# NEURAL DIFFERENTIATION OF MESENCHYMAL STEM CELLS IS DEPENDENT ON THE NEURON RESTRICTIVE SILENCER FACTOR

**By**

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## ABSTRACT

## NEURAL DIFFERENTIATION OF MESENCHYMAL STEM CELLS IS DEPENDENT ON THE NEURON RESTRICTIVE SILENCER FACTOR

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Mesenchymal stem cells (MSCs) exist as an adult stem cell in major reservoirs primarily in the bone marrow and adipose tissue. Under normal physiologic conditions, MSCs serve mainly as the progenitor cell for adipocytes, chondrocytes, and osteocytes. The plasticity of MSCs has led researchers to investigate differentiation beyond their canonical lineages and since, in vitro studies have shown that MSCs can be induced to differentiate into renal cells, beta/islet cells, hepatocytes, cardiomyocytes, and even neurons. Differentiated MSCs exhibit changes in gene marker expression, morphology, and even gain functional characteristics. Previously, our lab has shown that neural-like characteristics can be induced in MSCs by exposure to the cyclic adenosine monophosphate (cAMP) elevating compounds, forskolin and isobutylmethylxanthine (IBMX). In addition to short-term neural-like morphology changes, MSCs gain expression of neural markers as well as sensitivity to dopamine. However, a molecular mechanism to explain why cAMP elevating compounds would have a proneural effect in MSCs is lacking.

Differentiation of stem cells into a mature phenotype is strongly driven by transcription factors within a cell. Some transcription factors control regulation of so many genes required for the mature differentiated cell type that they are termed master transcriptional regulators. For example, during osteogenesis, the master transcriptional regulator Runx2 is essential for differentiation of MSCs to osteocytes. Yang et al. demonstrated that silencing the master transcriptional regulator, NRSF, in MSCs could induce several neural characteristics. Therefore,

I hypothesized and went on to show that forskolin and IBMX could be driving neural-like differentiation of MSCs by regulating NRSF.

Neural differentiation of MSCs has also been studied from a tissue engineering perspective. In particular, it has been demonstrated in several types of stem cells that culture on very soft substrates can promote neural differentiation. This phenomenon shows that stem cell differentiation can also be influenced by physical characteristics in its environment. However, the molecular mechanisms explaining how cells can sense and respond to soft surfaces to affect differentiation are still vaguely characterized. We hypothesized that since soft surfaces induce neural-like differentiation in stem cells that maybe soft surfaces were somehow affecting NRSF. We go on to show that soft PDMS somehow affects NRSF within MSCs and that this is the main driver of neural-like differentiation from soft surfaces.

The aims of both projects show that neural differentiation in MSCs can be induced by both small molecules and the physical environment. Seemingly disparate stimuli are connected due to their ability to downregulate expression of NRSF. These studies highlight the role of transcription factors in determining stem cell fate and show that their modulation can even transdifferentiate cells across their germ line barriers.

**DEDICATED TO MY FRIENDS AND FAMILY**

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## KEY TO ABBREVIATIONS

ATP: adenosine triphosphate

AFM: atomic force microscopy

b-FGF: basic fibroblast growth factor

BMP: bone morphogenetic protein

BDNF: brain derived neurotrophic factor

B-TRCP: beta-transducin repeat containing e3 ubiquitin protein ligase

cAMP: cyclic adenosine monophosphate

COREST: co-repressor of expression silencing transcription

CREB: cAMP response element binding protein

DNA: deoxyribonucleic acid

DMEM: dulbecco's modified eagle medium

ESC: embryonic stem cell

ECM: extracellular matrix

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

FGF-2: fibroblast growth factor-2

FI: forskolin and IBMX

GFAP: glial fibrillary acidic protein

G9a: histone-lysine N-methyltransferase

HAT: histone acetyltransferase

HDAC: histone deacetylase

HGPS: Hutchinson-gilford progeria syndrome

IBMX: isobutylmethylxanthine

iPSC: induced pluripotent stem cell

kPa: kilo pascal

LEF: lymphoid enhancer-binding factor

LMNA: lamin A/C

LMX1a: LIM homeobox transcription factor 1-alpha

MAC: methylacrylamide chitosan

MASH1: mammalian achaete scute homolog-1

MAPK: mitogen activated protein kinase

MeCP2: methyl-CpG-binding protein 2

mRNA: messenger ribonucleic acid

MSC: mesenchymal stem cell

MYOD: myogenic differentiation

NA/K: sodium/potassium

NEUROD: neurogenic differentiation

NGF: nerve growth factor

NRSE: neuron restrictive silencer element

NRSF: neuron restrictive silencer factor

NSC: neural stem cell

NSE: neuron specific enolase

NT: neurotrophin

NURR1:  nuclear receptor related 1 protein

PDMS: polydimethylsiloxane

PDE: phosphodiesterase

PITX3: pituitary homeobox 3

PKA: protein kinase A

PPARγ: peroxisome-proliferator-activated receptor gamma

PSC: pluripotent stem cell

p-SMAD: phospho-SMAD

REST4: repressor of expression silencing transcription factor-4

RHO GTPase: ras homologous guanine triphosphate

RILP: rab-interacting lysosomal protein

RUNX2: runt-related transcription factor 2

SBE: smad binding element

SEM: standard error of the mean

shRNA: short hairpin ribonucleic acid

siRNA: small interfering ribonucleic acid

SMURF1: SMAD specific e3 ubiquitin protein ligase 1

SOX: Sry-related HMG box

TAZ: Tafazzin

TBR1/2: T-box brain 1/2

TCF: T-cell factor

TF: transcription factor

TH: tyrosine hydroxylase

TPH: tryptophan hydroxylase

TRK: tropomyosin receptor kinase

TUJ1: beta-III-tubulin

VMAT2: vesicular monoamine transporter 2

YAP: yes-associated protein

# CHAPTER 1. INRODUCTION

## 1.1 Origin of Mesenchymal Stem Cells

The discovery of Mesenchymal Stem Cells (MSCs) stemmed from 19th century studies of bone marrow transplantation[1] that established that certain elements within the marrow could grow bone "organoids," later named ossicles, when transplanted heterotopically into mice[2]. These ossicles were found to be miniature bone fragments that themselves contained marrow-like elements. However, due to the cellular heterogeneity of the bone marrow, the existence of the "bone" stem cell could not be pinpointed to a single cell. Though it was established that bone marrow contained some kind of bone regenerating potential, serious work in this field did not make many breakthroughs until the 60s[3]. Friedenstein et al. showed that certain stromal-like cells within the marrow could be separated from the hematopoietic cells of the marrow based to their ability to adhere to tissue culture plastic and form fibroblastic colonies. Transplantation of these cells into various animal models revealed their ability to differentiate into adipocytes, chondrocytes, and osteocytes[4]. However, again due to the heterogeneous nature of the stromal-like cells, it could not be ruled out that several species of progenitors were giving rise to separate differentiated cells or if it there was a single multipotential cell. Finally, work by Pittenger et al.[5] in the late 90s showed that from a single cell colony, it was possible to differentiate into adipocytes, chondrocytes, and osteocytes in vitro importantly demonstrating that MSCs had multipotent plasticity.

### 1.2 Native Physiologic Roles of MSCs

The canonical role of MSCs is to serve as the progenitor cell for adipocytes, chondrocytes, and osteocytes (Figure 1.1). Not surprisingly, the major niches that MSCs operate in are adipose

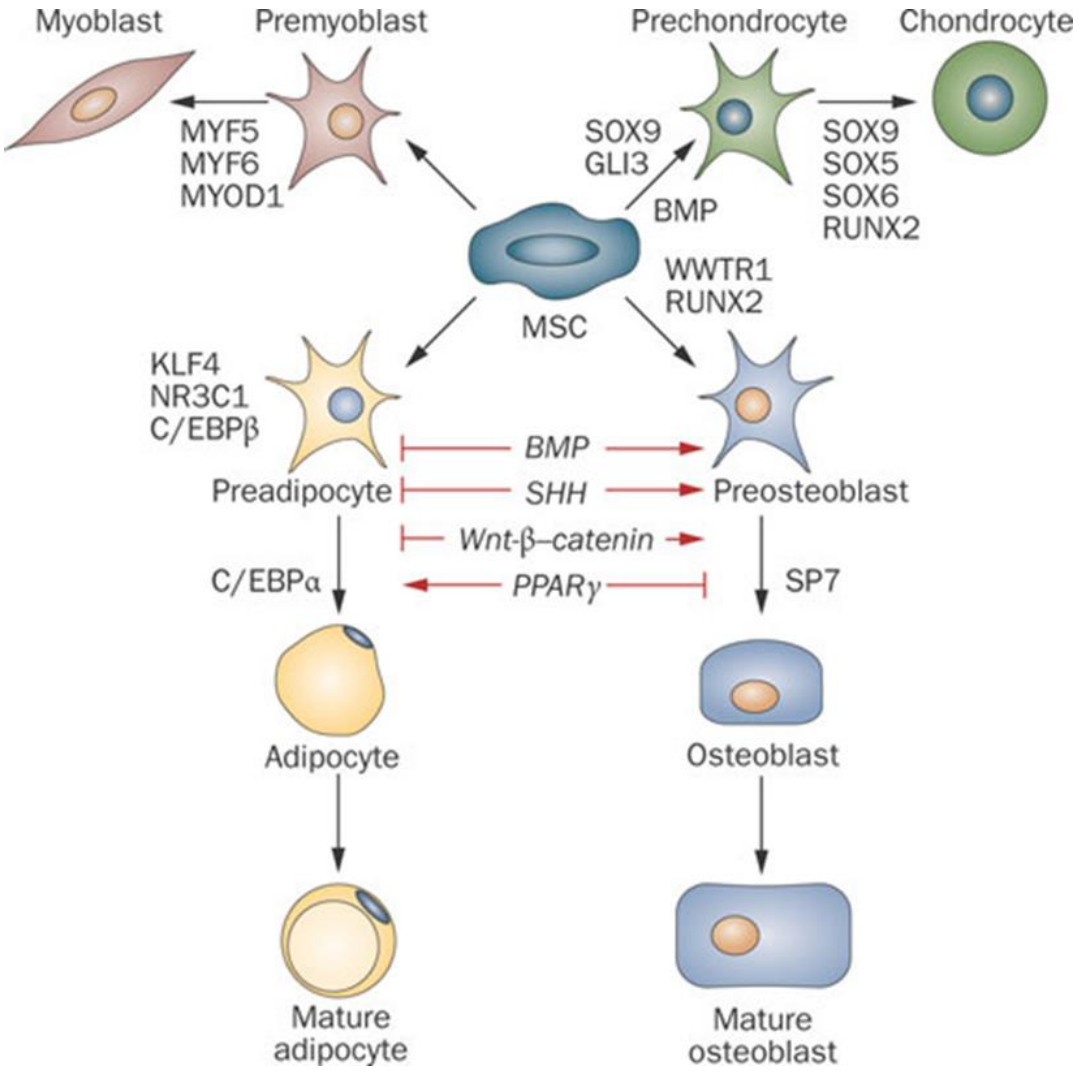


**Figure 1.1 MSCs are the progenitor for several mesodermal cell types.** These cell types are all of the mesodermal germ line. Takada, I., et al., Nat. Rev. Rheu. (2009) 8, 442-447.

tissue and bone marrow. Especially in bone marrow, MSCs have been shown to exert a strong paracrine effect on the environment secreting cytokines that promote the growth and

development of other cells and tissues. MSCs also have an immunoregulatory role and modulate the immune response[6-8]. Despite having tripotential differentiation, it appears that MSCs have priority function in the development and maintenance of bone. Most mutations that negatively affect MSC function are embryonic lethal but some mutations often result in organisms with bone softening and defects in skeletal formation[9]. However, not all defects in the MSC population affect bone. Interestingly, cell models of the premature aging disease, Hutchinson-Gilford Progeria syndrome (HGPS), show that the dominant mutation in the gene *LMNA* causes severe defects in mesenchymal lineages as well as depletion of the mesenchymal stem cell pool[10].

Due to their ease of isolation and proliferative nature, MSCs make for a convenient cell line for studying stem cells in vitro. The two main reservoirs of MSCs in mammals include the bone marrow as well as adipose tissue, which are relatively abundant sources. Other tissues, including tooth pulp[11], placenta[12, 13], isolations from the perivascular region[14], have given rise to MSC-like colonies that display multipotential plasticity. However, due to the limited yield from these sources they are not commonly used in lab settings. Nevertheless, isolation from the various tissues shows that MSCs probably play many undiscovered roles in several niches.

In addition to their plasticity, growth properties, and accessibility, MSCs are also an uncomplicated stem cell to handle in tissue culture compared against embryonic stem cells, induced pluripotent, hematopoietic stem cells, and neural stem cells. We rather easily harvest them from rat tibias and femurs post-euthanasia[15]. From there, the cells adhere to tissue culture polystyrene with no additional substrate required nor any other cell feeder layer. Cell medium is also very simple consisting of DMEM with 10% FBS. No other growth factors are needed to

sustain growth however, bFGF (FGF-2) is sometimes used by other labs to prevent senescence of the cells[16].

These properties of MSCs have also made them a suitable cell type for therapeutic uses. MSCs could potentially be isolated from a person, expanded and cultured *ex vivo*, then re-implanted therapeutically. MSCs also have specific advantages against other cell types. Firstly, MSCs do not carry the risk of teratoma formation that is seen in the transplantation of embryonic stem cells and induced pluripotent stem cells[17-19]. Second, transplantation of MSCs would not have to be matched. Since the donor of the cells is also the recipient, transplantation would be autologous[20], circumventing host rejection and the need for modulation of the immune system.

The highly plastic nature of MSCs has attracted the attention of much research especially in vitro work aimed at differentiation outside of the canonical lineages. There has been some success with MSCs gaining function of various other cell types including ectodermal and endodermal types. MSCs can be driven to differentiate into cardiomyocytes with exposure to 5-azacytine[21] and seem to improve vascular function in transplantation models[22]. MSCs have also been differentiated into insulin-producing islet cells that continue to secrete insulin upon transplantation[23]. MSCs can also be induced into hepatocytes upon exposure to hepatocyte growth factor[24]. Of particular interest is the study of neural differentiation in MSCs.

### 1.3 Neural-like Differentiation of MSCs

Stem cell replacement therapy has been theorized to be a treatment for many neurodegenerative diseases including Alzheimer's Disease, Parkinson’s Disease, and Amyotrophic Lateral Sclerosis. However, neural stem cells, especially human, are very hard to

obtain. Inducing neuronal differentiation from other cell sources has been a principal aim in stem cell biology.

Many labs have documented neural-like differentiation of MSCs[25-39]. Early studies often identified that upon induction, MSCs appeared to take on neural-like morphology. Others have shown induced expression of several neural markers and genes important for neural function. Of these, genes for neural morphology (TUJ1)[39], biosynthesis (TH)[27], growth (BDNF) and function (ion channels) are often screened for and detected. Somewhat more convincing is the ability for induced MSCs to acquire neural functions such as response to neurotransmitters[39] (communication) and generation of spontaneous Na+/K+ currents[40].

Despite these observations, there remains a large amount of skepticism about the extent to which neural differentiation can occur in MSCs[41-43]. Neural differentiation would indicate that transdifferentiation of MSCs across the mesoderm-ectoderm barrier is occurring, a process that has never been documented to happen under normal physiological conditions. Another confounding issue is that there is no standardization of methods used to induce neural differentiation resulting in the use of a wide array of growth factors, hormones, and small molecules to induce differentiation. Few studies investigate the underlying cell signaling changes and this has brought up the counterpoint that MSCs, under certain conditions, can be selectively induced to express neural-like characteristics without undergoing true differentiation.

However, it should not be ignored that MSCs can gain neural functions. Even if MSCs cannot undergo neural differentiation, their ability to selectively gain neuronal function can still be used therapeutically[44]. Transplantation of MSCs induced to secrete dopamine had a therapeutic effect in macaque-based Parkinson's Disease model[45]. Inducing MSCs to control

their microenvironment through the secretion of neurotrophic factors has also shown improvement in rodent models of Parkinson's[46].

### 1.4 Forskolin and IBMX Induce Neural-Like Differentiation by Activating CREB

Since there are so many different methods for inducing neural differentiation in MSCs, it is important to study the molecular mechanisms that are engaged and how they result in gene expression changes that culminate in cell lineage change. Previously, work in our lab by Linxia Zhang investigated neural differentiation of MSCs by inducing the cAMP pathway[39]. cAMP has an important role in neuronal differentiation[47-52] and it was hypothesized that it could induce neuronal differentiation in MSCs. To this end, the cAMP-elevating small molecules forskolin and IBMX [FI] were used to cause a large increase in intracellular concentrations of cAMP. Forskolin is a potent inducer of the enzyme, adenylate cyclase[53], that catalyzes the cyclization of ATP to cAMP while IBXM is a potent inhibitor of phosphodiesterases[54] that break the cAMP ring. Large increases in the concentration of cAMP activate the kinase, PKA. PKA then goes on to phosphorylate and activate the transcription factor, CREB[55, 56] (Figure 1.2), which in turn was hypothesized to mediate the neural inducing effect of FI by activating the expression of neural genes[39]. The weakness of this hypothesis is that, while CREB is certainly known to induce neural gene expression, it also controls the expression of hundreds of non-neural genes[57],

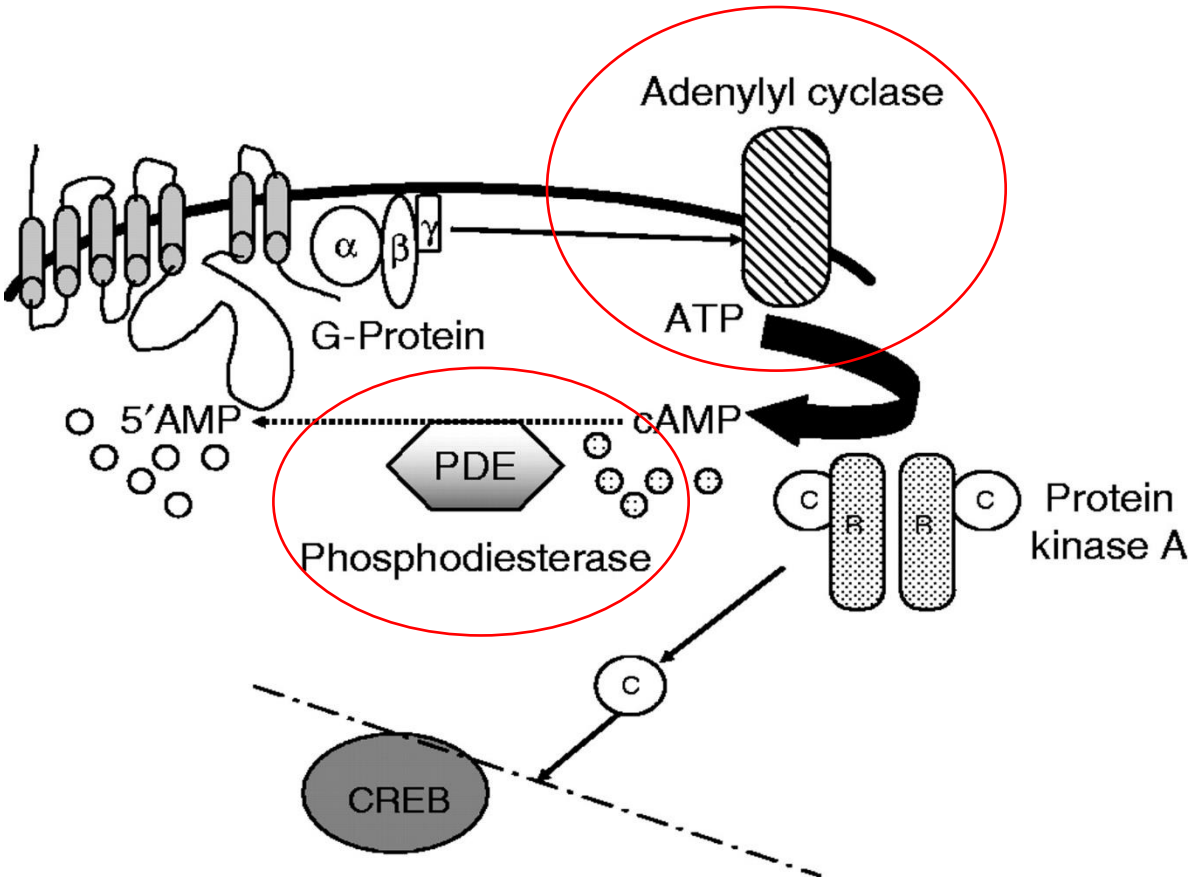


**Figure 1.2 cAMP activation of CREB**. Vezzosi and Bertherat. Euro. J. Endocrinology (2011) 2, 177-188.

especially in an MSC background. Additionally, activation of CREB provides no explanation for why MSCs could differentiate across the mesoderm-ectoderm germline barrier.

