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Reprograming Neuronal Cells by Overexpression of Fibroblast-Specific Transcription Factors

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COMMITTEE IN CHARGE OF CANDIDACY

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Abstract

In mammals, a complex system of regulatory signals distinguishes tissues, structures and functions. Combinations of transcription factors and co-factors regulate activation and repression of genes that result in cellular differentiation. Whole genome arrays allow the monitoring of genomic expression in specific tissues. Fibroblast microarray studies have shown candidate genes that may be involved in fibroblast identification, including genes that express transcription factors Prrx1, Snai2 and Twist1. A previous study showed that the Prrx1 and Snai2 could reactivate a fibroblast phenotype in hybrid cells that had lost fibroblast identity. Furthermore, overexpression of these factors in liver-derived cells strongly repressed liver gene expression and activated fibroblast expression. Based on these observations, expression plasmids containing Prrx1, Snai2 and Twist1, expression cassettes were transfected independently into mouse Neuro2A neuronal cells using standard transfection technique, followed by the selection of G418-resistant clones (pool and clones). Expression of essential fibroblast marker genes and neuronal genes was monitored in transfected cells and non-transfected cells using qualitative real-time polymerase chain reaction (RT-qPCR) on cDNA derived for isolated RNA. Results showed that, surprisingly, little activation of expression occurred for any of the fibroblast genes tested. Rather, strong repression of several fibroblast genes was observed. However, both Snai2 and Prrx1 did appear to strongly repress several neural genes tested, suggesting a partial reprograming of the Neuro2A cells away from a neural phenotype.

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Chapter 1

Introduction

1.1 Overview of Cell Reprogramming

Cell reprogramming technology has expanded rapidly over recent years. Many attempts have been carried out to reprogram cells since the 1960s, with most experiments designed to establish aspects of cellular development. Over the past decade, a large increase in research has occurred in this area with several significant developments (Mong et al.). Among them is the knowledge that pluripotent cells can be utilized to generate alternate cell lineages and that somatic cell programming is neither a unidirectional nor irreversible process.

Understanding cellular reprogramming requires deep knowledge of gene regulation. All multicellular genomes utilize specific combinations of gene expression to produce cell-specific functions. Expression of key genes drives normal cellular processes, and perturbations in gene expression can result in cancerous growth of cells. Gene regulation is both functional and structural (Schmidt and Plath). Therefore, the study of gene regulation is key when approaching the topic of cellular reprogramming.

A key finding was reversing the functionality of a fully differentiated mammalian cell back to a pluripotent stem cell using forced expression of some transcription factors (TFs) (Yamanaka). In this series of experiments, overexpression of Oct4, Sox2, Klf4, and c-Myc genes was able to reprogram somatic cells to an embryonic state (pluripotent stem cells) which could then be coaxed into becoming a variety of cell types using specific growth environments.

Stem cells play a crucial role in experiments designed to understand the reprogramming processes. These cells possess two unique traits that can be exploited when conducting

reprogramming experiments, and much effort has gone into understanding stem cell choice during development (Rosa and Brivanlou). Stem cells have a pluripotent potential and can, under appropriate conditions, differentiate into any cell type (or even an entire organism - a clone). Another aspect of stem cell is their self-renewing ability through cell division while maintaining an undifferentiated state.

1.2 Mechanisms controlling mammalian gene expression

Much is known about regulation of gene expression in multicellular organisms. Mechanisms of gene regulation include chromatin, DNA regulatory elements, non-coding RNA, transcription factors and co-factors. Chromatin (which includes DNA and histones) plays direct role in eukaryotic gene regulation by undergoing structural modifications that allow or limit access of genomic DNA to transcription machineries. Where genome access is granted, transcription is initiated and resulting mRNA molecule is translated into proteins which carry out functional roles in cellular metabolism, DNA synthesis, cell homeostasis and gene regulation (Narlikar et al.). Noncoding RNAs also play vital functions in splicing and translation as well as regulating gene expression.

Cis-regulatory DNA sequences and particular DNA binding domains of TFs which bind them lead to the transcription of genes. The TF domains are able to bind to target DNA regulatory sequences and participate in recruitment of the RNA polymerase complex to initiate transcription. Amino acids sequences of the TF binding domains are often mutated leading to altered ability to assist in establishment of gene transcription, which can ultimately affect cellular programming and cellular identity. The highest risk is gene mutation that results in incomplete gene activation (Mong et al.). Epigenetics also plays a critical role in gene regulation through

DNA methylation and through modification of histones modification by acetylation, phosphorylation, methylation and ubiquitination of specific amino acids.

Cis-acting DNA regulatory elements are responsible for the control of eukaryotic gene expression and are therefore important determinant of cellular identity. These cis-acting elements are classified into four major categories: promoters, enhancers, silencers, and response elements. However, several other processes regulate gene expression, including DNA looping, transcriptional, post-transcriptional, and post-translational regulation. Those are vital for genetic regulatory elements to function properly since they aid in gene expression (Kim et al.).

1.3 Chromatin Remodeling and Histone Modifications

Chromatic remodeling is a genetic process that is necessary for cell reprogramming. (Kanherkar et al.). Histone modification plays a key role in the chromatin remodeling through epigenetic regulation. The assessment of histone modification in a cell type or tissue can be determined through the chromatin immunoprecipitation (ChIP) assay using antibodies to chromatin-modifying proteins that identify bound regions of DNA. This process keeps track of the interactions of DNA-proteins and allows the analsis of chromatin structures that are usually around a particular DNA sequence (Grath and Dai).

The ChIP Assay is potentially able to identify histone modifications during the process of reprogramming. In this procedure, protein-DNA crosslinking occurs using glutaraldehyde (Geiman and Robertson), followed by shearing of DNA and then use of antibodies to precipitate proteins-DNA complexes (Fang et al.). Crosslinks are then chemically removed and the attached DNA sequenced. In this way, interaction of chromatin modifying proteins with DNA control elements of key regulatory genes can be established during cellular reprogramming.

During recent years, several biotechnology companies have developed procedures to identify histone modifications and subsequent effects on gene expression of cultured cells or tissues. Each method uses algorithms to identify genome-wide data (Alvarez Palomo et al.). These modifications include histone deacetylation, methylation, and phosphorylation by specific enzymes (Wilmut et al.).

1.4. Reprogramming of Cells by Targeting Candidate Gene Expression

Typically, only a small percentage of the selected cells are converted into pluripotent stem cells (iPSCs) in the cell reprogramming attempts. Long before the current methodologies were available, the frequency of conversion was diminishingly small (Budniatzky and Gepstein). However, these experiments served as the template for current methods which provide the basis for reprogramming of even fully differentiated cells to a pluripotent state. Some of the innovations utilized the overexpression of primordial cells (Masserdotti et al.).

It is important to note that extensive epigenetics changes occur during reprogramming, starting from the initiation of the process. These processes are those that primarily create the embryonic stem cells (ESCs) resembling iPSCs (Takahashi). The resulting product should be molecularly, functionally, and structurally determining factor of the procedure success.

Many studies have shown that the frequency of reprogramming of cells by either forced expression specific transcription factors and/or the introduction of compounds that can drive cellular differentiation result in only a small percentage of cells that respond. Although many studies do not support the idea of this process being stochastic, clonal analysis shows that the reactivation of pluripotency takes place in varied and distinct periods preceding the initiation in the specificity. The formation of iPSCs is not clearly understood due to the complexity of the

process post-initiation (Gao et al.). This complexity includes large-scale changes in gene expression patterns. Therefore, the analysis of processes in single cells responsible for the formation of iPSCs is very difficult (Abdelalim et al.) and limited studies on this topic have adequately addressed the issue.

The understanding of gene expression profiles during formation of iPSCs is the most appropriate method to explain the mechanism of cell reprogramming. This technique gives an insight into how cells are separated into distinct cell types at each point in cell differentiation. Typically, marked differences in gene expression profiles are observed as cells differentiate. Furthermore, the method also gives the implication that the activation of certain genes does not mean that the cell reprogramming will be successful. Rather, it suggests that it is the activation of other groups of genes that leads to the successful formation of iPSCs.

After initial steps in reprogramming, cells enter into another complex phase leading to further transformations. This step results in the activation of endogenous pluripotency circuitry that normally hinders the successful formation of iPSCs and results in often unpredictable percentages of iPSCs (Polo et al.). Studies aimed at understating this post-initiation phase offer an understanding of critical genes that are activated at key points in the process.

Recent whole-genome fibroblast analyses identified candidate genes that can potentially serve as master regulators of fibroblasts identity (ref). These studies compared gene expression profiles of fibroblasts to that of hepatoma cells and fibroblast x hepatoma hybrid cells. Most notable are genes Prrx1 and Snai2, which were shown to activate several downstream fibroblast marker genes such as Bmp3, Twist, Shox2, C-fos, Slug, Sema3A, Sppl, and Col1a1in the cell hybrids which had previously turned off these genes (Ray et al.).

The ability of Prrx1 and Snai2 to reprogram cells was further explored by over-expression of these genes in a rat liver-derived hepatoma cell line, Fg14. Results showed that both Prrx1 and Snai2 activated expression of several fibroblast marker genes (Twist, cFos, Shox2a, and Bmp3 but not others (Sema3a and Spp1) in the liver-derived cells. Importantly, repression of several liver-specific genes was observed by overexpression of these transcription factors (Alzahrani).

Moreover, suppression of either Prrx1 or Snai2 in fibroblasts using RNAi knockdown experiments was found to reprogram the fibroblasts to an earlier linage that could then be coaxed into expressing linage specific marker genes for adipocytes, chondrocytes or osteocytes. These findings indicate that the inhibition of a single TF gatekeeper in a cell engaged in lines is appropriate for the acquisition and reprogramming of cell plasticity without permanent genetic modification (Ray et al.).

