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14. ABSTRACT The research detailed in this final report is to study how to improve the rate of peripheral nerve regeneration. The slow rate of nerve regeneration in limbs results in poor prognosis for patients suffering from severe injuries, leading to muscle impairment, and in extreme cases, atrophy. For our research, we will study the rate at which nerves regenerate in a rat model. We will excise the peripheral nerve, and study how to modulate the nerve regeneration through the use of a decellularized nerve graft and stress altered cells (SACs), a cell type we have identified that show stem-cell like qualities after undergoing physical and chemical stresses.					
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1. Introduction:

This report summarizes the results of our funded studies (Award #W81XWH-13-1-0298) involving an innovative approach to improve the rate and quality of peripheral nerve regeneration. Prior methods have resulted in very slow nerve regeneration with variable degrees of success. This commonly results in pain and muscle impairment with possibly atrophy and loss of function at the treated site. We proposed to study the rate of nerve regeneration and quality of functional improvement using nerve grafts transplanted into a rat model, comparing implantation of decellularized nerve grafts alone, to the same grafts after being seeded with recently described stem cells, referred to as SAC cells, that can be generated upon exposure of mature fully differentiated somatic cells to varying sub lethal stresses. We proposed that these “Stress Altered” cells (SACs) mimicked cells recruited by the body in response to injury, and possessed the potential to mature into all of the cellular elements normally present in peripheral nerve.

2. KEYWORDS:

peripheral nerve repair, nerve injury, decellularized nerve allograft, neural regeneration, stem cells, stress altered cells, peripheral nerve injury model, nerve graft

3. OVERALL PROJECT SUMMARY:

This comprehensive final report summarizes the overall progress and accomplishments of the studies proposed and undertaken. The overall goals of our proposal were three specific aims, which were to be undertaken as nine separate tasks that aligned with our Statement of Work (SOW). Each aim has been restated below, and the accomplishments, challenges and modifications are presented. The progress as related to each task, is summarized and problems encountered as well as decisions to alter some specifics of the tasks to overcome these hurdles are described.

Overall summary: With some modifications, we were able to accomplish several facets of Aim 1, specifically tasks:

1a-d (Tissue Harvesting and Cell Culture) and 2 (Stress Alteration of Human foreskin fibroblasts, (HFFs rather than mice spleen

lymphocytes as originally proposed. This is explained in detail below). Task 2a – To induce mechanical stress alteration was accomplished (of human foreskin fibroblasts - HFFs) as well as Task 2c – To induce low pH stress alteration (of HFFs). We were unable to accomplish task 2b - Induce hypoxia stress alteration using either the originally proposed mouse spleen lymphocytes or the HFFs, for reasons explained below. Consequently, we were unable to study the effects of the combination of hypoxia with either mechanical stress 3a or acid stress 3c.

As a result of attempting to perform tasks 1, 2 and 3 using the originally planned CD45+ lymphocytes, and being unsuccessful with tasks 2 and 3, we decided to change the cell type studied from mouse derived lymphocytes to commercially purchased human foreskin fibroblasts as explained in more detail below.

Concerning Aim 1, we also accomplished Task 4 - Stress Alteration Analysis, of the HFFs.

We were unable to accomplish tasks 3a and 3c because the HFF cells that we ultimately elected to were found to be extremely resistant to any effects of hypoxia.

Tasks 4 a, and c were accomplished to limited degrees as discussed in more detail below. We elected to focus analysis of the stress treated cells on immunohistochemical analysis and quantitative PCR analysis, rather than FACCS analysis. By this time, we had acquired the ability to perform quantitative PCR analysis and felt that the results would be much more precise, with less likelihood of false positives and generally more accepted than either immunohistochemistry alone, or FACCS sorting. Consequently, we did proceed with immunohistochemical analysis as originally proposed, but altered our plan from FACCS analysis (4b) to the more précised quantitative PCR analysis.

After reviewing the results, (both positive and negative), as related

to tasks 1 - 4, we felt that the tasks accomplished in Aim 1, were more significant than those not accomplished, and therefore proceeded to Aim 2.

In regard to Aim 2, we were unable to achieve satisfactory seeding of the decellularized nerve grafts, consistently seeing insufficient penetration of the seeded cells into the body of the decellularized nerve grafts. We attempted several variations of seeding without substantial success. We even performed some subcutaneous implantation of seeded grafts, as had been planned in Aim 3, to see if the few cells that had not significantly invaded the parenchyma of the nerve grafts in vitro, would multiply and do so in the in vivo chemical environment in which the grafts were implanted.

