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Final Report

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A. Specific aims, summarized from the original proposal

The main hypothesis of this project is that Neural Stem Cell (NSC) differentiation is regulated by specific sets of mRNAs which in turn are modulated post-transcriptionally by unique groups of microRNAs. This fundamental idea led us to propose two specific aims (1) identify the specific mRNAs required for neural stem cell (NSC) differentiation and (2) determine mechanisms by which specific mRNAs are modulated by microRNAs during NSC differentiation. I will briefly discuss the accomplishments of this project and the future direction we will pursue.

Aim 1.

Specific mRNAs are required for neural stem cell differentiation. We will identify relative expression levels of mRNAs during differentiation of two different clones derived from E13.5 rat cortices which have either neurogenic (cell clone L2.2) or multipotential (L2.3) phenotypes upon differentiation. Microarray studies will identify mRNAs associated with cell fate decision. These genes will be tested by siRNA knockdown assays in cell culture to determine their ability to control NSC differentiation for potential therapeutic purposes. siRNA knockdown assays will modulate specific genes altering the developmental outcome of the cell, by hindering differentiation or skewing it towards a specific phenotype. Finally, we will compare differentiation of NSCs in culture with those transplanted into spinal cord or brain to determine host tissue effects on microRNA regulation.

Aim 2.

Specific mRNAs are modulated by specific microRNAs during NSC differentiation. We believe that a subset of the genes regulating neural stem cell differentiation will be specific targets of the microRNAs involved in NSC differentiation. The matching of microRNA and mRNA will be approached by two distinct perspectives, a bioinformatic and a molecular approach. By matching the lists of differentially expressed mRNA and microRNA, we will elucidate a series of computationally predicted genes that are regulated by a specific group of microRNAs. Predicted microRNA/mRNA interactions will be tested in culture, confirming microRNA targets which regulate NSC differentiation.

B. Overall summary of progress made during the contract period

Aim. 1

Regarding Specific Aim 1 we have completed profiling mRNA and microRNA expression patterns during differentiation of the neurogenic and multipotential NSC clones by microarray analysis. Triplicate cultures were prepared from a neurogenic L2.2 NSC clone and a multipotential L2.3 NSC clone prior to (0 days) or 1 or 3 days following bFGF withdrawal. Low molecular weight fractions of RNA were prepared and assayed on the NCode microRNA microarray (Invitrogen) by Dr. L. Goff. We also prepared high molecular weight fractions from the same samples, labeled them by incorporation of biotinylated nucleotide into a cDNA reaction, and hybridized them to the Applied Biosystems 1700 rat genome survey microarrays. In collaboration with Rebecka Jörnsten, from the Statistics Department at Rutgers, data from both sets of arrays were quantile normalized and filtered by ANOVA at 5% FDR (mRNA) or 10% FDR (microRNA), yielding 1,337 significantly regulated mRNAs and 45 regulated microRNAs.

Validation by qPCR has been completed on a selected set of differentially expressed transcription factors, including members of the basic Helix-Loop-Helix (bHLH) family, and several of the microRNAs. For example, Neurogenin2 and Pax6 are both significantly regulated upon differentiation. These results correlate well with the expression patterns seen on our

microarray analysis. These results give us confidence that the exploratory lists of gene expression changes detected on our microarrays are reasonably accurate.

We hypothesized that siRNA knockdown assays of specific transcription factors will modulate specific genes altering the developmental outcome of the cell, by hindering differentiation or skewing it towards a specific phenotype. I will expand later in this report on the work pertaining to this second portion of the first aim and initially focus on the second aim. This way I can explain why we chose the genes that we have begun to interrogate.