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1.5. Neuronal Cell Development

Neural cell formation, especially that of neurons and glial cells, during embryogenesis is essential for the survival of the developing embryo. In the process of embryo formation, the neural system is usually the first to develop and the last to complete development (Buganim et al.). During the initial stages, the neural system forms a neural plate that folds, resulting in the formation of a neural groove and a neural tube over time.

1.6. Stages of the Neurogenesis

During the development of the nervous system and neurons, four phases are involved. These stages include neurogenesis, cell migration, differentiation, and outgrowth (Crespo et al.). The neurogenesis process forms the foundational basis of the neural and nervous system. It may be defined as the first and the initial stage of neural development. Undifferentiated cells tend to undergo mitosis to give rise to neuroblasts or stem cells (Gage). These will eventually be differentiated into different neuron types. Neuroblasts are considered to perform more work than stem cells in the process of embryonic development. Stem cells do not take a significant role until adult neural development.

Cell migration is the second stage of neurogenesis. In this phase, the nervous system is involved to perform the corresponding regulatory functions. The differentiation of actions becomes more identified, which is a natural step of development. Cells always move from the ventricular region to other systems. In other zones, they are tasked with the establishment of cell populations, which is a natural step of the whole structure.

The third stage is the migration of cells into the marginal zone leading to differentiation. In this phase, the nervous system cells start to be task-specific in nature (Chatterjee and Ahituv). Neural cells differ according to the functions they perform. This differentiation stage takes place concurrently with the fourth phase. The fourth phase includes formation of dendrites and axons.

1.7 Neurological Diseases

Neurological disorders are complications that affect the nervous system and the puerperal system. They are the problems that make it impossible for the nervous system to perform its natural functions. The most common disorders are Alzheimer's disease, Epilepsy, Multiple Sclerosis, Parkinson's disease, and the Common Migraines (Chatterjee and Ahituv). Each

neurological disorder is characterized by different features in its progression. Symptoms can either be psychological or physical, and mood swings are the most common psychological symptom. Some of the main physical features of neurological disorders are partial or complete paralysis, seizures, unexplained pains, the loss of sensation, cognitive inabilities, and muscle weakness.

1.8. Reprogramming of Neuronal Cells

Early attempts to reprogram neuronal cells were unsuccessful, largely due to a poor understanding of the mechanisms of cell differentiation. However, more recent attempts have been successful (Gascon et al.). Pluripotent cell-specific factors have been utilized to reprogram fibroblasts to neural cells directly. Vierbuchen et al. (2010) was able to show direct conversion of fibroblasts to functional neurons by the introductions of a several transcription factors (Ascl1, Brn2 and Myt1).

Specific molecules, mostly neuron-specific transcription factors, have been shown to generate several different types of neurons. Neurog2 and Ascl1, which play a central role in neuronal function (see Figure 1), are the most commonly used proteins. (Masserdotti et al.).

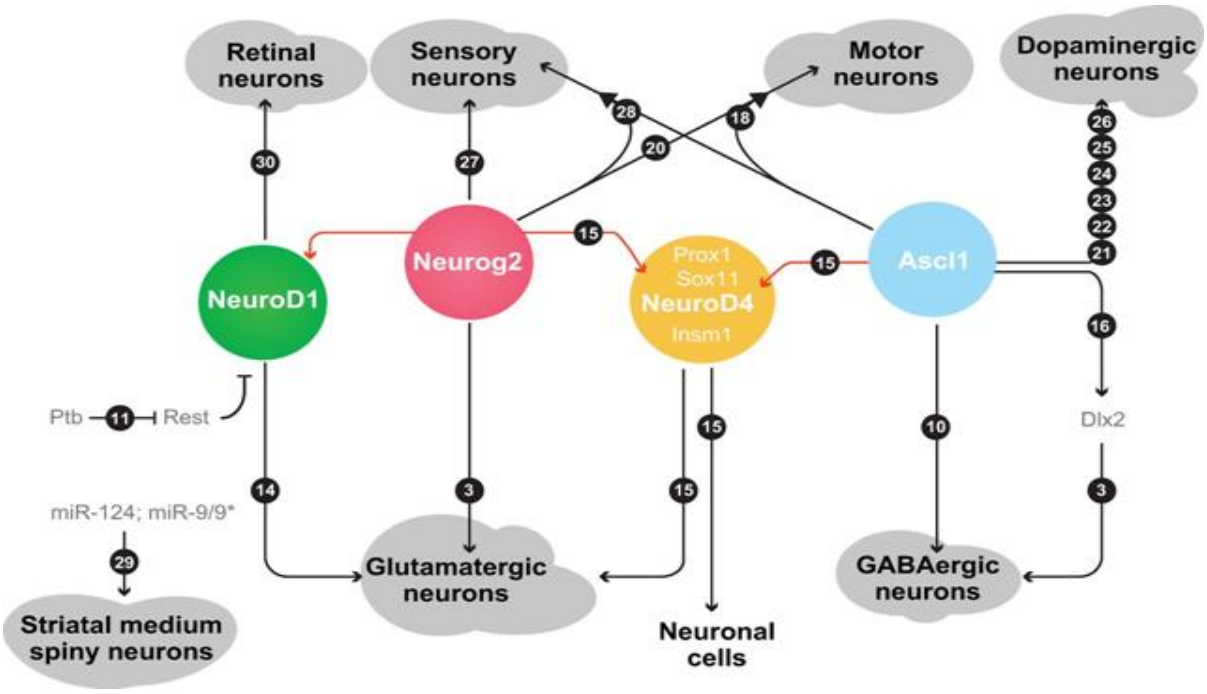


Figure 1. Specific molecules, which are mostly transcription factors, can generate several different types of neurons. Neurog2 and Ascl1 are the most commonly used. Red lines connect Neurog2 or Ascl1 with their downstream targets (Masserdotti et al.)

The key question in this study was whether key fibroblast-specific transcription factors Snai2, Prrx1 and Twist1 have the ability to reprogram neuronal cells using mouse Neuro-2A cells as a model. To that end, expression plasmids were introduced into Neuro-2A cells and a selection scheme used to identify cells which had uncorroborated plasmid sequences. Pooled transfectants and individual clones were screened for overexpression for the transgene followed by expression profiling for both neuron-specific and fibroblast-specific for transcription.

Chapter 2

MATERIALS AND METHODS

2.1 Cell culture

In this study, a mouse neuronal cell line (Neuro2A) was used. This is a continuous cells line that grows well in culture. Cells were maintained in a medium containing 1:1 Ham F12 / Dulbecco modified Eagle medium (FDV) with 10% bovine fetal serum (FBS) plus 5 ug/ml penicillin and streptomycin (GIBCO). All cells were incubated at 37 ° C in a moist 5 per cent CO_2 in a humidified incubator. Cells were split 1:5 as needed and transferred to fresh plates.

2.2 Cell Transfection

Cells were transfected with candidate genes by us lipofection using commercially available reagents (Invitrogen). In this process, liposomes introduce DNA into the cells. The liposomes trap the DNA and then fuse into the cells with the target cell membrane. Expression vectors containing candidate genes (Pprx1, Snai2 and Twist1) purchased from Origene, Inc. were introduced by lipofection into the mouse Neuro2A cells. 6-well cell culture plates were used to transfect candidate genes. Briefly, 0.5 ml of FDV media without antibiotics and streptomycin (Pen / Strep) was mixed with DNA (1 μg / μl) and the solution mixed at room temperature with 5 μl Lipofectamine Plus reagent (Invitrogen, Inc) followed by 5 μl of Lipofectamine LTX reagent. The solution was gently mixed by pipetting and incubated for30 min at room temperature before applying to the cultured Neuro2A cells.

The day before transfection, cells were cultured in a 6-well template at a density of 10^5 /well. The media on cell plates was removed and replaced with the transfection mixture. Plates

were regularly rocked at 37° C in a 5 % CO_2 incubator for 6-8 hours. After 6-8 hours the medium was replaced with FDV containing 10 % fetal bovine serum plus penicillin and streptomycin and incubated at 37 ° C in 5% CO_2incubator for two days. For stable transfection, cells were divided into full medium plus 500 μg / ml G418 (typically several replicate T-25 flasks at 1:20, 1:10, and 1:5 dilutions) and incubated for 2-3 weeks. After that, G418 resistant clones were pooled (10—50 clones per pool) or individually picked and transferred into larger plates until the cells could be lysed and the RNA extracted.