Regardless of variations in seeding techniques and seeding density as well as implantation of various configurations were not able to identify any significant penetration, ingrowth and engraftment of the seeded, stress altered cells (SACs) in any of the decellularized nerve grafts studied. At that point, we elected to not proceed with the third phase of the study, as we had altered many variables related to seeding of the grafts with SACs, with no success, and the goals of the study as designed were then deemed to be unachievable.

Detailed review of the Specific Aims:

Specific Aim 1: To explore the most effective/efficient approach(es) to induce stress altered stem cells (SACs) by comparing the effects of different stresses and combinations of stresses, and evaluating stem cell yield and marker expression as a function of time.

Rationale: Our approach to generating stress altered pluripotent stem cells was based on what was at that time, a report accepted by NATURE, accepted to be published in January, 2014. Some of the work presented in the report had been performed in my lab in Boston, while much of the data was acquired in laboratories at the

RIKEN institute in Japan. Based on the data reported by my Japanese colleagues, for this proposed research study, we proposed to focus our initial efforts to effectively generate the SACs by exposing CD45+ lymphocytes procured from neonatal mice spleens, to 3 different stresses as described in the proposal.

Negative results: As a result of several successful isolations per week, 2X/month, for 6 months, were we indeed able to procure large quantities of CD45+ neonatal murine lymphocytes as outlined for in Task 1 (successful completion of task 1a-d). Unfortunately these somatic cells did not respond to mechanical stress and low pH in the manner that we anticipated and had been described in the NATURE report. After more than 50 stress exposures to either low pH or mechanical stresses described, we experienced virtually no success in generating SACs cells as described in the Nature report. Analysis of the treated cells exhibited no evidence of expression of the generally accepted embryonic stem cell markers Oct4, nanog or sox-2 via either immunohistochemical analysis or quantitative PCR analysis. It is important to note that by this time, several months after publication of the NATURE report, several other independent labs and investigators also reported having minimal success in replicating the results as reported in Nature using CD45+ lymphocytes.

Modification of the approach: Since my lab in Boston had experienced positive results in generating SAC cells upon stress exposure of human foreskin fibroblasts (HFF cells), and such cells were readily available as a commercial product, we elected to refocus our efforts on exposing mature somatic human foreskin fibroblasts (HFF cells) to the stresses described. Consequently, beginning in month 3, we studied the potential of mechanical trituration of the cells (as a mechanical stress), hypoxia and exposure to an acidic solution composed of [0.2mM] ATP, having a pH of 5-6, to create SACs. Since we were now planning to study

human cells, we planned to change the graft recipient model from rats, to nude (athymic) rats, in order to be able to implant human cells that would not be rejected. We also felt that using human cells as a mature somatic source would move us more quickly toward human application, if successful.

Since it has been reported that HFF cells have a potential to be contaminated with very small amounts of stem or progenitor cells, we first undertook negative sorting of the purchased HFFs for the stem cell marker SSEA-1, that is occasionally expressed by such cells.

Results:

A. Negative sorting for SSEA-1. We found that there were virtually no cells contained within our purchased HFF cells that expressed stem cell marker SSEA-1. That is, the HFF cells that we acquired appeared to be a reasonably pure population of mature somatic cells with virtually no contamination of stem cells, and would be suitable for the studies proposed.

B. After several attempts, to expose the cells to hypoxia we could find no indication of cell injury or death of HFF cells after several weeks of exposure. We then increased the degree of hypoxia; that is, we further reduced the oxygen content by exposing the HFF cells to an absolute anoxic environment (zero oxygen) by placing the cells inside of an anoxia chamber that was equipped with a catalytic converter to remove any water and thus any potential for oxygen contamination. We found that the HFF cells survived very well with no indication of injury after more than a week in this environment. We concluded that HFFs, were extremely resistant to hypoxia. Consequently, hypoxia would not be an efficient stress for studying the generation of SACs.

Next modification: upon concluding that hypoxia would not be a significant stress for altering human foreskin fibroblasts (HFFs),

we then focused our efforts on stressing the HFF cells with an acidic ATP solution alone or in combination with mechanical trituration, or with mechanical trituration alone, as described in tasks (task 2a,c and task 3b).

