 0.38	1.08 ± 0.31 *
Stance Width	1.41 ± 0.28	1.04 ± 0.33	1.2 ± 0.21 *
Axis Distance	1.58 ± 0.25	1.13 ± 0.36	1.35 ± 0.23 *
Midline Distance	1 ± 0.22	1.25 ± 0.27	0.92 ± 0.17
SFI	9.02 ± 0.63	5.41 ± 0.63	7.29 ± 0.55 *
Wet Muscle Mass Ratio (GM)	0.52 ±0.02	0.50 ±0.01	0.51 ±0.05
Gastrocnemius CAMP Ratio	0.29 ± 0.05	0.27 ± 0.04	0.39 ± 0.05 *
Myelin Thickness (µm)	1.14 ± 0.22	0.69 ± 0.09	0.88 ± 0.13 **
Axon Diameter (µm)	2.29 ± 0.28	1.96 ± 0.24	2.36 ± 0.36 **
Fiber Diameter (µm)	3.93 ± 0.28	2.86 ± 0.25	3.84 ± 0.3 **
G Ratio (AD/FD)	0.58 ± 0.02	0.68 ± 0.02	0.61 ± 0.01 **

*p<0.05, **p<0.01

Table 2. Preliminary results of functional and histological analysis at the end of 4 months post nerve injury. ANA plus AFS cells group showed significant improvement in gait function, compound evoked muscle action potentials (CMAP), myelin thickness and axon diameter compared to ANA group alone (*p<0.05, **p<0.01), closely resembling the best outcomes obtained from autograft group.

Progress Months 24-36

Histology :

The gastrocnemius and tibialis muscles from both the experimental and contralateral side were harvested and weighed. The ratio of the experimental and contralateral muscle weights was calculated to measure the recovery of atrophy. 14μm sections of muscle were cut and stained with α-bungarotoxin (Thermo Fisher, NY) to visualize neuromuscular junction morphology following nerve injury and repair as previously described. 10 consecutive slides per animal were analyzed for each group.

Statistical analysis

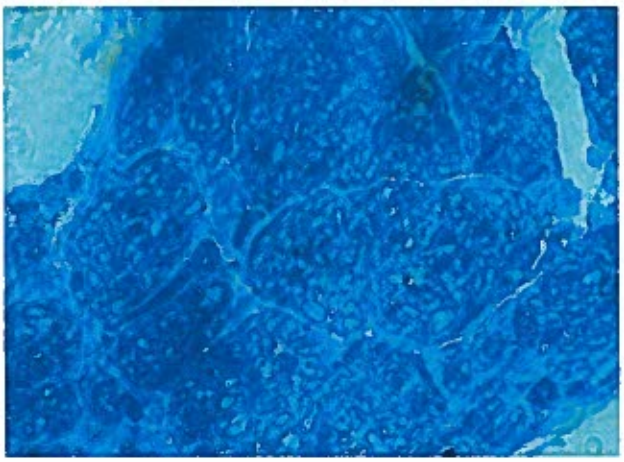
Results were reported as mean values and the standard error of the mean (SEM). One-way ANOVA test with Bonferroni multiple comparisons was used to determine the statistically significant differences between experimental groups. The following conventions were used:significant, *$p < 0.05$; very significant, **$p < 0.01$; and extremely significant, ***$p < 0.001$

Histologic results of nerve autograft v. nerve allograft plus AFS cells. Cross sections of the distal part of the regenerated nerves were evaluated by light and electronic microscopy. ANA plus AFS group showed significantly higher value of myelinated axon area per nerve, axon diameter, fiber diameter and myelin diameter compared with ANA alone, which closely resembled the outcomes obtained from autograft group. (Table 1).

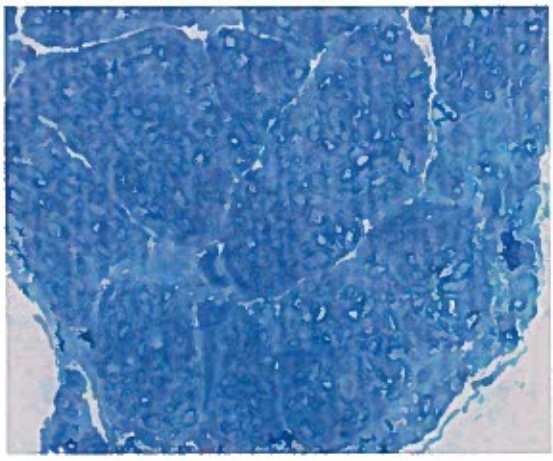
Histology of sciatic nerve graft at 4 mo post-injury/repair.

H&E stains of nerve cross sections:

Autograft –1000X at 4 mo.



AFS seeded ANA,1000X at 4 mo.



Distal Nerve Stump Histological Outcomes			
	Autograft	ANA	ANA+AFS
Myelin Thickness (μm)	1.64 ± 0.22	0.89± 0.09	1.47 ± 0.13**
Axon Diameter (μm)	2.29 ± 0.28	1.96 ± 0.24	2.36 ± 0.36*
Fiber Diameter (μm)	3.93 ± 0.28	2.86 ± 0.25	3.84 ± 0.3**
G Ratio (AD/FD)	0.58 ± 0.02	0.68 ± 0.02	0.61 ± 0.01
Myelinated axon area (%)	82.63± 7.54	11.78 ± 2.96	55.66 ± 7.89**

Table1. * indicated significance compared with ANA group (* P<0.05, ** P<0.01).

Electronic microscopy revealed greater myelinated axon surface and myelin thickness in ANA plus AFS cells treated group (Figure 2.1.1), indicating enhanced regenerating ability of the axons.

Neuromuscular junction morphology analysis

Cross sections of gastrocnemius and tibialis anterior muscle were assessed at the junctions where tibial and common peroneal nerves enter the muscles. There were no significant differences in the number and shape of NMJ between ANA plus AFS group and autograft group.(P= 0.69) (autograft vs. ANA+AFS vs. ANA: 45 ± 9 vs. 39 ± 9 vs. 28 ± 8, Figure 8) The NMJs of ANA group demonstrated a flat synapse outline and fewer neuromuscular junctions compared with autograft and ANA plus AFS groups.(p<0.05)

Autograft

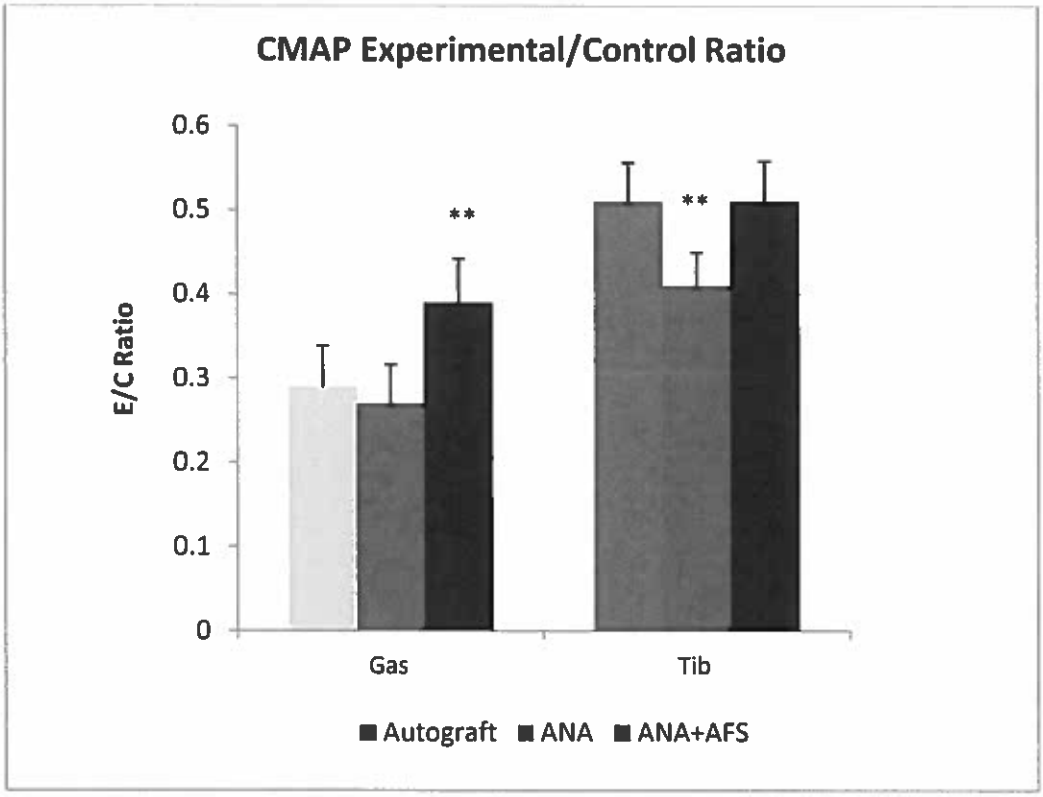
Allograft

Allograft + AFS

Fluorescent microscopy representative pictures of neuromuscular junctions in gastrocnemius muscle. Magnification: 200X

Functional recovery of the innervated muscles following nerve transection/repair using the different constructs also was evaluated by studying compound motor action potentials elicited by nerve stimulation above the repair site four months after nerve repair.
Electrophysiology analysis comparison among autograft, ANA and ANA plus AFS cells groups. The Cadwell EMG Sienna Wave System was used for the electrophysiology testing. 12 weeks after the nerve autograft, ANA and ANA plus AFS cells implantation, rats were anesthetized with isoflurane and the regenerated sciatic nerve was exposed. Electromyographic analysis was examined by stimulating the regenerated nerve distally (suture sites were taken as referral points) with a monopolar cathodic electrode at 1mA, the anode was placed on the rat chest. Muscle contractions were recorded by electrodes placed into the gastrocnemius muscle (medial and lateral) and tibialis muscle of both experimental and control limbs.
Compound evoked muscle action potentials (CMAP) was recorded by three consecutive stimulations that were averaged for CMAP delays and amplitudes measurement.

