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Final Report

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1. Original aims of the project.

1. Identify miRNAs that are regulated upon differentiation in culture. We will focus on stem cell-derived isolates that are in the pathway leading to radial glial cells, with the intent of enhancing differentiation towards oligodendrocytes. We will identify miRNA changes associated with specific stages of differentiation, including embryonic source tissues, neurospheres, BLBP$^+$ radial glial-like cells (similar to RG3.6), and more differentiated progeny of these cells. We will compare these patterns to mature cell types collected from adult spinal cord.
2. Restrict differentiation of stem cells by antisense inhibition or overexpression of stem cell-specific miRNAs. We will use 2'-OMe-antisense RNA electroporated into stem cell cultures (Poy et al., 2004) to reverse translational inhibition by selected miRNAs. We will use double-stranded siRNA mimics of specific miRNAs to overexpress. Differentiation will be detected by immunocytochemical staining for cell type-specific markers. Finally, we will transplant "primed" cells into spinal cord and detect differentiation with immunohistochemistry.

2. Project successes.

As proposed, we exploited a set of rat neural stem cell (NSC) clones to investigate microRNA-modulated differentiation mechanisms. Briefly, early rat cortical cells (E14.5 forebrain) were enriched for radial glial phenotype in the presence of FGF2 and LIF for 2 d and then stabilized by infection with a v-myc-expressing retrovirus (20). One clone expressed the NSC marker nestin and the radial glial marker BLBP (FABP7) and could differentiate into a mixture of cells expressing markers for astrocytes, oligodendrocytes, and neurons (13). Another clone, L2.2, was more neurogenic, producing TuJ1$^+$ and Pax6$^+$ cells upon differentiation by withdrawal of bFGF ultimately producing GABA-ergic cells similar to interneurons, having electrical activity and molecular markers characteristic of cells derived from ganglionic median eminence (Li et al., submitted).

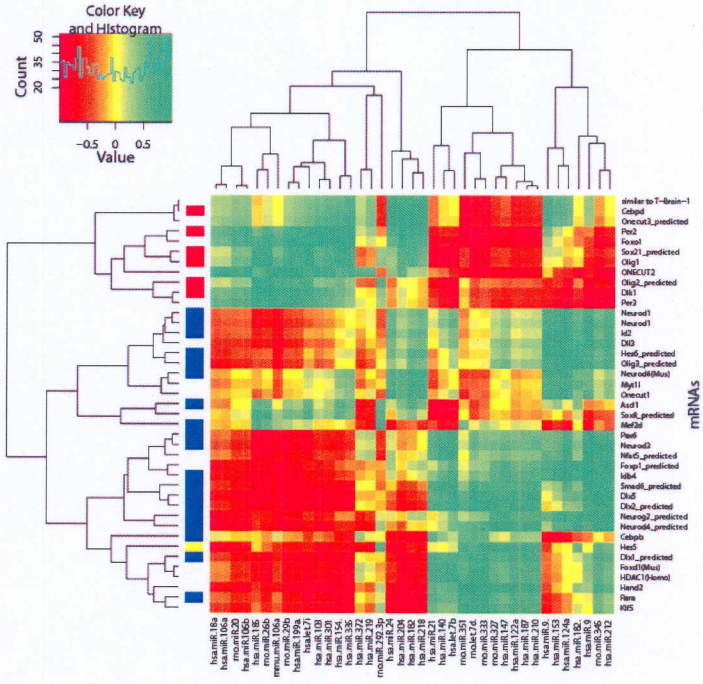

Fig. 1. Cross-correlation analysis of selected transcription factor mRNAs and significantly-regulated microRNAs during differentiation of neurogenic (L2.2) and multipotential (L2.3) cells. Selected mRNAs were labeled red or blue (left of the heatmap) for gliogenic or neurogenic activity based on results from the literature. As shown in the color key, green indicates similar patterns of mRNA and microRNA expression (positive correlation). Red indicates inverted patterns or negative correlation.

These two cell clones provided a model system for NSC differentiation that was either restricted to neurons (L2.2) or not restricted (L2.3).

