THE DEVELOPMENTAL IMPLICATIONS OF THE REGULATORY RELATIONSHIP BETWEEN CJUN AND OCT4 IN MURINE EMBRYONIC STEM CELLS

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ABSTRACT

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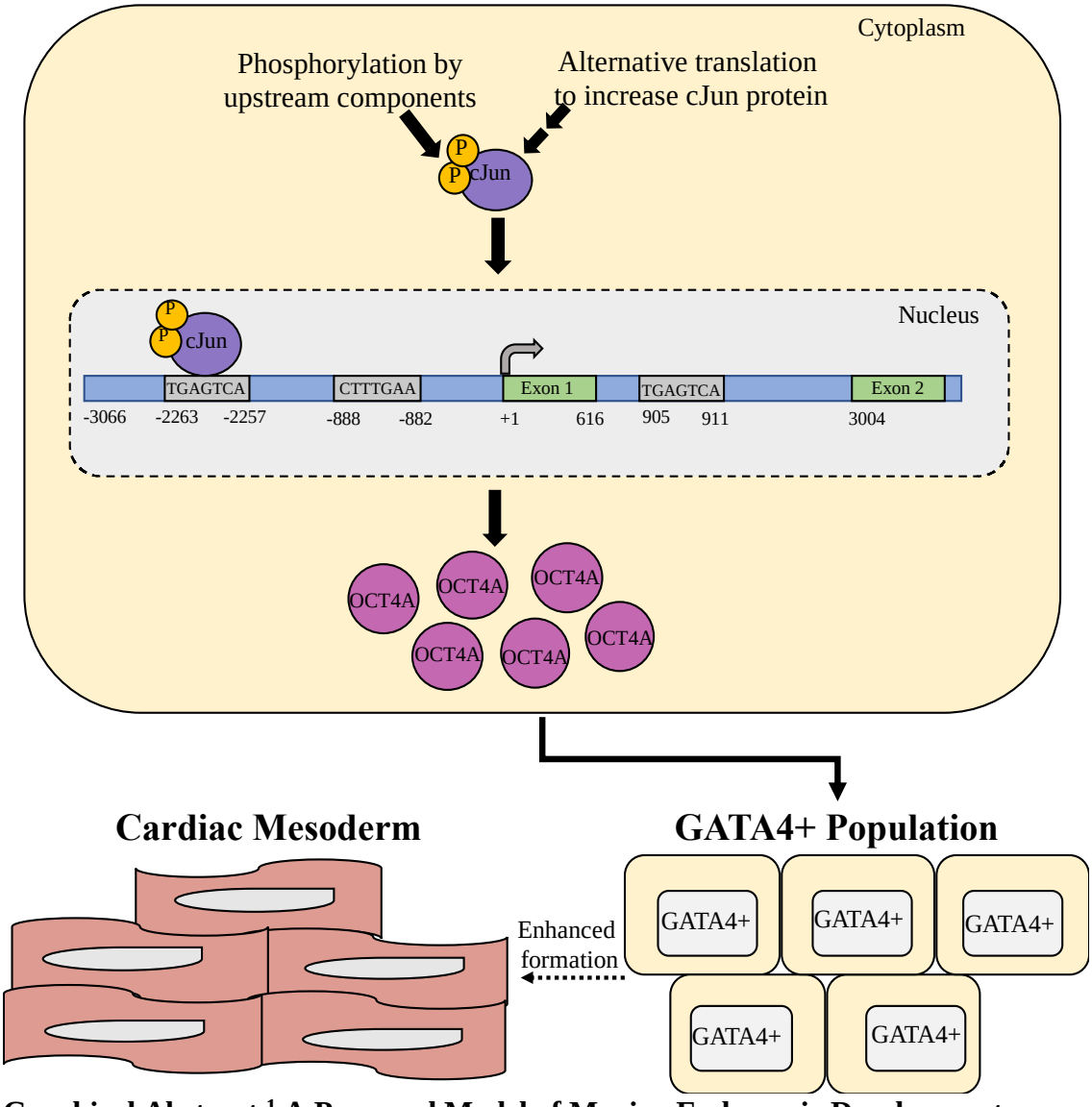
As cells transition from the point of fertilization through the process of embryonic development, many molecular changes occur that affect cell fate. At the blastocyst stage, the earliest distinction, two separate cell populations arise. The trophectoderm cells will generate all of the extraembryonic tissues while the inner cell mass will yield all of the embryonic tissues. These cells, which generate the organism, are termed pluripotent at this stage and found within the inner cell mass (ICM). A variety of genetic mechanisms that regulate this event have been characterized. Here, we examined the effect of cJun expression in regulating Oct4, a gene known to be a master regulator of pluripotency. Luciferase assays were performed to validate our bioinformatics analysis of the 3 kb *Oct4* promoter, which identified a cJun binding site approximately 2500 bp upstream of the transcription start site. These experiments demonstrate transient expression of cJun off a reporter plasmid significantly increases luciferase activity of the Oct4-luciferase reporter construct, while expression of the transcriptionally inactive cJun mutant L40/42A represses it. Additionally, we corroborate previous reports that cJun can synergize with Beta-catenin, a member of the Wnt pathway, to significantly increase the promoter activity of *Oct4*. A role for regulation of endogenous *Oct4* expression was

