FINAL REPORT 06-2920-SCR-E-0

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BODY OF REPORT

**Summarize progress made during grant period:**

The overall goal of the proposal was to identify key supplementation factors to improve myelination of oligodendrocytes derived from murine embryonic stem cells. In this regard, the aim of the proposal was to a) develop a differentiation system to generate oligodendrocytes from embryonic stem cells b) perform a biochemical characterization of the cells during the differentiation process and c) identify key supplementation factors to promote myelin synthesis of murine embryonic stem cell derived oligodendrocytes.

During the grant period, we have developed a differentiation system to generate oligodendrocytes from embryonic stem cells. In this process, two differentiation systems were compared viz. directed differentiation and the traditional embryoid body system for embryonic stem cell differentiation. A comparison of the two techniques showed that embryoid body mediated differentiation in the presence of 0.1 μM retinoic acid (RA) showed higher A2B5 expression, an oligodendrocyte marker, as compared to the directed differentiation process on day 8 of differentiation. In addition, a biochemical analysis of the A2B5 positive sorted cell population showed higher lactate production as compared to the non-sorted population. The progress made based on the results obtained showed the generation of a homogenous population of oligodendrocytes derived from embryonic stem cells and a preliminary metabolic characterization of the sorted cells. This analysis is the first step towards further metabolic characterization of the sorted population and identification of biochemical supplementation factors to improve myelin synthesis of embryonic stem cell derived oligodendrocytes.

**Tasks addressed during the grant period:**

During the grant period, the tasks addressed included the development of a differentiation system to generate oligodendrocyte-like cells from embryonic stem cells. In addition, oligodendrocyte positive cells were sorted to generate homogenous populations from murine embryonic stem cells and a temporal profile of the differentiated cells as well as the oligodendrocyte positive sorted cells were carried out. In this work, lab personnel were trained and assigned specific tasks including embryonic stem cell culture and performing biochemical assays as requirement of the completion of a Senior Year Undergraduate Project in the Biomedical Engineering Department.

**Results, Data and Discussion:**

As proposed in the fellowship grant proposal, the first step was to identify a suitable differentiation system for generating oligodendrocyte-like cells from embryonic stem cells. In this regard, two differentiation systems were used viz. the hanging drop differentiation method that consisted of generating embryoid bodies from undifferentiated embryonic stem cells. Embryoid bodies were individually plated in each well of tissue-culture treated six well plates on day 4 of differentiation. Media was changed on day 8 of differentiation and cells were exposed to 0.1 μM retinoic acid until day 11 of differentiation. For the directed differentiation system, cells were cultured at a density of $10^5$ cells/well in polystyrene six well plates. Media was changed every two days and cells were

exposed to N2 supplement for 10 days. A2B5, a neural progenitor marker was used to identify oligodendrocyte-like cells derived using the two experimental systems.

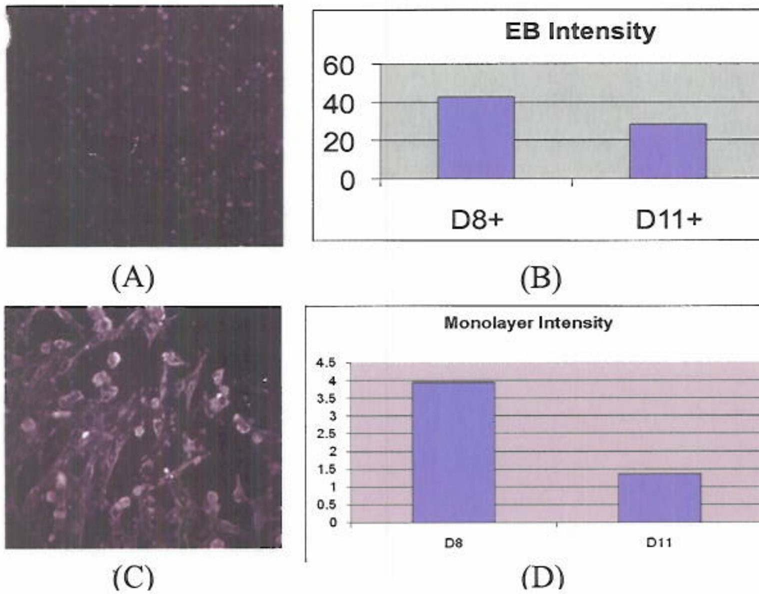



**Figure 1:** Neural progenitor cell marker analysis for the two differentiation systems. Immunostaining of differentiated cells on day 8 of differentiation for A2B5 marker for (A) Embryoid (Magnification 20X) and (B) Monolayer Culture of differentiated ES cells (Magnification 10X). A2B5 fluorescence quantification on day 8 and 11 of differentiation for (C) Embryoid Body Mediated Differentiated Cells (D) Monolayer Culture Configuration of day 8 and day 11 differentiated cells.