Electrophysiological analysis of CMAP indicated that ANA plus AFS cells group had significant higher experimental/control ratio of wave potentials on gastrocnemius muscle compared with autograft and ANA groups. (Left CMAP (mv) autograft vs. ANA vs. ANA+AFS: 10.14±3.52 vs. 9.20±3.33 vs. 10.32±2.7; Right: 34.25±8.25 vs. 33.45±4.2 vs. 26.37±6.17. $p<0.01$) CMAP ratio of tibialis muscle had no significant differences between autograft and ANA plus AFS groups but was significantly higher than ANA group alone. (Left: 12.00±1.39 vs. 11.20±2.17 vs. 13.17±5.80; Right: 23.24±6.69 vs. 26.75±5.78 vs. 25.60±7.34. $p<0.01$)



Mean amplitudes of compound muscle action potential (CMAP) after stimulation of regenerating and contralateral control sciatic nerve with a monopolar electrode proximally. B. Ratio of amplitude of experimental to contralateral CMAP of gastrocnemius and tibialis muscle in ANA, ANA plus AFS and autograft groups.

Muscle atrophy after autograft, ANA or ANA+ AFS cells implantation was analyzed by excising the gastrocnemius muscle and tibialis muscle at the end of 4 months and calculating the ratio of the mass of the experimental muscle vs. the mass of the muscle in the control side (E/C ratio). There was no significant

difference among autograft, ANA and ANA plus AFS groups on E/C ratio of gastrocnemius muscle and tibialis muscle. (gastrocnemius muscle weight E/C ratio, autograft vs. ANA vs. ANA+AFS: 0.51 ± 0.03 vs. 0.50 ±0.04 vs. 0.51±0.05; tibialis muscle: 0.65 ± 0.05 vs. 0.60 ± 0.06 vs. 0.6± 0.04,

Walking track analysis after 4 months recovery

Gait analysis of 24 parameters at the end of 4 months following injury indicated that there were no significant differences in stance/swing ratio, stride time, stance factor, swing stride percentage, brake stride percentage, propel stride percentage, stance stride percentage, brake stance percentage, propel stance percentage, hind limb shared stance percentage, step angle, stide length, max dA/dT among three groups.

Baseline	Autograft	ANA	ANA+AFS	4mons	Autograft	ANA	ANA+AFS
Stride(s)	0.48	0.45	0.432932	Stride(s)	0.54	0.52	0.50
Stance/Swing	2.79	2.76	2.630303	Stance/Swing	1.86	1.79	1.76
StanceWidth(cm)	2.64	3.02	2.92197	StanceWidth(cm)	3.73	3.16	3.51
Paw Area at Peak Stance in sq. cm(cm^2)	3.81	3.31	3.304318	Paw Area at Peak Stance in sq. cm(cm^2)	2.71	2.64	2.48
StanceFactor	1.02	1	1.000909	StanceFactor	0.85	0.83	0.83
Overlap Distance(cm)	1.85	1.84	1.389921	Overlap Distance(cm)	1.47	0.79	0.98
Ataxia Coefficient	0.44	0.36	0.482045	Ataxia Coefficient	0.47	0.46	0.65
Midline Distance (cm)	2.23	2.32	3.161136	Midline Distance (cm)	2.25	2.9	2.22
Axis Distance (-cm)	1.31	1.58	1.359167	Axis Distance (-cm)	2.08	1.79	1.83
%SwingStride	26.49	26.71	27.92348	%SwingStride	35.25	36.32	36.91
%BrakeStride	13.45	15.61	20.95833	%BrakeStride	17.02	18.63	24.11
%PropelStride	60.04	57.67	51.11288	%PropelStride	47.7	45.05	38.97
%StanceStride	73.51	73.29	72.07652	%StanceStride	64.71	63.68	63.08
%BrakeStance	18.3	21.44	28.5487	%BrakeStance	26.32	29.96	38.04
%PropelStance	81.7	78.69	70.73125	%PropelStance	73.68	70.16	61.95
% Hind limb Shared Stance	65.4	66.29	65.3417	% Hind limb Shared Stance	67.58	67.05	69.7
StepAngle(deg)	68.94	64.1	63.52901	StepAngle(deg)	62.37	62.92	61.73
PawAngle(-deg)	9.18	6.19	-7.89836	PawAngle(-deg)	18.48	17.87	16.53
StrideLength(cm)	12.14	11.12	10.82803	StrideLength(cm)	13.45	13.18	12.62
Paw Drag(-)	8.45	9.14	-11.2632	Paw Drag(-)	11.72	11.29	12.24
SFI(-)	4.84	7.55	5.271452	SFI(-)	43.7	40.92	38.42
MAX dA/dT (cm^2/s)	375.58	317.98	288.6579	MAX dA/dT (cm^2/s)	241.17	225.1	211.24
MIN dA/dT(-cm^2/s)	43.64	37.79	46.7363	MIN dA/dT(-cm^2/s)	23.05	20.68	33.31

The autograft group showed significant better recovery at stance width, overlap distance, ataxia coefficient, axis distance, SFI compared to ANA and ANA plus AFS groups. ANA plus AFS group exhibited better functional recovery in stance width, overlap distance, midline distance, axis distance, paw angle, paw drag than ANA group alone and didn't show significant differences from autograft group in these parameters, indicating preferred regenerating ability of AFS cells at the end of 16 weeks following a long nerve gap injury. In addition, the ratio of 4 months post-surgery to the baseline was significantly higher than allograft alone, suggesting an overall better sciatic function recovery than ANA group. (*$p<0.05$, **$p<0.01$ in all indices)

Task 2.1 complete

Task 2.2 (months 12-24) – Motor end plate preservation to increase functional recovery following denervation/reinnervation of the affected muscle will be studied in a separate cohort of rats. This group (n=10) will be subjected to nerve injury and repair using a 15 mm nerve defect and autologous nerve repair as in 2.1. A beta 2 agonist (fenoterol) will be administered via an osmotic minipump to the denervated gastrocnemius complex at a dose rate of 1.4 mg/kg/day in a total volume of 24 microliters. This drug and dosing regimen has been demonstrated to reduce and reverse muscle wasting in rats (Ryall 2003). It is hypothesized that it may reverse the loss of NMJ surface area and number following denervation. This may allow greater recovery following reinnervation.

A control group of injured rats (n=10) treated with vehicle for the beta2 agonist only will also be studied. Muscle force generation and histology to examine neuromuscular junction density will be performed at 120 days.

- An amendment requesting additional rats to pursue this study was approved by the Wake Forest IACUC. Accordingly, this amendment is being prepared for submission to the USAMRMC ACURO so that these studies can be initiated.

Progress Months 24-36 - These studies were delayed pending approval of an extension of the animal care and use committee approval for this research. Protocol approval is only good for three years. These protocols were approved by the Wake Forest IACUC on 23/06/2016. The ACURO reviewed and approved this protocol on 25/08/2016. These studies will be initiated within this quarter.

A no-cost extension of the award through 31/08/2017 was received on 02/08/2016 to allow completeion of the proposed studies.

Task 2.3 (months 18-36) – Large gap nerve repairs will be studied in nonhuman primates. The nerve reconstruction constructs utilized in study 2.1 [ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2)] will be employed bilaterally in a randomized fashion (right arm v. left arm) to repair a large gap nerve defects (6 cm) in macaca fasciculata monkeys. Electrophysiologic testing as well as functional assessments (grasp and pinch ability) will be assessed longitudinally on a bimonthly basis (beginning 3 months post surgery) for 12 months following large nerve gap repair of the median nerve. At the end of 1 year, the animals will be euthanized. The median nerve from the elbow to the wrist crease will be removed bilaterally for histologic study and the muscle tissue of the thenar complex will be recovered bilaterally.

- The results from Task 2.1 are encouraging and procedures are underway to procure test subjects through the Wake Forest School of Medicine Non-Human Primate Program and the Wake Forest University Animal Resources Program. Vervet monkeys will be used instead of m. fasciculate because they are less expensive, they are available immediately and will not require quarantine, and they are of comparable size.
- An extension of the original contract will be required to complete these studies because they require at least a 12 month follow-up period to appropriately assess functional recovery.

Progress Months 24-36 - These studies were delayed pending approval of an extension of the animal care and use committee approval for this research. Protocol approval is only good for three years. These protocols were approved by the Wake Forest IACUC on 23/06/2016. The ACURO reviewed and approved this protocol on 25/08/2016. These studies will be initiated within this quarter.

A no-cost extension of the award through 31/08/2017 was received on 02/08/2016.

KEY RESEARCH ACCOMPLISHMENTS:

Cell seeding of the acellular allografts for peripheral nerve repair.

- This methodology is being compiled as a manuscript for submission.

All test groups of animals in Task 2.1 (rat studies) were successfully treated using the appropriate nerve repair constructs as originally proposed. The functional outcomes of these large gap nerve repairs have been compiled and the results are being prepared for submission for publication.

CONCLUSION:

Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The ability to incorporate cells into nerve scaffold poses a research challenge. Current techniques are inadequate. The current research has tried two innovative approaches which have not been successful. This potential pitfall was recognized in the research plan and the project pursued methods to increase the permeability of the nerve epineurium. **This obstacle was overcome through an innovative combination of techniques utilizing injection of cells into the body of the nerve and increasing the porosity of the epinurium using microneedle punctures.** The increased porosity of the epineurium insures appropriate nutrition of the implanted cells via diffusion. These constructs have been demonstrated to retain viability following implantation into a nerve defect and offer improved outcomes compared to unseeded nerve allografts for segmental nerve defect repairs.