### 1.5 Soft Surfaces and Neural Differentiation

Neural differentiation often solely focuses on soluble factors. However, in the field of biomedical engineering, physical cues from the environment and their effects on cell growth and differentiation are frequently studied. One of the more interesting phenomena documented is that cells can sense the stiffness of the physical environment. The stiffness of the substrate can

influence differentiation of the stem cell. Discher et al. reported that culturing MSCs on very soft surfaces promoted neural gene expression as well as neural morphology change[58]. Specifically, MSCs grown on polyacrylamide gels with a surface modulus of <1 kPa, reflecting that of brain tissue, increased expression of neural markers including microtubule-associated protein 2 and neural structural proteins such as β-III tubulin (TUJ1) and neurofilament. Since then, others have shown that soft surfaces have a neural-inducing effect on several types of stem cells including ESCs[59], iPSCs[60, 61], and adult NSCs[59, 62].

More importantly, soft surfaces have been shown to increase neuronal function. Keung et al. reported that soft ECM promotes dopaminergic differentiation of human PSCs (hPSCs) as assayed by the expression of the dopaminergic marker and dopamine biosynthetic enzyme, tyrosine hydroxylase[61]. Application of this finding could specifically lead to use of soft surfaces to enhance dopaminergic neuron studies. Similarly, soft surfaces were recently used to promote the generation of motor neurons from PSCs improving overall yield and specificity and shortening the generation time[60]. Importantly, soft surfaces assisted in the development of neuronal morphology and improved the cell's ability to generate action potentials. Given that generation of subtypes of neurons is a tedious and inefficient process, soft surfaces could be applied to promote in vitro culture of all neural cells.

Very soft surfaces (<1 kPa) appear to have a neural-inducing effect on stem cells, but to date, it remains unclear the extent to which the cells mature. Current work shows that hESCs and hPSCs respond to soft surfaces by differentiating into functional neurons; however, while very soft surfaces have a neural-inducing effect on NSCs and MSCs, it is unclear if soft surfaces alone can drive neural differentiation in these cell types to fully functional neurons. Indeed, Keung et al. found that very soft surfaces promoted expression of pan-neuronal markers including TUJ1,

but did not promote differentiation of neuronal subtypes suggesting that only early neuronal induction was occurring.

Currently, research regarding the cellular mechanisms that respond to soft surfaces is superficial. While some work has implicated YAP/TAZ[60] and BMP-SMAD[63] signaling, how these changes affect stem cell differentiation are poorly characterized.

### 1.6 Transcription Factors and Reprogramming

Despite CREB being a weak explanation, the overall idea that FI could be affecting a transcription factor to drive neural-like differentiation is an important end point when characterizing these mechanisms. Transcription factors can control entire programs of gene expression that are specific to cell types. Often in the normal physiological development of a cell, activation of terminal transcription factors complete cell differentiation by expressing genes specific for the mature phenotype. Transcription factors are also of particular interest when specifying cell fate because their exogenous expression, or repression, can directly convert cells from one type to another, even across germline barriers.

Exogenous expression of transcription factors to convert cells from one type to another began in the late 80's when MyoD was used to convert fibroblasts to muscle cells[64]. Since then, several transcription factors have been identified that can be used to convert cells from one differentiated type to another (Figure 1.3). This line of research reached a pinnacle when Yamanaka et al. used exogenous expression of transcription factors to reprogram fibroblasts into induced pluripotent stem cells[65].

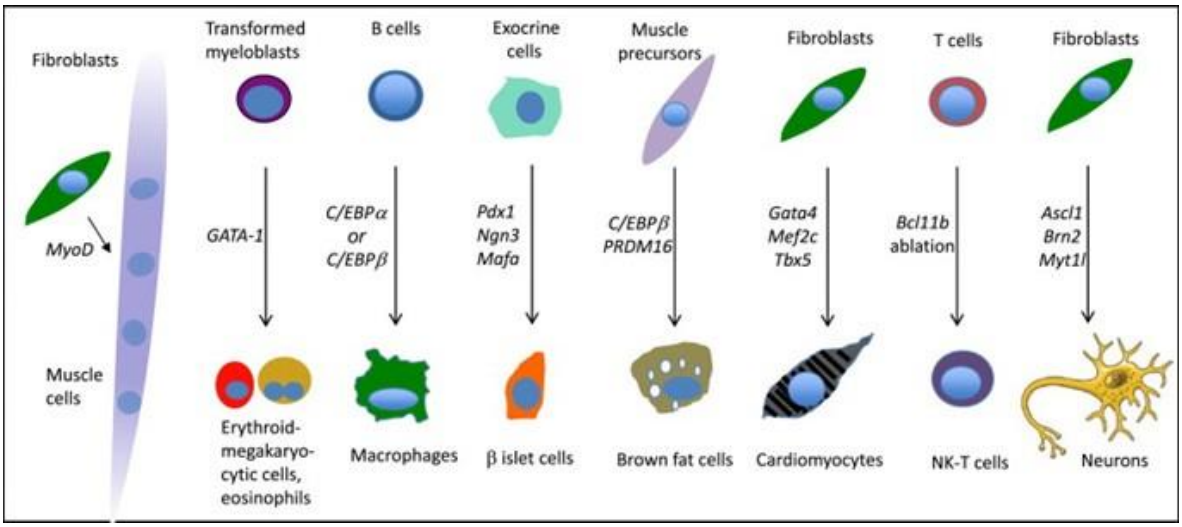


**Figure 1.3 Transdifferentiation using exogenous expression of transcription factors.** Cells reprogrammed in this way can even cross germline barriers

Transcription factors that can reprogram cells from one type to another have the ability to do so because they are often master transcriptional regulators[66]. MyoD, is considered a master transcriptional regulator because it controls expression of many genes responsible for myogenesis. In MSCs, osteogenesis is largely controlled by the master transcriptional regulator, runx2[67], while chondrogenesis is dependent on sox9[68]. PPARγ is agreed to be the master regulator of adipogenesis[69]. Many cell types have had their master transcriptional regulators identified to date.

In the case of neural cells, master transcriptional regulators have been harder to identify, presumably because of the variety of the types of neurons. In the cases of reprogramming to neuronal cells, several transcription factors are often used simultaneously. In the conversion of MSCs to dopaminergic neurons, Caiazzo used exogenous expression of mash1, nurr1, and lmx1a in combination[70]. NeuroD and its family of transcription factors represents a potential master regulator for neuronal cells[71], however, it does not appear to induce a specific type of cell,

instead, some sort of early stage neural-like cell. While many master transcriptional regulators act as gene activators, there also exist master transcriptional regulators that act as gene repressors. Interestingly, disrupting master transcriptional repressors can also induce "differentiation" through gene silencing or ablation. Case in point, the Neuron Restrictive Silencer Factor, NRSF from here onward, directly opposes neural gene expression especially in non-neural tissue. Yang et al. silenced NRSF with shRNA in MSCs to induce neural differentiation[40]. By silencing one transcription factor, this research group was able to induce pan-neural effects including gene expression, morphology changes, and gain of spontaneous generation of Na+/K+ currents.

### 1.7 The Function and Roles of NRSF

NRSF is a master transcriptional repressor that is ubiquitously expressed in NSCs as well as in non-neural tissue where it prevents neural differentiation and gene expression. NRSF was first identified to be a transcriptional repressor of the type II sodium channel gene[72]. After this discovery, it was clear that NRSF was a coordinate repressor for several neural genes[73-75] and was able to restrict neural gene expression to neurons. During normal neuronal development, NRSF is temporarily downregulated to allow for expression of neural genes to complete neuronal maturation[76, 77]. NRSF has distinct regions that mediate its function[78] (Figure 1.4). Through its DNA-binding domain, NRSF binds to a conserved 21-bp neuron restrictive silencer element (NRSE)[79] that is often found on the promoters of neural genes. The DNA binding and sequence specificity of NRSF is conferred by multiple zinc finger domains in the DNA binding domain. The N-terminal domain of NRSF recruits HDACs as well as the mSin3 co-repressor[80]. Additionally, the C-terminal domain indirectly recruits HDACs through binding of its co-

repressor, CoREST[81]. The CoREST can form a complex with HDACs as well as the methyl-DNA recognition co-repressor, meCP2. Additionally, NRSF can recruit the histone methyltransferase G9a by forming a complex with the chromodomain containing protein CDYL[82].

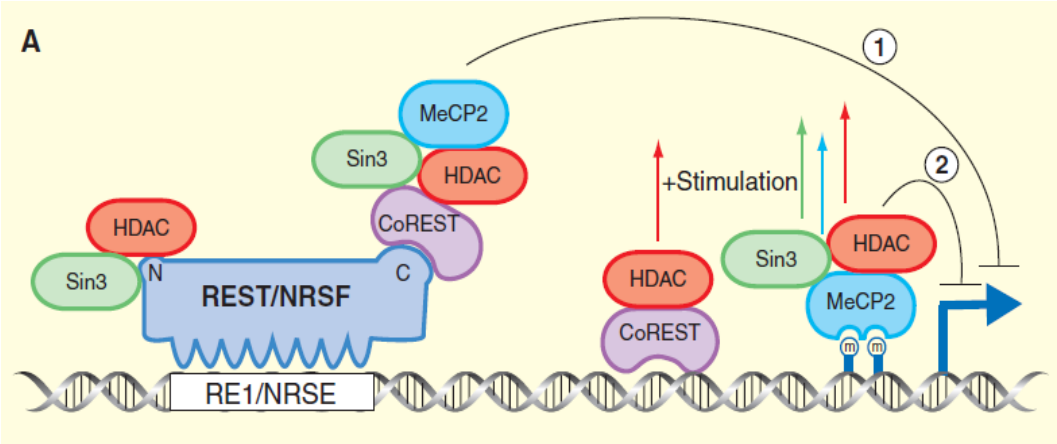


**Figure 1.4 Diagram of NRSF and how its domains mediate its functions.** Coulson, J. Current Biology. (2005) 15, 665-668.

The activity and expression of NRSF is also regulated very tightly. Since NRSF is a transcription factor, its localization to the nucleus is important for its function. A key regulator of nuclear import includes the REST-interacting LIM domain protein, otherwise known as RILP[83-85]. RILP interacts with a nuclear localization signal in the 5$^{th}$ zinc finger domain of NRSF[83] and helps the import of NRSF into the nucleus. Dysregulation of RILP has been associated with progressive myoclonus epilepsy (PME), a type of familial epilepsy[86].

NRSF is subjected to several spliced isoforms. However, of these only REST4 directly interferes with its activity. REST4 is C-terminal truncated form of NRSF and cannot recruit

coREST. Because it can still bind to NRSEs, REST4 acts in a dominant interfering manner on NRSF.

NRSF has been implicated in several neural diseases. In neuropathic pain, inflammation induced upregulation of NRSF may contribute to repression of potassium channel expression[87, 88] that may contribute to an underlying mechanism. NRSF dysregulation of sodium channel expression may also play a role in neuropathic pain specifically in dorsal root ganglion[89]. NRSF activity has been shown to be dysregulated in the neurodegenerative disease, Huntington's Disease[90, 91]. In the non-disease state, the protein, huntingtin, sequesters NRSF in the cytoplasm allowing the expression of neuronal genes including the important neurotrophin, brain-derived neurotrophic factor (BDNF). However, mutant huntingtin protein no longer sequesters NRSF in the cytoplasm, causing NRSF to accumulate in the nucleus where it inappropriately represses neuronal gene expression including BDNF. Downregulation of BDNF is proposed to contribute to the neurodegenerative state of Huntington's disease.

NRSF has also gained attention in brain cancers including glioblastoma[92, 93], neuroblastoma[94, 95], and medullablastoma[96]. Surprisingly, NRSF is upregulated in these cancers and has taken on an oncogenic role, although a mechanism explaining the cancer promoting effects of NRSF has not been established. However, NRSF's role in promoting "stemness" in NSCs, suggests that NRSF could be supporting the proliferative nature of brain cancers though it isn't necessarily a driver of oncogenesis itself[95]. Recently, it was found that siRNA-mediated downregulation of NRSF in glioblastoma cells strongly reduced their proliferation ability as well as their ability to initiate tumor formation in mice[92]. Interestingly, this method of treating glioblastoma is not necessarily lethal but elicits its effect by attacking the stem cell nature of a small population of glioblastoma cells by forcing their differentiation.

## 1.8 Hypothesis

It is clear that master transcriptional regulators play an important role in determining cell phenotype and contribute powerfully to the normal homeostasis of a cell. It is also plausible to think that molecular signaling converges on expression (or repression) of master transcriptional regulators turning them into coincidence detectors for cell fate decisions. Since NRSF coordinates so many genes specific to neurons, it is easy to see how pan-neural effects can be induced through one regulator. Therefore, when it comes to characterizing neural differentiation, especially in vitro, it is essential to understand what is happening to the activity of transcription factors, in particular NRSF.

## CHAPTER 2. FORSKOLIN AND IBMX INDUCE NEURAL-LIKE DIFFERENTIATION OF MESENCHYMAL STEM CELLS THROUGH DOWNREGULATION OF NRSF

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### 2.1 Abstract

Mesenchymal stem cells (MSCs) are multipotent adult stem cells exhibiting a high proliferation rate and plasticity as compared to other adult stem cell lines. MSCs naturally serve as precursors for adipocytes, chondrocytes, and osteocytes, and provide trophic support for cells of the immune system in the bone marrow. In vitro experiments have shown that MSCs can be induced to gain characteristics of neural cells including generation of Na+K+ currents, expression of neural specific structural proteins, and exhibition of neuronal morphology upon induction. Previously, we demonstrated that the cAMP-elevating agents, forskolin and IBMX, induced neural-like differentiation of MSCs, including expression of neural markers and increased sensitivity to neurotransmitters. However, due to the broad range of effects that forskolin and IBMX can elicit through the intracellular second messenger, cAMP, a better mechanistic understanding is required. Here, we show that neural induction by forskolin and IBMX is dependent on downregulation of expression of the master transcriptional regulator, NRSF, and its downstream target genes. Since silencing of NRSF is known to initiate neural differentiation, it suggests that forskolin and IBMX result in transdifferentiation of MSCs into a neural lineage.

## 2.2 Introduction

Mesenchymal stem cells (MSCs) constitute part of the adult stem cell niche serving mainly as the progenitor cell for adipocytes, chondrocytes, and osteocytes[5]. In vitro, they have been induced into renal, hepatocytic, cardiac, and pancreatic cells[21, 97-100]. However, research from several labs has demonstrated that MSCs can differentiate beyond these canonical lineages. Combined with their relative ease of attainment from bone marrow and adipose tissue[5, 101, 102] and their high proliferation rate, MSCs are an excellent candidate for cell replacement therapies. Autologous grafting of MSCs remains a principal objective of the field and clinical trials have already demonstrated their safety[99, 103].

Among the potential conditions that MSCs aspire to treat, neurodegenerative diseases including Parkinson's Disease attract a considerable amount of attention. Interestingly, MSCs can be induced to express neural markers and function[26-28, 33, 39] Numerous laboratories have been able to force MSCs to express neural characteristics including neural gene expression, neurotransmitter biochemistry, morphology, and electrophysiology. Additionally, MSCs have a lower risk of post-implantation tumorigenesis as compared to embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

Neural differentiation of MSCs is a controversial topic in stem cell biology because it requires transdifferentiation across the mesoderm-ectoderm barrier, an unnatural phenomenon. Additionally, several studies have questioned the extent to which MSCs "differentiate" into neurons[41, 104-106]. Nevertheless, these doubts do not nullify observations that MSCs can acquire genuine neural characteristics. Thus, how and why MSCs would have neural potential becomes an interesting question.

Previously, our laboratory showed that forskolin and IBMX (FI), chemical agents that increase intracellular concentrations of the second messenger, cyclic adenosine monophosphate (cAMP), could induce neural differentiation of MSCs including expression of neural markers, a change in cell morphology, and increased sensitivity to the neurotransmitter dopamine[39] While cAMP is known to play a role in neural differentiation of MSCs[25, 29, 107], how it induces differentiation of MSCs is unclear. Rises in intracellular levels of cAMP canonically signal through protein kinases to activate the transcription factor CREB. We originally hypothesized that FI activation of CREB drove expression of neural genes that accounted for the phenotypic changes in the MSCs. However, CREB is involved in the development of tissues derived from the endoderm, ectoderm, and mesoderm and because of its pleiotropic nature it is unclear why FI treatment would favor neural differentiation.

Transcription factors are critical for specifying cell lineage. Indeed, reprogramming cells with forced expression of transcription factors can transdifferentiate cells from one lineage to another, even across the germ lineage barrier[64, 65, 70]. By exogenously expressing transcription factors, cells can be directly differentiated or even reprogrammed across germ line barriers[65]. To better understand neural induction of MSCs with FI we asked if FI could be affecting neural-specific transcription factors. Previously, Yang et al. demonstrated that knockdown of the master transcriptional repressor of the neural phenotype, the neuron restrictive silencer factor (NRSF), induces neural gene expression, gain of neuronal morphology, and causes the cells to generate spontaneous action potentials[40]. NRSF is a transcriptional repressor that is ubiquitously expressed in NSCs as well as in non-neural tissue. NRSF binds to a conserved 21-bp neuron restrictive silencer element that is often found on the promoters of neural genes where it then recruits histone deacetlyases and DNA methylases to turn off gene expression[78] . NRSF is

expressed in MSCs where its importance in keeping the neuronal phenotype shut off is demonstrated in silencing experiments. Silencing of NRSF alone results in BMSCs that spontaneously fire Na+ currents, a distinct gain of neuronal morphology, and expression of a variety of neural genes including BDNF and NSE[40] . Because of the importance of NRSF in neural differentiation, we questioned whether FI-induced differentiation affects NRSF expression to promote neural differentiation in MSCs.

Given that both FI as well as knockdown of NRSF in MSCs cause neural differentiation, we hypothesize that FI had a regulatory effect on NRSF. We report that FI downregulates expression of NRSF and that this event is responsible for the expression of neuronal genes and for the increase in sensitivity to neurotransmitters in MSCs. Knockdown of NRSF recapitulates the changes observed during FI induced differentiation and overexpression of NRSF is able to block expression of neuronal genes in FI-treated MSCs. We propose that the mechanism behind FI induced neural differentiation of stem cells requires the downregulation of NRSF

## 2.3 Materials and Methods

### 2.3.1 Materials

For detailed information on materials, antibodies, and primer sequences see tables 1, 2, and 3 in the supplemental figures section.

### 2.3.2 Mesenchymal stem cell culture and isolation

MSCs were isolated from animals using procedures approved by the Institutional Animal Care and Use Committee at Michigan State University. MSCs were derived from bone marrow isolated from 4 to 6 week-old Sprague-Dawley female rat as previously described10. Femurs and

tibias were removed from 4 to 6-week-old rats. The two ends of the bone were cut open and the marrow was flushed with 10mL of DMEM using a 25g needle and syringe. The cell suspension was filtered through a 70-um nylon mesh to remove bone debris and blood aggregates. Cells were cultured in low glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and free from antibiotics. Cells were incubated in a humidified atmosphere containing 5% CO2 at 37C. Non-adherent cells from the flushed marrow were removed after 48h after isolation. Media was replaced every 3 days until the cells reached 80–90% confluence. Confluent cells were detached by 0.25% trypsin–EDTA (Invitrogen) and plated for further experiments. Neural differentiation was induced by culturing MSCs in the presence of growth media supplemented with 10uM forskolin (Sigma) and 100uM isobutylmethylxanthine (IBMX) (Sigma) for up to 5 days.