Results: We found that employment of mechanical trituration alone was more effective than only exposure of the mature cells to the acidic solution described. The most effective mechanical trituration for generating SA Cells was via pipetting through a fire polished pipet having an internal diameter of 500-600 microns for 25 minutes. Using this stress, we were able to generate spheres composed of cells that expressed high levels of the stem cell markers Oct4, Nanog and Sox2. Although we were able to do so consistently, we were not able to generate these cells after every stress treatment, being successful after only about 10 - 15% of our stress treatments. The presence of the stem cell markers was confirmed using both immunohistochemistry and real time quantitative PCR. When exposing cells to the acidic ATP solution alone, we were able to generate such spheres less than 5% of the time, the most effective range of pH for this solution being 5.1 – 5.5. When we exposed mature somatic cells to the combination of mechanical and acid stresses, we were more effective in generating SACs than with either stress alone, being successful in between 20 -25% of the treatments.

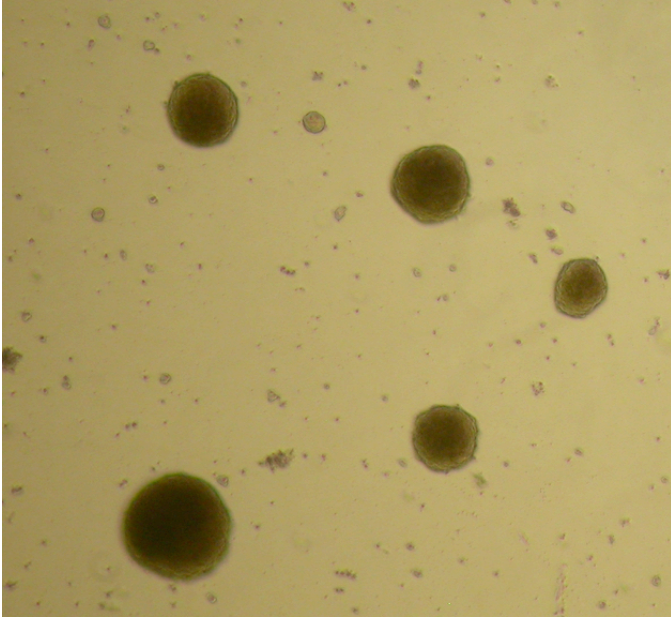


Figure 1: Spheres generated from the application of a combination of 2 stresses, acid exposure and mechanical trituration. The HFF cells were suspended at a cell concentration of 1 million cells/cc in a solution containing ATP titrated to a pH of 5.3 and then tritured for 25 minutes through a pipet with an internal diameter of 500 microns. By day 10, as evidenced by the use of quantitative PCR, the spheres were positive for the stem cell markers Oct-4, Nanog and Sox-2. The levels of OCT4 expression varied from 10 - 25x control levels. Expression of nanog was found to be from 50 to 300 x control levels, and expression of Sox2 was in between these two ranges.

After 11 days in vitro, cells growing as floating spheres and expressing the stem cell markers noted above were then maintained in a neural enhancing media for 5-7 days, at which time they expressed the neural stem cell marker, Nestin as evidenced by immunohistochemistry and quantitative PCR analysis. The level of nestin expression using PCR analysis was in the order of 20-50x control.

In an attempt to further demonstrate pluripotency, we undertook several in vivo implantations of spheres that had been grown for

10-14 days in vitro, into nude mice. It was our expectation that these spheres would develop into teratomas within 8-12 weeks after implantation.

Results: After more than 100 implants of what we believed to be pluripotent spheres, we were unable to identify any evidence of teratoma formation.

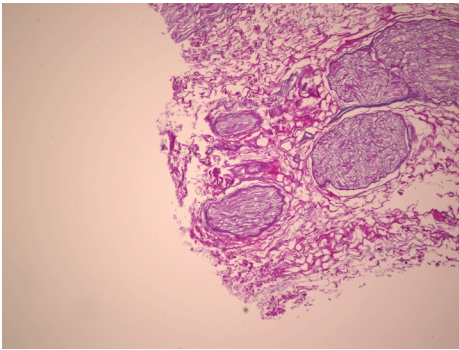


Figure 2: This image shows the H&E stain of a tissue like mass on the dorsum of a nude mouse after subcutaneously implanting an a mesh of PGA fibers seeded with STAP cells. In neither this, nor any of the H&E sections were we able to detect the presence of any tissue containing cells representative of the three germ layers (endoderm, mesoderm, or ectoderm).

Rationale for proceeding: While the ability to form teratomas is considered by some to be a hallmark of pluripotency, the PI is in disagreement with this belief. I believe that teratoma formation is an unnatural phenomenon, and would be a particularly bad characteristic to exhibit. I believe that it is rather the hallmark of an uncontrolled biologic process. I believe that the creation of teratomas by pluripotent cells is representative of a process that has gone awry. In a controlled environment, pluripotent cells should indeed be able to generate any of the three germ layers, but in a directed fashion, as a result of the chemical environment into

which they are delivered. Consequently, I viewed this as a positive characterisitc, rather than a negative finding, if indeed we still were able to cause the mesodermally derived HFF cells to dedifferentiate and express markers normally associated with stem cells, and then mature into the cellular components of mature nerves.