Aim. 2

It is well documented that microRNAs exhibit temporal and tissue specific expression patterns, and have been implicated in developmental roles, including adipocyte, hematopoetic and neuronal differentiation (Brennecke, Hipfner et al. 2003; Krichevsky, King et al. 2003; Kuwabara, Hsieh et al. 2004; Sempere, Freemantle et al. 2004; Krichevsky, Sonntag et al. 2006). We hypothesized that the expression of specific combinations of microRNAs determines the final phenotypic state upon differentiation. We were particularly interested in identifying specific groups of microRNAs involved in neural differentiation with the hope that if we were to exogenously express these clusters of microRNAs we would be able to direct NSC differentiation towards determined neural phenotypes.

We have cross-correlated the expression patterns of a subset of transcription factor mRNAs and microRNAs from the list of significantly expressed genes (Fig.1). Focusing on this specific subset allows us to interpret predicted microRNA-mRNA mechanisms in the context of NSC differentiation. For example, we hypothesize that several of the negatively correlated microRNA-mRNA combinations, seen as red on the heatmap, would be indicative of mRNA degradation by a specific microRNA via the RISC complex. Furthermore, when looking at positively correlated transcription factor

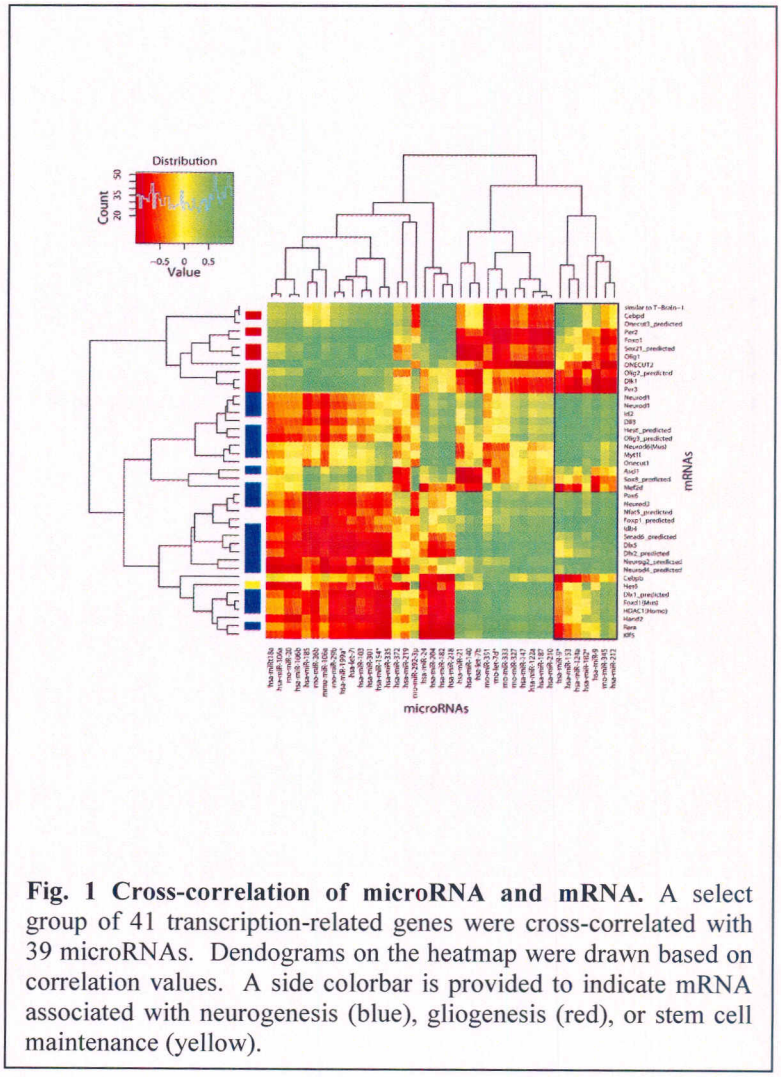

Fig. 1 Cross-correlation of microRNA and mRNA. A select group of 41 transcription-related genes were cross-correlated with 39 microRNAs. Dendograms on the heatmap were drawn based on correlation values. A side colorbar is provided to indicate mRNA associated with neurogenesis (blue), gliogenesis (red), or stem cell maintenance (yellow).