As proposed in aim 1, we profiled gene expression in both cell clones at 0, 1 or 3 days following bFGF withdrawal to identify differentially-expressed mRNAs and microRNAs. The mRNAs were profiled using Applied Biosystems 1700 Rat Genome arrays which identified 2,003 significantly regulated mRNAs (by ANOVA at 5% FDR). Analysis of mRNA expression patterns confirmed the phenotypes observed by immunostaining and also uncovered novel transcription networks likely to distinguish the cells using a bioinformatics approach (4). MicroRNAs were profiled used the Invitrogen NCode microRNA arrays [which we developed (3) and are produced by Invitrogen under license] to identify an exploratory set of 39 regulated microRNAs (10% FDR). Approximately 20 of the 39 putatively-regulated microRNAs were tested by qPCR with the Applied Biosystems TaqMan microRNA assays, which confirmed the regulation predicted by the microarray. As we had done earlier with human embryonic stem cell microRNA and mRNA profiles (11), we hypothesized that microRNAs and mRNAs were coordinately regulated during NSC differentiation, so we examined expression data by cross-correlation. Expression profiles for each mRNA across the two cell types and time points were correlated with profiles for each microRNA and a matrix was constructed containing every possible correlation value. This matrix was then clustered hierarchically and drawn as a heatmap. Examining all mRNAs and microRNAs reveals distinct patterns of specific mRNAs and microRNAs, but this view of the data was too complex to display here. We selected a set of transcription factors (TF) that were previously studied for their role in NSC differentiation and these correlations were re-drawn as a subset of the heatmap (Fig. 1). Again, results show distinct patterns of correlation, but now the clustering correctly partitions TF that are gliogenic (tagged red in the figure) from those that are neurogenic (blue). Interpretation of this heatmap predicts specific subsets of both mRNAs and microRNAs that are coordinately regulated during neurogenesis and gliogenesis. Analysis of the microRNA expression patterns in coordination with mRNA changes reveals a cluster of microRNAs and mRNAs that correlate with a neurogenic phenotype. This cluster includes the archetypical neuron-enriched microRNAs, mir-9 and mir-124 (1, 2, 7-9, 19). This result not only confirms the selective regulation of a neurogenic microRNA cluster but also demonstrates our ability to culture neural precursors and assess gene regulation using traditional functional genomics and bioinformatics techniques.

Parallel projects growing out of this grant focused on a similar approach to study microRNA and mRNA coordinate regulation in human embryonic stem cells (11) and mesenchymal stem cells (5, 10), as well as a chapter summarizing our methods (Goff et al., in press).

As proposed in aim 2, this cluster of regulated microRNAs was tested for its ability to enhance production of neuronal markers by fluorescence-activated cell sorting (FACS). Techniques were updated as new, effective reagents became available. A set of four microRNAs found to correlate with neurogenesis in the rat L2.2/L2.3 model (mir-9, mir-124a, mir-153, mir-187) were tested for their ability to enhance neurogenesis in the multipotential precursor (L2.3). To test whether exogenous microRNAs affected differentiation, Ambion Pre-Mir microRNA mimics were transfected into cells using an Amaxa Shuttle 96-well nucleofector. After plating cells, FGF was withdrawn to initiate differentiation. After 3 days, cells were stained and quantified by FACS for TuJ1, a common marker for the neuronal pathway. Addition of the four Pre-mirs increased the number of cells expressing neuronal marker (Fig. 2, upper left). Testing individual microRNA mimics, we found that three of the four were capable of acting alone (Fig. 2, lower left). To test if endogenous microRNAs were required for neuronal differentiation, Ambion Anti-Mirs for all four microRNAs were used in similar experiments, and we found that a mixture of all four antagonists reduced the differentiation into neurons and also increased the number of cells

expressing the immature marker nestin (Fig. 2, right). These results indicate that microRNAs are not only regulated by but also affect differentiation processes in developing CNS precursors.