Results: Spheres generated from mature HFF cells after exposure to the stresses described, were then exposed to a neuro differentiating media between days 4-11. These spheres were shown to express the neural stem cell marker Nestin in amounts of up to 30x the controls, using Quantitative PCR. Consequently, we concluded that we had sufficient evidence that we could indeed generate stem cells from mature mesodermally derived somatic cells, and that these cells could then be driven toward a neural lineage, and that we should proceed to the next phase of our proposed studies. We also found that the cells contained within the speres studied did not possess the ability to generate what we felt to be an abnormal tissue; that is, teratomas.

Overall conclusion: Some of the components of specific aim 1 were met, while others were not. We felt that we had generated sufficient evidence in the studies undertaken in phase I to conclude that the concept was valid, and that the potential to achieve the desired outcome still existed in spite of any negative data. Consequently, we elected to proceed with the next phase of the proposed studies to enhance peripheral nerve repair.

1. We found hypoxia to be an impractical stress to convert HFF cells to stemness.
2. We found that we could consistently generate spheres that expressed generally the accepted stem cell markers, Oct -4,

nanog and sox2.

3. We found that such spheres containing stem cells would express the neural stem cell marker, nestin after being incubated in an environment that favored neural tissue development.
4. We found that when implanted, these spheres did not generate teratomas, but concluded that this was unnecessary, and even positive if indeed they could be driven to neural tissue without the uncontrolled formation of teratomas.

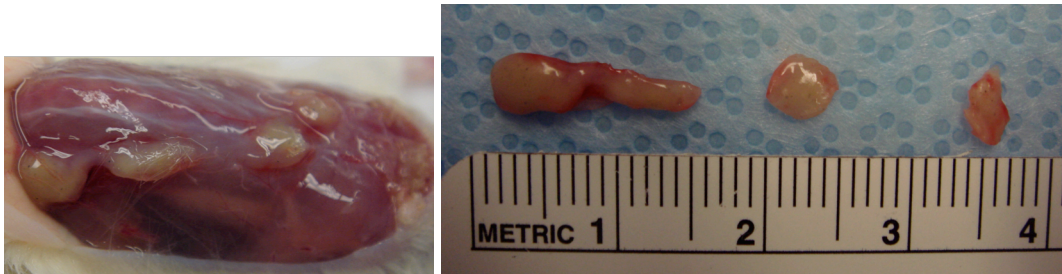
Specific Aim 2: Develop methods for optimized the seeding of stress altered stem cells (SACs) and mesenchymal stem cells into decellularized nerve grafts ex vivo.

Specific Aim 3: Assess the rate and degree of functional nerve repair of decellularized scaffolds seeded with experimental stem cells in a long graft rat sciatic model compared to controls.

The media in which the SACs were being maintained was a 1:1 Dulbecco's Modified Eagle Medium:F12 media with vitamin B27. As noted above, the media was changed to a neural induction media in order to provide environment to drive the SACs to differentiate into neural cells. We cultured the SACs in this neural induction media for an additional 5-7 days. Immunocytochemistry and qPCR analysis of the collected SACS kept in vitro in this manner demonstrated the presence of nestin. The SACs were plated on ornithine-coated chamber slides in neural induction media. Media were exchanged every 3 days. After 7 days, the cells were fixed on 4 % paraformaldehyde for 30 minutes. We tested for expression of the neural makers Nestin, SOX1, SOX2, and PAX6.

While many of the samples were positive for expression of nestin as described above, none of the samples expressed the markers SOX1, SOX2, or PAX6.

In addition to immunohistochemistry and qPCR analysis, we conducted a teratoma analysis, as it is commonly believed that teratoma formation is an additional hallmark of pluripotency (the formation of the three germ layers: endoderm, mesoderm, and ectoderm). We seeded Day 11 SACs on PGA fibers (biodegradable fiber) and cultured them for 5 days until the SACs formed a matrix within PGA fiber. The fibers were then implanted subcutaneously in nude mice for 12 weeks.



In addition, the Day 11 SACs were seeded on decellularized scaffolds (provided by AxoGen) and implanted them subcutaneously in nude mice for 12 weeks.