### 2.3.3 Cell Transformation

Plasmid complexes were prepared in Opti-MEM (Gibco) with 1ug of plasmid, 1 uL of P3000 reagent, and 1uL of Lipofectamine 3000 (Invitrogen) per sample. 10^6 cells/mL were reverse transfected with prepared plasmid complexes growth medium free of antibiotics. After 16h, the medium was replaced and the cells were grown for another 24h before sample collection or treatment. For silencing experiments, siRNA was complexed with Lipofectamine 3000 in Opti-MEM. 10^6 cells /mL were reverse transfected with siRNA (0-50nM) for 16h. Afterwards, medium was refreshed and the cells were cultured for an additional 48h before sample collection or assays.

### 2.3.4 Western Blotting

Whole-cell extracts were prepared by lysing cells with RIPA buffer (50mM Tris pH 8.0, 150 mM NaCl, 1% IGEPAL (NP-40), 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate) on ice for 30 min. Nuclear fractions were prepared by swelling cells in a hypotonic buffer (10mM HEPES (pH 8.0), 1.5 mM MgCl2, 10 mM KCl) on ice then lysing with a dounce homogenizer. The nuclei were spun down and incubated on ice in a high salt buffer (20 mM HEPES pH 8.0, 1.5 mM MgCl2, 420 mM NaCl, 25% glycerol) to extract the protein. Lysates were mixed with 5X SDS protein loading buffer (50mM Tris pH 7.0, 25% glycerol, 2% SDS, 0.025% bromophenol blue) and denatured at 95C for 5 min. 20ug of each sample lysate was separated by electrophoresis on an 8% Tris–HCl gel and transferred to a nitrocellulose membrane. Membranes were then blocked in 5% milk and 0.05% Tween 20–TBS (Tris buffered saline) for 1 h and incubated with primary antibodies against tyrosine hydroxylase or GAPDH (Cell Signaling) or NRSF/REST (Millipore) overnight at 4C. Anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Thermo Scientific) was added the second day after primary antibody incubation. The blots were incubated for 90min and then washed three times with 0.05% Tween 20–TBS. The blots were then visualized by SuperSignal west femto maximum sensitivity substrate (Thermo Scientific). Image of full blots are included in the supplemental figures section.

### 2.3.5 Real Time PCR

mRNA samples were prepared with the RNeasy Mini Kit (Qiagen). mRNA was then reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real Time PCR was used to quantify gene expression for *TH*, *TUJ1*, *NSE*, *DRD1*,

*DRD5*. Primer sequences are provided in Table 128, 29. cDNA from samples was mixed with iQ Sybr Green Supermix (BioRad) and run on a MyIQ single detection Thermal Cycler. Data was transformed using the DDct method.

### 2.3.6 NRSF Subcloning

pHR'-NRSF-CITE-GFP was a gift from Jay Nadeau (Addgene plasmid # 21310). NRSF was cloned out using PCR and the resulting fragment was cloned into a pCMV-Myc-N plasmid (Clonetech). Overexpression of NRSF was confirmed with western blotting against NRSF as well as the myc tag (Supplemental Figure 2.1).

### 2.3.7 Calcium imaging

Calcium imaging was performed according to the protocol by Tropel et al.[28] and modified by Zhang[39]. Cells were cultured in four-well chambered cover-glass (Lab-Tek) coated with poly-L-lysine (Cultrex). After neural induction with FI or transformation with lipocomplexes, the cells were stained with 4 uM Fluo-4 (Invitrogen) in ACSF–HEPES (artificial cerebral spinal fluid with HEPES: 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl2, 2.5 mM CaCl2, 1 mM NaH2PO4, 26.2 mM NaHCO3, 11 mM dextrose, 10 mM HEPES, pH = 7.4) for 30 min at 37C. Excess dye was removed by washing cells with PBS twice and placing into a 37C chamber on the stage of Olympus FluoView 1000. Then, 0.5 ml ACSF–HEPES was added to the well to begin imaging. Images were captured every 1.137 s and fluorescence intensity is represented by a spectral table (warmer colors represent higher intensity whereas cooler colors represent lower intensity). After 15–20 images, 0.5 ml ACSF–HEPES buffer containing the following neurotransmitters were added: 200 uM glutamate (final concentration 100 uM), 200 uM

dopamine (final concentration 100 uM), or 200 uM ATP (final concentration 100 uM). A total of 200–300 images were recorded and the data was analyzed by the FluoView 100 software. Changes in the fluorescence intensity of the Ca2+signal are represented as F/F0. The percent of responsive cells is calculated as the number of cells with a F/F0 signal greater than 20% of the total number of cells.

### 2.3.8 Statistical Analysis

Gene expression data were determined as statistically significant by Tukey's Test following one way ANOVA for groups with multiple means. For experiments comparing two samples a student's t-test was performed. Results were presented as the average of the data set +/– the SEM (standard error of the mean). Similarly, statistical significance of calcium release quantification is also represented as the average of the data set +/– the SEM.

## 2.4 Results

### 2.4.1 FI Causes Downregulation of NRSF in MSCs

We found that inducing neural differentiation in MSCs with FI strongly downregulated NRSF protein expression after 24h, which continued over the 5 day treatment course (Figure 2.1a). Concomitantly, NRSF expression in the nuclear fraction was strongly downregulated in the FI-treated MSCs as compared with the controls (Figure 2.1b). Thus, FI has a strong effect on the protein expression and localization of NRSF.

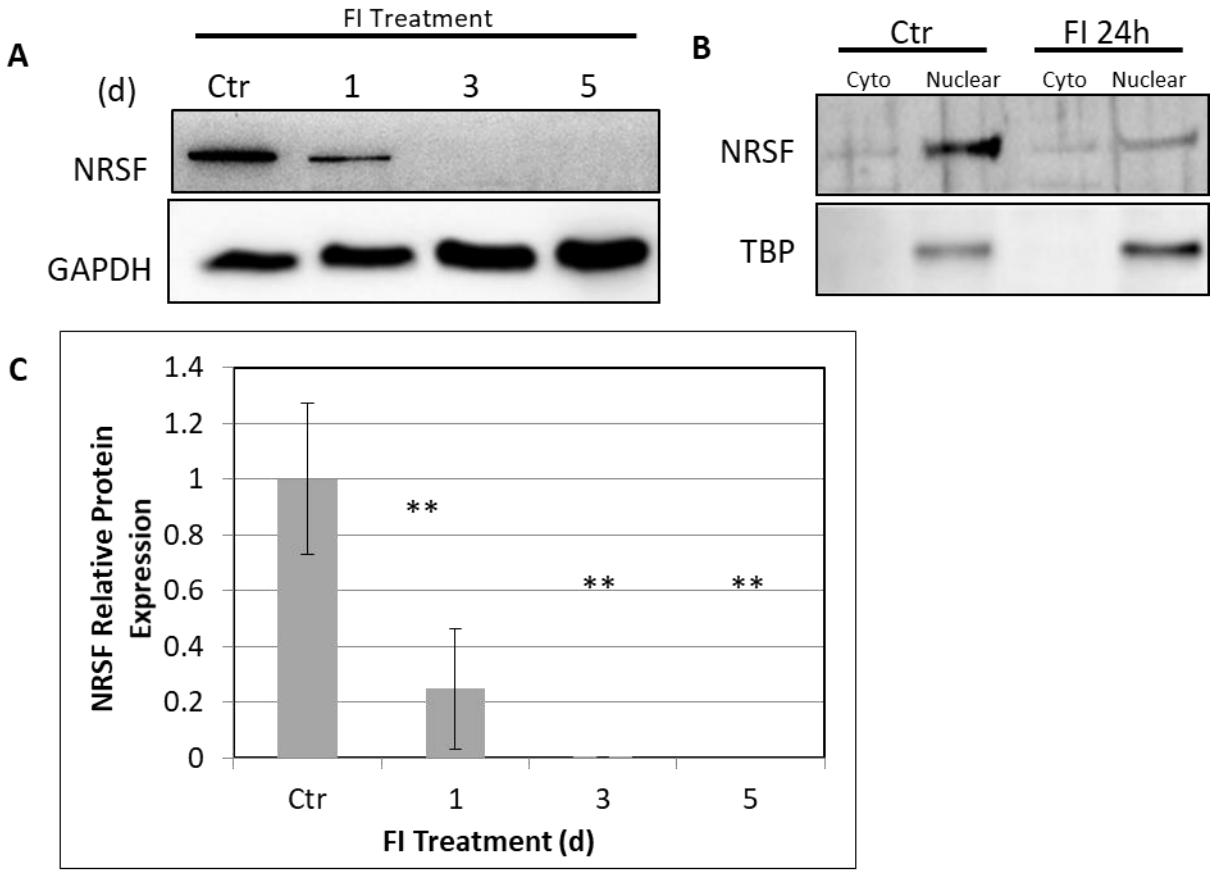



**Figure 2.1. FI downregulates NRSF protein in MSCs.**
**A.** Treatment of MSCs with FI over 5 days shows a marked reduction in NRSF protein expression. Full length blots are included in supplementary figure 2. **B**. This effect occurs within 24h. **C.** NRSF is also reduced in the nuclear fraction. **$p < 0.01$ as compared to control using Tukey's test following one-way ANOVA with N=3.

### 2.4.2 FI Treatment Induces Gene Expression of Several NRSF Repressed Target Genes

Given that FI strongly downregulates NRSF, we determined if FI caused an increase in the expression of known NRSF-repressed genes. TUJ1 and nse are well characterized genes, regulated by NRSF, that are commonly used as neural markers. Since FI-induced MSCs were previously shown to express dopamine sensitivity[15], we assayed for tyrosine hydroxylase (th) expression. th is the rate-limiting enzyme for dopamine synthesis that is specific to dopamine producing neurons and is known to be repressed by NRSF[108]. The gene expression levels of th,

TUJ1 and nse increased 24 hours after FI treatment reflecting the corresponding decrease in NRSF protein expression. This continued through the three days of treatment (Figure 2.2a), suggesting a relationship between FI and NRSF repression of gene expression. Since we previously demonstrated that FI induced dopamine sensitivity in MSCs we investigated changes in gene expression that could explain this gain of dopaminergic function. We measured the expression of dopamine receptor genes (*drd*). Notably FI induced expression of drd1 and drd5 (Figure 2.2b) as well as several common markers of dopaminergic differentiation (Supplemental Fig. 2.2).

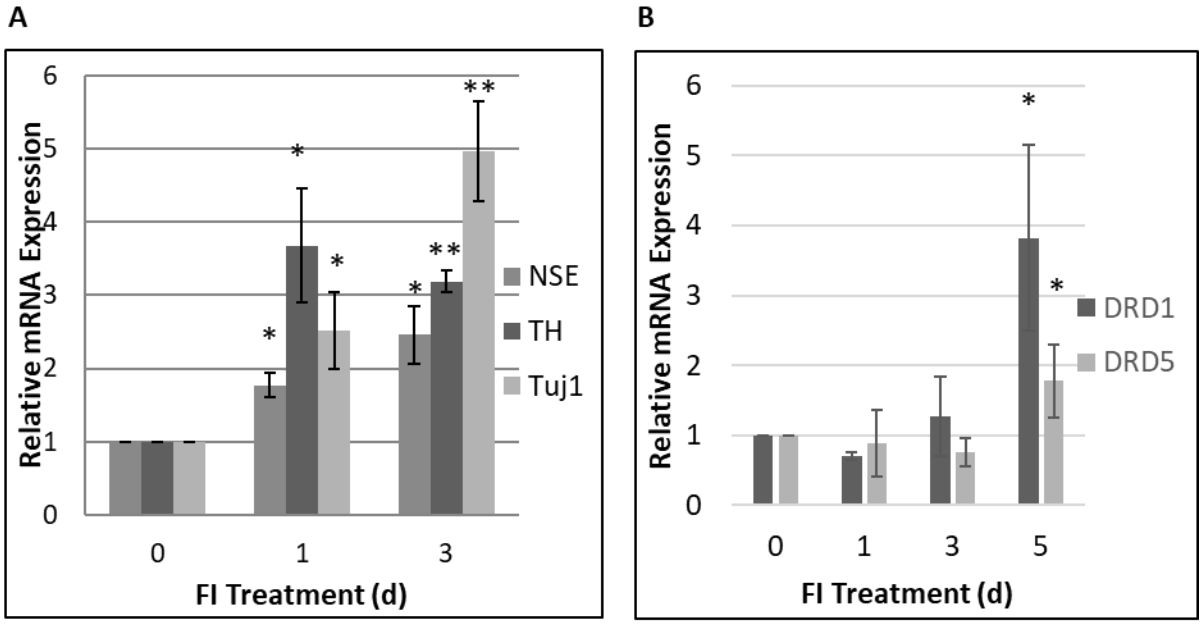



**Figure 2.2 FI de-represses NRSF-dependent gene expression.**
**A.** Treatment of MSCs with FI induces gene expression of several neural markers that are known targets of NRSF. **B.** FI treatment induces expression of dopamine receptor genes. * $p < 0.05$; **$p < 0.01$ as compared to day zero control using Tukey's test following one-way ANOVA with N=3.

### 2.4.3 Knockdown of NRSF with siRNA Reproduces FI-Induced Gene Expression

To demonstrate that the neural gene expression induced by FI in MSCs was the result of NRSF downregulation and not off-target effects of increased intracellular cAMP concentrations, we knocked down NRSF using siRNA. After silencing for 3 days we found that 10 and 50nM of siRNA strongly downregulated NRSF protein levels (Figure 2.3b) and de-repressed gene expression of th, TUJ1, and nse, (Figure 2.3a.). At 50nM of siRNA, the NRSF gene expression reduced by over 90% (Figure 2.3c), while 1nM of siRNA downregulated NRSF expression, it did not significantly de-repressed th, TUJ1, or nse expression, suggesting that NRSF-dependent repression is dose-dependent.

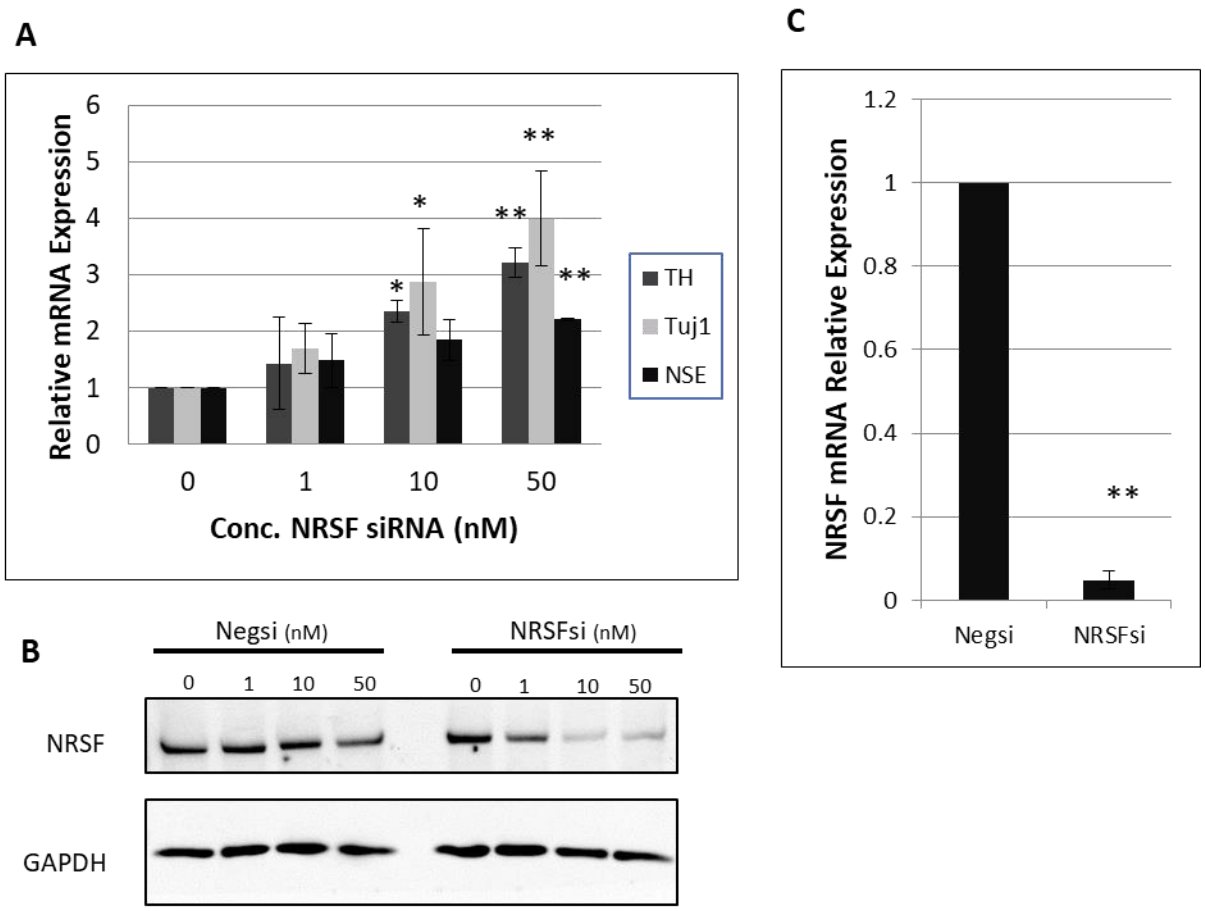



**Figure 2.3 NRSF silencing de-represses FI induced genes.**
**A.** Knockdown of NRSF protein using siRNA induces expression of same neural markers that FI induces, in a dose dependent manner. **B.** Expression of NRSF protein levels with increasing concentration of siRNA. **C.** Knockdown of NRSF mRNA expression with 50 nM NRSF siRNA. Full length blots are included in supplementary figure 4. * $p < 0.05$; **$p < 0.01$ as compared to 0 nM siRNA treatment using Tukey's test following one-way ANOVA with N=3.

### 2.4.4 Overexpression of NRSF Downregulates Target Gene Expression and Blocks FI Induced Gene Expression

To determine if neural-like differentiation of MSCs was specifically the result of the downregulation of NRSF by FI, NRSF was overexpressed in MSCs by cloning murine NRSF into a pCMV myc-N-terminal vector. We overexpressed myc-NRSF in MSCs, treated the cells with FI for three days and observed that NRSF-dependent gene expression did not increase and in some cases decreased below baseline (Figure 2.4a). FI treatment over the three days did not downregulate NRSF protein expression in the overexpressing cells (Figure 2.4b) showing that FI induced neural gene expression is due to the de-repression of NRSF and not off-target gene activating effects of FI.

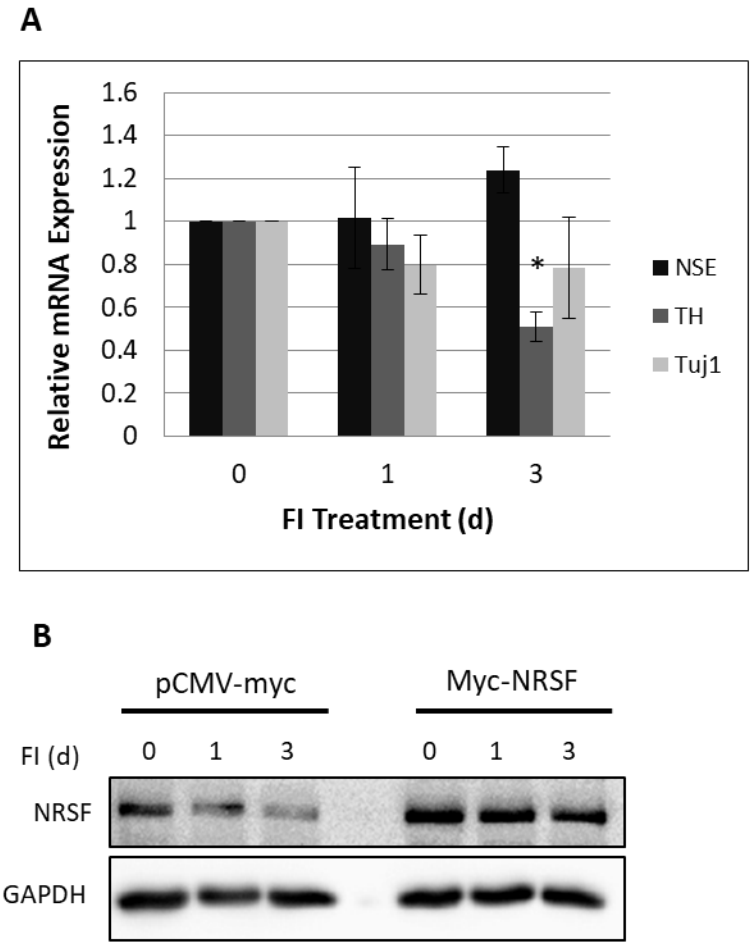



**Figure 2.4 NRSF overexpression attenuates FI-induced gene expression.**
**A.** MSCs were transfected with a vector overexpressing NRSF then treated with FI. Gene expression of several NRSF targets inducible by FI are no longer induced in MSCs overexpressing NRSF. **B.** Immunoblot of NRSF expression remains elevated during the course of the FI treatment in MSCs overexpressing NRSF vs. those transformed with an empty vector. Full length blots are included in supplementary figure 5. * $p < 0.05$ as compared with its level on day 0. Statistics were performed using Tukey's test following one-way ANOVA with N=3.