In-vivo assessment of these constructs was evaluated using a rat sciatic nerve model. The animals in which a nerve allograft that was seeded with AFS cells demonstrated improved recovery compared to animals receiving nerve allograft alone. This recovery was comparable to that achieved using nerve autograft, the current clinical gold standard for repairing large nerve gaps.

These constructs will be tested in a preclinical non-human primate model.

PUBLICATIONS, ABTRACTS, AND PRESENTATIONS:

Abstract submitted to the Orthopaedic Research Society Annual Meeting in 2016 entitled: "Regeneration of large-gap peripheral nerve injuries using acellular nerve allografts plus amniotic fluid derived stem cells (AFS)".
Authors: Ma A, Marquez-Lara AJ, Martin E, Smith TL, Li Z.
Presented at the Ortohopaedic Research Society Annual Meeting in Orlando FL in March of 2016.

Abstract submitted to the Federation of American Societies for Experimental Biology annual meeting in 2016 entitled: "Regeneration of Large-gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS)" Authors: Xue Ma, MD, PhD, Alejandro Jose Marquez-Lara, MD, Eileen Martin, Thomas L. Smith, PhD, Zhongyu Li, MD PhD
Presented in San Diego, Ca in April of 2016.

In-Progress Report- Ft Detrick, MD, 04 February, 2016.

Abstract submitted to both American Association of Hand Surgery(AAHS) and the American Society of Peripheral Nerve (ASPN) "In vivo tracking of amniotic fluid derived stem cells on acellular nerve graft" has been accepted as an oral presentation at both the AAHS and ASPN 2017 annual meeting in Hawaii. Copy attached.

INVENTIONS, PATENTS, AND LICENSES:
Nothing to report

REPORTABLE OUTCOMES:
Nothing to report

OTHER ACHIEVEMENTS
Nothing to report

CHALLENGES:
Because of the extended timeline required to achieve seeding and incorporation of AFS into the nerve allografts, we requested and received a contract extension in order to complete SOW task 2.3. These non-human primates will be acquired in the current quarter.

The Wake Forest Institutional Animal Care and Use Committee and ACURO approved a change of species of non-human primate from macaca fasciculate to vervet monkeys (Chlorocebus pygerythrus). This change was requested to reduce the acquisition costs of test subjects and expedite the enrollment of test subjects. Vervet animals are readily available on our campus and can be enrolled immediately. They are comparable in size to the Cynomologous monkeys originally proposed for use in these studies.

REFERENCES

1. Ma J, Shen J, Garrett JP, Lee CA, Li Z, Elsaidi G, Ritting A, Hick J, Tan KA, Smith TL, Smith BP, Koman LA. Gene expression of myogenic regulatory factors, nicotinic acetylcholine receptor subunits, and GAP-43 in skeletal muscle following denervation in a rat model. J Orthop Res. 2007 Nov; 25(11):1498-505.

2. Ma J, Smith BP, Smith TL, Walker FO, Rosencrance E, Koman LA. Juvenile and adult rat neuromuscular junctions: density, distribution, and morphology. Muscle and Nerve 2002; 26: 804-809.

3. Ryall JG, Plant DR, Gregorevic P, Sillence MN, Lynch GS. Beta-2 agonist administration reverses muscle wasting and improves muscle function in aged rats. J Physiol 2003, 555(1): 175-188.

4. Stone AV, Ma J, Callahan MF, Smith BP, Garret JP, Smith TL, Koman LA. Dose and volume dependent response to intramuscular injection of botulinum neurotoxin-A optimizes muscle force decrement in mice. JOR 2011 Nov; 29(11):1764-70).

5. Stone A, Ma J, Whitlock PW, Koman LA, Smith TL, Smith BP, Callahan MF. Effects of Botox and Neuronox on muscle force generation in Mice. J Orthop Res 2007 Dec; 25(12): 1658-1664.

6. Whitlock PW, Smith TL, Poehling GG, Shilt JS, Van Dyke ME. A naturally-derived, cytocompatible, and architecturally-optimized scaffold for tendon and ligament regeneration. Biomaterials, 2007 Oct; 28(29):4321-9.

APPENDICIES: (attached)

Orthopaedic Research Society Annual Meeting 2016 abstract
Federation of American Societies for Experimental Biology annual meeting 2016 abstract
American Association for Hand Surgery annual meeting abstract 2017
Peripheral Nerve Society Annual meeting abstract 2017
Scientific Research Grants:

1.) American Society for Surgery of the Hand – In-vivo tracking of Amniotic Fluid Derived Stem cells on Acellular Nerve Graft. PI - Xue Amy Ma, MD, PhD
2.) Effect of Amniotic Membrane and Amniotic fluid Stem Cells on Schwann cell Neurotrophic Cytokine Production. PI- Xue Amy Ma, MD, PhD

COLLABORATIVE AWARDS:

Dr. Z Li : CO-PI

"Acceleration of Regeneration of Large-Gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS)"

ERMS/Log Number - OR120157 and OR120157P1

Insert Award Number – W81XWH-13-1-0309 and W81XWH-13-1-0310

PI: Thomas Smith, PhD and Zhongyu Li, MD, PhD **Org:** Wake Forest University Health Sciences **Award Amount:** $939,786

Study/Product Aim(s)

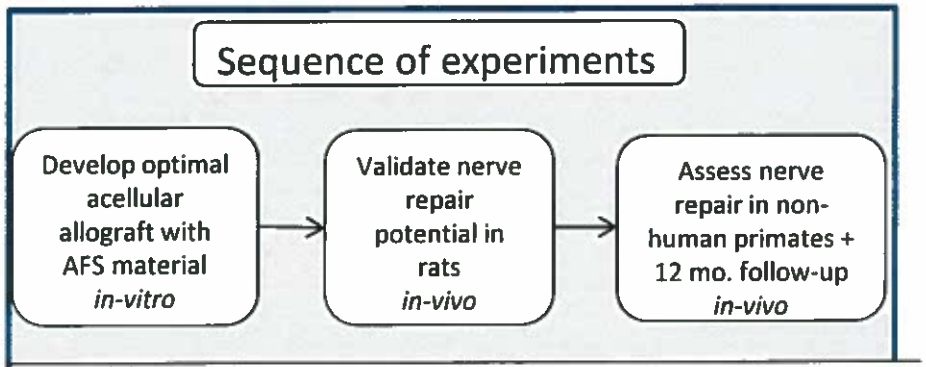
Specific Aim 1 (SA1): To demonstrate the ability to seed Acellular Nerve Allografts (ANA's) with AFS using sub-atmospheric pressure (SAP) *in-vitro*

Specific Aim 2 (SA2): To establish the feasibility of using AFS seeded ANA's in large gap nerve repairs *in-vivo.*

Approach – Partnership: Basic Scientist + Hand Surgeon

SA1: Establish the feasibility of using AFS seeded ANA's in large gap nerve repairs *in-vivo.* Cell culture techniques will be employed to seed commercially available ANA's with commercially available AFS. Both ANA's and AFS materials are FDA approved

SA2: Establish the feasibility of using AFS seeded ANA's in large gap nerve repairs *in-vivo.* Rats will be studied first to establish optimal nerve construct. Non-human primates will be studied as a pre-clinical model.



Accomplishments:

1. Gait analysis of all cohorts of rats complete as is functional testing of final group
2. Evaluation of histology and functional recovery being performed in final rat cohort
3. Electron Microscopy of seeded nerve constructs removed from implanted rats
4. MRI images of Fe-labeled AFS cells demonstrate cell viability after implantation
5. IACUC protocol renewal approved by ACURO
6. No-Cost Extension of Contract to allow enrollment of Non-Human Primates approved

Timeline and Cost

Activities CY	13	14	15	16
Specific Aim 1.1				
Specific Aim 1.2				
Specific Aim 1.3, 2.1, 2.2				
Specific Aim 2.3				
Estimated Budget ($K)	**$94.5**	**$261.8**	**$219.3**	**$144.9**

Updated: August 31, 2016

Goals/Milestones

1.1 – Cell seeding using SAP – **Completed**

1.2 - AFS seeded onto ANA - **Completed**

1.3 – Study migration of Schwann cells onto the AFS seeded scaffold. **Completed**

2.1 – ANA with AFS studied using Lewis Rats with large nerve gaps. **Completed**

2.2 – Motor end plate preservation to increase functional recovery of rats – (USMRMC ACURO approval obtained. IACUC being renewed

2.3 – Large gap nerve repairs will be initiated in Q1 in non-human primates.

Comments/Challenges/Issues/Concerns

- If timelines change – Acquisition of NHP's pending results from 2.2
- Spending is under budget because non-human primate experiments have not yet begun.

Budget Expenditure to Date

Projected Expenditure: $913,675

Actual Expenditure: $720,549

I. Abstracts presented for DOD project

Abstract accepted for Orthopedic Research Society 2016 annual meeting (ORS 838) and Experimental Biology 2016 annual meeting (EB 6688)

Regeneration of Large-gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS)

Xue Ma, MD, PhD, Alejandro Jose Marquez-Lara, MD, Eileen Martin, Thomas L. Smith, PhD, Zhongyu Li, MD PhD
Department of Orthopedic Surgery, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Introduction: Surgical reconstruction of peripheral nerve lesions in the extremities is challenging and often results in impaired functional recovery. The "gold standard" for successful nerve repair is a primary tensionless epineural repair which often is not possible. Nerve guidance tubes as well as acellular nerve allografts (ANA) have been developed to provide repairs comparable to those obtained with autografts. In order to promote nerve regeneration across large nerve gaps, regenerating axons are capable of extending the gap distance for nerve recovery when in the presence of Schwann cells. Tissue engineering strategies have attempted to mimic this cell environment by adding other supportive types of cells such as stem cells to the nerve allograft.
Hypothesis: We hypothesized that acellular nerve allografts (ANA) can be seeded with amniotic fluid-derived stem cells (AFS) to promote and accelerate nerve regeneration. The presence of the AFS provides support for the regenerating axons without the requirement of becoming Schwann cells.