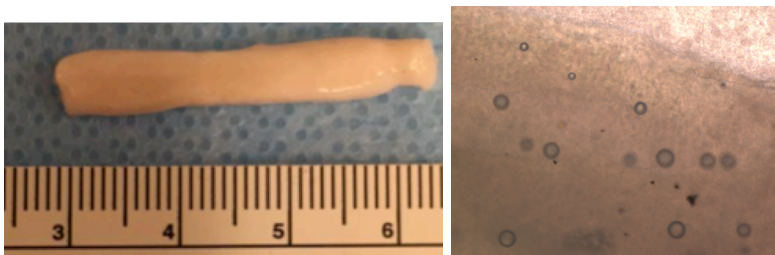


Figure 3: The left image above shows the decellularized scaffold before seeding with SACs. The right image above shows the decellularized scaffold after seeding with SACs. It is important to note that the SACs had difficulty attaching to the nerve grafts. As a result, we decided to inject the grafts with SACs instead of seeding

them on the surface.

There was some tissue formation observed on the decellularized scaffolds after 12 weeks. After harvesting these scaffolds and performing H&E staining on them, it was found that the seeded scaffolds did not produce a nerve graft.

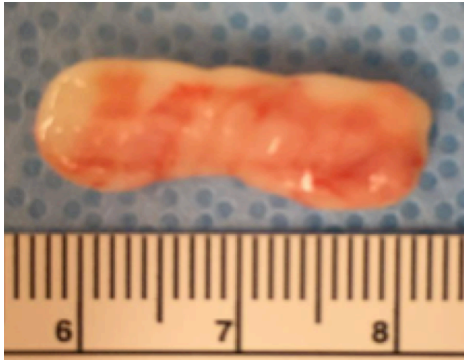


Figure 4: The image above shows the graft after removal from nude mouse.

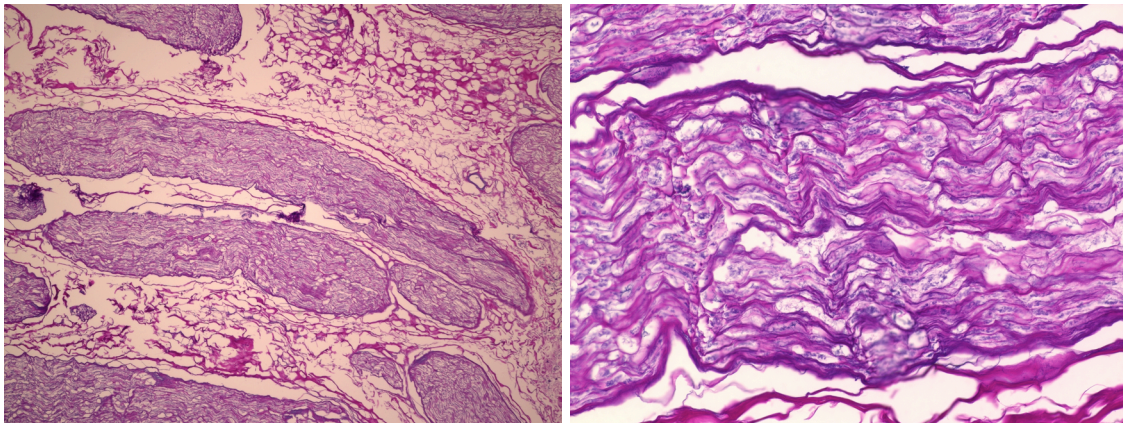


Figure 5: The images above show the H&E stains of the SAC seeded nerve graft after subcutaneous implantation for 12 weeks in the nude mouse. Again, no peripheral nerve regeneration was detected.

4. KEY RESEARCH ACCOMPLISHMENTS:

Although we were not successful in producing viable nerve grafts for peripheral nerve regeneration, a standardized method of producing stress-altered cells was established. This method combined the use of mechanical trituration as a stress in combination to exposure of the cells to an acidic ATP solution described above, and consistently resulted in the generation of SACs (consistently, but not always). While we were unable to effectively seed the decellularized nerve grafts with the SACS that were generated, we attributed this to the very dense nature of the decellulrized grafts, rather than an inherent problem with the concept. It is still our belief that the use of an appropriately configured synthetic graft material that is saturated with a decellularized, pulverized nerve matrix will result in the outcome that we had originally hoped for.

5. CONCLUSION:

We were unable to generate peripheral nerves using SACs. The procedure of forming SACs was standardized. However, effectively using these SACs to seed onto the PGA fiber and nerve grafts to produce teratomas and viable nerves, respectively, was

unable to be done. Our next project includes figuring out how to properly seed these SACs so that teratoma formation and nerve regeneration can be observed.