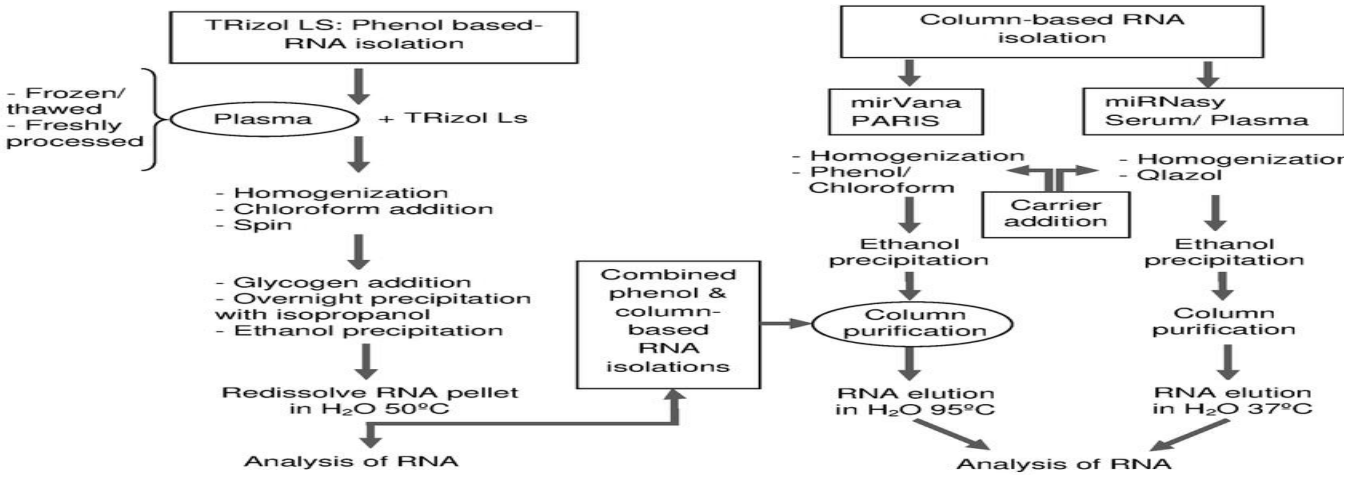
To monitor transfection efficiency, the cells were transfected with a Green Fluorescent Protein (GFP) plasmid. Normally, a 4-6% transfection efficiency was noted by counting the number of positive GFP cells 48 hours post-transfection. Also, a no-DNA control plate was used to ensure that no G418-resistant cells were present in the transfecting cell line.

2.3 RNA Isolation

RNA was extracted from 70-80 percent confluent monolayers (approximately 10^7 cells) using a Qiagen RNeasy Mini Kit (Cat # 74104) following the manufacturer's DNAse I (Cat # 79254) digestion step as per protocol. Briefly, the nutritional medium was extracted from the crop dish, and cells were lysed by treated by 350 ul strongly denaturing guanidine isothiocyanate (GITC) containing RLT buffer and beta-mercaptoethanol and mixed by rocking the pan, then scraped and transferred in a 2 ml collection tube to a Qiashredder column. For homogenization, samples were centrifuged for 2 minutes at 15,000 rpm. By pipetting, the flow-through was combined with 70 percent ethanol, the mixtures were transferred in collection tubes to RNeasy columns and centrifuged for 15 seconds at 10,000 rpm and the flow through discarded.

RW1 buffer was then used to wash the resin and centrifuged 10,000 rpm for 15 secs, followed by digestion with DNase I in the RDD buffer (Qiagen Cat # 79254) at room temperature for 15 minutes. To prepare DNaseI, it connected 10 ul of DNAseI to 70ul RDD (supplied with DNaseI kit). RW1 buffer was then added to the RNeasy column and for 15 seconds centrifuged at 10,000rpm. The RNeasy column was moved to a new 2 ml collection tube, and after each wash the RNeasy column was washed twice with 500ul RPE, and centrifuged. Finally, the RNeasy column was transferred to a 1.5 ml tube, and the RNA was elucidated by adding 40 µl RNase free water into the column and centrifuging for one minute at 14,000 rpm. The RNA samples were collected in tubes with microfuge and deposited at 70°C. The final concentration of the RNA was calculated using a Bio line spectrophotometer with a nanodrop plate reader.

Figure 2. A flow chart showing RNA Isolation:



2.4 Synthesis cDNA

To produce cDNA from RNA, the MasterAmp Higher Fidelity RT-PCR package (Applied Biosystems, part#4368814) was used. Reactions mixtures included RT buffer, 25uM dNTP blend, 10uM RT random primers, MultiScribe, and sterile nuclease-free water and 1.8 μg RNA in a final 20ul volume. A Bio-rad Thermal Cycler was used to synthesize cDNA using 25 ° C for 10 min, followed by 37 ° C for 2 hours, 85 ° C for 5 min, then 4 ° C before storing at -20 ° C. The cDNAs were diluted 1:40 for use in the RT-qPCR.

2.5 Primer Design

Primer design was performed using NCBI software. This program was optimized to identify primers that hybridize at temperature levels around 55 and 65 ° C to specific gene objectives and produce short amplicons when using qPCR. Usually, primers' melting temperature can be determined using closest adjacent thermodynamic measurements. Primers accuracy can be enhanced by ensuring the 3 'ends are not quite sticky. Intermolecular homology inside the primers should be avoided to minimize the chances of snapping back formation that interferes with the annealing (Thoo and Brown).

The primer pairs were purchased via Integrated DNA Technologies Inc. Three primers were designed to be evaluated for each gene, and the primer set that produced optimal amplification with the lowest background signal for both parental neuronal cells and fibroblast were used for further experiments. We controlled the expression of several genes in hepatic and fibroblast function. Tables 2 and 3 show primary pairs used in this analysis and estimated melt and annealing temperature.

Table 1. Neuronal gene primer sets used in this study

Primer	Sequence	TM °C	Annealing temperature °C
Zfp593 Set 4	GGC AGG AAG AAG GGT ATA AGA TG CAG TGA GGC GAG AAG GAT TG	54.89 55.52	70.2
Zfp593 Set 5	CAG GCG TCG AAA TCT CAT AGT CTA TGC TCT GGT GTC CAC TTC	54.53 55.06	70.1
Foxd1 Set 3	GCT AAG AAT CCG CTG GTG AA CTG CTG ATG AAC TCG CAG AT	55.01 55	70.8
Foxd1 Set 4	CTT CGG ATT CTT GGA CCA GAC AAG TCA GGG TTG CAG CAT AG	55.28 55.06	70.7
Dkk1 Set 1	GGA GAA ATG GAA TAG TCG GTG ATA G AGG GAG AGA GAG AGA GAG AGA A	54.76 55.45	69.8
Dkk1 Set 2	CTG AGA TGT GCT CTC TGC TTA G GTG TGT GAG AGA GAG AGA GAG A	54.84 54.84	70.1
Syt6 Set 3	CTG CAT ACC TCG TGT CTT ACT C GTT AGG GCA GCA GAG AAA CT	54.77 54.73	70.0
Syt6 Set 4	CTA CAA CGA GGC CAT CAT CTT TCG TTG TGG CCA ACT CTA TC	54.61 54.68	70.3
Phox2a Set 3	CCT TCT GGA CCT GGC ATT ATC TAG GGA TCA GAC ACT GGG TAG	55.26 55.07	70.7
Phox2a Set 5	GCC CTG AAG ACA AAC CTC TT CCG GGA TAG GGA GGG ATA AT	54.99 55.37	70.9

Table 2. Fibroblast gene primer sets used in this study

Primer	Sequence	TM °C	Annealing temperature °C
Col1a1Set 4	AGA CCT GTG TGT TCC CTA CT GAA TCC ATC GGT CAT GCT CTC	55 55.42	70.8
Bmp3 Set 1	GGT GGG AAA TGA CAG CAA TAA C GGC AAG ACA CCT AGT GAG AAA	54.31 54.47	69.8
Bmp3 Set 3	AAC TGT GTC CTA GCC ATT CTT AC GTC TGT CTG TCT GTC TCT CTC T	54.49 55.09	70.8
Spp1 Set 1	CTT TCA CTC CAA TCG TCC CTA C CAG AAA CCT GGA AAC TCC TAG AC	55.07 54.8	70.1
Spp1 Set 5	AGA CCG TCA CTG CTA GTA CA CAG TCC ATA AGC CAA GCT ATC A	55.01 54.53	70.2
Sema3a Set 4	GAA AGC AAC GCC GAC AAA G GGT CCT CCT GTT TCT ACC TTT C	55.11 54.85	70.2
Sema3a Set 5	CAC GGA TTC ATG CAA ACT CTT C GCC ATC TCC ATC GTC ATC TTT A	54.43 54.51	69.9
m c-Fos Set 4	GAG CTG GTG CAT TAC AGA GA GTG TGT TTC ACG AAC AGG TAA G	54.47 53.99	69.4
c-Fos Set 5	ACC TGA GAG CTG GTA GTT AGT TCC AGC ACC AGG TTA ATT CC	54.70 54.83	70.6
Shox2 Set 4	CTG GGT TGG GAG GAA TCA AA GTC AAA GTC AGG CCC ATA TCA	54.85 54.67	70.5
Shox2 Set 5	GAA GAG CAA GAA GAG GGA AGA C GGA ATC ACT GTC TGT GGT ATC G	55.06 55.07	70.3

2.6 Quantitative-Polymerase Chain Reaction (RT-qPCR)

RT-qPCR assays were performed in triplicate for each gene tested All the cDNA samples were diluted and concentration (5 ng/µl) was standardized among the samples. A final volume of 20 µl of reaction mixture contained 2 µl of specific cell line cDNA template (5 ng/µl), 2 µl of both forward and reverse gene-specific primers (0.5 µM) (IDTDNA), 6 µl of sterile nuclease free water, and 10 µl of Fast SYPR© Green Master Mix (Applied Biosystems), with the total volume of the reaction mixture at 20 ul. The list of primers used for genes and their Tm are described in Tables 1 and 2. An Applied Biosystems StepOne qPCR machine was used to amplify cDNA. Typical reactions included 40 cycles, with the first step at 95° C for 3 min, followed by second step at 5° C above melting temperature (Tm) for the primers 30 min for extension/ annealing, and an extension step at 60° C for 60 min. Also, the control used in the RTqPCR experiments contained 2 µl of gene specific primer (0.5 uM) (IDTDNA), 8 µl of sterile nuclease free water, and 10 µl of Fast SYPR© Green Master Mix (Applied Biosystems), but were without target cDNA.