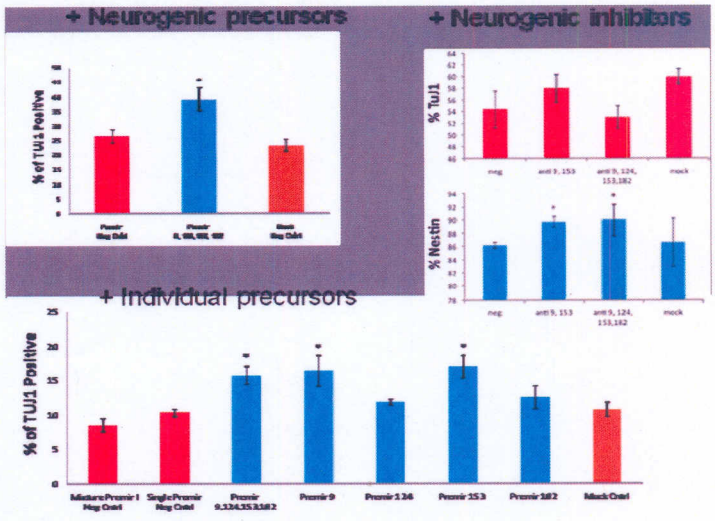

Fig. 2. FACS analysis of multipotential (L2.3) cells treated with (upper left) a mixture of four neurogenic microRNA precursor mimics, (lower left) individual microRNA precursor mimics, or (right) a mixture of four microRNA antagonists.

A prediction from these results is that specific microRNAs and mRNAs are co-regulated by common or interacting transcriptional mechanisms. This observation arose through the fortuitous discovery that one allele of the neurogenic mir-9 is found immediately adjacent to a gene encoding Mef2c, a TF known to be expressed in muscle and brain (6, 12, 17). Mef2c forms heterodimers with the neurogenic factor MASH1 (18) and also mediates neuronal survival under specific conditions (16). Of the three genomic loci encoding mir-9, our studies found that only the locus adjacent to Mef2c is regulated during neurogenesis. Furthermore, this Mef2c is also regulated during NSC neurogenesis. The NSC-regulated mir-9 gene contains an active Mef2 binding site within its promoter sequence. This indicates that a neurogenic microRNA is in turn regulated, at least in part, by a neurogenic transcription factor (Mef2c).

A second prediction is that microRNAs may affect TF activity *indirectly*, by modulating binding partners to change specificity or regulatory effect. Mef2c is known in muscle to be capable of changing from a positive-acting factor to a transcriptional inhibitor upon binding one of several molecules, including Type IIA HDACs. We found that several neurogenic microRNAs are bioinformatically predicted to target HDAC4 and HDAC5 transcripts, suggesting that microRNAs may mediate this switch. In model cell culture systems, mir-9 overexpression represses protein production from luciferase mRNAs containing HDAC4 3'UTR sequences (not shown). We are currently working to determine if Mef2c binds with HDAC4 prior to neurogenesis in our precursor cultures. This is an excellent example of a neurogenic microRNA effecting an amplified switching of an inhibited to an active neurogenic transcription factor, substantiating our hypothesis that small microRNA changes modulate differentiation pathways. Our investigation of Mef2c has turned out to be fortuitous since other have recently demonstrated that gene therapy with an altered Mef2c produces neurons from precursors and those neurons are effective in a mouse model of stroke (14, 15)

This work has been presented at several conferences (Society for Neuroscience Annual Meeting, Cold Spring Harbor Symposium on Quantitative Biology, International Society for Stem Cell Research, Society for Developmental Biology) and will be prepared for submission as soon as possible. To be honest, I've been waiting until we had the complete picture of the role of Mef2c, HDAC4 and mir-9 before we submit this work to have the strongest impact.

3. Project challenges.

We proposed to focus on gliogenesis with the idea that restoring oligodendrocytes after a demyelinating injury would be a more directly efficacious strategy for therapeutic cell transplant. However, analysis of our most interpretable data led us instead to focus on neurogenesis. Essentially, the techniques available to us were more successful when applied to questions of neurogenesis. We have recently produced a new series of immortalized precursor cells and this time we believe we have produced "glial-restricted" precursors, so we are now ready to turn focus to gliogenesis in ongoing projects.