### 2.4.5 FI-Induced Dopamine Sensitivity in MSCs is Dependent on NRSF Downregulation

As previously reported by Zhang et al., MSCs exhibit some sensitivity to neurotransmitters, in particular, dopamine. When exposed to dopamine, up to 80% FI-induced MSCs respond to dopamine by releasing calcium waves into their cytosol suggesting that FI induced MSCs gain neural-like signaling function. To determine if FI induced sensitivity to dopamine is dependent on NRSF, we used siRNA to knockdown NRSF protein expression. After 72h the cells were stained with a calcium sensitive dye, Fluo-4, exposed to dopamine, and imaged with an Olympus Fluoview 1000 confocal microscope to observe calcium release in real time. When induced with dopamine, up to 78% of cells knocked down with NRSF siRNA became sensitive to dopamine and released calcium (Figure 2.5a) into the cytosolic space. Cells that exhibit an increase in fluorescence intensity of 20% or greater at any point up to 60 seconds after exposure to dopamine (Figure. 5b), over the fluorescence intensity at t=0 sec were counted as sensitive to dopamine. Less than 30% of the negative control cells transfected with scrambled siRNA gained dopamine sensitivity (Figure 2.5c). This is in agreement with our previous results[15] showing that less than 40% of the uninduced MSCs showed dopamine sensitivity. In addition, the negative control cells that responded to dopamine showed far less intense calcium release.

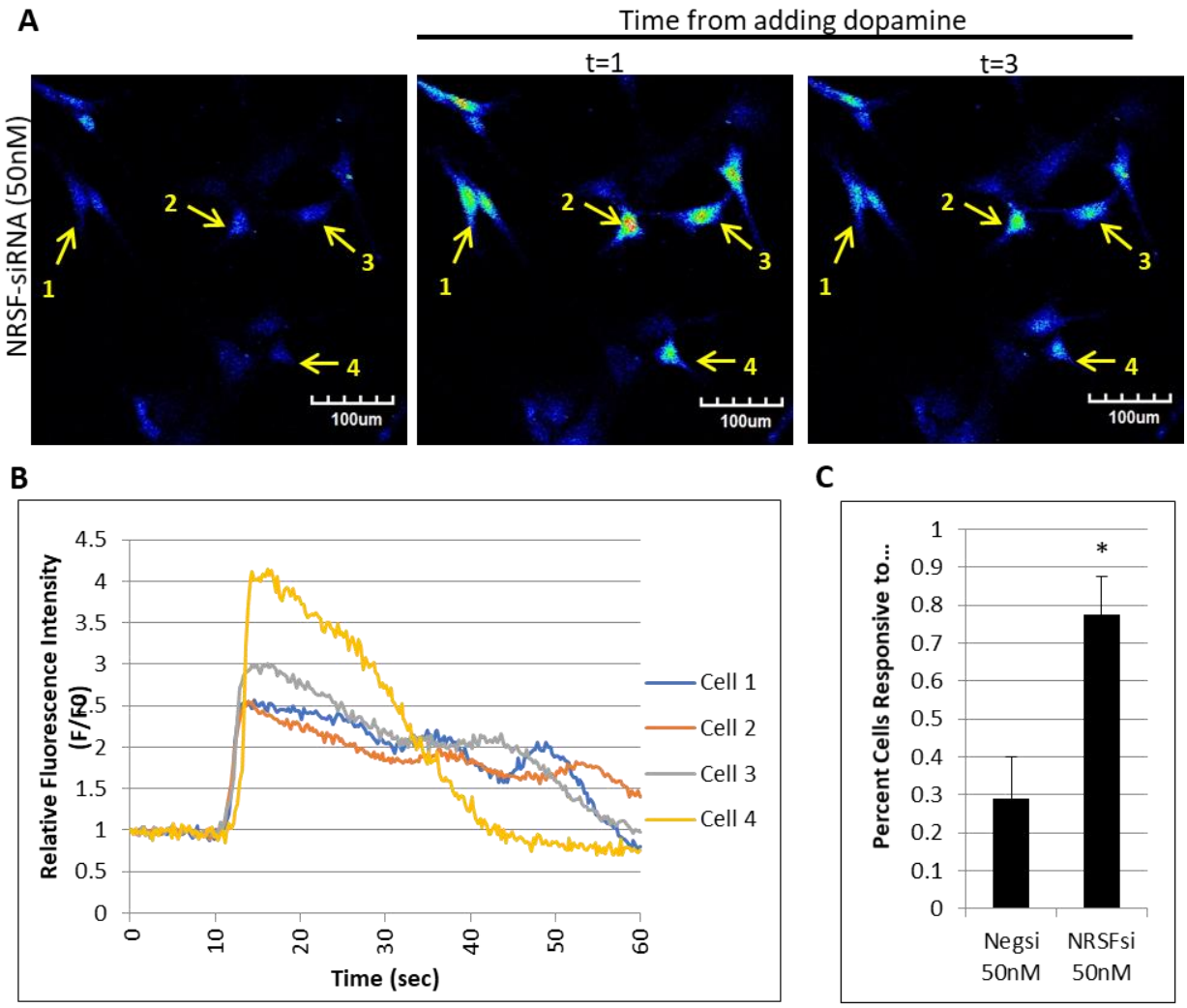



**Figure 2.5 Knockdown of NRSF with siRNA Induces Dopamine Sensitivity in MSCs.** **A.** Dopamine sensitivity in MSCs silenced with 50nM of siRNA against NRSF. Increase in fluorescence intensity represents increase in calcium release via the fluo-4 dye. **B.** Individual fluorescence intensity of select cells over time (sec) of experiment. **C.** Quantification of cells responsive to dopamine exposure. Increase of fluorescence intensity of 20% over initial resting fluorescence intensity (F/Fo) was counted as a positive response. * $p < 0.05$ using students T-test with N=3.

To determine if NRSF inhibits MSC sensitivity to dopamine, MSCs were transfected with either pCMV-myc empty vector or pCMV-myc-NRSF and induced with FI for 4 days. MSCs overexpressing NRSF and induced with FI appeared to almost completely lose their dopamine sensitivity, with <10% of the cells responding to dopamine (Figure 2.6a). In the empty vector expressing cells, FI induced an increase in dopamine sensitivity as expected with >50% of cells responding by releasing calcium (Figure 2.6c). Taken together, FI increases dopamine sensitivity in MSCs in a NRSF-dependent manner. In addition, a small percentage of MSCs in the silencing experiments exhibited sensitivity to glutamate (Supplemental Figure 2.3). This mirrors our previous study with FI that showed ~20% of the induced cells were sensitive to glutamate.[39]

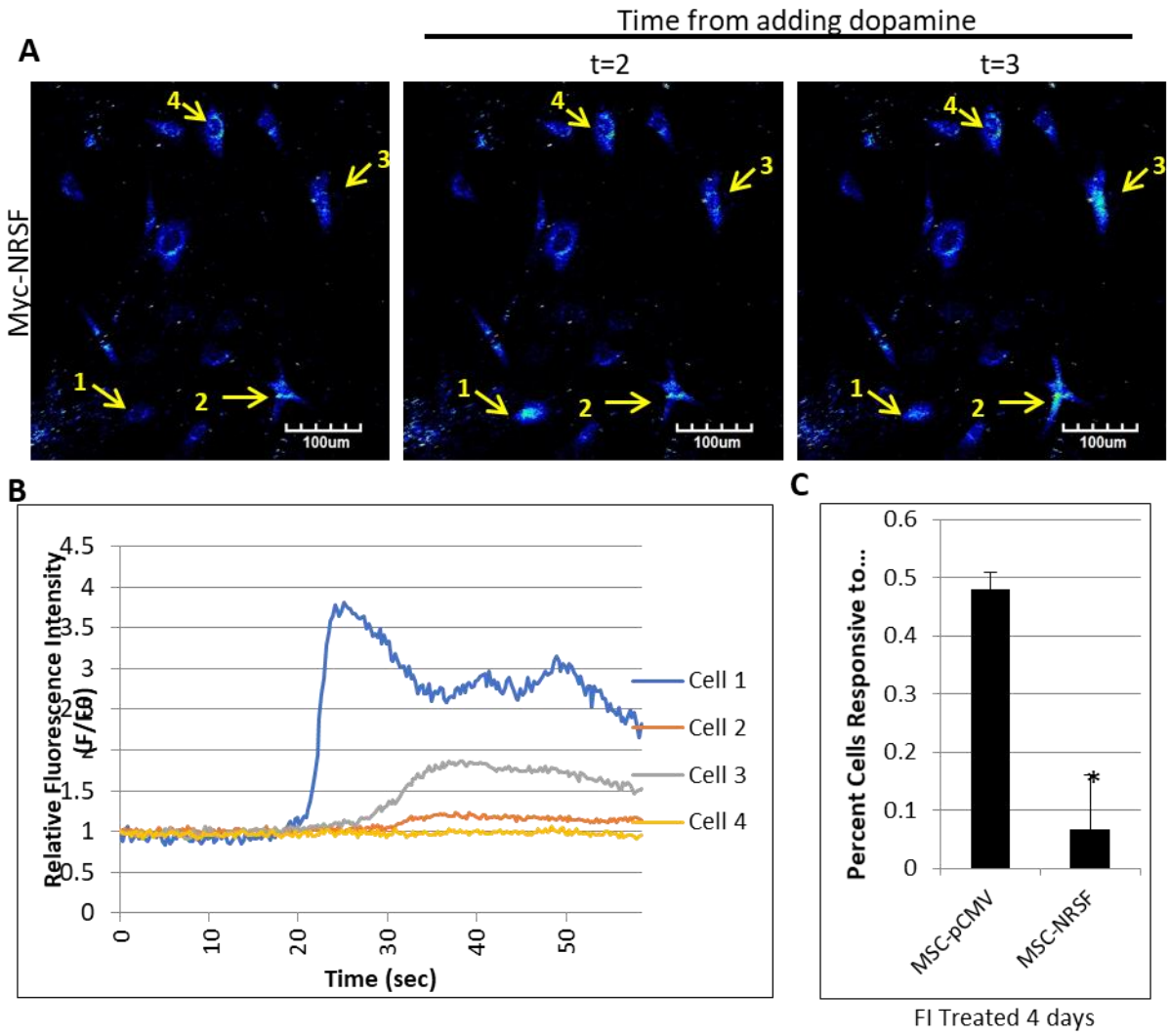



**Figure 2.6 Overexpression of NRSF In MSCs Abolishes FI Induced Dopamine Sensitivity.**

A. MSCs overexpressing NRSF from a pCMV vector are unable to respond to dopamine after being induced with FI for 4 days. Again, an increase in fluorescence intensity represents calcium release via the fluo-4 dye. B. Individual fluorescence intensity of select cells over time (sec) of experiment. C. Quantification of cells responsive to dopamine exposure. Increase of fluorescence intensity of 20% over initial resting fluorescence intensity (F/Fo) was counted as a positive response. * $p < 0.05$ using students T-test with N=3.

### 2.4.6 FI Downregulates NRSF Protein Levels through a Post-Translational Mechanism

It is not known how FI downregulates NRSF protein expression. Since FI activates the PKA-CREB signaling pathway, the downregulation of NRSF likely occurs through an indirect mechanism. MSCs induced with FI over a period of days did not significantly modulate NRSF mRNA levels (Figure 2.7a), suggesting that the protein expression of NRSF is strongly regulated through a post-translational mechanism. The ubiquitin-proteasome system can downregulate protein expression by tagging target proteins with polyubiquitin chains which are recognized by the proteasome and subsequently degraded. Using the proteasome inhibitor MG-132 NRSF is strongly upregulated in the MSCs within 3 hours of treatment suggesting that it is regulated by the ubiquitin-proteasome system (Figure 2.7b). This is in agreement with Westbrook et al.[109] who previously demonstrated that NRSF is regulated by the E3 ubiquitin ligase B-TRCP in stem cells and cancer cells. Inducing MSCs with FI increased B-TRCP protein expression (Figure 2.7c) which remained elevated during the course of the treatment suggesting that FI may downregulate NRSF protein expression by upregulating B-TRCP.

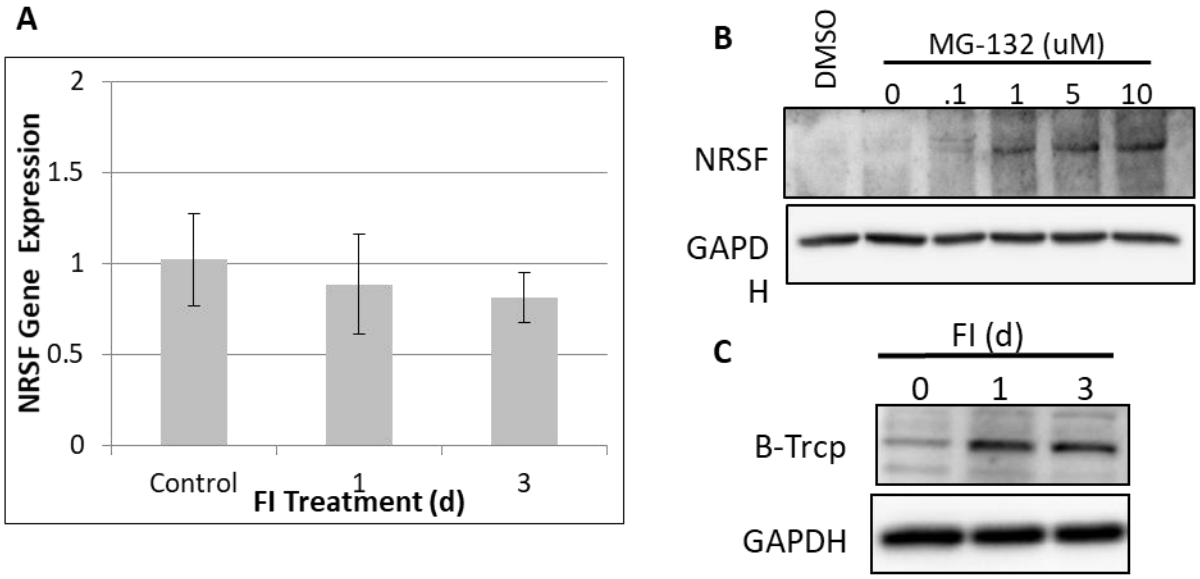



**Figure 2.7 FI Induced Downregulation of NRSF involves a post-translational mechanism.**
**A.** FI does not downregulate mRNA expression of NRSF despite a large decrease in protein expression. No statistically significant changes in gene expression for N=3. **B**. NRSF protein expression is elevated in the cells treated with proteasome inhibitor for 2h suggesting that it is negatively regulated by the ubiquitin-proteasome system. Full length blots are included in supplementary figure 7. **C.** FI induced MSCs show increased protein expression of the E3 ligase B-Trcp.

## 2.5 Discussion

Studies of neural differentiation of MSCs use various factors for induction, including soluble chemicals (BHA, B-ME), growth factors (bFGF), hormones (RA), and morphogens (BDNF)[26, 28, 105]. Despite a lack of consistency in the differentiation protocols, these studies demonstrated induction of several neural-like characteristics in MSCs, including neural gene expression, morphology changes, and functional changes with some cells able to produce action potentials[40]. Most report expression of TUJ1 and other common neural markers, such as TH and

NSE as indication of neural differentiation. Additionally, Tropel et. al. used calcium release as a measure of neurotransmitter sensitivity and Zhang et al. were able to replicate this effect[28, 39]. Notably, bFGF, a biological molecule, used by Troppel and FI, a chemical mixture, used by Zhang both induced MSCs to gain dopamine sensitivity, suggesting that the various differentiation protocols are likely activating a common program that controls the neural phenotype. We propose that these differentiation methods ultimately converge on the master transcriptional regulator, NRSF, to induce neural differentiation.

Previously, our laboratory reported that FI induced neural-like differentiation of MSCs and that CREB was a key transcription factor in this process.[39, 110] However, due to the highly pleiotropic nature of CREB and its importance to other non-neural cell lineages, we investigated possible mechanisms induced by FI that might be specific to neural differentiation. We hypothesized that NRSF, whose expression is a major hurdle in the development and maturation of NSCs to functional mature neurons[76, 111], might also play a role in the neural differentiation of MSCs. We demonstrate here that downregulation of NRSF is necessary and sufficient to express neural characteristics in MSCs and mediates the neural differentiation induced by cAMP-elevating compounds, forskolin and IBMX.

We demonstrate that cAMP-elevating compounds do not directly induce a neural phenotype but de-repress it through the down-regulation of NRSF. It is also noteworthy that FI appears to control NRSF expression indirectly as its gene expression levels are unchanged after 3 days of FI treatment (Figure. 7a), suggesting an involvement of a posttranslational regulatory mechanism in the downregulation of NRSF induced by FI. Importantly, we observed an increase in protein expression of the E3 ubiquitin ligase B-TRCP, which is known to be a post-translational regulator of NRSF expression[109] . Our results suggest that FI treatment is activating

physiologically relevant molecular machinery to induce neural differentiation. Furthermore, FI's ability to partially transdifferentiate MSCs across the mesoderm-ectoderm barrier relies on NRSF and further suggests that FI is partially reprogramming MSCs through modulation of transcription factors.

While our evidence suggests that FI specifically downregulates NRSF post-translationally, it is possible that other neural differentiation inducers downregulate NRSF through transcriptional processes. NRSF is regulated by the SMAD family of TFs, notably SMAD1/5/8[112] and its promoter region contains two SMAD binding elements required for its expression. SMAD has been implicated in neural differentiation, and differentiation protocols used to generate neural cells from iPS or ESCs frequently use SMAD inhibitors to facilitate differentiation[113-115] . This suggests that the SMAD signaling pathway is important for NRSF dependent neural differentiation and suggests a common pathway that various inducers could act on to cause neural differentiation. To this end, the MAPK pathway is also important for neural differentiation. While MAPK signaling is somewhat ubiquitous and is generally associated with growth, it also engages in morphogen dependent differentiation. bFGF is a common inducer of neural differentiation in MSCs and neurotrophins such as NGF, BDNF, and NT-3-4 signal through the MAPK pathway through Trk receptors. Thus, not surprisingly SMADs activity can be inhibited upon phosphorylation by MAPK[114, 116]. Therefore, it is plausible that neural inducers signal through MAPK kinase to affect NRSF expression via the SMAD signaling pathway.

In neural cells, expression of NRSF is regulated on several levels. At the transcriptional level, NRSF expression can be induced by SMAD proteins and TCF/Lef[112, 117]. It is regulated at the protein level by the ubiquitin-protease system as it is a substrate of the E3-ubiquitin ligase,

B-TRCP[109]. Finally, the cellular localization of NRSF is important for its function. Being a transcriptional repressor, NRSF functions maximally when it is nuclear and allowed access to DNA. Regulatory mechanisms that prevent nuclear import of NRSF are important for the homeostasis neural cells. Indeed, the huntingtin protein has been shown to sequester NRSF in the cytoplasm to permit expression of neuronal proteins, most notably, BDNF. Mutations in huntingtin that affect this binding show increased NRSF in the nucleus and repression of neuronal genes that contribute to the Huntingtin's Disease pathophysiology[90, 91]. Whether these mechanisms hold in MSCs needs to be more rigorously tested but could provide a possible explanation for why MSCs exhibit any neural competency.