Methods: *In vitro* study: $1X10^6$ "Off the shelf" AFS cells were injected underneath the epineurium of the ANAs using a 26 G syringe. Seeded grafts were placed vertically at the bottom of a small centrifuge tube covered with DMEM containing 20% FBS for overnight then transferred to a 48 well plate for additional 48 hours. *In vivo* study: ANA with AFS cells for long gap nerve repairs were studied using Lewis Rats. A large gap nerve injury (1.5 cm) was created in the sciatic nerve, and the gap was repaired immediately with an ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2), or with an autograft (Group 3). Outcome assessments include walking track analysis (DigiGait Imaging system, Figure 1) to document the return of motor control at 1 month and 2 months post- injury.

Results: *In vitro* AFS cells seeding to ANA: DAPI staining on longitudinal and cross sections of ANAs showed cells spread evenly through the nerve fibers (Figure 2.) *In vivo* gait analysis of 23 parameters of the autograft, ANA and ANA plus AFS cells groups at 2 months post-injury indicated that there were no significant differences in stride, stance/swing ratio, paw area at peak stance, stance factor, midline distance, % swing/stride, % brake/stride, % propel/stride, % stance/stride, %brake/stance, % propel/stance, % hind limb shared stance, step angle degree, stride length, MAX dA/dT and MIN dA/dT among groups.The autograft group showed greater stance width, overlap distance, axis distance, paw angle and paw drag compared to the ANA and ANA plus AFS cell groups.($p<0.01$ in all indices, Figure 3) ANA plus AFS cell group showed reduced swing time, %swing/stride at the end of 2 months compared with 1 month time point (1 month vs. 2 months: 0.17±0.01s vs.0.14±0.02s; 37.76±3.97% vs. 33.37±4.78%; $p<0.01$, $p<0.05$) In addition, ANA plus AFS cell group

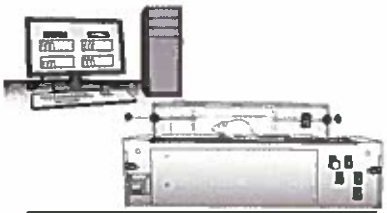
demonstrated a more robust motor function recovery compared to ANA alone group (paw angle and paw drag value are close to autograft group), indicating AFS cells facilitated the nerve regeneration 2 months following injury. We will keep tracking the motor function recovery as well as the histological outcomes till the end of 4 months following injury.

Discussion: We have developed an effective and consistent method to seed the ANA with AFS cells. The cells are viable 72 hours after seeding and spread through the entire ANA evenly. The seeding method could potentially prolong the time of the AFS cells staying in the ANA thus support and enhance the host nerve regeneration.

Significance: The findings of the study may have a direct impact on the future of stem cell therapies to facilitate nerve regeneration in patients who sustain peripheral nerve injuries.

Acknowledgements: The study is funded by Department of Defense USAMRAA (W81XWH-13-1-0310)

Figure 1.



DigiGait Imaging System

Figure 2.

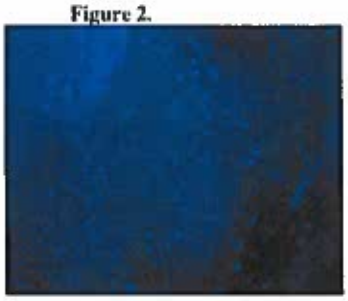


Figure 2. Cross section of a sciatic nerve allograft seeded with $1X10^6$ AFS cells -DAPI staining showed AFS cells nuclei appeared bright blue. Magnification X100

Figure 3.

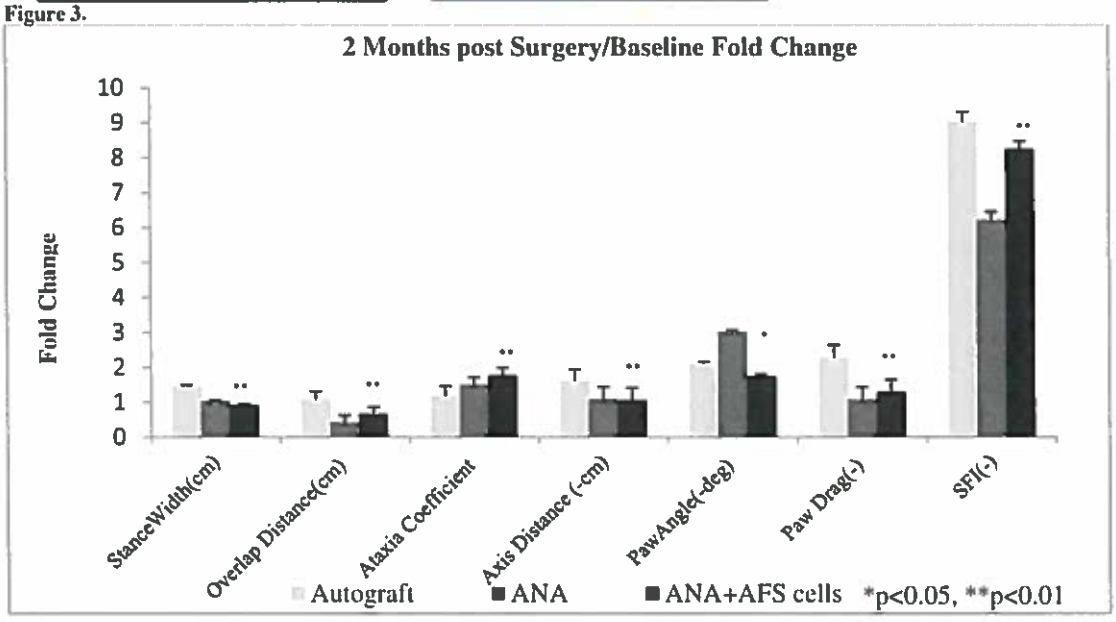


Figure 3. ANA+AFS cells group demonstrated differences in stance width, overlap distance, ataxia coefficient, axis distance, paw angle, paw drag and SFI compared with autograft group at the end of 2 months post- surgery.

Abstract accepted for American Society for Surgery of the Hand 2016 annual meeting (ASSH 7147)

In vivo tracking of amniotic fluid derived stem cells on acellular nerve graft

Xue Ma, MD PhD, Tianyi David Luo, MD, Thomas L. Smith, PhD, Zhongyu Li, MD PhD

Hypothesis: Amniotic fluid derived stem (AFS) cells can be seeded to nerve allografts to promote nerve regeneration; their impact on the regenerating nerve and nerve bed and can be tracked by MRI imaging over time.

Methods: Cell labeling: Commercially available AFS cells (Nutech™) were labeled using supraparamagnetic micron sized iron oxide (MPIO) (Bangs Laboratories, Fishers, IN) containing magnetite cores encapsulated with styrene/divinyl benzene and coated with dragon green fluorescent dyes, at a ratio of 1.5×10^6 AFS cells to 20 µL of 3×10^8 MPIOs for 2 weeks. The cells were visualized by fluorescence microscopy to confirm the presence of MPIOs in the AFS cells. Cell viability and proliferation assay: Following labeling, 5×10^3 cells were plated per well in 96-well plate; unlabeled AFS cells will serve as the control. Cell viability at 1, 3, 5, and 14 days were evaluated using CCK-8 assay (Sigma-Aldrich, St. Louis, MO).Six replicates were tested for each group. Cell differentiation assay and neurotrophic factors quantification: AFS cells were cultured in neurogenic induction media (contains forskolin, neuregulin-β1 (Sigma-Aldrich, St Louis, Mo) and 50% of volume of rat primary Schwann cell conditioned media) for 2 weeks and the morphology changes over time were recorded. The conditioned media was collected and neurogenic growth factors were analyzed using Quantibody Human Growth Factor Array (Ray-Biotech, GA). MRI study: MPIO labeled AFS cells (1.5×10^6) were injected to an acellular nerve allograft (ANA) and cultured for 36 hours then the ANA was implanted to repair a large nerve defect (1.5 cm) of the sciatic nerve in a Lewis rat model. The fate of the labeled AFS cells was evaluated by MRI at 1 week, 2 weeks and 4 weeks post- surgery. Histology: contiguous frozen sections were stained with Prussian blue and nuclear red in order to identify the MPIO-labeled AFS cells incorporated into the nerve graft. Real time PCR analysis: transplanted AFS cells that have incorporated into the allograft identified by immunohistochemistry were isolated. Neurogenic conversion of cells in vivo was confirmed with real-time PCR using human primers for neurogenic lineage markers.

Results: The MPIO labeled AFS cells are viable at the end of 14 days. (Figure1.) There were no apparent differences of proliferation rate and morphology between the AFS and AFS plus MPIO groups. (Baseline OD: AFS vs. AFS+MPIO: 0.41±0.03 vs. 0.40± 0.02, p=0.55; 1 week OD: 0.78±0.19 vs. 0.61±0.11, p=0.07; 2 weeks OD: 1.42±0.07 vs. 1.36±0.25, p=0.58)

9T MRI imaging showed MPIO labeling, with a strong decrease in signal, appearing as fuzzy dark spots in T2 weighted images at 1 week post-surgery, indicating AFS cells' involvement in sciatic nerve repair and regeneration (Figure 2.). The other MRI endpoints studies are currently underway.