Chapter 3

RESULTS

The purpose of these experiments was to determine whether fibroblast transcription factors have the ability to reprogram neuronal cells into a fibroblast phenotype. These fibroblast-specific transcription factors were identified due to their being highly expressed in fibroblasts, poorly expressed in hepatoma cells and repressed in fibroblast x hepatoma cell hybrids (termed FR cells). This analysis used genome-wide expression profiling using whole-genome microarrays to compare parental and FR cell gene expression. Using a criterion of at least 5-fold repression in the hybrids compared to the fibroblast cells, 566 fibroblast-specific genes were identified. We further applied a criterion of the gene encoding a product that served as a transcription factor or played a role in a signal transduction pathway. Several genes were identified, including fibroblast lineage-specific transcription factors Prrx1, Shox2, Snai2, c-Fos, and Twist. Based on results from other studies, we focused on three fibroblast-specific genes Prrx1, Snai2 and Twist1.

Plasmids containing mouse Prrx1, Snai2 and Twist1 expression cassettes were purchased from Origene and introduced via lipofection into the neuronal cell line Neuro2A. Transfected cells were selected using G418 to select for the neuro gene expressed from the same plasmids. Each transfection resulted in 30 to 100 G418 resistant clones. These clones were either pooled (>30 clones per pool) or picked individually and expanded in cell culture until harvesting of total RNA. We first determined the reliability of the RNA extraction in generating reproducible levels of RNA. To his end, we quantified each RNA sample, generated cDNA using 1.8ug of RNA, then diluted samples and compared GAPDH expression levels using RT-qPCR. Results show

that the Ct values for GAPDH levels were consistently between 18 and 22 cycles for Neuro2A cells pooled transfectants and clones (Figure 3). Therefore, the protocol used resulted in reasonably consistent reproduction of RNA, and subsequent generation of cDNA.

3.1. Regulation of Neuro2A gene expression by Prrx1

We asked whether the introduced Prrx1 gene was overexpressed in the transfected cells. Using mouse Prrx1 specific primers, results show that the pooled N2A-prrx1 transfectants overexpressed the Prrx1 gene by over one 1000-fold in the pooled cells and between 10 and 40-fold in the two clones analyzed (Figure 4). Therefore, overexpression of the Prrx1 gene in Neuro2A cells was successfully established.

Analysis of fibroblast and neuronal gene expression was carried out. To determine if fibroblast- specific genes were activated in the N2A-pprx1 transfectants, a panel of fibroblast-specific genes (Col1a1, Bmp3, Ssp1, Sema3a, c-Fos and Shox) were chosen. RT-qPCR results were normalized to Gapdh levels for each sample and compared to expression levels in the non-transfected Neuro2A cells, and most surprisingly showed a >2-fold repression (Figure 5). In contrast, the N2A-pprx clone 3 showed strong (>50 fold) activation of two genes, Col1a1 and Bmp3 (Figure 5).

We next asked if overexpression of Prrx1 could repress neuron -specific gene expression. A panel of genes to be tested were chosen based on a literature search and applying the criteria of moderate to high expression and neuron specificity in expression. The genes chosen included Syt6, Phox2a, Foxd, Dkk1 and Zpf593. Primers were designed to amplify each cDNA, and in some cases two primer sites for the same gene applied to account for differential splicing reported for several of the genes.

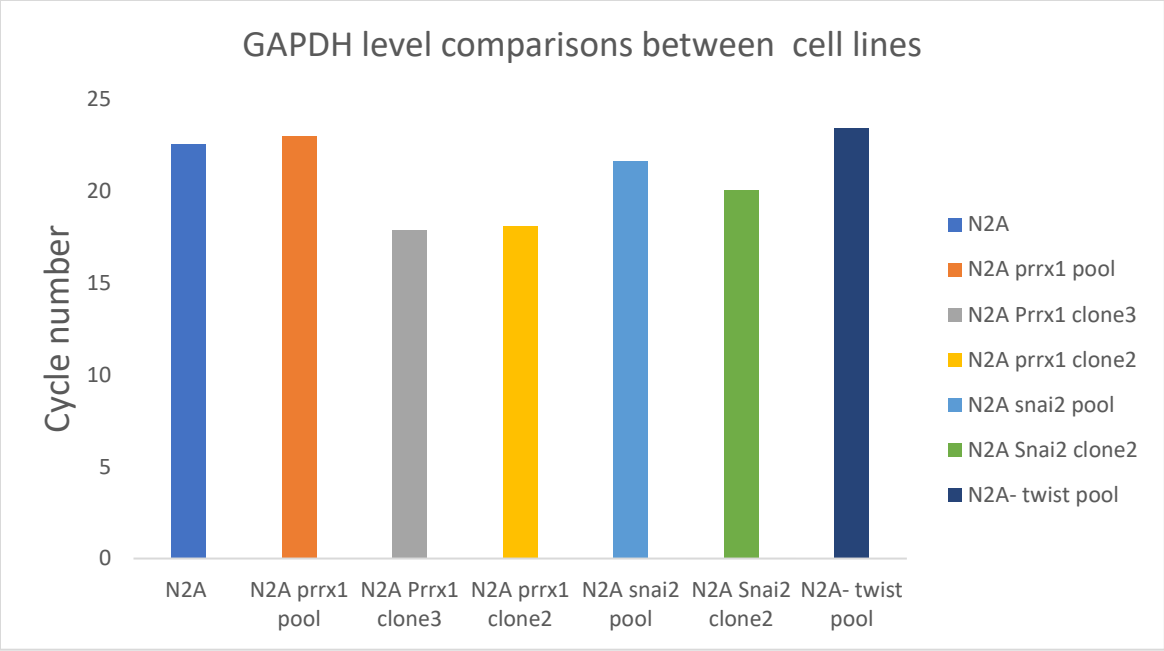


Figure 3: Gapdh level comparisons between cell lines. Comparative Gapdh levels are shown from the indicated cells lines using the cDNA prepared from total RNA.

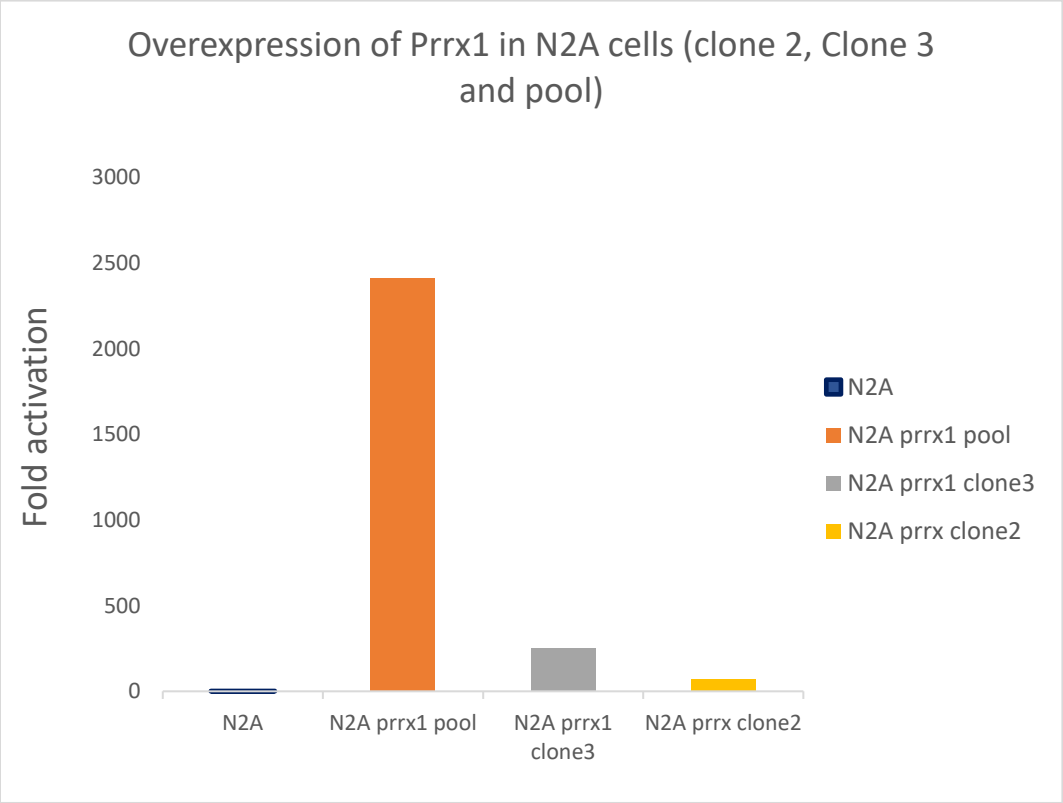


Figure 4: Over-expression of Prrx1 in Neuro2A cells. Prrx1-transfectd Neuro2A cells (pooled and individual clones) were tested for prrx1 expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