4. Implications for future research and/or clinical treatment.

Understanding the role of microRNAs in the differentiation of neural precursor cells is likely to be important for developing cells suitable for therapeutic transplant. We already know that exogenous addition of a mixture of four microRNAs increases the differentiation of precursors towards a neuronal cell ($TuJ1^+$). Furthermore, this is likely to work in concert with MEF2C, a key transcription factor in neuronal fate specification. Recent publications by others show that MEF2C-induced cells, transplanted into a stroke model, produce viable neurons and augment behavioral recovery(15). Our belief is that a transient addition of microRNA to precursor cells will be a more acceptable and approvable strategy to achieve similar results in stroke as well as spinal cord injury.

5. Plans to continue this research, including applications submitted to other sources for ongoing support.

Based on this project and the results generated, we already obtained additional funding to support ongoing research into the role of microRNAs in neural differentiation. Grants were awarded by NIH (Grumet, PI; Hart, Co-PI) and two awards from the New Jersey Commission on Science & Technology (Hart, PI). Another grant submitted to NIH was not funded but was given encouraging reviews and will be resubmitted in the fall. This award was the seed that will guide all research in my laboratory for the foreseeable future.

6. List and include a copy of all publications emerging from this research, including those in preparation.

Published:

Lakshmipathy, U., B. Love, L.A. Goff, R. Jörnsten, R. Graichen, R.P. Hart and J.D. Chesnut (2007) MicroRNA Expression Pattern of Undifferentiated and Differentiated Human Embryonic Stem Cells., Stem Cells Dev 16: 1003-1016.

Goff, L.A., J. Davila, R. Jörnsten, S. Keles and R.P. Hart (2007) Bioinformatic analysis of neural stem cell differentiation., J Biomol Tech 18: 205-12.

In preparation:

Goff, L.A, U. Lakshmipathy, and R.P. Hart. The Analysis of MicroRNAs in Stem Cells. Invited chapter for "Stem Cell Research and Therapeutics," edited by Y. Shi and D. Clegg, Springer, In press.

Goff, L.A, S. Boucher, C. Ricupero, S. Fenstermacher, M. Swerdel, L. Chase, C. Adams, J.D. Chesnut, U. Lakshmipathy and R.P. Hart. mRNA and miRNA Regulation of microRNA Expression in Differentiating Human Multipotent Mesenchymal Stromal Cells. Experimental Hematology, In press.

Li, H., J. Davila, L. Goff, M. Swerdel, R.P. Hart, M. Plummer, and M. Grumet. Functional differentiation of an embryonic cortical neuronal precursor clone is promoted in co-culture with glial cells via secreted factor independent mechanisms. Submitted.

Goff, L.A., C. Camarillo, C. Ricupero, J. Davila, M. Swerdel, H. Li, M. Grumet and R.P. Hart. MEF2C regulates mir-9 in a feed-forward, pro-neurogenic mechanism. In preparation.