As shown in Figure 1(A) and 1(B), the intensity of A2B5 immunostaining was higher for RA treated embryoid body cultures on day 8 of differentiation as compared to monolayer culture configurations on same day of differentiation. As shown Figure 1(C), the RA treated embryoid cell cultures showed highest intensity on day 8 of differentiation with reduction in A2B5 intensity on day 11 of differentiation. For the monolayer cultures, the average intensity due to A2B5 showed a similar trend as in the embryoid body cultures. However, the fluorescence intensity was ~10 fold lower as compared to the embryoid body cultures. Also, the average intensity value was higher for the unsupplemented monolayer cultures as compared to N2 supplemented monolayer cultures (data not shown). In all cases, the immunostaining analysis was performed and compared to undifferentiated embryonic stem cells as negative controls.

Based on identification of day 8 RA treated embryoid body mediated differentiated cells as A2B5 positive cells representing an oligodendrocyte phenotype, a temporal analysis of the embryoid body mediated differentiation system was carried out and the results are shown in Figure 2.

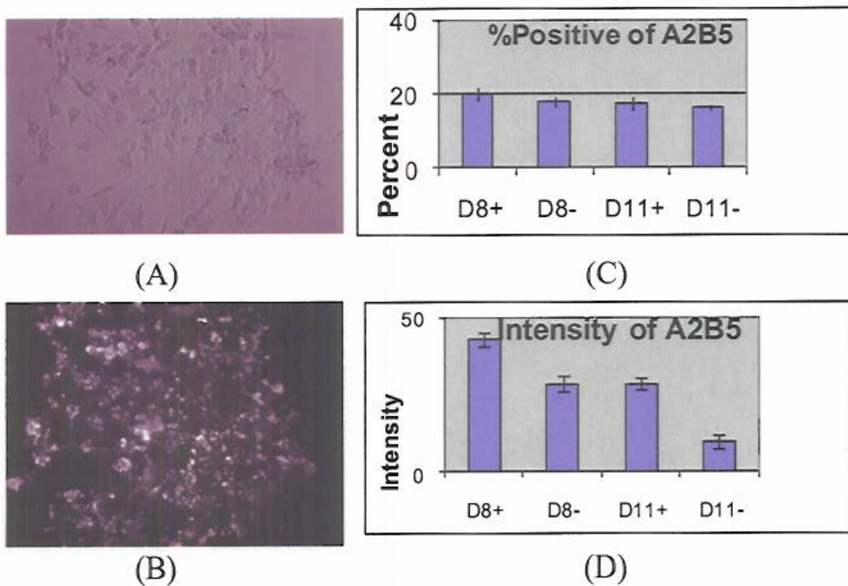
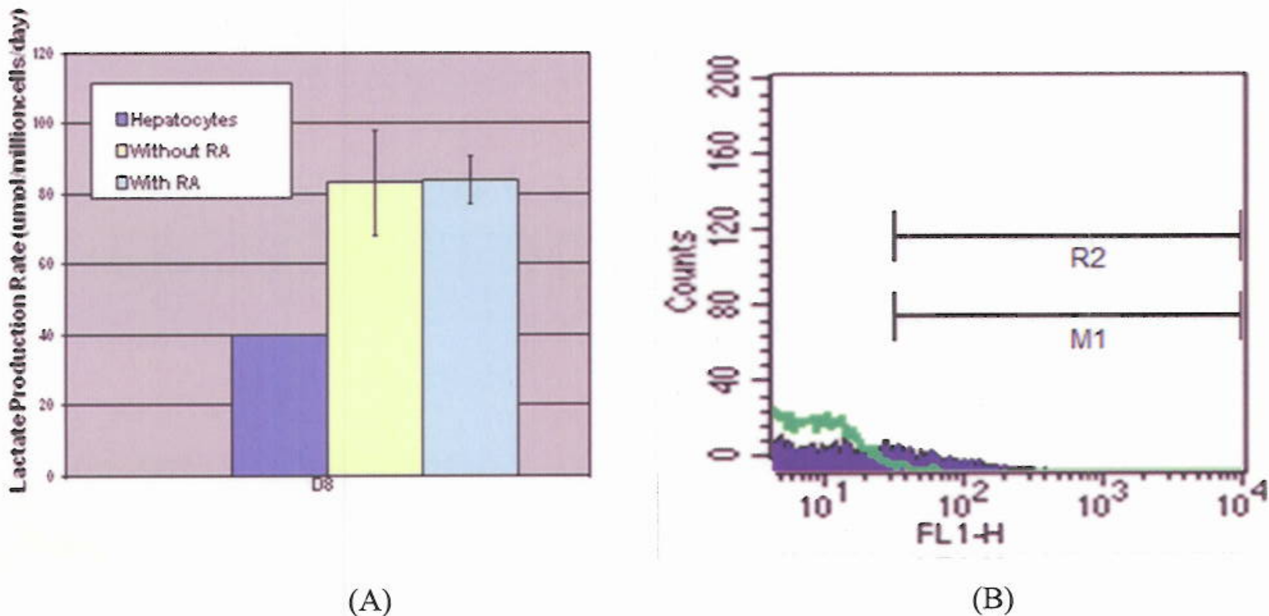