While our results demonstrate that NRSF is critical for FI induced neural differentiation of MSCs, the molecular mechanism explaining FI-dependent downregulation is still incomplete. It is encouraging that FI caused increased expression of B-TRCP as this E3 ligase is a known regulator of NRSF and is important for neural differentiation in neural progenitor cells[109]. Future work is needed to determine if and how FI induced neural differentiation depends on B-TRCP activity.

We show that FI causes MSCs to gain sensitivity to dopamine and several markers of the dopaminergic neuronal subtype. Interestingly, although it is known that NRSF represses the tryptophan hydroxlase (TPH) gene important for serotonin synthesis[118] , neither FI nor NRSF silencing caused an expected increase in tph expression (data not shown) suggesting that FI favors induction of MSCs towards the dopaminergic lineage and that TPH is dependent on other factors. The ability of FI to increase TH expression could be of clinical relevance to modulate dopamine production to treat pathologies caused by the lack of dopamine. This implicates a potential role for FI in controlling the dopaminergic phenotype associated with pathologies,

including schizophrenia, Parkinson's Disease, addiction, and depression. Our results suggest that FI could be a useful approach to modulate dopamine behavior in stem cells.

We previously observed that treatment of MSCs with FI temporarily induces a dramatic change in cell morphology resembling neuron-like structures[15] . However, this effect is transient lasting only 12-24h. We observed no change in cell morphology during NRSF silencing over 72h (data not shown) suggesting that the FI induced early morphology change does not involve NRSF. However, this does not preclude a possible role of NRSF in morphology changes on a longer time scale as Yang et al.[40] showed that NRSF knockdown in MSCs over 14 days displayed significant morphology changes.

Transcription factors are key determinants of cell fate and artificially affecting their expression is well documented to transdifferentiate cells from lineage to lineage. While our study uses cAMP-elevating compounds, forskolin and IBMX, to induce differentiation of MSCs, the study underscores the importance of downregulating NRSF as the critical mechanism for induction. Our results suggest that chemical induction of neural differentiation is not due to off-target or non-specific effects,but is dependent on changes in transcription factors. This further supports the claim that MSCs can, to an extent, undergo neural transdifferentiation by depression of NRSF.

In conclusion, we show that FI induces neural-like differentiation of MSCs through NRSF and that downregulation of NRSF is necessary for induction of the neural phenotype in MSCs. Finally, we hypothesize that various neural induction protocols ultimately converge on downregulating NRSF to induce the neural phenotype.

# CHAPTER 3. SOFT SURFACES DOWNREGULATE NRSF TO INDUCE NEURAL DIFFERENTIATION IN MSCS

## 3.1 Abstract

Neural differentiation of stem cells is largely dependent on extracellular signals within the cell microenvironment. These extracellular signals are mainly in the form of soluble factors that activate intracellular signaling cascades that drive changes in the cell nucleus. However, it is becoming increasingly apparent that physical characteristics of the microenvironment provide signals that can also influence lineage commitment. Very low modulus surfaces have been repeatedly demonstrated to promote neurogenesis. The molecular mechanisms governing mechano-induced neural differentiation are still largely uncharacterized; however, a growing body of evidence indicates that physical stimuli can regulate known signaling cascades involved in native neural differentiation. Among these, SMAD signaling has been implicated as a possible driving mechanism. Understanding how the physical environment affects neural differentiation at the molecular level will enable research and design of materials that will eventually enhance neural stem cell (NSC) differentiation, homogeneity and specificity.

## 3.2 Introduction

It is a documented phenomenon that various kinds of stem cells including iPSC[59, 61, 119], NSCs[59, 120], and MSCs[58] show enhanced neural differentiation when cultured on soft surfaces for an extended period. This effect could have practical applications for tissue engineering as a strategy for improving the efficiency of neural cell culture. However, a more complete understanding of the molecular mechanism is needed to explain this effect, and to what extent soft surfaces contribute to differentiation. While some well-known molecular signaling components have been implicated, including BMP, SMAD[63], and YAP/TAZ[60] components, their response to soft surfaces on overall gene regulation within the cell is still unclear. Current research shows that stem cells cultured on soft surfaces have altered BMP and YAP/TAZ signaling. Since these mechanisms converge on SMAD, a signal-transducer with a well-studied role in neural differentiation[113, 121], studying its downstream effects could reveal how SMAD drives differentiation induced by soft surfaces.

Inhibition of SMAD is important for proper neural development from the embryonic state. This also holds in vitro as inhibition of SMAD by small molecules can be utilized to improve the efficiency of neuronal differentiation[115]. Studies have shown that cells cultured on soft surfaces show disrupted BMP-SMAD signaling. Zouani et al. showed that these cells are unresponsive to BMP2 treatment and have downregulated amounts of p-SMAD[122] while Du et al. demonstrated that soft surfaces cause internalization of BMPR1A[63]. The consequence of which also results in lower levels of p-SMAD. Du et al. further demonstrate that on soft surfaces, lower levels of p-SMAD lead to reduced import of SMAD to the nucleus. Additionally, Sun et al. showed that stem cells cultured on soft surfaces have altered cellular localization of SMAD with more rigid surfaces promoting nuclear localization and softer surfaces promoting

cytoplasmic localization[60]. However, it was concluded that import/export of SMAD was a result of the effects of soft surfaces on YAP/TAZ function, a mechanism not mutually exclusive with the previous results.

NRSF is the master transcriptional regulator of the neural phenotype[73, 74] found mainly in non-neural cells and neural stem cells. Downregulation of this transcription factor occurs naturally during maturation of neurons[78]. Forced silencing of this transcription factor in MSCs gives rise to several neural characteristics, including marker expression, morphology, and generation of Na+/K+ currents[40]. Additionally, in our lab, we have shown that forskolin and IBMX downregulate NRSF to render MSCs sensitive to dopamine, indicative of a gain of neural communication (Thompson et al., *in press*). Interestingly, the gene promoter region of NRSF contains two SMAD binding elements (SBEs)[112], both of which are responsive to SMAD1/5/8 and which contribute to its upregulation. BMP2 has been shown to activate these SBEs in astrocytes to upregulate NRSF.

While signal transducers are clearly important for cell differentiation, they ultimately control the activity of transcription factors that regulate large programs of gene expression that determine cell fate. Indeed, dozens of transcription factors have been studied that underlie many types of neural cells. However, few transcription factors are able to drive differentiation across germ line barriers. Of note, is the effect of soft surfaces on mesenchymal stem cells, a mesodermal type. Engler's original observation on soft polyacrylamide gels suggested that MSCs were becoming more neural like, however, to date, there is no record of MSCs transdifferentiation into neural cells under physiological conditions. Transdifferentiation of MSCs to neural-like cells has only been demonstrated using exogenous expression or repression of transcription factors in cellular reprogramming experiments.

Since soft surfaces appear to act through molecular mechanisms that converge on SMAD, and SMAD can potentially affect cell type through regulation of NRSF, we aim to show that stem cells cultured on soft surfaces regulates NRSF to drive neural differentiation. PDMS is a common polymer used to coat medical devices that has minimal toxicity when used as a substrate for cell culture. The stiffness of PDMS can be easily tuned down to 1 kPa by adjusting the amount of cross-linker used during its curing. Due to these properties of PDMS we cultured MSCs on very soft PDMS (1:80) and demonstrated neural gene expression and downregulation of NRSF activity. Additionally, the very soft PDMS caused MSCs to gain dopamine sensitivity akin to our previous experiments wherein NRSF expression was modulated.

## 3.3 Materials and Methods

### 3.3.1 Mesenchymal stem cell culture and isolation

MSCs were isolated from animals using procedures approved by the Institutional Animal Care and Use Committee at Michigan State University. MSCs were derived from bone marrow isolated from 4 to 6 week-old Sprague-Dawley female rat as previously described[15]. Briefly, femurs and tibias were removed from 4 to 6-week-old rats. The two ends of the bone were cut open and the marrow was flushed with 10mL of DMEM using a 25g needle and syringe. The cell suspension was passed through a 70-um nylon mesh to remove bone debris and blood aggregates. Cells were cultured in low glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and free of antibiotics. Cells were incubated in a humidified atmosphere containing 5% CO2 at 37C. Non-adherent cells from the flushed marrow were removed after 48h after isolation. Media was replaced every 3 days until the cells reached 80–

90% confluence. Confluent cells were washed with PBS (no CaCl2) detached by 0.25% trypsin–EDTA (Invitrogen) and plated for further experiments.

### 3.3.2 PDMS Preparation

PDMS was prepared from the Sylgard 184 manufacturer's kit. We prepared regular PDMS at the specified crosslinker to base ratio of 1:10 and also very soft PDMS at a crosslinker to base ratio of 1:80. This is the lowest ratio we could use and still have the PDMS cure properly. Crosslinker and base was mixed for 5 minutes and degassed in a vacuum chamber for ~10 minutes before pouring. For cell lysates, PDMS was poured into 10cm Pyrex dishes. For immunostaining and calcium imaging, PDMS was poured onto 1mm thin glass coverslips. 1:10 PDMS was cured in a dry oven overnight at 70C. 1:80 PDMS was cured on top of a hot plate at 100C overnight, about 16h. After curing, PDMS was O2 plasma treated for 60 sec, 30W. Immediately following plasma treatment, PDMS was then incubated with poly-L-lysine for 1h at room temperature. PLL was then removed then washed several times with PBS. After drying, PDMS was sterilized under UV light for 30 min.

### 3.3.3 Nuclear Isolation

Nuclear fractionation for protein lysates was performed according to NE-PER Nuclear and Cytoplasmic Extraction Reagents. Briefly, cells were harvested by washing once with PBS then incubating with .05% trypsin-EDTA until cells lifted off substrate. Detached cells were collected and residual trypsin was neutralized with cell medium containing 10% FBS. Cells were pelleted in a centrifuge at 200g for 5 min and the excess trypsin was aspirated. Cell pellet was resuspended and washed with PBS and pelleted again to remove traces of FBS. After the

wash was aspirated, the cell pellet was vortexed and incubated in ice cold CER I solution for 10 min to swell the cells. CER II solution was then added and the cells were vortexed again extensively to disrupt the cellular membrane. The solution was then centrifuged at 16,000g for 5 min to pellet the nuclei. The supernatant was collected and kept as the cytosolic fraction while the pelleted nuclei were then resuspended in Nuclear Extraction Buffer. Nuclei were incubated on ice for up to 1h with vortexing every 10 min in order to extract nuclear proteins. After extraction, the solution was spun down again at 16,000g for 5 min to pellet the nuclei. The separated supernatant was collected as the nuclear extract.

### 3.3.4 Calcium Imaging

We chose Lab-Tek II Chamber Slide system to culture our cells for confocal imaging. However, due to the thickness of the glass plate of the chamber combined with the PDMS, we found that the PDMS needed to be first poured onto thinner coverslips. Uncured PDMS was poured onto 24X50mm, 1mm thin cover glasses then cured as describe above. Vacuum grease was used to seal the chambers to the PDMS before carrying on with the ordinary protocol (Supplemental Figure 3.1). Calcium imaging was performed according to the protocol by Tropel et al.[28] and modified by Zhang[39]. Cells were cultured in four-well chambered cover-glass (Lab-Tek) coated with poly-L-lysine (Cultrex). After neural induction with FI or transformation with lipocomplexes, the cells were stained with 4 uM Fluo-4 (Invitrogen) in ACSF–HEPES (artificial cerebrospinal fluid with HEPES: 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl2, 2.5 mM CaCl2, 1 mM NaH2PO4, 26.2 mM NaHCO3, 11 mM dextrose, 10 mM HEPES, pH = 7.4) for 30 min at 37C. Excess dye was removed by washing cells with PBS twice and placing into a 37C chamber on the stage of Olympus FluoView 1000. Then, 0.5 ml ACSF–HEPES was added to the well to

begin imaging. Images were captured every 1.137 s and fluorescence intensity is represented by a spectral table (warmer colors represent higher intensity whereas cooler colors represent lower intensity). After 15–20 images, 0.5 ml ACSF–HEPES buffer containing the following neurotransmitters were added: 200 uM dopamine (final concentration 100 uM) or 200 uM ATP (final concentration 100 uM). A total of 120 images were recorded and the data was analyzed by the FluoView 100 software. Changes in the fluorescence intensity of the Ca2+signal are represented as F/F0. The percent of responsive cells is calculated as the number of cells with a F/F0 signal greater than 20% of the total number of cells.

### 3.3.5 Atomic Force Microscopy

Substrate modulus of PDMS was measured using an atomic force microscope. The "tapping method" was employed to generate force curves from which the stiffness of the surfaces was approximated[123]. PDMS samples cured at 1:10, 1:35, and 1:70 were measured by Liu. From these measurements, a logarithmic curve was generated and we approximated the stiffness of the 1:80 PDMS sample from it.

### 3.3.6 Cell Transformation

Plasmid complexes were prepared in Opti-MEM (Gibco) with 2ug of plasmid, 2.5 uL of P3000 reagent, and 2.5uL of Lipofectamine 3000 (Invitrogen) per sample. About 10^5 cells/mL were seeded onto PDMS and grown for 4-5 days with medium changed after the third day of initial plating. On the day of transfection, cell medium was changed from normal growth medium to normal growth medium plus half volume of OptiMEM. The resulting transfecting medium contained 5% FBS. Lipocomplexes were added and the transfection took place for 16h

hours at 37C. Afterwards, the medium was replaced with normal growth medium and the cells were further cultured for 3-4 more days. After this time, assays were performed as described below.

### 3.3.7 Immunostaining

Cells were washed with PBS and then fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature. After washing 3 times with PBS, fixed cells were permeabilized with .1% triton-X100 in PBS for 15 minutes at room temperature. Cells were further washed 3 times with PBS to remove the triton X-100. Cells were then blocked in PBS with 10% normal goat serum and 1% BSA for 1 hour at room temperature. After blocking, cells were incubated for primary antibody overnight at 4C. Following overnight incubation, cells were washed 3 times with PBS and incubated for 1h at room temperature with appropriate secondary antibody. Cells were washed once more then fixed to a microscope slide with Prolong Diamond with DAPI. This was allowed to bond overnight at 4C before imaging.

### 3.3.8 Western Blotting

Whole-cell extracts were prepared by lysing cells with RIPA buffer (50mM Tris pH 8.0, 150 mM NaCl, 1% IGEPAL (NP-40), 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate) on ice for 30 min. Nuclear fractions were prepared by swelling cells in a hypotonic buffer (10mM HEPES (pH 8.0), 1.5 mM MgCl2, 10 mM KCl) on ice then lysing with a dounce homogenizer. The nuclei were spun down and incubated on ice in a high salt buffer (20 mM HEPES pH 8.0, 1.5 mM MgCl2, 420 mM NaCl, 25% glycerol) to extract the protein. Lysates were mixed with 5X SDS protein loading buffer (50mM Tris pH 7.0, 25% glycerol, 2% SDS,

0.025% bromophenol blue) and denatured at 95C for 5 min. 20ug of each sample lysate was separated by electrophoresis on an 8% Tris–HCl gel and transferred to a nitrocellulose membrane. Membranes were then blocked in 5% milk and 0.05% Tween 20–TBS (Tris buffered saline) for 1 h and incubated with primary antibodies against tyrosine hydroxylase or GAPDH (Cell Signaling) or NRSF/REST (Millipore) overnight at 4C. Anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Thermo Scientific) was added the second day after primary antibody incubation. The blots were incubated for 90 min and then washed three times with 0.05% Tween 20–TBS. The blots were then visualized by SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

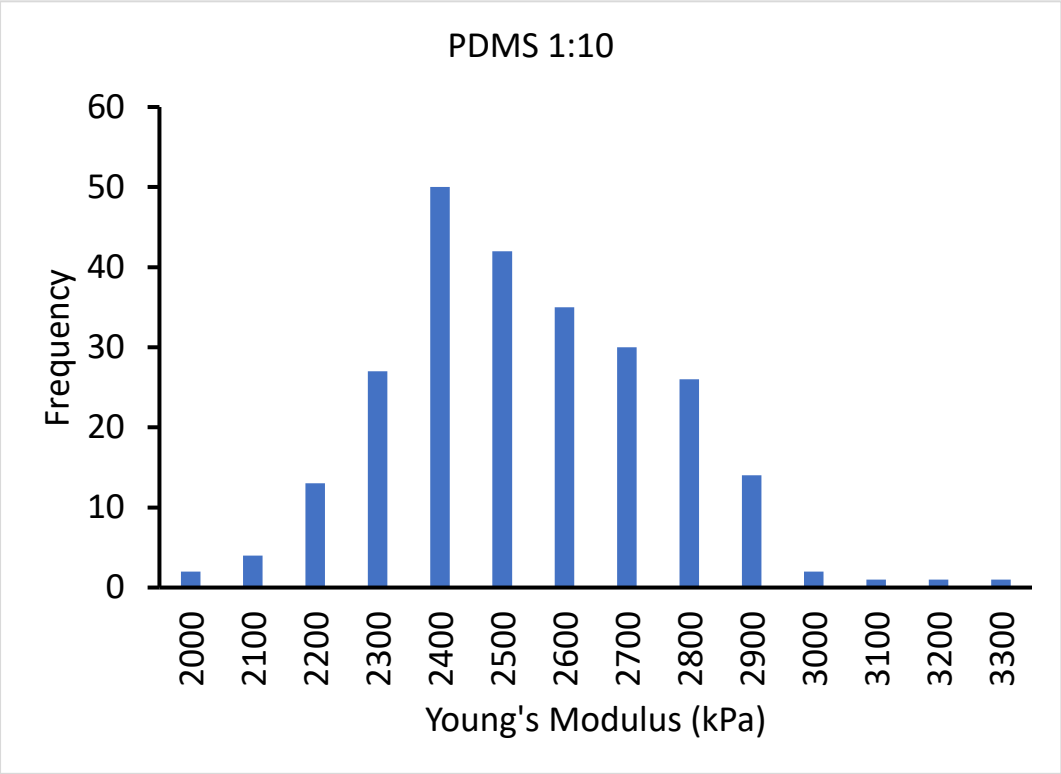
## 3.4 Results

### 3.4.1 PDMS Substrate Modulus by AFM

Pouring of PDMS using the manufacturer's instructions for a 1:10 ratio of crosslinker to base and curing in a dry oven at 70C overnight reliably produces surfaces with a modulus of ~2MPa. This has been reproduced many times over by other labs[124, 125]. However, making softer PDMS gels by using a lower ratio of crosslinker to base is not as reliable and issues with proper curing have been noticed. When the ratio of crosslinker to base is reduced below 1:50 we have noticed that curing overnight at 70C does not always completely cure our samples and either longer curing times or higher temperatures are needed. The lowest ratio of crosslinker to base we have been able cure properly is 1:80. At this ratio, we must pour and cure the PDMS in glass dishes or on glass slides at 100C for ~12-16 hours. Surface modulus must be checked again due to the differences in curing conditions. Using an AFM with the tapping method to generate a force curve, we calculated the Young's Modulus of PDMS mixed with cross-linker at

a 1:10, 1:35, and 1:70 (Figure 3.1). The averages found were 2485kPa, 268kPa, and 4.83kPa, respectively. After deriving a logarithmic curve from these data points, we extrapolated the data for our 1:80 surface to produce a stiffness of 1.88kPa (Figure 3.2). For the 1:80 PDMS, the softness should be low enough to observe neural changes within MSCs cultured.