Summary Points: 1. AFS cells are viable after infused with MPIO and attached to ANA.

2. MRI is an effective way to track the AFS cells longitudinally in rat model, thus have the potential to directly impact AFS cell delivery strategies for peripheral nerve regeneration in clinical use.

Figure 1.

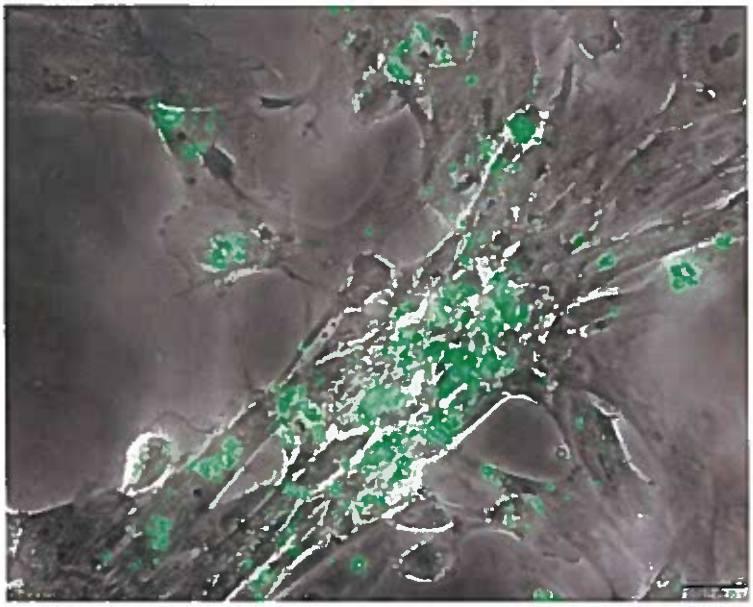


Figure 1. AFS cells infused with MPIO for 14 days.
Magnification: 400X

Figure 2.

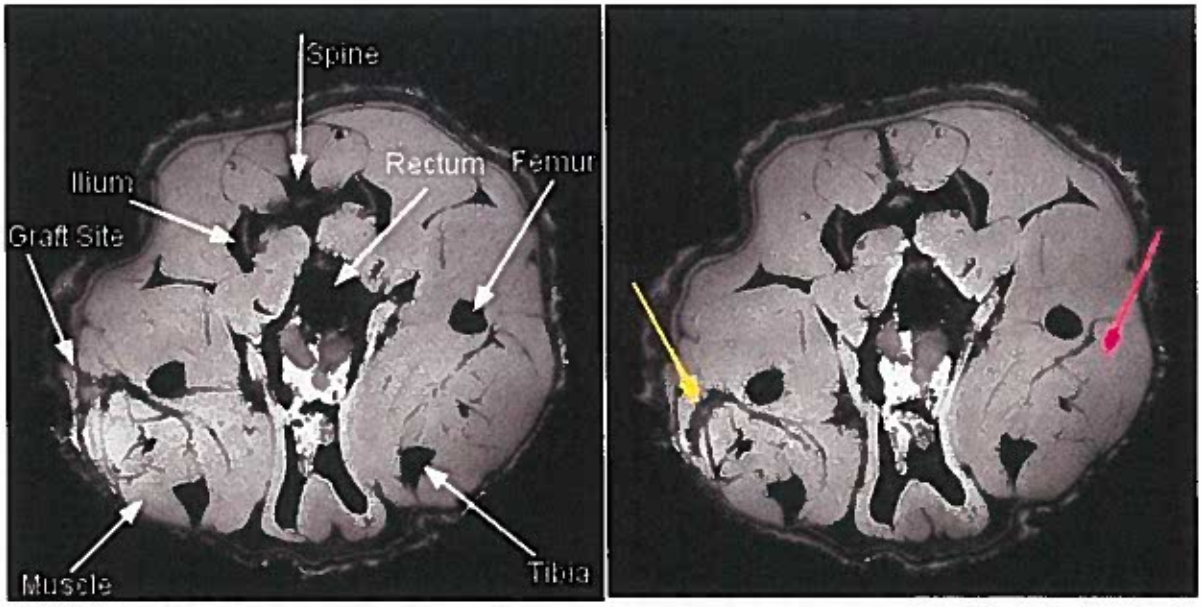


Figure 2. 1.5 x 10^6 AFS cells were labeled with MPIO particles and seeded onto the ANA graft; the graft was implanted to the left sciatic site of the rat for 1 week. Left image shows bone and air are black and the bladder is very bright. The MPIO signals on the graft side (yellow arrows), but not in the same areas contralaterally (pink arrows) suggest MRI effectively tracks the temporal location of AFS cells seeded on the nerve allograft during the nerve regeneration in vivo .

Abstract accepted for American Association for Hand Surgery 2017 annual meeting (AAHS 23778) and American Society for Peripheral Nerve 2017 annual meeting (ASPN 23910)

In vivo tracking of amniotic fluid derived stem cells on acellular nerve graft

Xue Ma, MD PhD, Tianyi David Luo, MD, Thomas L. Smith, PhD, Zhongyu Li, MD PhD

Introduction: Traumatic transections of peripheral nerves are associated with poor nerve regeneration. The use of nerve grafts with stem cells provides an alternative to autograft for nerve repair. The purpose of this study is using MRI to track the fate of amniotic fluid derived stem (AFS) cells that are seeded to nerve allografts and elucidate the mechanisms of their impacts on the regenerating nerve.

Methods: AFS cells were labeled using supraparamagnetic micron sized iron oxide (MPIO) coated with fluorescence dye. Labeled cells were plated and viability was assessed. Next, cells were cultured in neurogenic induction media; the conditioned media was collected to evaluate the neurogenic growth factors. Differentiated cells were confirmed with real-time PCR for neurogenic lineage markers.

MPIO labeled AFS cells were injected onto an acellular nerve allograft (ANA) and implanted to repair a 1.5 cm sciatic nerve defect in 10 rats. Labeled AFS cells were evaluated by MRI at 1, 2, and 4 weeks post-surgery. Intensity of the MPIO regions was quantified using ImageJ. Contiguous frozen sections were stained for iron to identify the labeled AFS cells incorporated into the nerve graft. Co-localization of the transplanted cells was confirmed using human specific nuclear antibody (Anti-NuMA).

Results: Labeled AFS cells demonstrated viability at 14 days (Figure 1). Proliferation rate and morphology between the control and labeled cells demonstrated no significant difference ($p=0.58$). Cells differentiated towards Schwann-like cells after being cultured in neurogenic induction media. NGF and NEFL gene expression were elevated by magnitudes of 202.60 ± 1.89 and 30.62 ± 1.99, respectively ($p<0.005$) compared to control. Cytokine quantification analysis of AFS cells showed significantly increased BDNF, β-NGF, β-FGF, GDNF, NGF R, NT-4 and TGF-β production. (Fold change compared to undifferentiated control: 10.25 ± 1.96, 383.06 ± 12.93, 3.95 ± 1.06, 5.78 ± 1.33, 46.84 ± 3.67, 2.69 ± 0.77, 25.39 ± 3.74, $p<0.001$ respectively).

7T MRI demonstrated MPIO labeling, with a strong decrease in signal, appearing as fuzzy dark spots in T2-weighted images at 4 weeks post-surgery Therewas no significant difference of average normalized hypointense region volume between 2 weeks and 4 weeks post-injury (0.47 ± 0.06 and 0.52 ± 0.12, respectively, Figure 2). Cell integration was confirmed by iron and Anti-NuMA staining.

Conclusions: AFS cells maintained viability after labeling and can be used to augment nerve repair by seeding onto ANAs. Cytokine analysis suggests a paracrine-mediated effect on nerve repair. MRI can effectively track the AFS cells longitudinally in the rat model, thus has the potential to monitor AFS cell delivery strategies for nerve regeneration in clinical use.

Figure 1.