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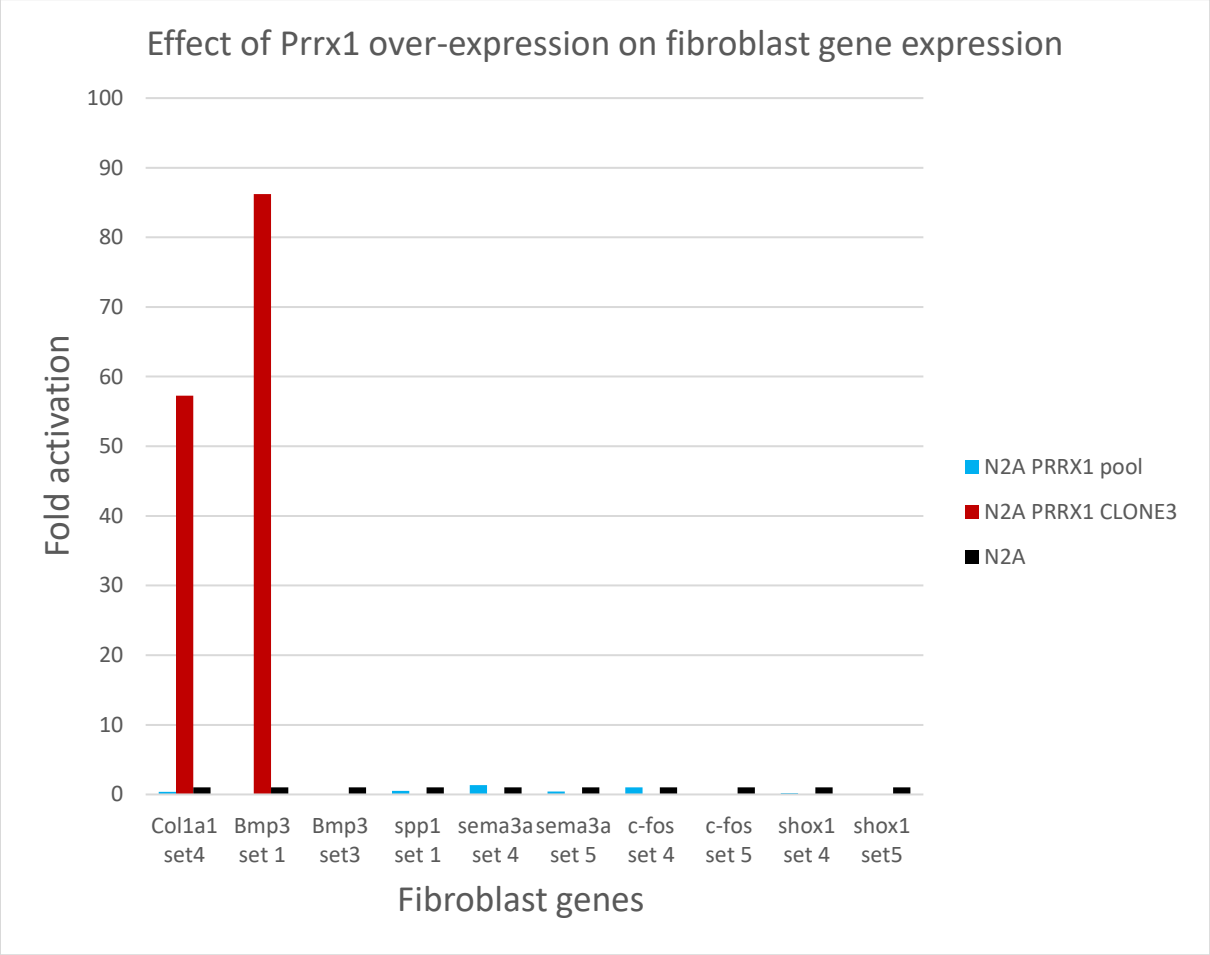

Figure 5. Effect of Prrx1 over-expression on fibroblast gene expression in Neuro2A cells. Prrx1-oversexpressing Neuro2A cells (pooled and individual clones) were tested for fibroblast gene expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

Results from the pooled transfectants show that expression of some genes (Zfp593, Dkk1, Foxd) was only modestly affected while others (Syt6, Phox2a) were repressed ~ 5-fold (Figure 6). However, the two clones analyzed showed strikingly results, with all five neuronal gene tested being repressed between 5 and 50-fold in both clones (Figure 6). These results suggest that Prrx1 overexpression can strongly repress neuronal gene expression.

3.2 Regulation of Neuro2A gene expression by Snai2

We asked whether the introduced Snai2 gene was overexpressed in the transfected cells. Using mouse Snai2-specific primers, results show that the pooled N2A-snai2 transfectants overexpressed the Snai2 gene by over one 400-fold in the pooled cells and ~25-fold in the N2A-snai2 clone chosen (Figure 7).

Analysis of fibroblast and neuronal gene expression was then carried out. To determine if fibroblast- specific genes were activated in the N2A-snai2 transfectants, we tested the same panel fibroblast- specific genes (Col1a1, Bmp3, Spp1, Sema3a, c-Fos and Shox) as used in the prrx1 experiments described above. As described previously, RT-qPCR results were normalized to Gapdh levels for each sample and compared to expression levels in the non-transfected Neuro2A cells. Surprisingly, results showed repression rather than activation of most fibroblast genes, with most displaying 2 to 20-fold repression in both the pooled transfectants as well as in the clone (Figure 8).

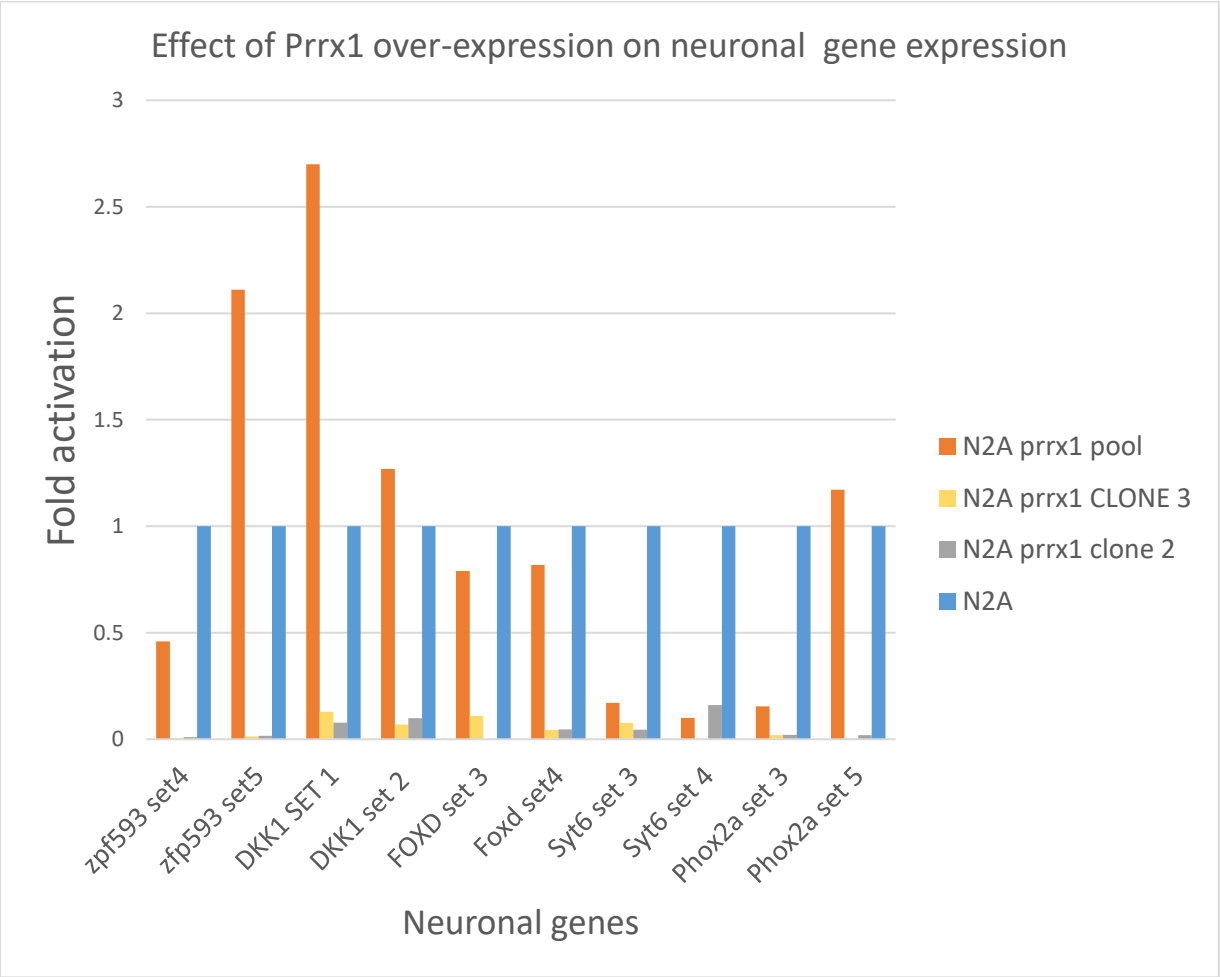

Figure 6. Effect of Prrx1 over-expression on neuronal gene expression.

