MicroRNA dynamics in early neuronal differentiation with correlation to protein output and cell biology

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ABSTRACT

Many studies reveal a hierarchy of complex interactions involving miRNA regulation of mRNA and subsequent protein output in dynamic biological processes. Exposure of NTERA2 cells to retinoic acid induces cellular differentiation into the neuroectodermal lineage. We harvested cell cultures for miRNA, mRNA and protein expression studies throughout a 14-day differentiation time course study. This unique approach utilized an integrated analytical sample workflow based on real-time PCR quantification of 377 miRNAs and 10 mRNA-protein pairs. Our data indicate dynamic regulation of miRNA species throughout the time course – many miRNA species are up-regulated during the differentiation process. Several up-regulated miRNA species such as miR-125b and miR-145 have been reported to interact with specific mRNAs and modulate their respective protein levels. For instance, while p53 mRNA levels rise upon differentiation, the p53 protein levels decline. Our data suggests that p53 mRNA translation is repressed by miRNA-125b cues. Our experimental approach and qPCR assay tools have great value for studying the interplay between miRNA dynamics and protein output.

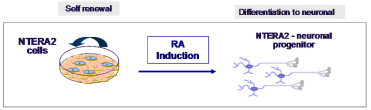
INTRODUCTION

Monitoring the molecular cues that direct cellular differentiation is an important undertaking for regenerative medicine. MicroRNAs (miRNAs) bind to mRNA and influence gene expression by destabilizing the target mRNA or repressing translation. miRNAs have a key regulatory role in cellular processes such as apoptosis, oncogenesis, differentiation and development. miRNAs have important functions in neuronal differentiation and recent reports detail essential roles of several miRNA species. The NTERA2 pluripotent human embryonic carcinoma cell line is a simple in vitro model system for characterizing molecular events occurring in early neuronal differentiation. Exposure of NTERA2 cells to retinoic acid (RA) induces cellular differentiation into the neuroectodermal lineage. We harvested cell cultures for global miRNA and key mRNA/protein pairs for expression studies throughout a 14-day differentiation time course study.

MATERIALS AND METHODS

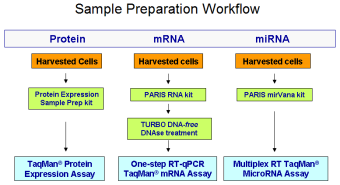
NTERA2 cells were cultured as specified by ATCC. The RA induction was commenced and cells harvested at days 0, 1, 4, 10 and 14. RNA was purified using Ambion PARIS™ kit procedures. Protein assay lysates were prepared using the PLA sample preparation lysis buffer – no purification steps were necessary. miRNA signatures of 377 targets were obtained using the MegaPlex™ Preamp Primer Pools and TaqMan® Array MicroRNA cards. In parallel, mRNA expression was measured using one-step RT-qPCR TaqMan® assays and protein expression profiles were generated using TaqMan® Protein Assays. The 10 protein/mRNA pairs quantified were ALCAM, CSTB, EGFR, ICAM1, LIN28, NANOG, NCAM1, OCT3/4, p53 and SOX2. Gene and miRNA expression relative quantification calculations were performed using the comparative Ct method with beta-actin and RNU48 designated as the endogenous references and day 0 the calibrator sample. The protein expression profiles were calculated using the ProteinAssist™ Software delta Cq squared method with day 0 lysates as the calibrator. Cluster and heat maps were constructed using DataAssist™ Software.

Figure 1. NTERA2 Retinoic Acid-induced Differentiation



NTERA2 cells as a model system for studying changes in gene and protein expression in response to retinoic acid induction. NTERA2 cells (1X10⁶) were cultured in T75 flasks in the presence or absence (untreated control) of 10uM trans-retinoic acid (RA) for 14 days. Cells were harvested by scraping at multiple time points and analyzed for protein, mRNA and gene expression. Harvest time points were at 0, 1, 4, 10 and 14 days. At each time point, the cells were split and lysates prepared separately as illustrated in the workflow depicted in Figure 3.

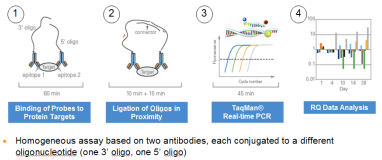
Figure 2. NTERA2 Cell Lysate Workflows



NTERA2 cells were harvested, lysed and processed in three parallel workflows. The mRNA and miRNA species were purified using the PARIS™ purification kits. The RNA was quantified by UV A260 and 10 ng used for each one-step RT-qPCR reaction. The protein lysates were used directly as sample input into the TaqMan® Protein Assay workflow. The assays were run using the Applied Biosystems StepOnePlus™ Real-Time PCR System. A 2 ng aliquot of purified RNA (mirVana™ PARIS™ kit) was converted to cDNA using the Megaplex™ Preamp primer pool A. The preamp miRNA cDNA was analyzed using the TaqMan® Array MicroRNA card version 2.0 on the Applied Biosystems 7900HT Real-Time PCR System.