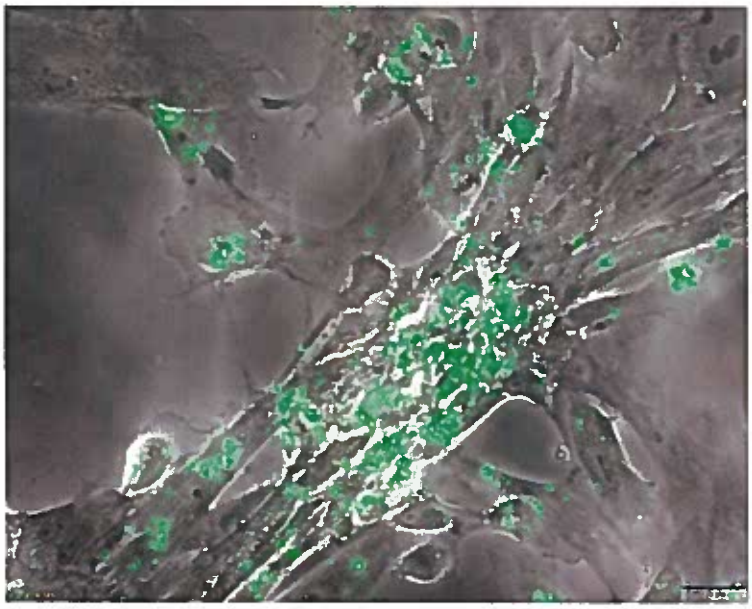
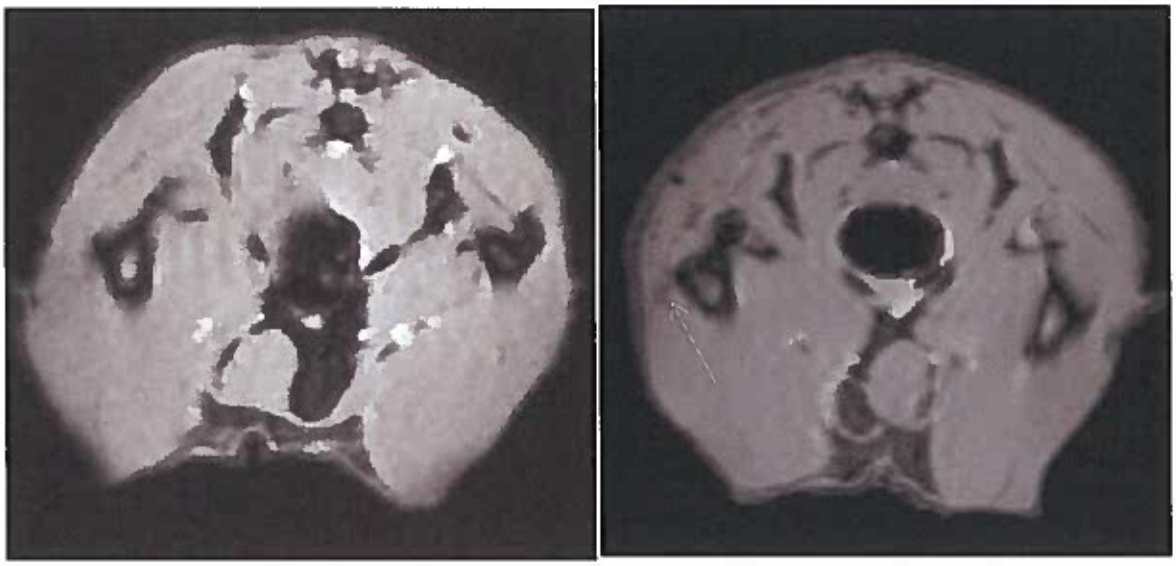


Figure 1. AFS cells infused with MPIO for 14 days.
Magnification: 400X

Figure 2.



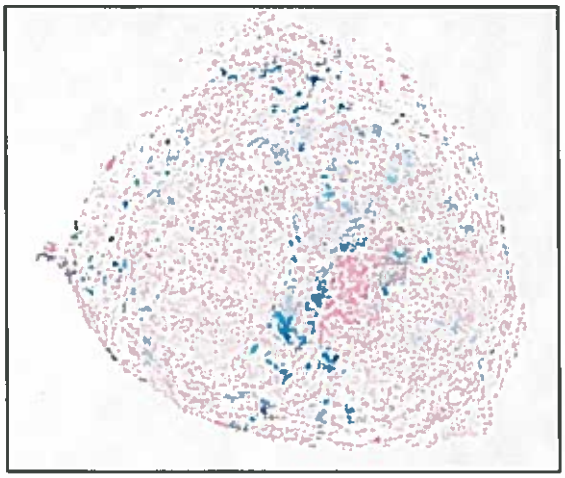


Figure 2. Longitudinal images of the same animal before injury (left) and 4 weeks post-surgery (right).1.5 x 10^6 AFS cells were labeled with MPIO particles and seeded onto the ANA graft; the graft was implanted to the left sciatic site of the rat for 4 weeks. The MPIO signals on the graft side (white arrow) suggest MRI effectively tracks the temporal location of AFS cells seeded on the nerve allograft during the nerve regeneration in vivo. Prussian blue staining for the iron particles indicated AFS cells co-localize with the hypointense region.

II. Grants funded as compliments to DOD grant

1. Resident award from American Society for Surgery of the Hand

In-Vivo Tracking of Amniotic Fluid Derived Stem Cells on Acellular Nerve Graft

A. Specific Aims

Peripheral nerve repair remains a challenging clinical problem. Surgical factors dictating the success of a "gold standard", tension-free primary epineural repair include both the length of the nerve defect and it's interaction with the regenerating soft tissues in the nerve bed. The results of nerve repair are generally suboptimal with poorer outcomes observed in patients with mixed nerve injuries, nerve gaps larger than 5.0 cm, and proximal nerve injuries.(1) Nerve repairs can be technically perfect followed by optimal post-repair rehabilitation programs, and still, the clinical outcomes often are unpredictable and disappointing to the patient and surgeon.(2)

Traumatic transections of peripheral nerves are associated with poor nerve regeneration especially when there is a long nerve gap between the injury and the distal nerve stump.(3) The environment at the site of the nerve repair also plays a role in nerve regeneration. Although the regeneration of axons is supported by resident Schwann cells changing to a phenotype supporting growth, the environment supporting neuronal growth must establish axonal contact in a timely manner.(4) The use of autologous nerve grafts provides cell rich material to promote axon regeneration. However, the use of autografts is limited by donor availability, morbidity at the donor site, and non-specific regeneration.(5,6) Several techniques have been described to isolate the repaired peripheral nerve from the surrounding soft tissues in an attempt to reduce the complication of fibrosis with adhesions hampering both functional and symptomatic outcomes.

Although animal studies using transplanted stem and precursor cells have been shown that these cells support surgical nerve repair, the clinical application of such strategies must continue to be studied to determine the optimal method for cell delivery and the fate of the transplanted cells.(3) Information that will support the safety and efficacy of cell replacement therapies for nerve repair includes: the ideal number of cells required for transplantation, the best method of cell delivery, the survivability of the transplanted cells, and the most appropriate cell type to use.

Walsh, et al. indicated that it is important to track the fate of cells that are transplanted for peripheral nerve repair.(3) They noted if cells are not labelled before they are delivered to the site of peripheral nerve injury, it is difficult to identify the mechanism of the cell therapy. For this reason, the proposed study will use micron sized iron oxide (MPIO) particles and 7 Tesla MRI to track the attachment of the cells to allograft nerve scaffolds.

The proposed study will use both in vitro and in vivo experimental methods. The in vitro study will determine if AFS cells change morphology and produce neurogenic growth factors capable of supporting nerve regeneration. The in vivo AFS cell tracking study will document the temporal location of transplanted AFS cells

in regenerating nerve tissue in order to determine if they are incorporated into the regenerating nerve tissue and if they induce host neuroregeneration.

The specific aim of this study is to determine the effect of amniotic tissues seeded into a nerve scaffold and whether they contribute to improved histological and functional outcomes following peripheral nerve repair in vivo. We hypothesize that amniotic membrane and amniotic fluid stem cells will accelerate the recruitment and proliferation of Schwann cells, thus facilitate the functional recovery of the nerve following injury.

Specific Aim 1: Quantification of neurotrophic factor expression produced by labeled AFS cells, in vitro. AFS cells will be labeled using supraparamagnetic micron sized iron oxide (MPIO) particles as previously described at our institution.(11) These labeled cells will be cultured in normal media; the conditioned media collected from the flasks after three days will be analyzed using proteomics to evaluate neurogenic growth factor production. Rationale: Labeled AFS cells are capable of neurogenic differentiation. Hypothesis: The expression of neutropic factors produced by labeled AFS cells will be quantifiable.

Specific Aim 2: Monitor the functional and histological outcomes following sciatic nerve transection and repairment with scaffolds with different amniotic tissues for peripheral nerve regeneration *in vivo*. MPIO particle labeled cells will be seeded on a peripheral nerve allograft and inserted into a critical sized sciatic defect (1.5cm) in a Lewis rat model. Longitudinal tracking of these cells in the nerve allograft and surrounding nerve bed will be achieved using 7 Tesla MRI. Labeled cells and ex-vivo histology and immunohistochemistry will be used to correlate cell localization(11) and neurogenic differentiation of the labeled cells on the nerve allograft. In addition, transplanted AFS cells will be identified using immunohistochemistry. Neurogenic conversion of these cells in vivo will be confirmed with real time PCR.(9) Rationale: this investigational paradigm will determine whether or not AFS cells are incorporated into the regenerated nerve tissue. Hypothesis: AFS cells contribute to nerve regeneration by mediating trophic, paracrine effects.

B. Background and Significance

Peripheral nerve injury remains a challenging clinical problem with residual functional deficits (motor and sensory) associated with attempted regeneration across irreparable nerve gaps. In addition to fibrosis in the nerve bed and at the site of injury, peripheral nerves have an inherent regenerative difficulty in overcoming gap defects. When a nerve defect is too extensive to be repaired primarily, nerve scaffolds (e.g. conduits, allograft) and autografts have been employed with encouraging clinical results.(8) Extensive basic science and clinical research has been undertaken to improve clinical outcomes.(12)

It is currently unclear how human stem cell therapies contribute to peripheral nerve regeneration. A variety of different sources of stem/precursor cells are under study to determine their potential for peripheral nerve repair (3). However, there are many unanswered questions regarding how cell transplantation therapies can be optimized for clinical use (3). Existing studies report regeneration of nerve lesions in the context of few remaining transplanted stem cells without obvious differentiation (~10% at 2 weeks), suggesting a paracrine supporting role of these cells (16). The study hypothesis is that AFS cells that attach to nerve allografts do not act as a primary cell source. Rather, their impact on nerve recovery is probably mediated by their trophic paracrine effects on the regenerating nerve and nerve bed.

The proposed study is designed to monitor the location of stem cell transplants incorporated into peripheral nerve scaffolds. To achieve this goal, longitudinal tracking of the AFS cells will be studied in vivo using MRI in order to document the temporal and spatial interaction of the AFS cells with the regenerating nerve tissue. In vivo studies including cell proliferation assays, cell differentiation and viability assays, and neurotrophic factor quantification will identify the mechanism(s) by which AFS cell therapy impacts the functional recovery of the nerve. The potential mechanisms that will be investigated are: 1) AFS cells as a source of cells to be incorporated into the regenerating tissue via neurogenic differentiation and/or 2) AFS cells exerting a paracrine effect, creating a neuregulin- rich milieu in the nerve bed that enhances host repair mechanisms.