Prrx1-oversexpressing N2A cells (pooled and individual clones) were tested for neuronal gene expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

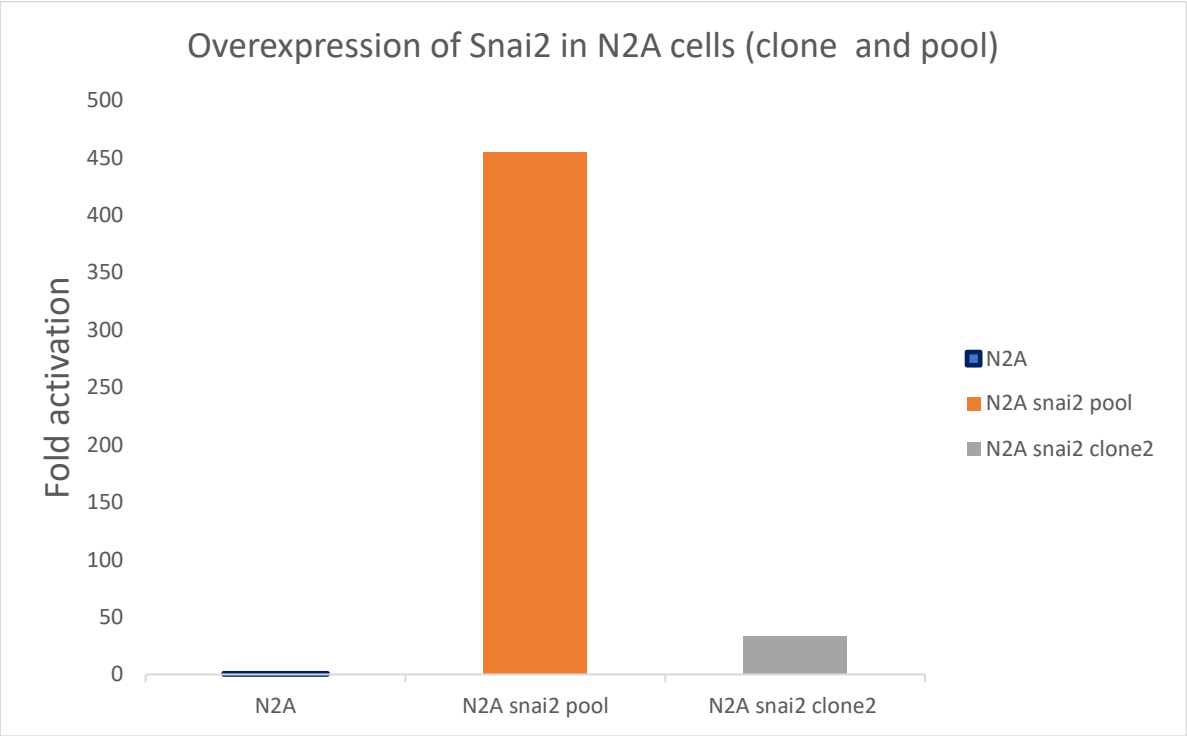

Figure 7. **Snai2 over-expression in N2A cells.** Snai2-transfected N2A cells (pooled and individual clones) were tested for Snai2 expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

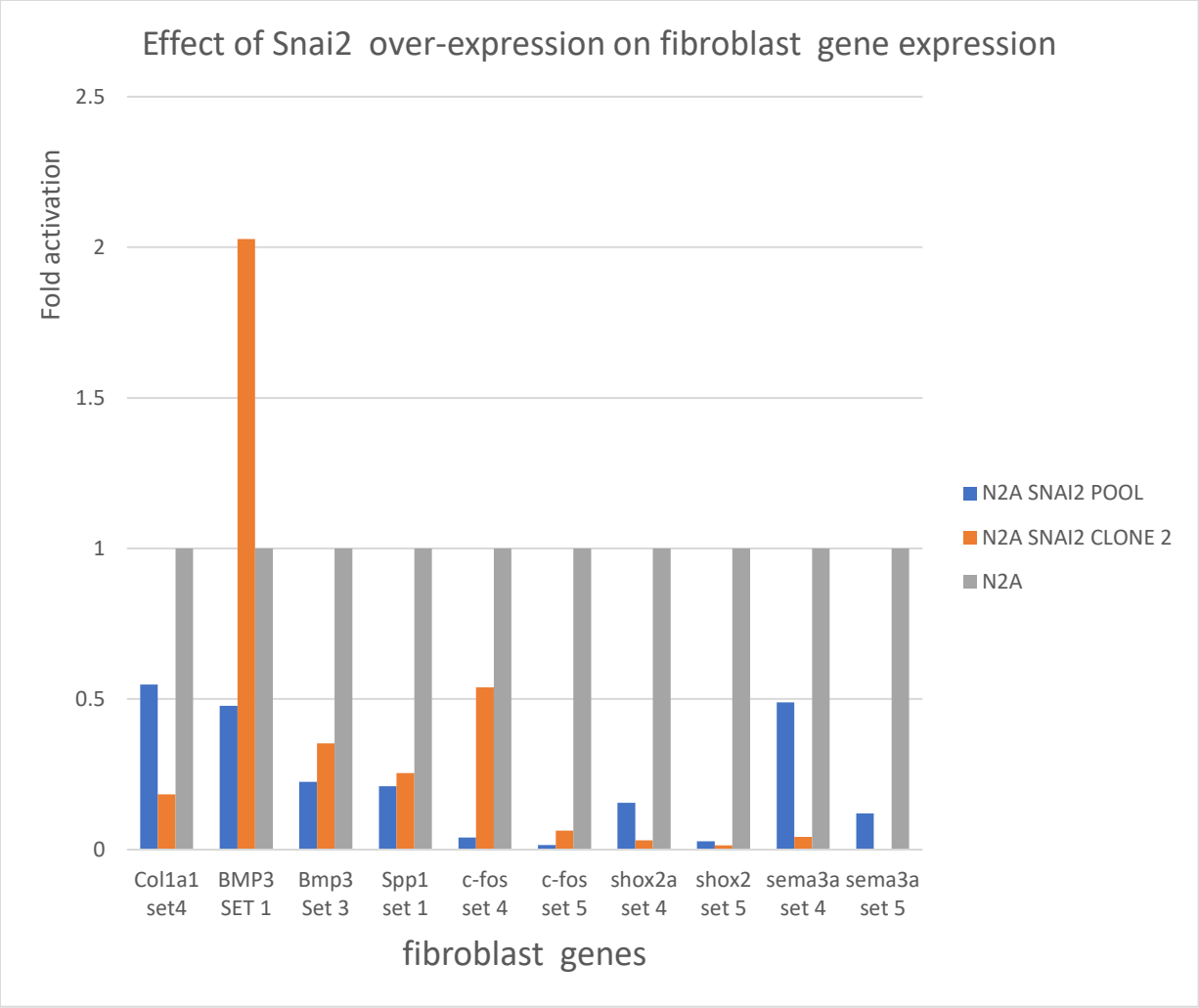

Figure 8. Effect of Snai2 over-expression on fibroblast gene expression. Snai2-oversexpressing Neuro2A cells (pooled and individual clones) were tested for fibroblast gene expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

We next asked if overexpression of Snai2 affected neuron -specific gene expression using the same gene panel as used on the Prrx1 experiments described above. Results from the pooled transfectants were highly variable, with expression of two genes, Zpf593 and Dkk1, unaffected and two genes, Syt6 and Doxd, showing modest effect or strong activation, depending on the primer sets used (Figure 9). However, as with the Prrx1 results, the N2A-snai2 clone showed strong (repression for each neuronal gene tested (with the exception of the Dkk1 gene using primer set #1 (Figure 9). These results suggest that Snai2 overexpression, like Prrx1, can strongly repress neuronal gene expression.

3.3 Regulation of Neuro2A gene expression by Twist1

We asked whether the introduced twists gene was overexpressed in the transfected cells. Using mouse Twist1-specific primers, results show that the pooled N2A-twist pooled transfectants failed to overexpress the Twist1 gene despite multiple trials (Figure 10).

Despite a lack of observing overexpression of the introduced twist gene in the transfected Neuro2A cells, analysis of fibroblast and neuronal gene expression was carried out. To determine if fibroblast- specific genes were activated in the N2A-twist1 transfectants, we tested the same panel fibroblast- specific genes (Col1a1, Bmp3, Spp1, Sema3a, c-Fos and Shox) as used in the Prrx1 and Snai2 experiments described above. As described previously, RT-qPCR results were normalized to Gapdh levels for each sample and compared to expression levels in the non-transfected Neuro2A cells. Surprisingly, result showed repression rather than activation of most fibroblast genes, with most displaying 2 to 5-fold repression in both the pooled transfectants. Therefore, activation of fibroblast genes was not observed by overexpression of Twist1 (Figure 11).