mRNA and microRNA groups, green on the heatmap, it would be possible to identify potential transcriptional regulatory networks working upon specific microRNA subgroups. The side colorbar in Fig.1 indicates mRNAs that are associated with neurogenesis (blue), gliogenesis (red), or stem cell maintenance (yellow). Interestingly, the highest-level cluster separation adequately distinguishes between neurogenic and gliogenic mRNA. Furthermore, the close proximity of miR-9 and miR-124a, both well known neural microRNAs, along with a strong positive correlation to known neurogenic transcription factors, provides additional evidence that these microRNAs, along with other members of this cluster, are expressed during neuronal specification, and are required for acquisition of the neuronal phenotype.

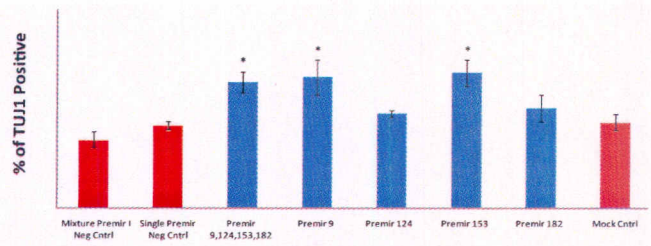

Fig.2 Exogenous expression of a neural cluster of microRNAs increases neuronal differentiation. Individual and a mix of PreMirs, microRNA mimics (Ambion), were nucleofected into L2.3 NSCs. Cells were assayed for TuJ1 expression by FACS. (n=3, *p<0.05)

Combined nucleofection of microRNA mimics of mir-9, mir-124, mir-153 and mir-182 into the L2.3 multipotential precursor cells increased the percentage of TuJ1+ cells produced upon bFGF withdrawal from 23.4±1.96% to 39.2±4.01%, ($p<0.033$, $n=3$), as assayed using flow cytometry (Fig.2). This represents a 40% increase in neurons. Individual nucleofection of these microRNAs showed that mir-9 and mir-153 was sufficient to cause an increase in $TuJ1^+$ cells. This suggests that these two microRNAs probably exert their activity on the same mRNAs during neurogenesis or at least similar genes from the same pathways.

We were interested in identifying which mRNAs are being regulated by these pro-neuronal microRNAs during neurogenesis and study these genes to complete specific aim 1. To interrogate this question we began to dissect the potential mRNA-microRNA interactions that were predicted from the cross-correlation matrix in Fig.1. One of the many potential interactions the matrix proposed was between mir-9 and several members of the Onecut family.

Onecut family members are a series of transcription factors that contain a cut and a homeobox domain(Hong, Kim et al. 2002). These transcription factors are associated primarily with endodermal development, specifically cell differentiation in liver and pancreas(Jacquemin, Lemaigre et al. 2003; Briancon, Bailly et al. 2004; Hara, Shen et al. 2007; Matthews, Lorent et al. 2008). Currently little is known about their role in neural development, except for a few studies which indicate a temporal and spatial specificity during neural development in the CNS (Jacquemin, Pierreux et al. 2003; Poustka, Kuhn et al. 2004). Onecut family members have been shown to regulate ngn3 and FoxA2, both genes have been associated with glial differentiation, specifically oligodendrocite specification (Jacquemin, Durviaux et al. 2000; Liu, Wu et al. 2002; Rausa, Tan et al. 2003; Norton, Mangoli et al. 2005).

In our model we observe a significant increase in the mRNA expression of all three family members in the multipotential clone L2.3, which produce a mixture of phenotypes upon bFGF withdrawal; but no difference in the neuro-restricted-precursor clone L2.2. The Onecut family members mRNA expression patterns anti-correlate with the expression of mir-9 in the L2.2 cell clone (NRPs Fig.3). This would suggest that mir-9 selectively targets Onecut family member 3'UTRs and regulates them in a negative manner during neuronal differentiation. In addition, Onecut2 has already been validated as a target of mir-9, (Plaisance, Abderrahmani et al. 2006), one of the pro-neuronal microRNAs. All the rest of the members of the Onecut family are predicted to be targeted by mir-9. These data would indicate that one possible factor regulating neuronal differentiation would be the inactivation of Onecut family members (glial factors) by an increased expression of mir-9 during neurogenesis.