**Figure 2:** Temporal Analysis of A2B5 immunostaining for embryoid mediated differentiation system (A) Transmitted light image and (B) Immunofluorescence image of RA treated day 8 differentiated cells (Magnification 20X). (C) Percentage of A2B5 positive cells and (D) Immunofluorescence intensity of differentiated cells on days 8 and 11 of differentiation in the presence and absence of RA (viz. D8+ corresponds to day 8 RA supplemented cultures and D8- corresponds to day 8 unsupplemented cultures).

As shown in Figure 2(A), morphological analysis of day 8 embryoid mediated differentiated cells reveals an elongated phenotype consistent with oligodendrocyte-like cells. Figure 2(B) shows cell staining for A2B5 oligodendrocyte-specific marker. Figure 2(C) shows the percent of A2B5 positive cells in the differentiated population. As shown, the highest percentage of positive cells (~20 %) is for day 8 RA treated embryoid mediated differentiated cells and significantly higher than other experimental conditions. Figure 2(D) shows that the A2B5 immunofluorescent intensity for the best experimental condition is consistent with percent of cells positive for the specific marker. Thus, based on temporal profile of immunofluorescence intensity and analysis, day 8 RA treated embryoid body mediated differentiated cells is the best experimental condition for generating oligodendrocyte-like cells.




(A) (B)

**Figure 3:** Metabolic and functional analysis of differentiated ES cells. (A) Comparison of lactate production rates for ES cell derived hepatocyte-like cells, day 8 embryoid body mediated differentiation without and with RA. (B) Comparison of isotype (control) and sorted A2B5 positive cells using immunocytochemical analysis.

Figure 3(A) shows the metabolic and functional analysis for the differentiated cells. As shown, embryoid body based derived oligodendrocyte-like cells have higher lactate production rates as compared to hepatocyte-like cells implying a more glycolytic phenotype. Also, while the A2B5 fluorescence intensity is significantly higher for cells exposed to RA as compared to without RA, the lactate production rate is similar implying the need for myelin-specific functional marker assays to correlate function with comprehensive metabolite measurements including carbohydrates, amino acids and ammonia. Figure 3(B) shows that A2B5 positive cells have higher percent positive cells (blue area) as compared to the controls (green line) implying the successful isolation of neural progenitor populations. The non-overlapping region corresponds to the homogenous population that can be sorted and further analyzed from a biochemical and functional standpoint.

Overall, in this study, we have shown feasibility of generating a population of oligodendrocyte-like cells from embryonic stem cells. In addition, preliminary biochemical analysis of the population has been carried out as a first step to further investigate the relationship between metabolic measurements and oligodendrocyte-specific function to identify key biochemical supplementation factors that can augment oligodendrocyte myelin synthesis.

Finally, in order to emphasize the utility of metabolic intervention in inducing differentiation or increasing differentiated function in embryonic stem cell derived oligodendrocyte-like cells, we have previously demonstrated the application of a similar methodology to hepatic differentiation of embryonic stem cells.