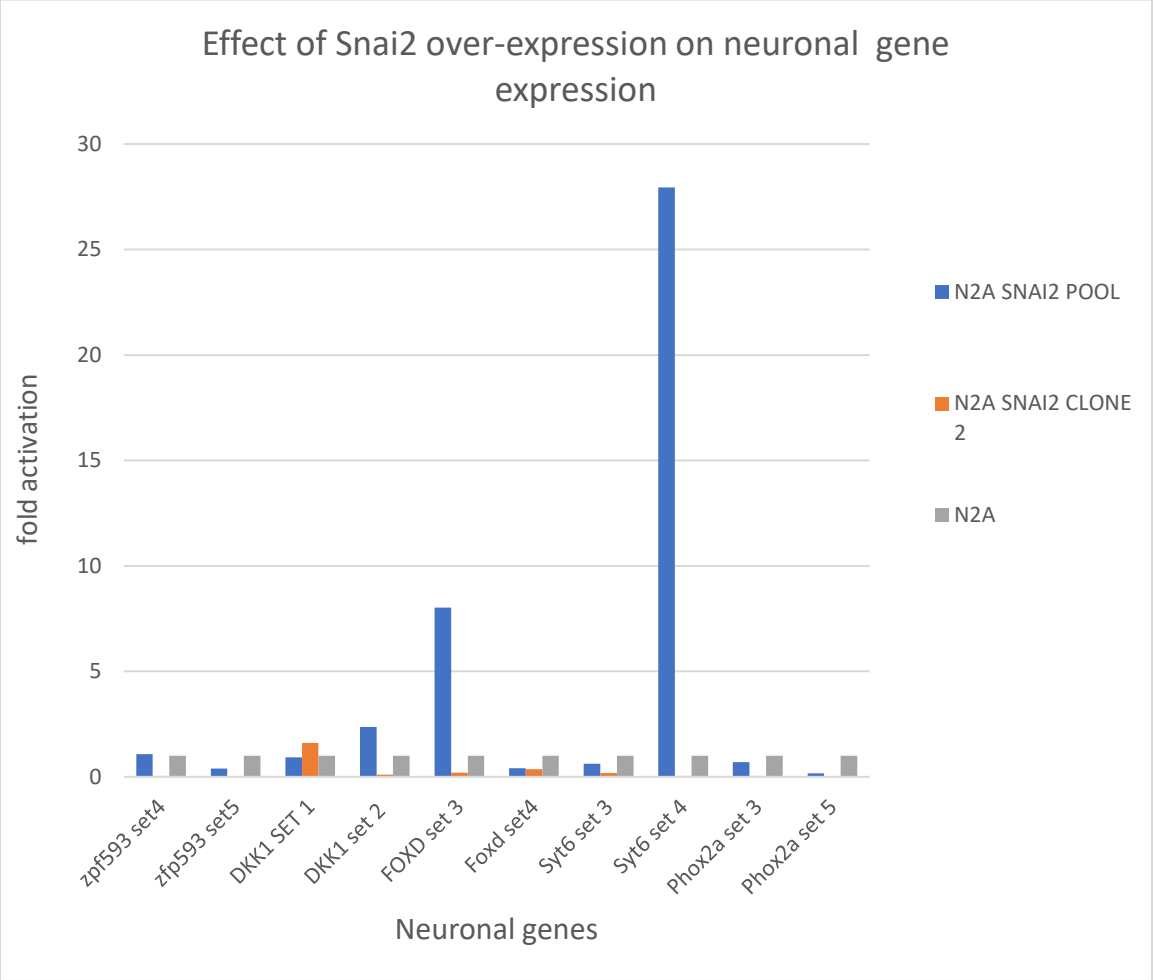

Figure 9. Effect of Snai2 over-expression on neuronal gene expression. Snai2-oversexpressing Neuro2A cells (pooled and individual clones) were tested for neuronal gene expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

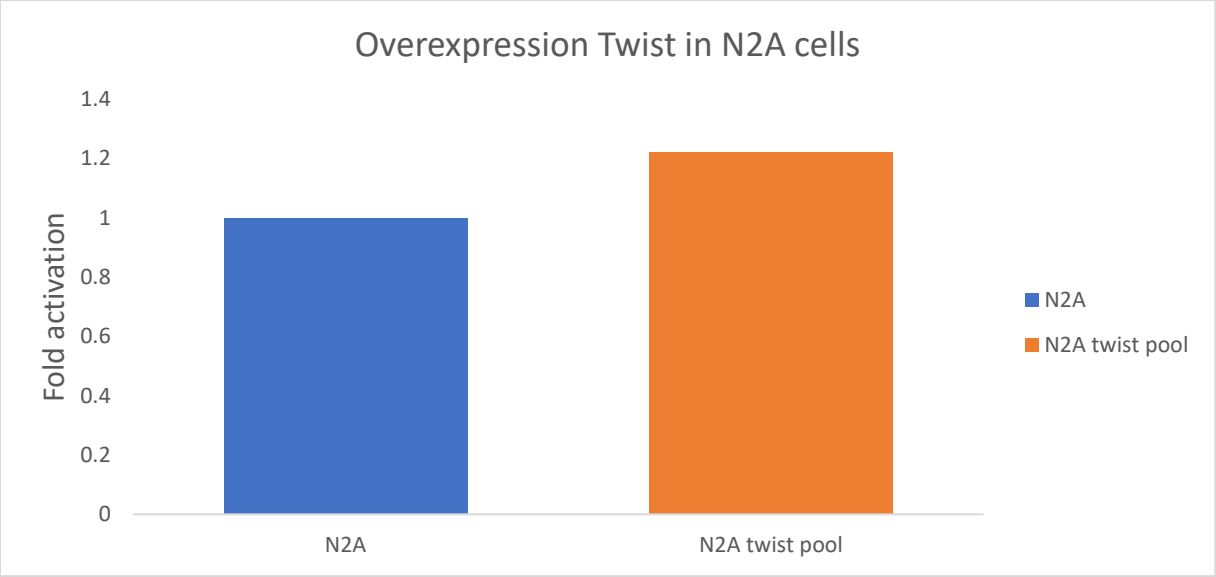

Figure 10: Over expression Twist in N2A cells. Twist1-transfectd Neuro2A cells (pooled and individual clones) were tested for mouse twist1 expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdhl evels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

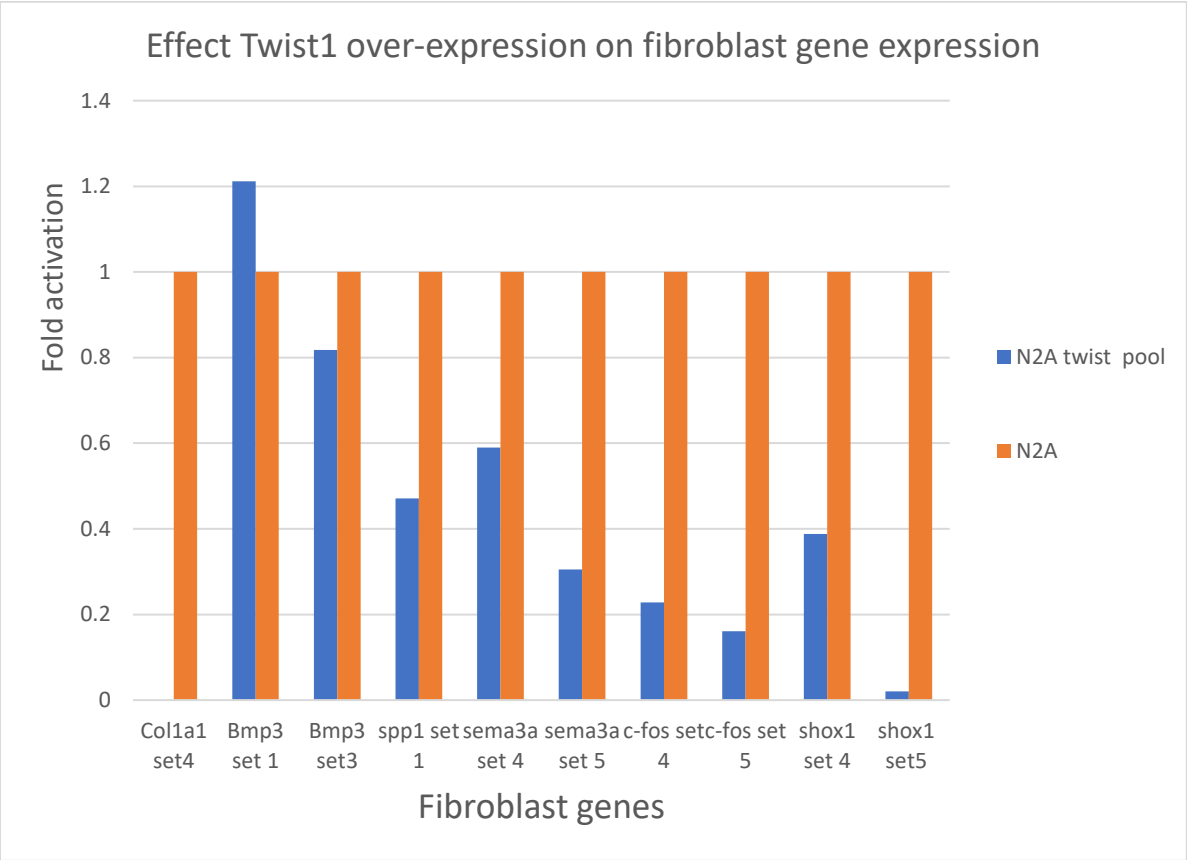

Figure 11. Effect of Twist1 over-expression on fibroblast gene. Twist1-transected N2A cells (pooled and individual clones) were tested for fibroblast gene expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial. and some (Cola1 and Shox1, with undetectable expression.

We asked if the N2A-twist cells exhibited repression of neuron -specific gene expression using the same gene panel as used on the Prrx1 and Snai2 experiments described above. Results from the pooled transfectants were highly variable, with expression of some genes (Dkk1, Foxd, and perhaps Syt6 and Phox2, depending on the primer set used) actually activated instead to repressed expression. Of those genes that were repressed, the level of repression was modest (Figure 12). Unlike the experiments with overexpression of Snai2 and Prrx1, clones were not tested, making it unlikely that strong repression effects would be observed