Currently, the ideal number of AFS cells required for transplant to support nerve regeneration in peripheral nerve injuries has not been established; numbers in the literature range from $4x10^3$ to $2x10^7$. (16, 17) The cell tracking and survival data described in the proposed study will provide evidence regarding the number of cells that are sufficient to maximize any therapeutic benefit of transplantation of seeded allograft with the goal of improving long term outcomes of nerve repair. In addition, information from the proposed study could possibly facilitate the standardization of a cell-loading strategy to ensure optimal cell delivery while addressing regulatory issues for all types of cell therapy for peripheral nerve regeneration. Therefore, an improved understanding of the mechanism(s) of the enhancement of peripheral nerve repair resulting from stem cell transplantation may enable more accurate clinical application in an area of medicine fraught with regulatory difficulty.

AFS cells have demonstrated multi-potency with neurogenic potential and have been suggested to provide biological augmentation of peripheral nerve regeneration.(9) AFS cells are commercially available for use in vivo and in vitro studies. Delivery of cells with proliferative capacity are expected to promote the microenvironment to cause the cells to differentiate into the required cell type.(10)

Because nerve autograft has limited availability, continued research has focused on the development of "next generation nerve guides" that incorporate growth factors and cell delivery. In this regard, animal models have demonstrated the functional and histological advantages of using stem cells to augment peripheral nerve regeneration.(3) However, the specific mechanism by which these cells achieve improved outcomes is currently unknown.

AFS cells have demonstrated a lack of immunogenicity (13) and have the potential to differentiate and take on nerve cell characteristics in the presence of biochemical cues in vitro.(9) However, in order to effectively deliver AFS cells as an "off the shelf" biological solution to facilitate peripheral nerve repair and regeneration, their mechanism of action must be elucidated. In addition, AFS cells have been shown to produce angiogenic and neurogenic growth factors in their undifferentiated form in vitro. Hence, these cells have been theorized to have the potential to support nerve regeneration by both supplying growth factors and possibly becoming incorporated into the regenerated nerve. A recent review of the use of stem cells in peripheral nerve regeneration has suggested that cell tracking may be useful in evaluating the exact contribution of transplanted cells in nerve regeneration, uncoupling their potential as a cell source and cellular drugstore.(14)

The proposed study will use commercially available non-tumorigenic, undifferentiated AFS cells (NuTech) that require minimal manipulation following isolation. The study will close the translational feedback loop by assessing the ability of the AFS cells to undergo neurogenic differentiation both in vitro and in vivo. In-vitro differentiation will be assessed after exposure of the AFS cells in tissue culture to neurogenic media for two weeks. Cells will be collected for real-time PCR to evaluate the expression of early neurogenic differentiation markers (Glial Fibrillary Acidic Protein, p75 neurotrophin receptor, and netrin-1), and the culture media will be analyzed for analysis of neurogenetic growth factors that might be elaborated by cells and released into the media.

Examination of in-vivo differentiation of the seeded nerve allografts will be achieved by surgically implanting the seeded allografts to repair sciatic nerve defects in rats. The labelled AFS cells will be isolated from the harvested scaffolds at the repair site. In addition to histological and immunohistochemical analysis, real-time PCR using human and rat primers for the same differentiation markers in vitro will be performed to assess the induced host neurogenic response.

In particular, the use of AFS cells, having the potential for both neurogenic differentiation and neurotrophic factor production in-vitro, look promising. To our knowledge, the proposed study is the first study to perform in vivo tracking of AFS cells on a devitalized nerve allograft scaffold. The specific aim of this is to demonstrate the temporal localization of these cells in the nerve scaffold during regeneration and to determine whether or not these transplanted cells become incorporated in the regenerating nerve tissue. Cellular migration to the nerve bed is the location where the immune-modulatory potential of AFS cells may play a role in reducing fibrosis in the regenerating nerve bed.(15) **The findings have the potential to directly impact AFS cell delivery strategies for peripheral nerve regeneration.**

C. Preliminary Studies

This project will compliment a larger DOD-funded study currently underway in our department investigating the use of AFS cell seeding on devitalized, acellular nerve allografts to accelerate functional and histological outcomes following nerve repair. In March 2013, Thomas L. Smith, PhD and Zhongyu Li, MD, PhD, both mentors for Dr. Ma, received funding from the Department of Defense Office of Congressionally Directed Medical Research Programs to study commercially available amniotic fluid derived stem cells (AFS) and their ability to incorporate into commercially available acellular nerve allografts. The DOD study investigates the motor functional and histological outcomes of acellular nerve allografts (ANA) seeded with AFS cells used to repair large gap nerve injuries. Together the combination of these two FDA-approved products, i.e. AFS cells and nerve allograft, have the potential to promote accelerated nerve regeneration following large gap nerve repair. The DOD protocol includes studies to develop sub-atmospheric pressure techniques to improve the process of seeding nerve allografts with AFS and to use these seeded allografts to repair large gap sciatic nerve defects in both a rat and primate model.

However, AFS cell differentiation and tracking experiments decided in the proposed study were not included in the DOD funding request. Therefore, there is no funding provided in the DOD grant to quantify neurotrophic factor expression by the AFS cells or to monitor the temporal location and phenotype of the AFS cells that are seeded on the allograft. Therefore, the proposal submitted to the AFSH is an independent assessment of the fate of AFS cells in peripheral nerve regeneration that will compliment data in the DOD study without duplication of experimental protocols.

Work accomplished on the DOD-sponsored study has developed a strategy for seeding the acellular nerve graft (ANA; AxoGen; Alachua, FL). A subatmospheric pressure seeding device developed in our laboratory has been used to seed $1x10^6$ 3T3 fibroblasts onto the nerve graft. Figure 1 demonstrates the penetration of these cells toward the center of the allograft 36 hour after seeding.

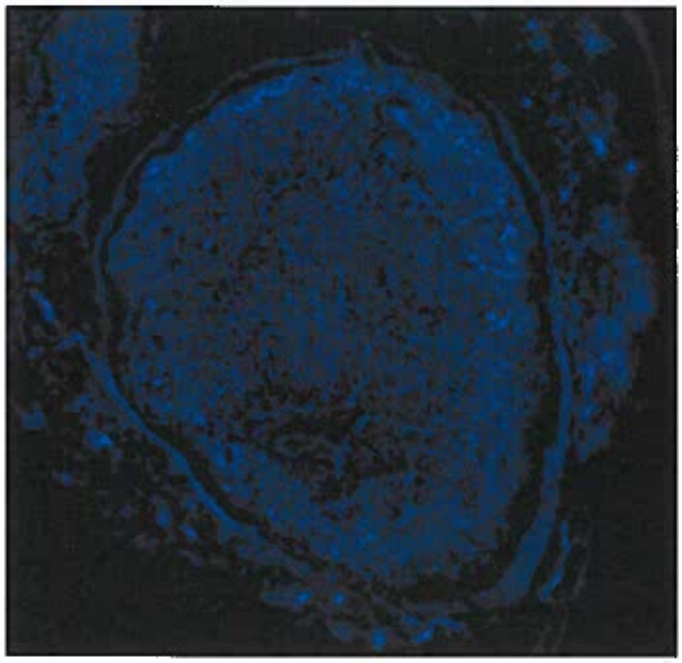


Figure 1. 3T3 fibroblasts seeded into a decellularized human nerve allograft. Cell nuclei are stained with DAPI and appear bright blue. (X200)

D. Research Design and Methods

Overview: Given the descriptive nature of this preliminary study, it is not possible to perform accurate power analysis; however, preliminary data from this study will be used to power future studies. In order to improve the translatable potential of our study, the nerve scaffold to be used is devitalized, acellular rat nerve allograft, processed by AxoGen Corp, FL (analogous to commercially available acellular nerve allograft).(8) The AFS cell source will be provided by NuTech, ™ (Birmingham, AL); these cells will be used for seeding in their "off the shelf" undifferentiated form.

Specific Aim 1: Quantification expression of neurotrophic factors produced by labeled AFS cells in vitro.

Cell Labeling: Commercially available AFS cells (Nutech™) will be labeled using MPIOs (Bangs Laboratories, Fishers, IN) containing magnetite cores encapsulated with styrene/divinyl benzene and coated with dragon green fluorescent dyes (wavelength, 480-nm excitation, 520-nm emission)[3], at a ratio of 1.5 million AFS cells to 20 µL of 3 x 10^8 MPIOs for 24hrs. After this process, the cells will be visualized by fluorescence microscopy to confirm the presence of MPIOs in the hAFS cells. The use of MPIOs has advantages over other tracking systems because the signal can be sustained in the cells for up to four weeks. In contrast, other chemical markers are limited to short term observations and also may affect the phenotype and viability of transplanted cells.(7)

Cell proliferation assay: Following labeling, 8 x 10^3 cells will be plated per 24-well plate, trypsinized and counted daily for 5 days; unlabeled AFS cells will serve as the control.

Cell differentiation and viability assay: Cells will be cultured in neurogenic induction media (contains platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), forskolin and neuregulin-β1 (Sigma-Aldrich, St Louis, Mo)for 2 weeks and the morphology changes over time will be recorded. Cell viability at 1, 3, 5, and 14 days of differentiation will be evaluated using CCK-8 assay (Sigma-Aldrich, St. Louis, MO). The cells will be collected at the end of 2 weeks for real-time PCR analysis to evaluate the expression of early neurogenic differentiation markers (Glial Fibrillary Acidic Protein, p75 neurotrophin receptor, netrin-1;Life Technology, Carlsbad, CA).