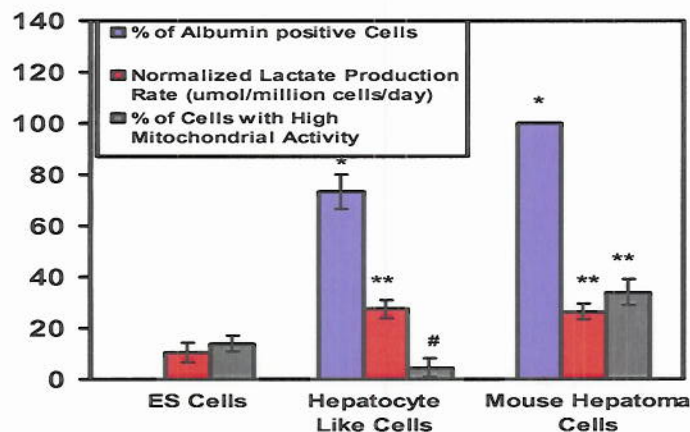
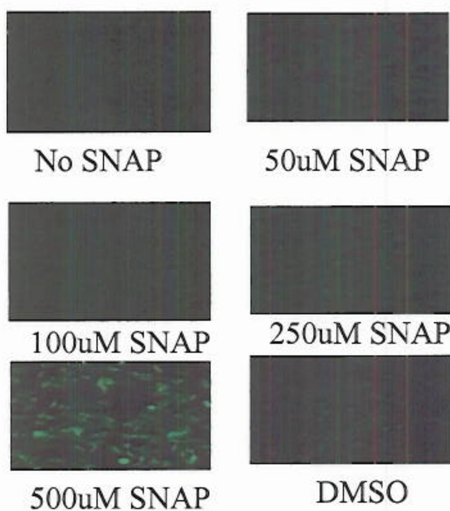


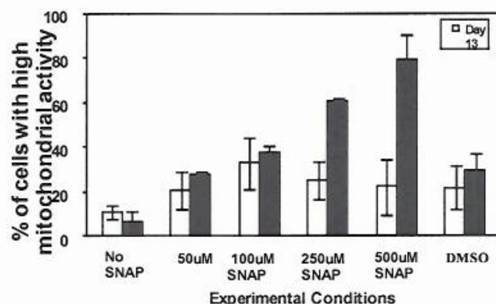

**Figure 4:** Comparison of functional, metabolic and mitochondrial capacity of undifferentiated ES cells, hepatocyte-like cells and mouse hepatoma cells.

As shown in Figure 4, functional and metabolic characterization of sodium butyrate mediated hepatocyte-like cells derived from embryonic stem cells has been performed previously in comparison to undifferentiated ES cells and mouse hepatoma cell populations. The results show that while hepatocyte-like cells have function (albumin secretion rate) and lactate production rate (indicative of glycolysis) comparable to mouse hepatoma cells, the mitochondrial activity is lower implying that further strategies have to be utilized to mediate increased differentiated function.

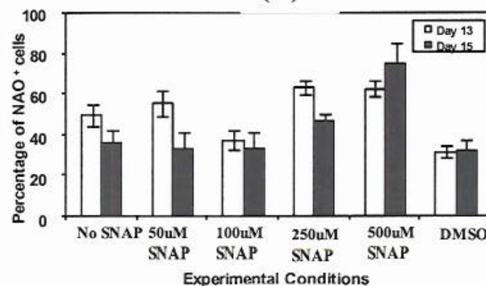
S-NitrosoAcetylPenicillamine (SNAP), a nitric oxide donor, has been shown to induce mitochondrial biogenesis, a phenomenon associated with mitochondrial proliferation and differentiation in various mammalian cell lines and liver developmental processes. As a result, we investigated the effect of different concentrations of SNAP on embryonic stem cell derived hepatic differentiation. As shown in Figure 5(A), 500 uM SNAP treatment results in highest percentage of albumin positive cells (~80%) as compared to other experimental conditions. In addition, hepatocyte specific functional augmentation is concomitant with increase in percentage of cells with high mitochondrial mass (indicated by NAO dye stain) and activity implying that metabolic regulation in differentiation augments differentiated function.




(A)




(B)




(C)

**Figure 5:** Functional and mitochondrial characterization of SNAP treated cells. (A) Intracellular albumin expression in cells exposed to different concentrations of SNAP (B) Percentage of NAO positive cells in SNAP treated populations (C) Percentage of cells with high mitochondrial activity in SNAP treated populations.

Thus, the feasibility of utilization of metabolic regulation in hepatic differentiation of embryonic stem cells can be extended to embryonic stem cell differentiation into oligodendrocytes to improve myelin synthesis.

Overall, the process of experimental design, conducting the experiments, data generation and results were performed in close consultation with the principal investigator with weekly meetings with Prof. Rene Schloss as the supervisor.

Future work in the proposed research include development of a comprehensive metabolic profile of the differentiation system that will provide insights into correlations between metabolite measurements viz. carbohydrates, lactate, amino acids, ammonia and oligodendrocyte-specific differentiated function. In addition, utilization of a panel of oligodendrocyte-specific markers including myelin protein synthesis will be a useful benchmark for future studies to improve myelination for identification of biochemical supplementation factors.

**Publications:**

We have attached a publication (currently under review) that supports the role of metabolic regulation in embryonic stem cell derived hepatocyte-like cells. This work is the basis for applying metabolic regulation in embryonic stem cell to oligodendrocyte-like cell differentiation.