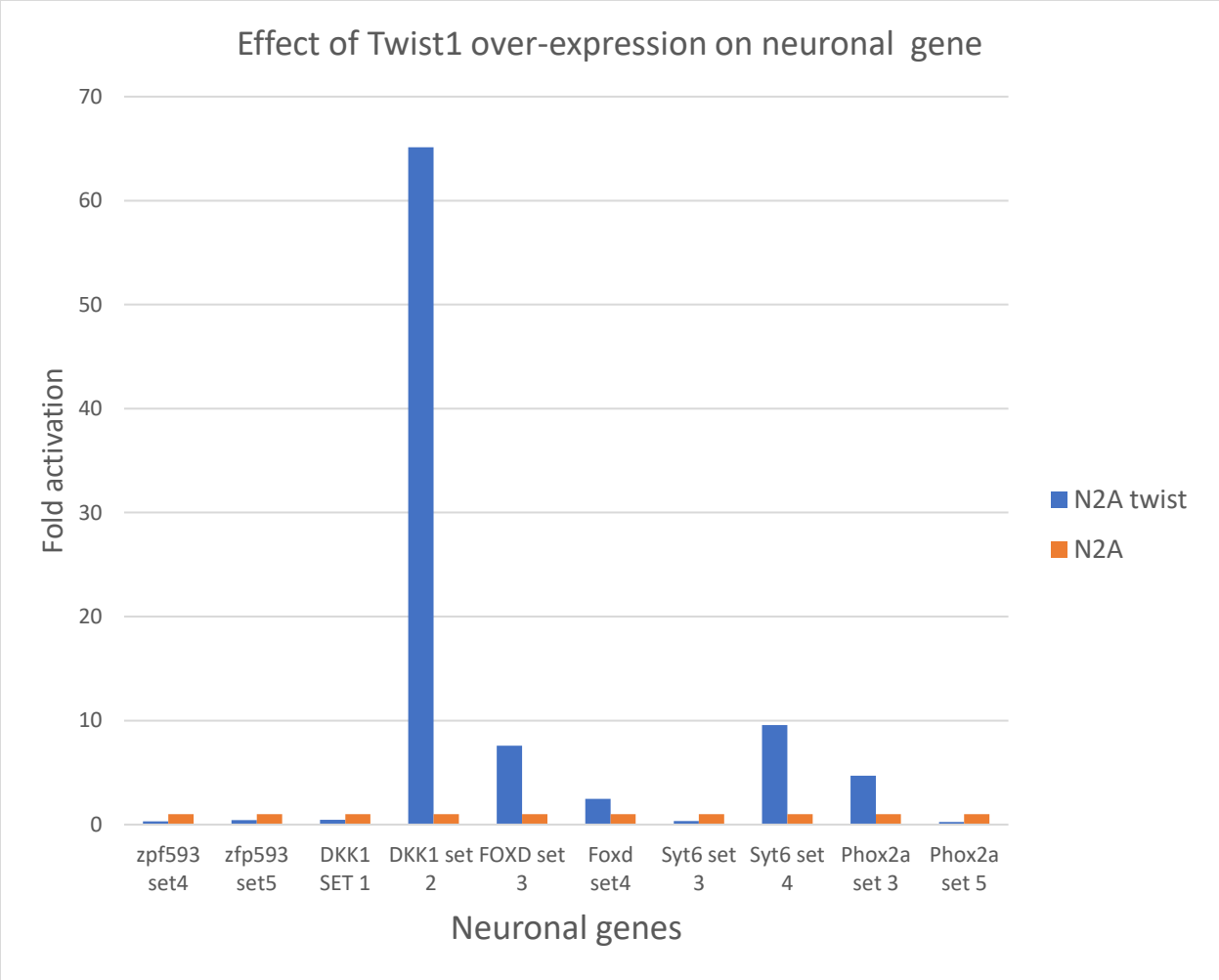

Figure 12. Effect of Twist1 over-expression on neuronal gene expression. Twist1-transected Neuro2A cells (pooled and individual clones) were tested for neuronal gene expression using qPCR of cDNA derived from the cells. The fold activation was normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 2 times, with triplicate reactions set for each trial.

Chapter 4

Discussion

In the past two decades, much progress has been made in our understanding of mammalian cell reprogramming. Most of these studies have dealt with reprogramming cells to an earlier developmental cell type and then coaxing them down specific lineages used cocktails of chemicals. For example, Marius Wernig and colleagues at Stanford University (Palo Alto,CA) described experiments showing that a cocktail of transcription factors (ascl1, brn2 and myt1) can convert mouse and human fibroblasts into functional neurons (Pang et al.; Vierbuchen et al.), and other transcription factors convert hepatocytes into neurons (Marroet al.). Importantly, the cells were found to retain their newly acquired phenotype even when the exogenous factors were inactivated suggesting that they were stably reprogrammed. These results show that it is possible to drive changes in cell states across germ layer barriers (mesoderm-ectoderm and endoderm-ectoderm).

In the current study, we asked whether certain transcription factors could reprogram cells into an alternate linage by the simple overexpression of single issue-specific transcription factors. The transcription factors chosen were those identified in our laboratory using a genome-wide screen for tissue specific transcription factors that were highly expressed in fibroblasts, poorly in hepatoma cells, and repressed in fibroblast x hepatoma somatic cell hybrids. Previous studies in our lab showed that overexpression of either of two of these genes, Snai2 and Prrx1, were found to be able to reprogram cell hybrids to a fibroblast phenotype. Furthermore, repression of these genes in fibroblasts followed by exposure to differentiation factors was found to reprogram these cells into other cell types, including chondrocytes and osteoblasts (Ray et al,). Subsequent studies in our laboratory showed that overexpression of these genes in a rat

hepatoma cell line led to repression of the hepatic phenotype and activation of the fibroblast phenotype based on gene expression analysis (Alzahrani) .

Here we asked whether these two genes, Snai2 and Prrx1, in addition to another candidate gene, Twist1, identified in the same genome-wide study, could reprogram neuronal cells. To that end, we introduced plasmids containing expression cassettes for these genes and monitored expression of a panel of both neuron- and fibroblast-specific genes in mouse Neuro2A neuronal. With Prrx1 and Snai2 experiments, both pooled transfectants and clones were monitored. For the N2A-twist cells, only a pool was analyzed due to technical difficulties.

Our results from the prrx1- transfected N2A cells (termed N2A-Prrx1) suggested that very little activation of fibroblast genes was detected. Of the six fibroblast-specific genes tested, only two (Cola1and Bmp3) genes were activated (albeit strongly, with >50-fold activation). The other genes tested actually showed a slightly reduced level of expression compared to the Neuro2A parental cells. Furthermore, this activation of Cola1and Bmp3 was noted in the N2A-Prrx1 clone analyzed but not in the pooled transfectants. Although this discrepancy could be due to the likely possibility that the pool contains clones with expression of the introduced Prrx1 expression plasmid ranging from high to undetectable, this seems unlikely since an average of high and low expressers should still result in a moderate level of average activation. It was also noted that the strong activation of the Bmp3 gene in the N2A-prrx1 clone was seen with one primer set but not the other. This may be due to known differential splicing of the Bmp3 gene such that the target sequences for primer set 3 were not in the final RNA product.

In contrast to the modest effects on fibroblast gene expression, neuronal gene expression was dramatically repressed. Each of the five genes tested were strongly repressed in the two

N2A-prrx1clones analyzed. Our results were very different depending on whether the clones or the pooled transfectants were examined. The N2A-prrx1 pooled cells showed modest activation or repression of a subset of the neuronal genes tested. In contrast, the two N2A-prrx1 clones analyzed showed strong repression of each of the neuronal genes.

The discrepancy in the above results comparing the pooled transfectants to the individual clones can be explained by the fact that we are observing gene repression rather than activation. In the latter case (activation), only a subset of clones needs to be expressing the transgenes to observe an average rise of target gene activation in pooled transfectants. However, in the case of gene repression, if even 50% the cells are overexpressing the transgene then the average level of target gene expression would be reduced by a maximum of 50% (or two-fold).

It was also noted that the N2A-prrx1 pooled cells had much higher levels of Prrx1 expression than that observed in the clones. This is may be due to the fact that the pooled cells had undergone many fewer cell division prior to harvesting. It is well known phenomenon that expression transgenes tend to decrease over cell passaging,

Similar results were found with the snai2- transfected Neuro2A cells (N2A-Snai2), showing, in the pooled transfectants, most fibroblast genes tested (Bmp3, Spp1, Sem3a) were repressed 4-8-fold and other genes tested (cFos and Shox) were repressed >50-fold. In the N2A-Snai2 clone tested (clone 2), all genes but Bmp3 were repressed, sometimes by as much as 50-fold. Therefore, rather than the expected activation of fibroblast genes, repression was the norm.

Analysis of neuronal gene expression in the N2A-Snai2 reflected those found in the N2A-prrx1 transfectants. The pooled clones showed activation of a few genes. However, the clone showed strong repression of most neuronal genes tested. This has led us to suggest that overexpression of Snai2, as with Prrx1, significantly depresses neural gene expression.

The results from Twist1 transfected Neuro2A cell (called N2A-twist) suggested that fibroblast-specific genes are only modestly affected, although c-Fos and Shox were repressed 5-45-fold. We found that reductions for c-Fos and Shox2 with each of the three transfected genes, which was not a general effect of overall gene expression caused by the introduction of a plasmid or the neo gene's presence as expression other genes were not affected or only modestly affected. The fact that a clonal cell was not tested prevents us from determining if Twist1 can suppress neuronal gene function.

Taken together, the above results suggest that overexpression of individual fibroblast – specific transcription factors is unable to activate fibroblast genes in the Neuro2A neuronal cells with some exceptions (such a Prrx1 activation of Bmp3 and Col1a1). In fact, a general trend of repressing expression of what is already low expression of these genes was observed. However, both Prrx1 and Snai2 overexpression were both found to strongly repress neuronal gene expression.

The question remains as to whether the observations made here suggest what could be considered reprogramming of cells. The loss of neuronal genes expression suggests that this might be the case. It would be worthwhile to conduct whole genome expression studies on N2A-pprx1 and N2A- snai2 transfectants to determine the extent of cellular reprogramming. It would also be important to conduct differentiation studies, as the Neuro2A cells are known to alter phenotype dramatically when serum starved and produce neurite outgrowth. It is predicted that neurite outgrowth would be truncated in the N2A-prrx1 and N2A-snai2 transfectants.

The importance of these findings comes from what has been accomplished to date. Previous studies have suggested that a reprogramming of fully differentiated mammalian cells required overexpression of a set of transcription factors. The results presented herein suggest that a single

factor can drive cellular identity. Such a phenomenon was observed by the ability of myoD to transform hepatoma cells to muscle lineage, although the phenotype was transient (Davis et al.).

Chapter 5

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