Figure 3. TaqMan® Protein Assays Schematic Overview

TaqMan® Protein Assays Utilize Proximity Ligation Assay Technology



- Homogeneous assay based on two antibodies, each conjugated to a different oligonucleotide (one 3' oligo, one 5' oligo)
- When the two conjugated antibodies bind and are in close proximity, the oligonucleotides can be ligated, serving as the template for real-time PCR amplification and quantification

Invented by Professor Ulf Landegren, Simon Fredriksson and colleagues at Uppsala University/Olink Biosciences

Fredriksson, S., et al. *Nature Biotechnology* 20, 473 - 477 (2002)

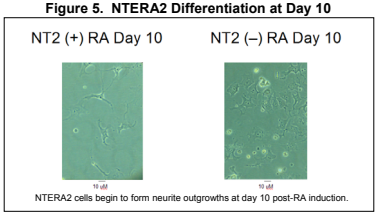
Figure 4. MicroRNA, mRNA-Protein Assay Sets

TaqMan® MicroArray Card A	377 miRNAs plus 3 endogenous references (U6, RNU44 and RNU48)
TaqMan® Protein Assays	10 protein targets
TaqMan® Gene Expression Assays	10 mRNA targets plus ACTB
10 protein-mRNA targets	ALCAM NANOG CSTB NCAM-1 EGF-R OCT3/4 p53 ICAM-1 SOX2 LIN28

Real-time PCR assays were conducted on each time point sample. The zero-day samples were used as the RNA calibrator controls for the delta-delta Ct relative quantification method. The miRNA endogenous reference was RNU48 and the mRNA reference was beta-actin (ACTB). The protein relative quantification method used the No Protein Control as the assay normalizer and the zero-day lysate sample as the calibrator for the Applied Biosystems ProteinAssist™ software inputs.

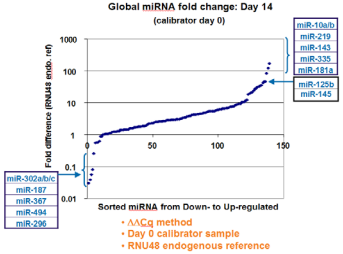
RESULTS

Figure 5. NTERA2 Differentiation at Day 10



NTERA2 cells begin to form neurite outgrowths at day 10 post-RA induction.

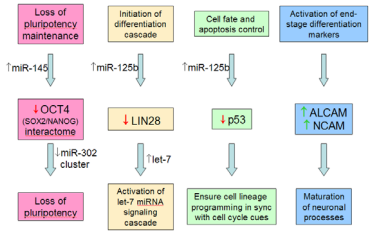
Figure 6. Global miRNA Expression at Day 14: Fold change relative to day 0



Most miRNA species are up-regulated by the differentiation process. The miR-10a/b were the highest up-regulated species, going from non-detected (Cq>32) to less than 20 at Day 14, at least a 4000-fold change using the delta-delta Ct method. Two other notable examples are miR-145 and miR-125b, both are reported to bind to specific mRNAs and prompt the differentiation process. The miR-302 cluster is highly down-regulated as cells lose pluripotency. This data suggests that the role of microRNAs in regulation increases as cells go down the differentiation pathway.

Figure 7. Biological Cascades in Neuronal Differentiation

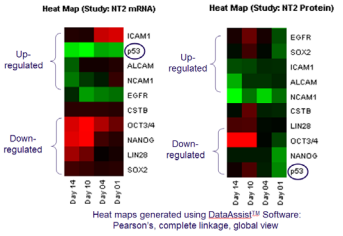
Execution of Early Neuronal Differentiation Cues



MicroRNAs are believed to be key initiators of the early differentiation cues. Here, we list miR-145 and miR-125b as major instigators of down-regulation of three key pathways.

Figure 8. Comparing mRNA to Protein Expression

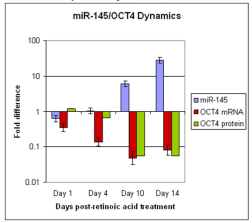
Global View



Most mRNA and their encoded proteins are coordinately regulated – however p53 is a remarkable exception. The p53 mRNA level increases while the protein level decreases.

Figure 9. Comparing miRNA, mRNA & protein expression miR-145 and OCT4 mRNA and protein

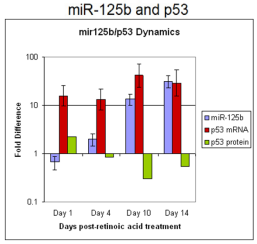
Loss of Pluripotency: miR-145 and OCT4



miR-145 has been reported to bind to and repress OCT4 mRNA levels (Xu et. al.)

Figure 10. Comparing miRNA, mRNA & protein expression miR-125b and p53 mRNA and protein

Cell fate and apoptosis control: miR-125b and p53



miR-125b shows significant translational repression of p53 mRNA

miR-125b has been reported to bind to and repress p53 mRNA function (Le et. al.)

CONCLUSIONS

- **We quantified miRNA, mRNA and protein using an integrated workflow and real-time PCR readout.**
- **We analyzed the global expression patterns of 377 miRNAs during early neuronal differentiation. We find that most miRNA species are up-regulated as the differentiation process occurs.**
- **TaqMan® Protein Expression Assays provide a simple, rapid and sensitive method for protein relative quantification in cell lysates.**
- **We quantified 10 mRNA-protein pairs and find most display coordinate regulation.**
- **We quantify a temporal series of specific miRNA-mRNA-protein interaction systems over the 14-day period:**
  - **miR-145 increases while the its target mRNA, OCT4 decreases. The OCT4 protein levels also eventually decrease.**
  - **miR-125b increases while its target mRNA, p53, actually increases. However, the protein levels decrease. This strongly suggests translational repression of the p53 mRNA.**

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