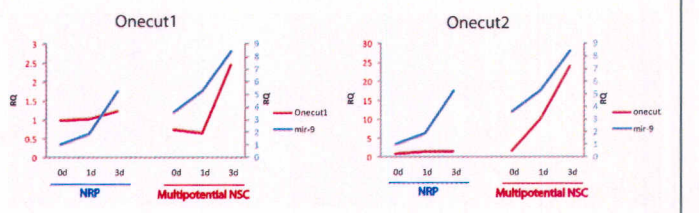

Fig.3 Onecut family mRNA expression relative to mir-9 expression during NSC differentiation. Onecut 1 and 2 mRNA and mir-9 expression after bFGF withdrawal from L2.2 and L2.3 cells was determined by qPCR. RQ values are plotted.

We have begun to interrogate the function of Onecut family members in glial differentiation and further assess their regulation by mir-9. Knock down of Onecut2 in the multipotential clone L2.3 lead to a significant increase in TuJ1$^+$ cells 3days post bFGF removal (Fig.4). Furthermore, at 5days post differentiation we see a trend that indicates a decrease in GFAP$^+$ cells. We have to repeat this assay in order to determine significance of this observation. This would support the hypothesis that the Onecut family, specifically Onecut2, is required in glial differentiation.

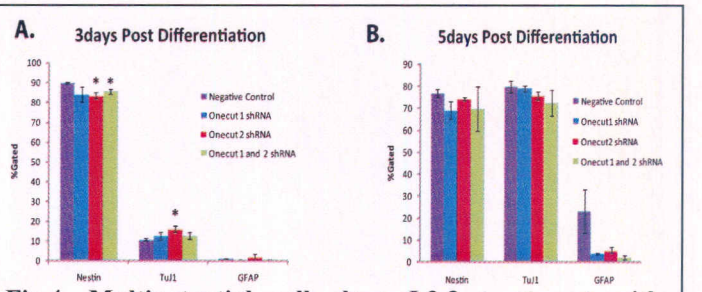

Fig.4. Multipotential cell clone L2.3 treatment with Onecut shRNAs. L2.3 cells were treated with shRNAs against Onecut family members and assessed for TuJ1$^+$, GFAP$^+$ and Nestin$^+$ cells by FACS.

A whole new set of questions arise from these data now that we know Onecut2 is involved in glial differentiation. For example, is Onecut2 involved in glial specification towards specific glial cell types, such as oligodendrocytes or Schwann cells? This is interesting to us because it entertains the notion that we could possibly direct glial differentiation by exogenously expressing Onecut2. The directed differentiation of NSCs to specific glial phenotypes, such as oligodendrocytes, would be very beneficial prior to transplantation therapies for conditions such as spinal cord injury or multiple sclerosis.

We have gathered data related to microRNA targeting of transcription factors and determined specific microRNAs that enhance NSC differentiation. We have also been able to predict specific microRNA:mRNA transcriptional networks necessary for determining NSC fate upon differentiation. These new networks have been dissected by siRNA knock downs of specific

genes associated with these pathways. Finally, we have begun to transplant NSCs into rat spinal cord and/or brain, to investigate in vivo effects upon these cells, in order to complete all aims proposed for this project.

We have proven that microRNA regulation during NSC differentiation affect transcription-factor-mediated cell mechanisms, which determines the cells final phenotypic fate. The microRNAs affecting these processes have great potential to be used for therapeutic purposes, specifically for SCI or other neuro-trauma related conditions. This work will allow for the harnessing of endogenous mechanisms to "program" stem cells prior to transplantation, in hope of increasing functional recovery in injured patients.