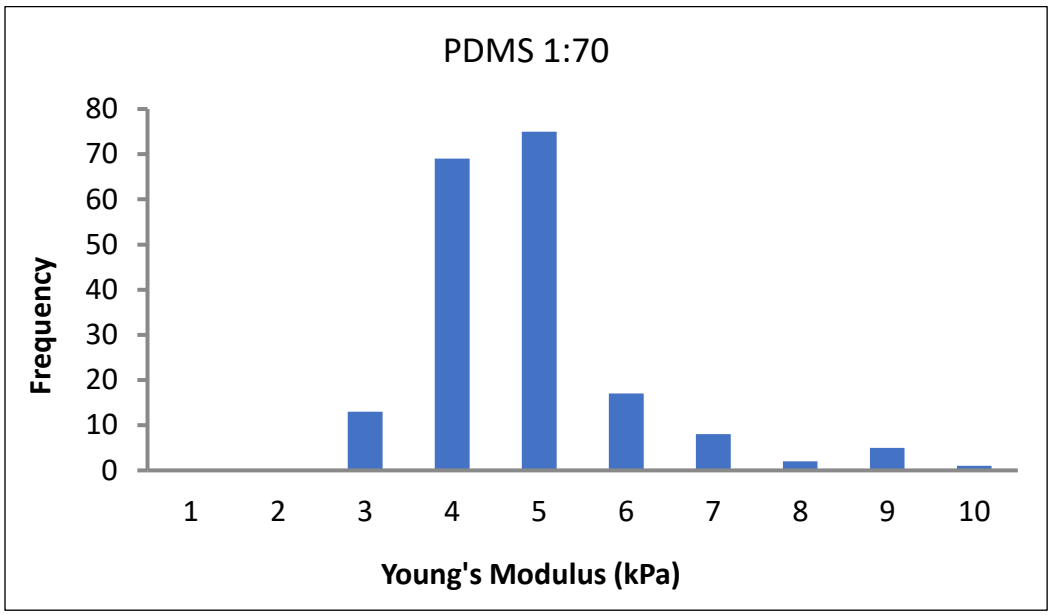
Average stiffness: 2485.86 kPa
Std error: 13.89 kPa




Average stiffness: 4.83 kPa
Std error: 1.12 kPa

**Figure 3.1 AFM Measurements for Young's Modulus for PDMS.** These measurements and calculations were performed by Chun Liu.

**Figure 3.1 (cont'd)**



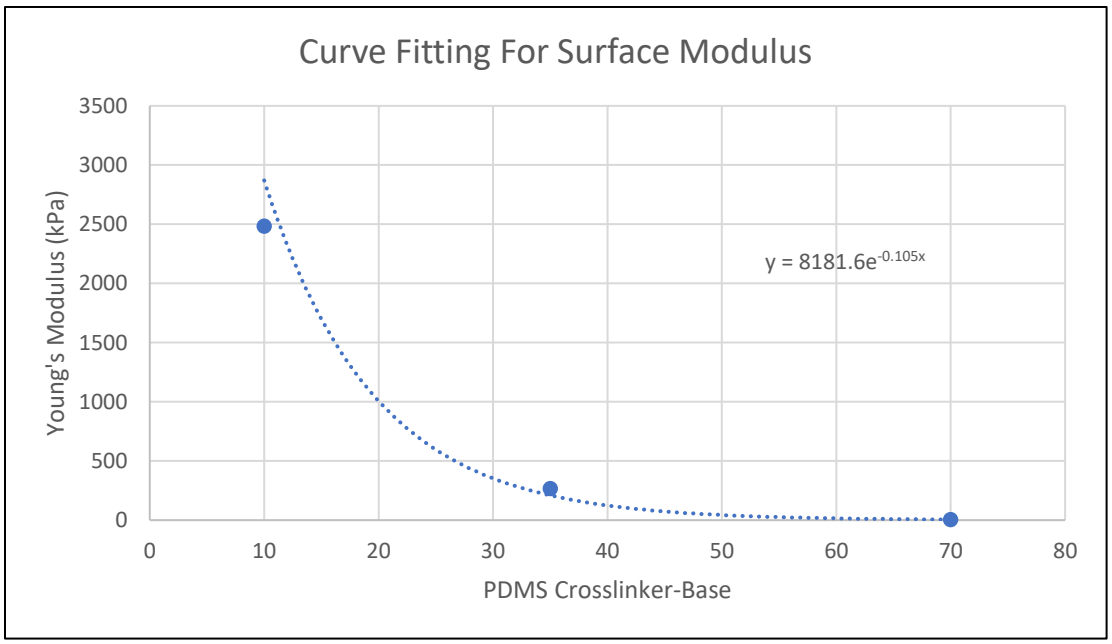




**Figure 3.2 Curve Fitting for Surface Modulus of PDMS.** Measurements for PDMS poured at a crosslinker-base ratio of 1:10, 1:35, and 1:70 were collected with AFM. From these data points, a logarithmic curve was derived and used to approximate the value of the 1:80 surface.

### 3.4.2 Protein Expression of Neural Markers on Soft Surfaces

MSCs show expression of several NRSF-dependent neural markers including TUJ1 and TH when cultured on soft PDMS (Figure 3.3). We chose these markers based on previous work showing that they could be expressed in an NRSF-dependent manner. Here we show that MSCs cultured on soft surfaces for over 1 week spontaneously upregulate protein expression of these markers. In addition, we also see that SMAD expression holds even on the different surfaces (Figure 3.3). This fits in better with establishing SMADs activity as a function of its localization.

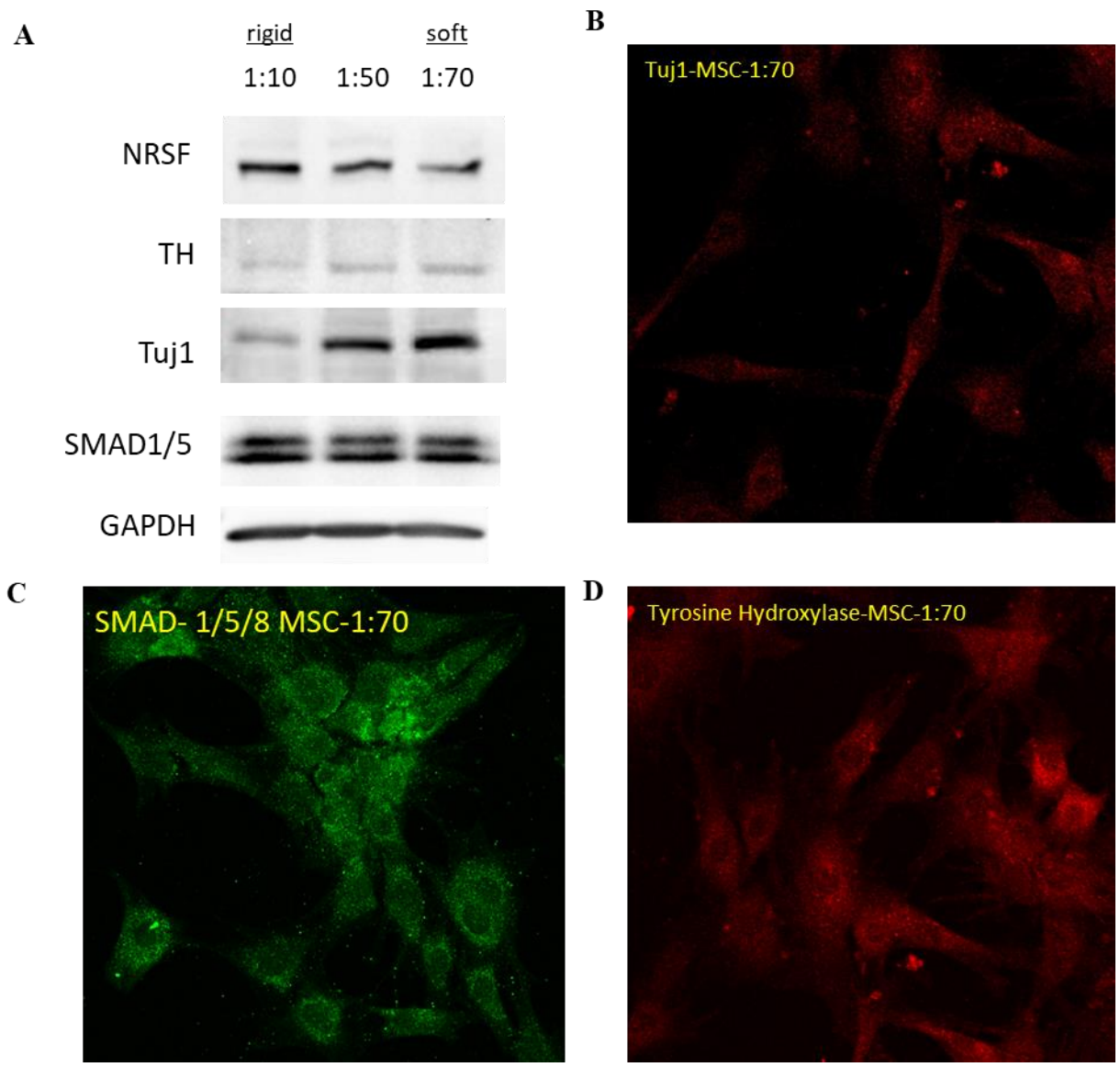



**Figure 3.3 Gene Expression of Neural Markers in MSCs Grown on Soft Surfaces. A.** Western blots for neural markers TUJ1 and TH, the master transcriptional regulator NRSF, and the signal transducer SMAD. All blots are normalized against separate GAPDH blots. GAPDH is represented by the blot for NRSF. **B, C, D.** Immunofluorescence for neural markers TUJ1, TH, and the signal transducer, SMAD.

In addition, immunofluorescence shows that gene expression is also properly distributed within the cells. Despite the increase in expression in neural markers, it should be noted that cell morphology does not appear to change if MSCs are cultured on stiff or soft surfaces.

### 3.4.3 NRSF Activity and Expression on Soft Surfaces

Importantly, NRSF expression was decreased when MSCs were cultured on 1:80 PDMS. However, NRSF expression was not eliminated. Interestingly, confocal imaging of NRSF protein levels revealed that when cultured on soft surfaces, NRSF showed up as predominantly nuclear in cells that had been cultured on the 1:10 PDMS while being mostly cytosolic when cultured on the 1:80 PDMS. This effect was not completely homogeneous across all cells per field of view. Cells were collected from their respective surfaces then fractionated into cytosolic and nuclear lysates.

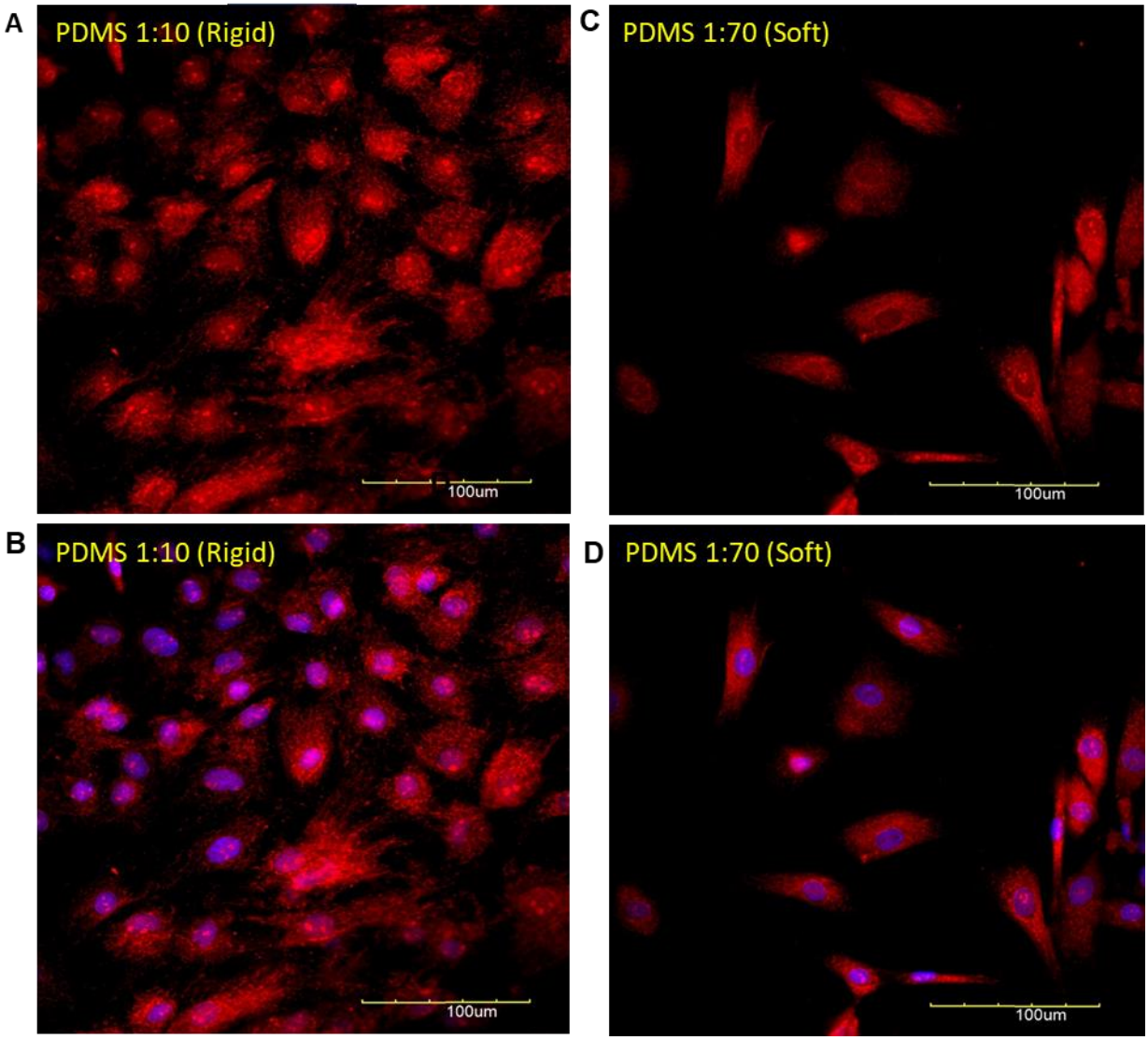



**Figure 3.4 Immunofluorescence of NRSF.** Immunofluorescence of NRSF expression in MSCs on stiff (**A,B)** or soft (**C,D)** surfaces. NRSF is in red while DAPI stain for the nucleus represented in blue. Images were taken with a 20X objective.

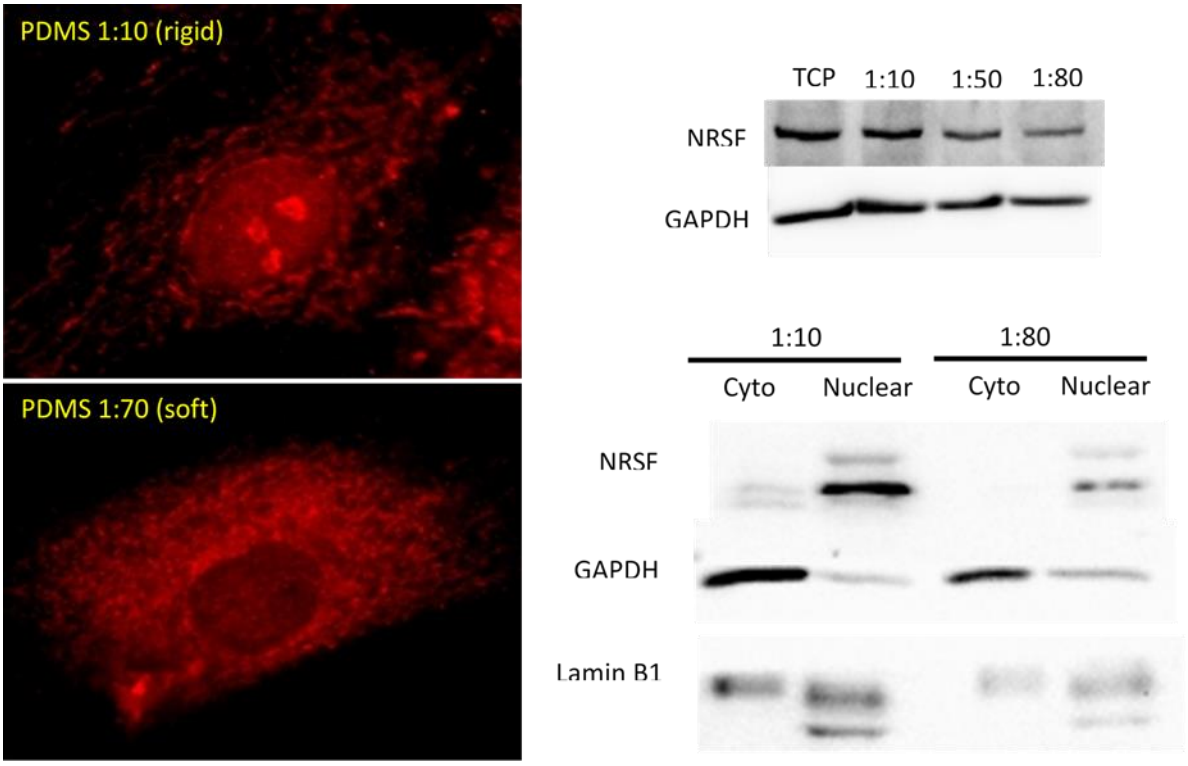



**Figure 3.5 Localization of NRSF.**
Immunofluorescence of NRSF expression in MSCs on **(A)** stiff or **(B)** soft surfaces. **C.** Western blotting for NRSF expression on PDMS of varying stiffness. Tissue culture plastic (TCP) is also used as a comparison. **D.** Comparison of NRSF expression in the cytosolic fraction vs. the nuclear fraction. Lamin B1 was used as a loading control for the nuclear fraction.

### 3.4.4 Calcium Imaging for Soft Surfaces

Here we set out to determine if culturing MSCs on soft surfaces could induce dopamine sensitivity. When cultured for up to 10 days, we noticed that cells grown on 1:10 surfaces seemed to lose their baseline response to dopamine. However, cells cultured on the 1:80 surfaces retained some 40% response to dopamine. It should be noted that the magnitude of the response to dopamine also seemed to be depressed. When cells were then induced to ATP after dopamine, many of the cells exhibited a full calcium release response showing that calcium release is not impaired in these cells.

### 3.5 Discussion

Studies of stem cells cultured on soft surfaces usually look for the expression of neural markers as an endpoint. Here we investigated the response of the master transcriptional regulator, NRSF, as this would give us a better explanation for the pan-neural effects seemed to be induced by soft surfaces. Culturing MSCs on soft surfaces for up to 10 days did result in downregulation of NRSF protein expression, although, it did not seem to decline as much as we anticipated. It is also noted that SMAD expression seems to be even in the course of these experiments. Interestingly, there seemed to be a change in the localization of NRSF protein in MSCs when cultured on soft vs. stiff substrates. This is significant due to the function of NRSF being dependent on its localization to the nucleus. Future work should investigate the mechanisms behind nuclear import of NRSF and if those mechanisms can be affected by substrate modulus.

Our results are in agreement with our previous study regarding neural-like differentiation of MSCs induced by FI. In addition to the rise in neural marker expression we also observed that MSCs cultured on 1:80 surfaces had a modest increase in cells that could respond to dopamine. This is significant because MSC response to dopamine is a hallmark in our experiments studying downregulation of NRSF.

Our results show that soft surfaces have an effect on NRSF. However, we do have to acknowledge that the response of NRSF was modest and we cannot yet conclude that soft surfaces cause neural differentiation. Interestingly, even though the field of tissue engineering has established a neural inducing effect of soft surfaces on stem cells, it has also been noted that complete conversion has not quite been proven. A hallmark of differentiated cells is their inability to revert back to a more undifferentiated state with the exception of during disease

states like cancer. Lee et al. demonstrated that neural differentiation of MSCs promoted by soft surfaces could be reversed simply by transferring the cells back from a soft to stiff substrate[126]. Additionally, MSCs grown on soft surfaces do not appear to alter their global DNA methylation patterns that are often seen when cells commit to another lineage, suggesting that the changes in gene expression while on soft surfaces is a transient effect[127]. Taken together, it does not appear that soft surfaces cause neural differentiation, but instead promote the effect.

In conclusion, our results further reinforce the neural inducing effect of soft surfaces on MSCs by showing altered regulation of NRSF. Though soft surfaces do not seem to provide enough stimulus to induce full differentiation in itself it would be easy to combine with other methods to attempt to produce a synergistic effect. Adding culture onto soft surfaces would be easy to implement especially with soluble factors to better improve neural cell culture.

# CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

## 4.1 FI Induced Neural Differentiation is Dependent on NRSF

The principal aim of this dissertation was to determine if there was a relationship between NRSF and neural induction in MSCs. This hypothesis was applied to neural induction by soluble factors, forskolin and IBMX, as well as by the physical environment ie. very soft PDMS. The observation that silencing NRSF could copy many of the characteristics induced by FI suggested that NRSF was somehow involved in the FI-dependent mechanism. Additionally, using overexpression of NRSF, this could block FI-induced neural-like differentiation of MSCs showing that downregulation of NRSF is necessary and sufficient.