Quantification of neurotrophic factors: The labeled cells will be cultured in 150mm diameter dishes until reaching 75% confluence. The medium will be removed, and the cells washed with FBS; serum free – MEM will be used for an additional 3 days. The conditioned media will be concentrated by lyophilization and neurogenic growth factor analysis will be analyzed using Quantibody Human Growth Factor Array (Ray-Biotech, GA).

Specific Aim 2: Monitor temporal location and phenotype of AFS cells seeded on a nerve allograft for peripheral nerve regeneration in vivo.

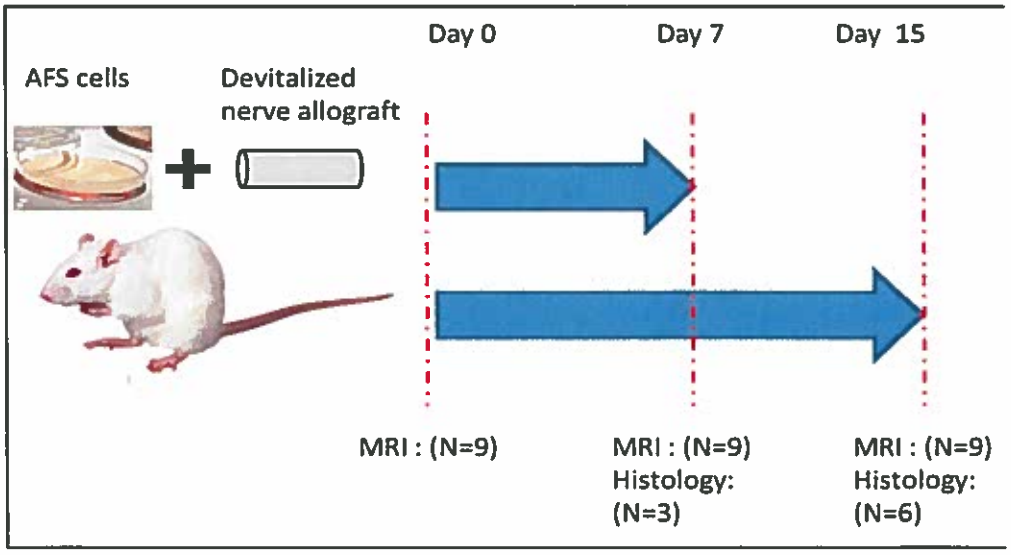


Figure 2. In-vivo experimental overview. All rats will undergo MRI in the first 24 hrs. Subsequent scanning will take place at 7 and 15 days with histological analysis at Dav 7 in three rats

Nine Lewis rats will be used in this preliminary descriptive study. The study has two time-points: 7 days and 15 days. The healing response of the nerve is expected to be 1mm per day. Therefore, these time-points will account for critical stages of healing across the defect site while remaining inside the window of the period including cell labeling. At Day 7 and Day 15, three rats will be euthanized; harvested tissue will be used for histology after MR imaging.

Surgical Procedure: Labeled cells will be seeded on the nerve allograft. The nerve allografts will be processed by AxoGen using donor sciatic nerves harvested from rats in our lab. Our laboratory has a material transfer agreement with AxoGen Corporation to provide nerve allograft for the proposed studies. These seeded allografts will be used for nerve repair. Following exposure of the sciatic nerve, a 1.5cm defect will be created. The seeded allograft will be sutured in continuity with the sciatic nerve using three radially placed 11-0 sutures (Johnson & Johnson). The hamstrings will be closed using 6-0 vicryl, with skin staples for skin closure.

MRI: Image acquisitions of the nerve graft will be taken on Day 1 for all animals (N=12). Subsequent imaging will take place at 1 week (N=9) and 2 weeks (N=6). Briefly, a 7.0T horizontal magnet small animal scanner (Bruker Biospin Inc, MA) with an actively-shielded gradient set capable of a maximum gradient of 400 mT/m will be used. A custom-made Litz volume coil with 25 mm ID (Doty Scientific, Inc, SC) will be used for both signal transmission and receiving. An ECG and respiration gated FLASH pulse sequence will be used for image acquisition with the following parameters: repetition time (TR) = 53.6 ms, echo time (TE) = 2.6 ms, flip angle (FA) = 30 degrees, number of excitations (NEX) = 4, matrix size = 256 × 192, slice thickness (thk) = 0.60 mm, and field of view (FOV) = 3.0 cm, giving an in-plane resolution of 117 × 156 μm.

The respiration and ECG of the rats will be monitored (SA Instruments Inc, Stoney Brook, NY) during scanning while they are anesthetized. Anesthesia will include induction using 3% isoflurane and oxygen at a flow rate of 3 L/min. Anesthesia will be maintained with a mixture of 1.5% isoflurane and oxygen at a flow rate of 1 L/min.

MRI analysis: Characterization of the size and intensity of the hypointense region, indicating the presence of the MPIO-labeled cells, will be achieved using contiguous MR images; these images will be analyzed individually using ImageJ (NIH). The total volume of the hypointense regions will be normalized at the first time-point in each animal. Briefly, the location and magnitude of the labeled cells at the repair site and the average intensity in the allograft will be divided by the average intensity of the adjoining nerve and soft tissues. This ratio will be recorded along with the area of the hypointense region. An average intensity for the entire volume will be calculated by multiplying the area of each slice's hypointense region by its average intensity, summing those products, and then dividing by the sum of the areas.

Histology: Contiguous frozen sections will be stained with DAPI and imaged by fluorescent microscopy in order to identify the MPIO-labeled AFS cells incorporated into the nerve graft. Sections also will be stained using Prussian blue and nuclear red counterstaining.

Immunohistochemistry: Co-localization of the transplanted cells will be confirmed using human specific nuclear matrix antibody (Anti-NuMA) analyzed by confocal microscopy. In contiguous 7 μm sections, transplanted AFS cells will be stained with antibodies that reflect neurogenic and Schwann cell

differentiation. Specifically, NT-3 (neurotropin-3), CNTF (ciliary neurotrophic factor), GFAP (glial fibrillary acid protein) and S100β (Schwann cell marker) will be assessed.(9;11)

Real time PCR analysis: Transplanted AFS cells that have incorporated into the allograft identified by immunohistochemistry (Anti-NuMA positive) will be isolated. Neurogenic conversion of cells in vivo will be confirmed with real-time PCR using human primers for neurogenic lineage markers. (Glial Fibrillary Acidic Protein, p75 neurotrophin receptor, netrin-1; Life Technology, Carlsbad, CA). To investigate whether transplanted AFS cells induce a host neuroregeneration, real-time PCR will be also performed using rat neurogenic-related primers. (18)

E. IACUC Approval

The laboratory animal care program of Wake Forest University Health Sciences (PHS Assurance #A3391-01) has been continuously accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) since April 8, 1966 (File # 8), and is a registered research facility (55-R-0001) in good standing with the USDA. External review of compliance with relevant laws, policies, and guidelines occurs during triennial site visits by AAALAC representatives, and periodic unannounced inspections by USDA veterinary medical officers. Internal compliance is conducted by a strongly proactive Institutional Animal Care and Use Committee. Animal housing and support space totaling 74,521 sq.ft. includes 16 housing buildings for nonhuman primates, 24 fly pens for pigeons, facilities for housing rodents, a barn and pasture for housing sheep/goats, a cage washing facility, offices, and other support areas.

The IACUC protocol for the proposed study is pending.

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G. Letters of Support

Shay Soker, PhD

L. Andrew Koman, MD

2. Scientific research grant from NuTech. Inc

Effect of Amniotic Membrane and Amniotic fluid Stem Cells on Schwann cell Neurotrophic Cytokine Production

Rationale In the previous study we have shown that co-culturing both rat and human Schwann cells with amniotic membrane and amniotic fluid stem cells facilitated Schwann cell growth.(Figure 1) The next step would be investigate the effects of these amniotic tissue and stem cells on the neurotrophic growth factors. We hypothesize that amniotic membrane and amniotic fluid stem cells will accelerate the neurotrophic cytokine production of Schwann cells, thus facilitate the functional recovery of the nerve following injury.

Research Design and Methods

Cytokine Mutiplex Assay

1.Nushield and Affinity membranes are incubated with DMEM (1 ml per 1 cm2 graft) on a plate rocker or shaker at a low speed for 72 hours at 4C. NuCel is spun down the mixture at 300g for 5 minutes then aspirated off the supernatant and replaced with an equal volume of DMEM, then incubated for 72 hours at 4C on a shaker. The pre-conditioned medium of Nushield, Affinity and NuCel are collected.

2.5000 Human Schwann-like cells (HSCs) are plated to each well for each condition (3 technical replicates). Nushield, Affinity and Nucel conditioned media are used to culture the HSCs for 2 weeks at 50, 25 and 10% by volume mixed with DMEM. Pico green ds DNA assay is performed to determine the viable cell number at baseline, 3 days, 1 week and 2 weeks' time point.

3.11 conditions are tested. Cell supernatants are collected at 3 days, 1 week and 2 weeks.

HSC alone(positive ctrl)

DMEM alone (negative ctrl)

HSC+Nushield media 50,25 10% (3 conditions)

HSC+Affinity 50,25 10% (3 conditions)

HSC+Nucel 50, 25 10% (3 conditions)

4.ELISAs for β-NGF and MBP are performed with cell supernatant following manufacturer's instructions. With the option of later performing a multiples quantibody array.

Clinical Significance. We hypothesize that amniotic membrane and amniotic fluid stem cells will support and facilitate Schwann cell recruitment and regrowth at the nerve injury site by providing trophic paracrine effects that have the potential to improve the regeneration of peripheral nerve and nerve bed.

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