C. Problems encountered in this report period

The biggest setbacks during this project involved the microRNA mRNA target validations in HeLa. We began the target validation in HeLa cells but shortly realized that it was not the best system to answer our questions. To validate specific microRNA-mRNA interactions involving translational regulation, we originally constructed a set of microRNA expression plasmids by cloning 1Kb regions surrounding mature microRNA sequences from the rat genome into pSI mammalian expression vector (Promega), intending to recapitulate Drosha processing of the primary transcript. We later changed this approach and begun to use synthetic microRNA precursors called PreMirs (ABI, Foster City, Ca) instead. qPCR assays indicated higher levels of expression were achieved with the PreMirs vs. our initial microRNA expression plasmids (data not shown). We also constructed a series of reporter plasmids by cloning the 3'UTR of several selected transcription factors into a luciferase reporter plasmid (pMir-Report, Ambion) to detect effects of microRNAs on reporter luciferase activity. Combining individual microRNA expression plasmids with a 3'UTR reporter plasmid should have allowed us to interrogate predicted microRNA regulation of mRNA translation directly. When performing these assays we were confronted multiple times with complications such as un-interpretable and non-reproducible data. We believe the problem was that the 3'UTR of our cloned genes had multiple response elements that were being regulated by endogenous microRNAs in HeLa. Furthermore, we were interested in interactions that occur during neural differentiation, by performing these assays in HeLa cells we were not replicating the correct context that occurs in NSCs. We changed our approach and began to validate microRNA:mRNA interactions under neural differentiation in either L2.2 (neuronal) or L2.3 (multipotential) cell clones. Also, instead of cloning the full length 3'UTR of our genes of interest into the pMir Reporter Construct, we only cloned the response element to which the microRNA binds. This way we can specifically focus on a small cis-element to which a specific microRNA binds. These elements are also mutated to demonstrate reversal of inhibition and sequence specificity (data not shown).

Another problem was that it took us some time to optimize transfection efficiencies in the NSCs we were utilizing. We were originally obtaining a low transfection efficiency (30%) but we were able to optimize this by using the 96 well nucleofector system by Amaxa. We are currently obtaining ~80% efficiency. This was critical for all of the exogenous expression assays.

D. Changes in research plan

There were no major changes to the research plan since the last progress report. The only major change was to our time table. Some of the necessary optimizations slowed down our progress. We are currently working on the last series of experiments proposed in specific aim 2.

E. List of publications emerging from this research, including those in preparation

Goff LA, Davila J, Jörnsten R, Keles S, Hart RP. Bioinformatic Analysis of Neural Stem Cell Differentiation. J Biomol Tech. 2007 Sep;18(4):205-12.

Li H, Han Y, Bi C, Davila J, Goff LA, Thompson K, Swerdel M, Camarillo C, Ricupero CL, Hart RP, Plummer MR, Grumet M. Functional differentiation of a clone resembling embryonic cortical interneuron progenitors 2008 (accepted for publication in Developmental Neurobiology)

"Coordinated regulation of microRNAs and mRNAs during neural stem cell differentiation." In preparation.

"Targeting of bHLH transcription factors by microRNAs during neural stem cell differentiation." In preparation.

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Matthews, R. P., K. Lorent, et al. (2008). "Transcription factor onecut3 regulates intrahepatic biliary development in zebrafish." Dev Dyn 237(1): 124-31.

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Plaisance, V., A. Abderrahmani, et al. (2006). "MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells." J Biol Chem 281(37): 26932-42.

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Rausa, F. M., Y. Tan, et al. (2003). "Association between hepatocyte nuclear factor 6 (HNF-6) and FoxA2 DNA binding domains stimulates FoxA2 transcriptional activity but inhibits HNF-6 DNA binding." Mol Cell Biol 23(2): 437-49.

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