## 4.2 The Role of cAMP in Induction of Neural Differentiation in Stem Cells

cAMP and cAMP-elevating compounds are frequently used in medium to induce neural differentiation and maturation in a variety of cell lines[48-51, 128-135]. It is assumed that activation of this signaling pathway contributes to neural induction through activation of the CREB transcription factor and CREB-dependent neural genes[136-138]. While this makes sense in a neural stem cell background, it is not very sufficient to explain why cAMP-elevating compounds can induce neural differentiation in MSCs. Here we show that cAMP downregulates NRSF and that this could be a more potent explanation for its effectiveness and inclusion in neural induction medium.

## 4.3 Criticisms Aimed at the Neural Differentiation of MSCs

Driving neural-like differentiation in MSCs through FI-induced downregulation of NRSF also addresses skepticism aimed at the capacity for MSCs to exhibit neural differentiation.

Again, since neural-like differentiation of MSCs would require transdifferentiation across the mesoderm-ectoderm barrier, there is still the question of if true differentiation is occurring[41, 42]. Previous criticisms claim that neural induction in MSCs could be the result of selectively driving expression of neural characteristics. Currently, exogenous expression of transcription factors is an accepted non-physiological based mechanism for transdifferentiation across the mesoderm-ectoderm barrier[70]. Since downregulation of NRSF is a natural event during differentiation and maturation of developing neurons and since knockout of NRSF has been shown to induce neural-like differentiation in MSCs, we argue that FI induced downregulation of NRSF is a mechanism that shares more in common with cell conversion or reprogramming.

### 4.4 Post-Translational Regulation of NRSF by an E3 Ligase

Since our results show that FI does not statistically significantly downregulate mRNA levels of NRSF, we hypothesized that FI downregulates NRSF at the post-translational level. Based on preliminary data from other projects, we wanted to show that the E3 ligase SMURF1 could ubiquitinate NRSF and cause its degradation through the proteasome, presenting a novel substrate interaction. Early experiments had shown that FI increased protein expression of SMURF1 (Figure 4.1a) and immunostaining had shown that SMURF1 localized to the nucleus (Figure 4.1b).

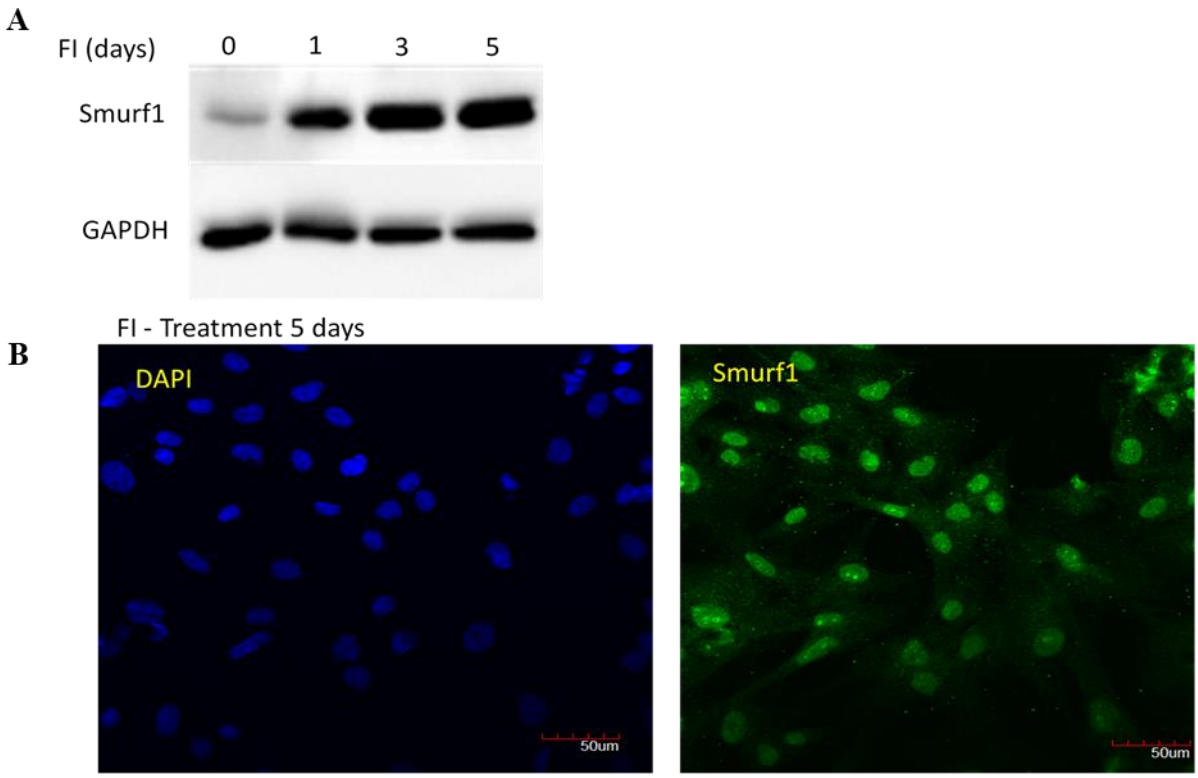



**Figure 4.1 SMURF1 Dynamics in MSCs During FI Treatment.** **A.** SMURF1 Protein expression assayed by western blotting during 0-5 days of FI treatment. **B.** SMURF1 immunostaining showing presence in the nucleus.

Additionally, the amino acid sequence of NRSF showed two possible binding for the WW domains of SMURF1 to interact with (Figure 4.2).

| Species | 131-134 | 163-166 |
|---|---|---|
| H. Sap | SGAPDIYSSN | RCKPCQYEAE |
| M. Mus | SAAPEIYSAN | RCKPCQYEAE |
| R. Nov | SAAPEVYSSN | RCKPCQYEAE |
| X. Laevis | N/A | RCKPCQYKAE |
| D. Rerio | | FCKPCQYQGE |
| T. Rubripes | N/A | N/A |
| Consensus Motif. | PXXY | |

**Figure 4.2 Amino Acid Sequence Homology of PXXY.** PXXY motifs are recognized by SMURF1. These domains were detected in several different species suggesting conservation.

However, attempts to reproduce the FI induced increase in SMURF1 were unsuccessful. After switching antibody lots to a well characterized monoclonal version, SMURF1 was reliably detected however, it was found that FI could not cause its upregulation (Figure 4.3a). Additionally, overexpression of SMURF1 was unable to downregulate NRSF levels (Figure

4.3b).

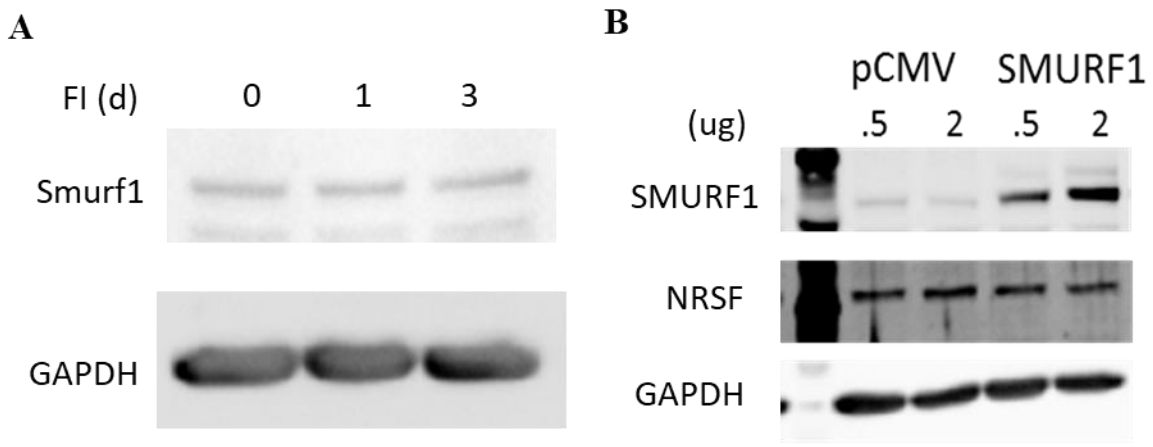



**Figure 4.3 Expression of SMURF1 and NRSF**
**A.** Western blotting for SMURF1 with the monoclonal antibody against the 1D7 motif showed that SMURF1 did not upregulate after treatment with FI. **B.** Overexpression of SMURF1 in MSCs has no effect on NRSF expression.

Since I could not reliably reproduce the SMURF1 response to FI using a more reliable antibody and because SMURF1 overexpression could not downregulate NRSF protein levels, I concluded that SMURF1 was not likely regulating NRSF and decided to look towards alternative ligases.

### 4.5 B-TRCP Is an Established Post-Translational Regulator of NRSF

Since Westbrook et. al. have previously shown that the E3 ligase, B-TRCP, is a negative post-translational regulator of NRSF[109], and we show that FI can induce B-TRCP expression in MSCs (Figure 2.7), we hypothesize that FI induction of B-TRCP is the mechanism that downregulates NRSF. Furthermore, we hypothesize that FI induction of B-TRCP is dependent on activation of CREB. Future experiments will be needed to verify this mechanism. However, the results could be interesting in that they would suggest that FI is inducing a native physiologic

signaling pathway for neural induction. Additionally, B-TRCP is well in agreement with our hypothesis that FI downregulates NRSF at the post-translational level.

### 4.6 Multiple Signals Could Converge on NRSF to Drive Neural Differentiation

Though we show that cAMP-elevating compounds can cause downregulation of NRSF, NRSF's overall expression is multifaceted. Our research highlights how cAMP-elevating compounds induce downregulation of NRSF through a post translational mechanism, however, we have ignored the regulation of NRSF at the transcriptional level. Immediately, the SMAD signaling pathway comes to mind since its role in BMP-controlled neural induction is well established. SMAD acts as a positive regulator of NRSF[112] and inhibition of SMAD is already a strategy used to drive neural differentiation in stem cells[115]. Interestingly, phosphorylation of SMAD by ERK[139] causes its inhibition as it disrupts SMAD's transport to the cell nucleus. This possibly provides a MAPK/ERK-dependent mechanism to downregulate NRSF expression. This hypothesized mechanism could possibly explain why several other types of soluble factors are also used to induce neural differentiation in a cAMP-independent manner. Growth factors such as BDNF and bFGF (FGF-2)[28] are commonly used in neural induction medium especially for MSCs. Much like cAMP-elevating compounds, growth factors can cause pan-neural effects in stem cells, but often drive differentiation through MAPK dependent signaling. Future work should be undertaken to determine if ERK mediated SMAD inhibition in neural induction also reduces expression of NRSF to drive neural differentiation. Such a possibility would cement NRSF's role as a master regulator as well as a major signal integrator and could also provide an explanation for why so many combinations of neural induction medium can induce differentiation.

### 4.7 Soft Surfaces Affect NRSF Expression to Drive Neural Differentiation

The neural inducing effect of very soft surfaces on stem cells seems to offer no immediate clues as to the signaling mechanisms. However, it should not be surprising that the few studies undertaken have implicated signaling pathways known to play a role in neural differentiation. Specifically addressing Du[63] and Sun's[60] works, soft surfaces seem to affect SMAD signaling mostly through BMPR or YAP/TAZ though neither's studies rule out the possibility that they can't run simultaneously. Their convergence onto SMAD signaling led us to hypothesize that rigid surfaces promote SMAD localization and, henceforth, NRSF expression (Figure 4.4)[140] When cultured on soft surfaces, SMAD transport is disrupted and NRSF expression cannot be activated (Figure 4.5)[140].

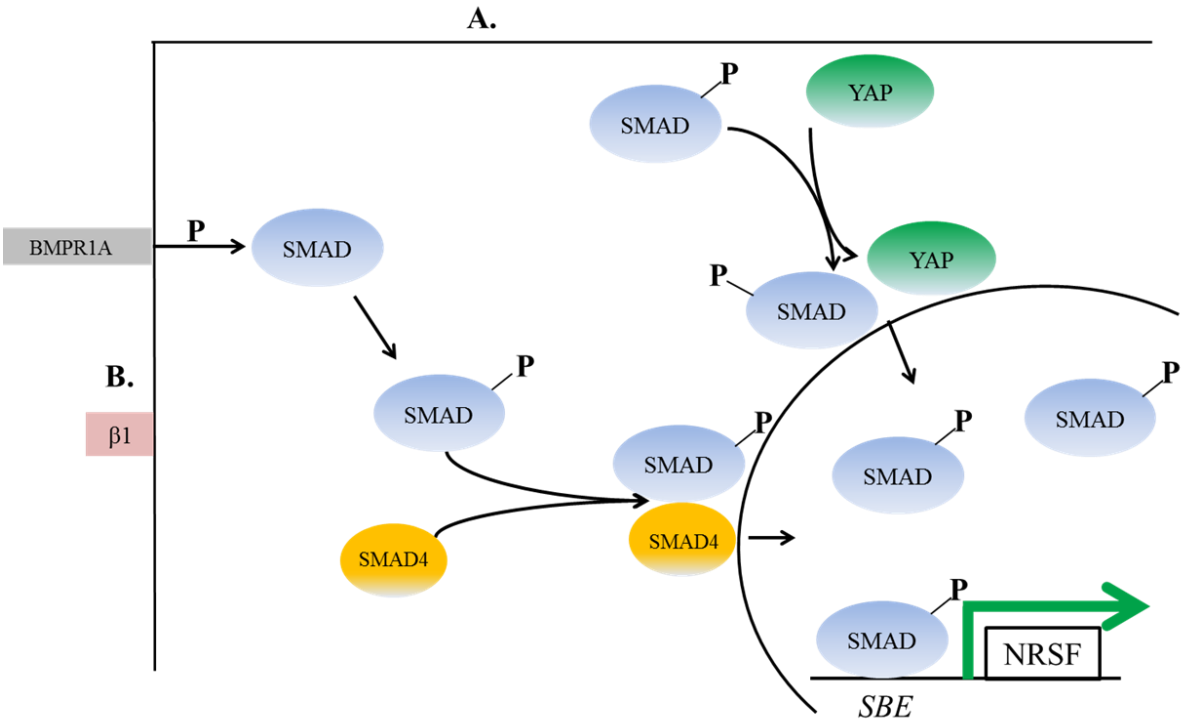



**Figure 4.4 Molecular Signaling Modulating SMAD on Stiff Surfaces. (A)** YAP mediates SMAD import into the nucleus to promote proliferation and inhibit differentiation. **(B)** Phosphorylation of SMAD by BMPR1A permits SMAD's import into the nucleus by SMAD4 (the co-SMAD). SMAD accumulation in the nucleus activates expression of NRSF thereby inhibiting neural differentiation

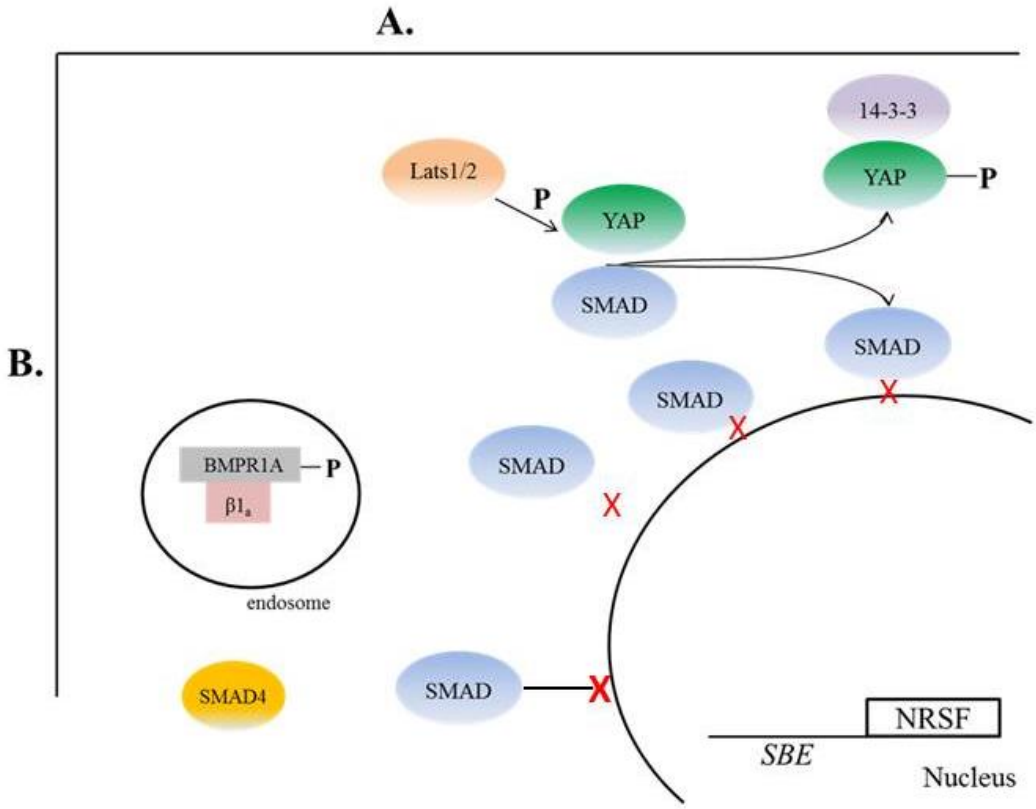



**Figure 4.5 Molecular Signaling Modulating SMAD on Surfaces. (A)** Phosphorylation of YAP by Lats 1/2 causes YAP to be bound and sequester in the cytoplasm by 14-3-3, reducing the ability of YAP to shuttle SMAD to the nucleus. **(B)** Soft surfaces increase BMPR1A internalization by endocytosis and reduce its ability to phosphorylate SMAD, thereby reducing the localization of SMAD on soft surfaces and its upregulation of NRSF expression.

What is also interesting about the effect of soft surfaces is that they induce pan-neural effects, much like FI. This suggests that soft surfaces are likely affecting cell signaling through a pleiotropic mediator and can lead us to hypothesize that NRSF is a potential target. The effect of soft surfaces on neural gene expression and dopamine response in MSCs copies what we have previously observed with our cAMP-elevating compounds. And like our previous experiments with FI, overexpression of NRSF from a vector prevented and eliminated response to dopamine showing again that downregulation of NRSF is necessary and sufficient for the neural inducing effect of soft surfaces.

## 4.8 Expanding the Understanding of Signaling Pathways Involved in Mechanotransduction To Further Highlight Its Importance

Mechanotransduction of the physical environment often implicates the cell cytoskeleton and its formation of focal adhesions. RhoGTPases are frequently studied due to their signaling and their effects on the cytoskeleton[141, 142]. A cell's ability to form focal adhesions on a substrate and subsequent activation of the focal adhesion kinase is also another well studied example of mechanotransduction[143]. Alternatively, our results highlight the importance of BMPRs at the cell membrane and show how external physical stimuli can be transduced to affect SMAD signaling. What is exciting about this result is that it implies that cellular sensing of the physical environment may have multiple mechanisms at its disposal and more than likely actively receives more physical stimuli as inputs completely aside from soluble factors. In the case of stem cell differentiation, it would be interesting to see a high throughput method used to screen the activity of transcription factors in response to culture on substrates with varying degrees of stiffness. Such an approach would undoubtedly reveal uncovered cell responses and would greatly facilitate research into how substrate stiffness affects molecular signaling and cell homeostasis.

## 4.9 Soft Surfaces in Design of Substrates for the *in vitro* Culture of Neural Cells

Interestingly, very soft surfaces specifically favor neuronal differentiation over glial differentiation. Leipzig[62] et al. corroborated Saha's[59] results and demonstrated that NSCs could be specifically differentiated into oligodendrocytes (~7 kPa), astrocytes (1–3 kPa) or neurons (<1 kPa) with varying degrees of Young's modulus on photopolymerizable methacrylamide chitosan

(MAC) surfaces. To date, surface modulus has been shown to affect a variety of cell lineages. These findings suggest that soft surfaces, i.e., with surface modulus of <7 kPa, could also be used to improve the specificity and homogeneity of differentiated neural cell cultures. Knowing how molecular signaling pathways are affected by soft surfaces could aid in the design of materials that can enhance neural differentiation.