References Cited

1. Cao, X., S.L. Pfaff, and F.H. Gage, *A functional study of miR-124 in the developing neural tube.* Genes Dev, 2007. **21**(5): p. 531-6.
2. Deo, M., J.Y. Yu, K.H. Chung, M. Tippens, and D.L. Turner, *Detection of mammalian microRNA expression by in situ hybridization with RNA oligonucleotides.* Dev Dyn, 2006. **235**(9): p. 2538-48.
3. Goff, L.A., M. Yang, J. Bowers, R.C. Getts, R.W. Padgett, and R.P. Hart, *Rational probe optimization and enhanced detection strategy for microRNAs using microarrays.* RNA Biology, 2005. **2**(3): p. e9-e16.
4. Goff, L.A., J. Davila, R. Jornsten, S. Keles, and R.P. Hart, *Bioinformatic analysis of neural stem cell differentiation.* J Biomol Tech, 2007. **18**(4): p. 205-12.
5. Goff, L.A., S. Boucher, C. Ricupero, S. Fenstermacher, M. Swerdel, L. Chase, C. Adams, J.D. Chesnut, U. Lakshmipathy, and R.P. Hart, *Differentiating Human Multipotent Mesenchymal Stromal Cells Regulate microRNAs: Prediction of microRNA Regulation by PDGF During Osteogenesis.* Submitted, 2008.
6. Ikeshima, H., S. Imai, K. Shimoda, J. Hata, and T. Takano, *Expression of a MADS box gene, MEF2D, in neurons of the mouse central nervous system: implication of its binary function in myogenic and neurogenic cell lineages.* Neurosci Lett, 1995. **200**(2): p. 117-20.
7. Kapsimali, M., W.P. Kloosterman, E. de Bruijn, F. Rosa, R.H. Plasterk, and S.W. Wilson, *MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system.* Genome Biol, 2007. **8**(8): p. R173.
8. Krichevsky, A.M., K.S. King, C.P. Donahue, K. Khrapko, and K.S. Kosik, *A microRNA array reveals extensive regulation of microRNAs during brain development.* Rna, 2003. **9**(10): p. 1274.
9. Krichevsky, A.M., K.C. Sonntag, O. Isacson, and K.S. Kosik, *Specific microRNAs modulate ES cell-derived neurogenesis.* Stem Cells, 2005.
10. Lakshmipathy, U. and R.P. Hart, *Concise Review: Micro RNA Expression in Multipotent Mesenchymal Stromal Cells.* Stem Cells, 2007.
11. Lakshmipathy, U., B. Love, L.A. Goff, R. Jornsten, R. Graichen, R.P. Hart, and J.D. Chesnut, *MicroRNA Expression Pattern of Undifferentiated and Differentiated Human Embryonic Stem Cells.* Stem cells and development, 2007. **16**(6): p. 1003-16.
12. Leifer, D., D. Krainc, Y.T. Yu, J. McDermott, R.E. Breitbart, J. Heng, R.L. Neve, B. Kosofsky, B. Nadal-Ginard, and S.A. Lipton, *MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex.* Proc Natl Acad Sci U S A, 1993. **90**(4): p. 1546-50.
13. Li, H., J. Babiarz, J. Woodbury, N. Kane-Goldsmith, and M. Grumet, *Spatiotemporal heterogeneity of CNS radial glial cells and their transitiion to restricted precursors.* Developmental Biology, 2004.
14. Li, H., J.C. Radford, M.J. Ragusa, K.L. Shea, S.R. McKercher, J.D. Zaremba, W. Soussou, Z. Nie, Y.J. Kang, N. Nakanishi, S. Okamoto, A.J. Roberts, J.J. Schwarz, and S.A. Lipton, *Transcription factor MEF2C influences neural stem/progenitor cell differentiation and maturation in vivo.* Proc Natl Acad Sci U S A, 2008. **105**(27): p. 9397-402.
15. Li, Z., S.R. McKercher, J. Cui, Z. Nie, W. Soussou, A.J. Roberts, T. Sallmen, J.H. Lipton, M. Talantova, S. Okamoto, and S.A. Lipton, *Myocyte enhancer factor 2C as a neurogenic and*

antiapoptotic transcription factor in murine embryonic stem cells. J Neurosci, 2008. **28**(26): p. 6557-68.

16. Mao, Z., A. Bonni, F. Xia, M. Nadal-Vicens, and M.E. Greenberg, *Neuronal activity-dependent cell survival mediated by transcription factor MEF2.* Science, 1999. **286**(5440): p. 785-90.
17. McDermott, J.C., M.C. Cardoso, Y.T. Yu, V. Andres, D. Leifer, D. Krainc, S.A. Lipton, and B. Nadal-Ginard, *hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors.* Mol Cell Biol, 1993. **13**(4): p. 2564-77.
18. Skerjanc, I.S. and S. Wilton, *Myocyte enhancer factor 2C upregulates MASH-1 expression and induces neurogenesis in P19 cells.* FEBS Lett, 2000. **472**(1): p. 53-6.
19. Smirnova, L., A. Grafe, A. Seiler, S. Schumacher, R. Nitsch, and F.G. Wulczyn, *Regulation of miRNA expression during neural cell specification.* Eur J Neurosci, 2005. **21**(6): p. 1469-77.
20. Villa, A., E.Y. Snyder, A. Vescovi, and A. Martinez-Serrano, *Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS.* Exp Neurol, 2000. **161**(1): p. 67-84.