Surfaces could then be better engineered to promote neural differentiation and function. Our results with soft surfaces show that substrate stiffness could now be a useful controllable tool for biomedical and tissue engineering approaches for cell culture.

### 4.10 NRSF, Cell Reprogramming, and Chromatin

An interesting question arises with the ability of MSCs to have the capacity for neural differentiation in the first place. Indeed, in our own experiments, we note that uninduced MSCs have a small baseline amount of neural gene expression (TUJ1, NSE, GFAP) and a percentage of them are able to respond to dopamine. Though neural differentiation of MSCs is supposed to be impossible in the face of the mesoderm-ectoderm germline barrier, many labs have been able to somewhat cross this barrier with many types of neural induction medium. This suggests that the mechanisms that make up the germline barrier are not as strong, especially when dealing with in vitro culture. One simple barrier that represses unintended neural differentiation of non-neural cells is simply compartmentalization of the pro-neural environment, ie. the brain. The pro-neural factors, growth factors, hormones, and morphogens that are required for complex differentiation of neural stem cells in to neuronal cells are strongly separated from other cell niches simply by the skull and the blood brain barrier. In the absence of exposure to these pro-neural factors, cells from other niches, ie. the bone marrow, did not need to evolve strong molecular mechanisms to

repress their potential for neural differentiation. A second mechanism for establishing the germline barrier is more intrinsic to individual cells. Here, organization of the chromatin can be used to restrict gene expression and even competency for a stem cell to differentiate into a specific cell type. However, chromatin barriers to restrict transdifferentiation of stem cells, have proven to be susceptible to exogenous expression of transcription factors.

### 4.11 NRSF as a Chromatin Regulator

A common theme in cell differentiation and reprogramming is that reprogramming happens at the level of chromatin structure. During somatic cell nuclear transfer as well as in induced pl[144]uripotent reprogramming[144-147], several epigenetic marks are reset. Additionally, heterochromatin is unwound and has the potential to be reorganized. During differentiation, chromatin organization appears to play a very important role in strongly silencing large amounts of gene expression. The organization of chromatin seems to even be purposeful within the nucleus. From the perspective of chromatin organization, the functional domains of NRSF stand out strongly. NRSF does not block transcription by inhibiting promoter regions of genes but instead recruits chromatin modifying enzymes, especially HDACs, to mark histones with repressive marks. The repressive marks of HDACs also help to compact DNA around histones keeping these sequences hidden from the transcriptional machinery. NRSF, through association with meCP2, also recognizes and is recruited to methylated DNA sequences, which are often associated as repressive marks[78]. Heterochromatin is a potent negative regulator of gene expression. Indeed, it can disable gene expression from entire chromosomes[148]. Heterochromatin represents an often understudied mechanism involved in stem cell differentiation. If we assume that heterochromatin formation represents a substantial portion of

the mesoderm-ectoderm barrier then it is plausible to hypothesize that NRSF, through its functional domains, is a major contributor to maintaining this barrier.

### 4.12 Why Dopaminergic Differentiation?

An outstanding issue regarding our work is that MSCs seem to have a predisposition towards expressing dopaminergic characteristics. Early on, we detected that FI induced not only expression of pan-neural markers such as TUJ1 and NSE, but also induced expression of genes important for the dopaminergic phenotype. Aside from increased TH expression, the strong upregulation of nurr1 also stands out as this is a transcription factor known to control expression of several dopaminergic genes (Figure 4.6).

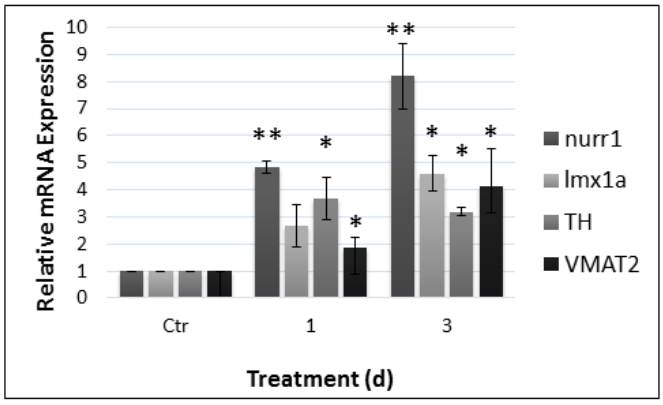



**Figure 4.6 Induction of Dopaminergic Genes by FI in MSCs.** Neural induction of MSCs by FI for up to 3 days shows upregulation of gene expression of a number of dopaminergic genes.

In combination with pitx3, nurr1 is considered a master regulator of the dopaminergic phenotype. Interestingly, this relationship between cAMP and dopamine-dependent gene expression has been hinted at in the literature[149] previously. However, the role of cAMP signaling is usually downstream of the dopamine receptors. Future work should be aimed to

address the source of dopaminergic potential, whether it be induced from the response to cAMP, or if it is an intrinsic potential of a population of MSCs.

### 4.13 Heterogeneous Differentiation of MSCs

FI induced differentiation in LZ's previous work showed that while most cells were dopamine responsive, a small population of cells had a response to glutamate[15], especially after 7 days. Additionally, more work by myself showed that silencing of NRSF with siRNA also induced a very small percentage of MSCs to gain glutamate sensitivity (Supplemental Figure 1.3). We still don't know if these are dopamine/glutamate double positive responsive cells or if they are distinct populations but it does suggest a degree of heterogeneous differentiation. The question becomes "is this a random response or is the population of MSCs used truly heterogeneous?"

### 4.14 Heterogeneity of MSCs

MSCs were originally characterized by their ability to adhere to tissue culture plastic and form fibroblast-like colonies[3]. Despite having multipotent plasticity for generating osteocytes, adipocytes, and chondrocytes, MSCs themselves are a heterogeneous cell population. Characterization of MSCs by surface markers has been difficult among the field, and there are several sets of markers to identify these cells but no true standard[15]. Further complicating matters of standardization, MSCs have been isolated from tissues other than the bone marrow and adipose tissue. Heterogeneous differentiation is an annoyance in in vitro cell culture but can be a dangerous response in vivo. This is illustrated well in attempts to treat animal models of cardiac infarction through cell replacement with MSCs. Though transplanted MSCs can

differentiate to smooth muscle to restore some of the damaged cardiac tissue, a large amount of transplanted cells also underwent chondrogenesis and osteogenesis[150]. In the case of the cells that underwent osteogenesis, the subsequent calcification of these cells proved lethal to the animals.

### 4.15 CRISPR to Eliminate Heterogeneous Differentiation

Both neural induction of MSCs with FI as well as NRSF knockdown with siRNA resulted in a small population of MSCs that could respond to glutamate. These results served as a reminder that heterogeneous stem cell differentiation is still a larger problem for the field of stem cell biology. However, this problem presented an opportunity for future investigation and spawned a project aimed at specifically addressing heterogeneous stem cell differentiation. Taken what was learned in this thesis about the importance of transcription factors to stem cell lineage commitment, a strategy was devised to restrict stem cell lineage choices in the hopes of preventing off-target stem cell differentiation. In the case of our glutamate sensitive population, it was determined that the TBR1/2 transcription factors[151-153] were required for the terminal glutamate-sensitive phenotype. Therefore, using CRISPR, TBR1/2 genes were targeted for knockout with the hopes of generating MSCs that could be induced into a purely dopamine-sensitive population. The results will hopefully provide a proof of concept approach for generating stem cells that more efficiently differentiate in homogenous populations.

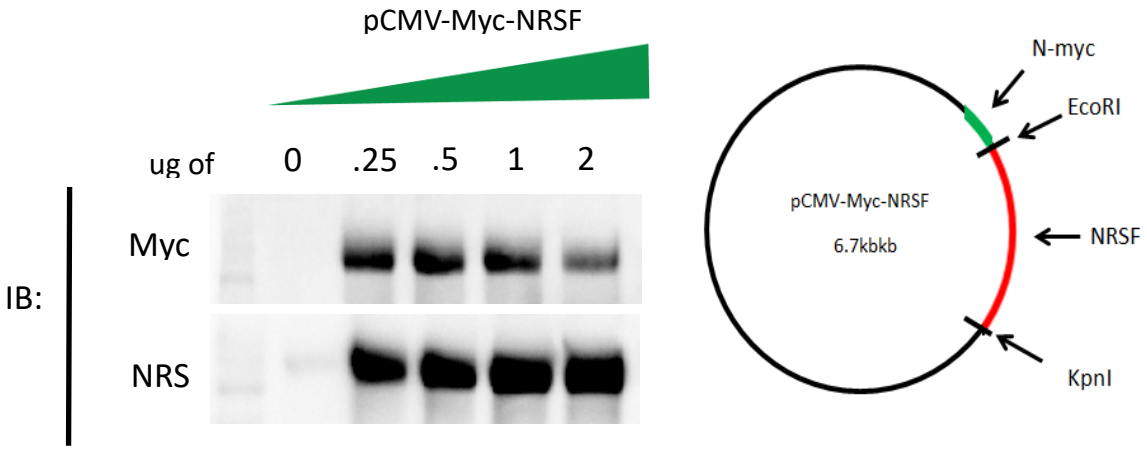
### 4.16 Overall Conclusion

The work in this thesis expands on previous work demonstrating induction of neural-like differentiation in MSCs with cAMP-elevating compounds. Here, I show that cAMP-elevating

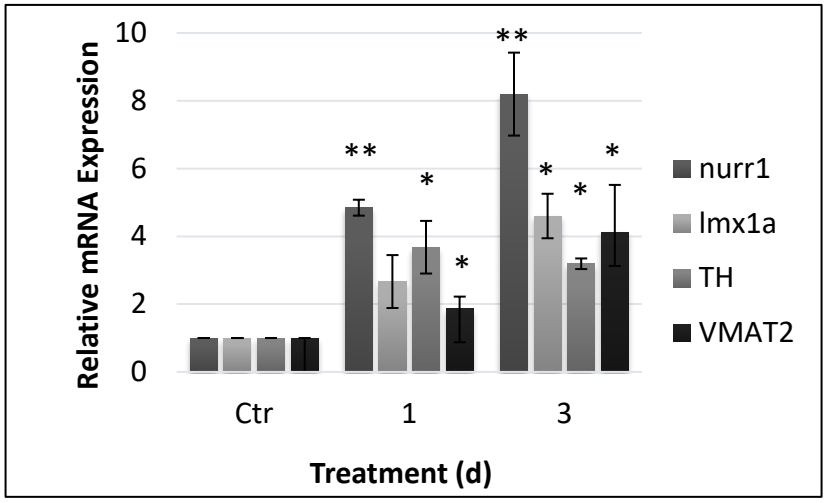
compounds elicit their effects by downregulation of NRSF. Since this hypothesis involves driving differentiation through the manipulation of a transcription factor, it is more related to cell reprogramming and hence, a better case for differentiation. Demonstrating that downregulation of NRSF is also a factor for the neural inducing effect of soft substrates also strengthens its case as a master regulator and a target for cell reprogramming. Using these results and combined with other protocols, methods for neural differentiation of MSCs should continue to be improved upon. Hopefully, this work will contribute to the broader goal of generating functional neural or neuronal cells from MSCs that have therapeutic effect in the treatment of neuronal diseases.

# APPENDIX

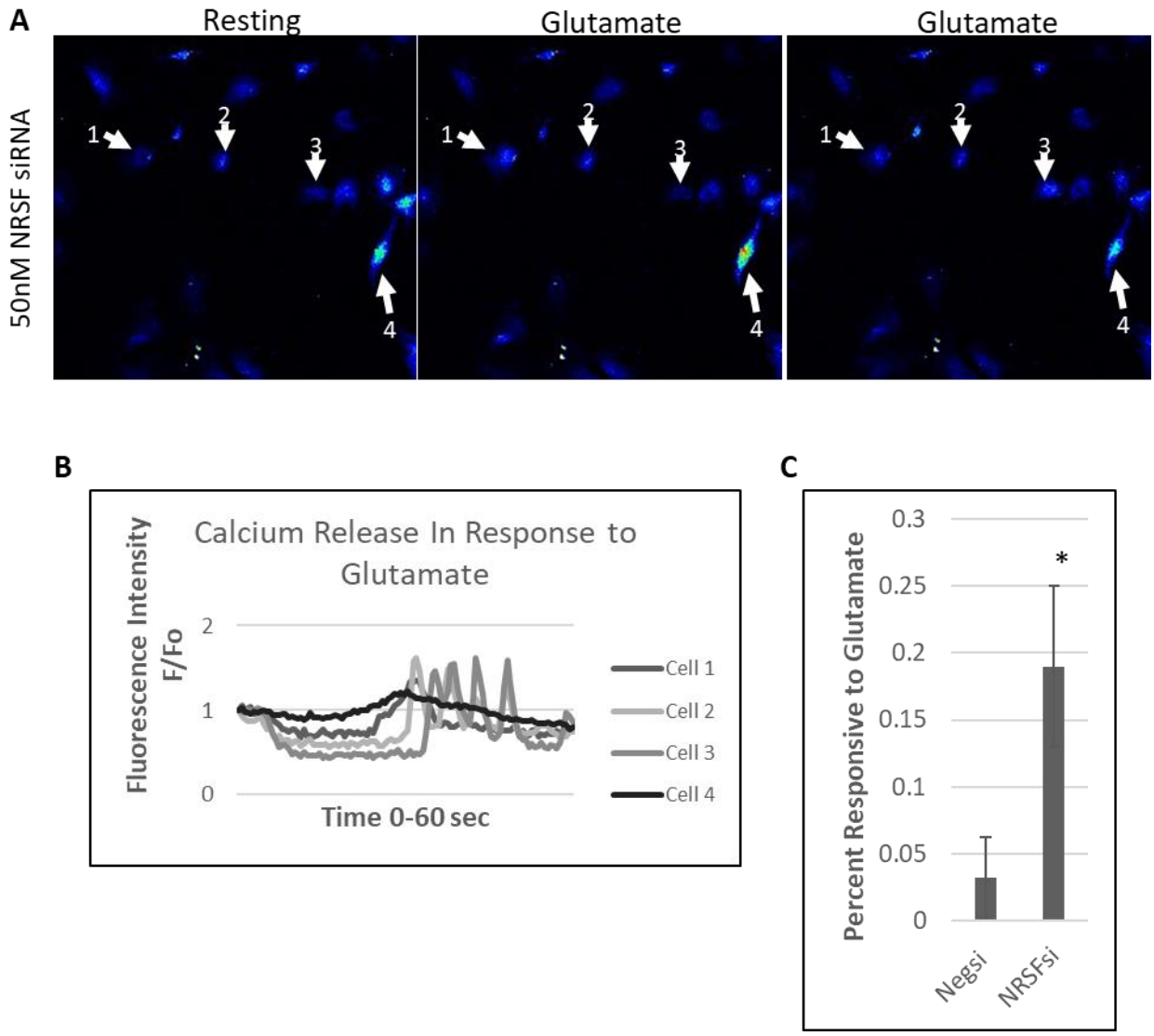
## SUPPLEMENTAL IMAGES




**Supplemental Figure 1.1 Overexpression of murine NRSF from pCMV-myc.** Murine NRSF was cloned out of pHR'-NRSF-CITE-GFP and subcloned into pCMV-Myc-N from Clonetech. Transient overexpression shows an increase in signal of a myc product at ~200 kDa. An increase in immunoreactivity for NRSF was also observed at ~200 kDa.




**Supplemental Figure 1.2 FI Treatment Induces Dopaminergic Genes In MSCs**
Neural induction of MSCs with FI induces dopaminergic transcription factors nurr1 and lmx1a, and functional dopaminergic markers, TH and VMAT2. * $p < 0.05$; **$p < 0.01$ as compared to control using Tukey's Test following ANOVA with N=3




**Supplemental Figure 1.3. Knockdown of NRSF with siRNA Induces Glutamate Sensitivity in MSCs.**

**A.** Glutamate sensitivity in MSCs silenced with 50nM. A small portion of MSCs with NRSF expression knocked down exhibit calcium release when exposed to glutamate. **B**. F/Fo intensity plot for cells 1-4. Fluorescence intensity of select cells. Cells that show greater than 20% increase in fluo-4 fluorescence are counted as responsive. Images were collected over 60 sec. **C.** Quantification of cells responsive to glutamate exposure. For negsi N=3; For 50nM NRSFsi N=6. * $p < 0.05$ using students T-test.

# TABLES

| Item | Company | Catalog Number |
|---|---|---|
| DMEM, low glucose | Gibco | 10567 |
| 70-uM nylon mesh | BD Falcon | 352350 |
| FBS | Gibco | 16000 |
| .25% Trypsin-edta | Gibco | 25200 |
| Forskolin | Sigma | F6886 |
| IBMX | Sigma | I5879 |
| Opti-MEM | Gibco | 31985 |
| Lipofectamine 3000 | Invitrogen | L3000 |
| Nitrocellulose | Biorad | 162-0112 |
| SuperSignal West Femto | Thermo | 34096 |
| Rneasy Mini Kit | Qiagen | 74104 |
| High Capacity cDNA RT Kit | Applied Biosystems | 4368814 |
| iQ Sybr Green Supermix | Biorad | 170-8882 |
| Chambered Cover-glass | Lab-Tek | 177429 |
| poly-L Lysine 70k-150k | Cultrex | 3438-200-01 |
| Fluo-4 | Invitrogen | F14201 |
| DAPI | Thermo Scientific | D3571 |

**Table 1.1 Materials**
List of reagents used

| Antibody | Dilution | Company | Catalog Number |
|---|---|---|---|
| NRSF | 1:2000 | Millipore | 07-579 |
| GAPDH | 1:10000 | Cell Signaling Technology | 2118 |
| TBP | 1:2000 | Cell Signaling Technology | 8515 |
| TH | 1:2000 | Cell Signaling Technology | 2792 |
| B-Trcp | 1:2000 | Invitrogen | 373400 |
| Goat-anti Rabbit | 1:10000 | Thermo Scientific | 31460 |
| Goat-anti Mouse | 1:2000 | Thermo Scientific | 31430 |

**Table 1.2 Antibodies**
List of antibodies used for blotting.

| Primers (RT-PCR) | Forward | Reverse | Reference |
|---|---|---|---|
| NSE | TTGTTCTCAGTCCCATCCAA | ACCACATCAACAGCACCATC | Thompson 2017 |
| Tuj1 | CAGACACAAGGTGGTTGAGG | AGTGGAGAACACGGATGAGA | Thompson 2017 |
| TH | GCCCCACCTGGAGTATTTTGTGC | GCTCCCCATTCTGTTTACATAGCCCGAAT | Thompson 2017 |
| NRSF | AGCGAGTACCACTGGCGGAAACA | AATTAAGAGGTTTAGGCCCGTTG | Spencer 2006 |
| 18S | ACTCAACACGGGAAACCTCA | AATCGCTCCACCAACTAAGA | Ferreira 2012 |
| Drd1 | AAGCTTCTCAAACTCACTGATTCC | AACTCAACTCCTACCCTTCCTTTC | Thompson 2017 |
| Drd5 | TGCCTCCATCCTGAATCTGTGTA | GGATGAAGGAGATGAGGATGGA | Thompson 2017 |
| Nurr1 | CCTGACTATCAGATGAGTGG | CAGTTTGGACAGGTAGTTGG | Thompson 2017 |
| VMAT2 | CGGGACAGCCGCCACTCGCGCAAACT | GTGTCCCCTTCTGACTCTCTGGCTGT | Thompson 2017 |
| Lmx1a | ATTGCGCCCAATGAGTTTGT | GTGGGCAACGTTGTATAGGG | Thompson 2017 |
| | | | |
| **Primers (Cloning)** | **Forward** | **Reverse** | **Reference** |
| EcoRI-mNRSF | ACTGTAGAATTCAGGTTATGGCCACCAGGTGA | KpnI-mNRSF | Thompson 2017 |
| KpnI-mNRSF | EcoRI-mNRSF | CATGATGGTACCCTCAGCTACTCCTGCTCCT | Thompson 2017 |

**Table 1.3 Primer Sequences**

These sequences were mostly designed by the author, Thompson. The sequences for the NRSF primer and the 18S loading control were from Spencer, 2006[154] and Ferreira, 2012[155].

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## BIBLIOGRAPHY


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