evaluated by immunoblot analysis of murine embryonic stem cells transiently transfected with GFP cJun, which demonstrated increased OCT4A expression 36 hours post-transfection. To assess the developmental implications of this regulatory relationship, we evaluated the effect of cJun overexpression on early differentiation by transiently transfecting mESCs and initiating embryoid body formation. When evaluated for germ layer marker expression by immunoblot analysis, it was revealed that an increase in cJun correlated with an increase in GATA4 expression, suggesting cJun expression could lead to selection toward the endodermal germ layer. In light of this, we carried out directed differentiation into cardiomyocytes, using a hanging drop intermediate, and found that an increase in cJun led to a significant increase in cardiomyocyte formation and beating. Overall, this suggests a new role for cJun in the regulation of potency and the formation of the endoderm during those early cell fate choices.

Key Words: pluripotent, cell fate, germ layer, cJun, Oct4

GRAPHICAL ABSTRACT



Graphical Abstract [1] A Proposed Model of Murine Embryonic Development

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TABLE OF CONTENTS

ABSTRACT ii
GRAPHICAL ABSTRACT iv
ACKNOWLEDGEMENTS v
LIST OF FIGURES viii
LIST OF APPENDICES ix
Appendix A ix
Appendix B x
INTRODUCTION 1
OCT4 as a Key Developmental Transcription Factor 1
The Regulatory Potential of cJun on *Oct4* 6
Embryoid Bodies as a Model for Murine Development 9
Statement of Aims 11
MATERIALS AND METHODS 14
Cell Culture and Maintenance of 293T Cells 14
Basic Cell Culture of J1 Murine Embryonic Stem Cells 14
Transient Transfection of 293T cells and J1 Murine Embryonic Stem Cells 15
Luciferase Assays in 293T Cells and J1 Murine Embryonic Stem Cells 15
Embryoid Body Formation 17
Nuclear Protein Extraction, BCA Protein Assay and Immunoblot Blot 18
Immunocytochemistry 20
Hanging Drop Embryoid Body Formation and Directed Differentiation to Cardiomyocytes 21
Data Analysis 21
RESULTS 24
A Regulatory Role for cJun in the Activation of the *Oct4* Promoter 24
Transient cJun Specifically Increases OCT4A Expression in J1 mESC 27

Transient cJun Expression is Associated with an Increase in GATA4 32
Transient cJun Expression Increases Cardiomyocyte Formation 37
Chemical Modulation of JNK Signaling Affects Potency, cJun Expression and OCT4 41
DISCUSSION 51
cJun Increases Oct4 Promoter Activity 51
Over-Expressed cJun Leads to Increased Expression of the OCT4A Isoform in Murine Embryonic Stem Cells 52
Overexpression of cJun results in increased GATA4 Expression 54
Overexpression of cJun During Embryoid Body Formation Increases Differentiation to Cardiomyocytes 56
Upstream Modulation of the JNK/cJun Signaling Pathway 59
Modulation of cJun Expression by Increased Translation Also Increases OCT4A Expression 60
Conclusions 61
LITERATURE CITED 63
APPENDIX A 73
Optimization of Luciferase Assays 73
Analysis of Embryoid Body Formation and Germ Layer Expression 77
Chemical Modulation of cJun/JNK Signaling 80
APPENDIX B 82
Antibody Information 82

LIST OF FIGURES

FIGURE 1. ALTERNATIVE SPLICING AND IRES IN MOUSE OCT4 GENE LEADS TO MULTIPLE ISOFORMS 6

FIGURE 2. IDENTIFICATION OF AP-1 BINDING SITES IN THE OCT4 PROMOTER OF MUS MUSCULUS 9

FIGURE 3. CJUN BINDS AND ACTIVATES THE OCT4 PROMOTER IN 293T CELLS 25

FIGURE 4. TRANSFECTED CJUN IS DETECTED BY WESTERN BLOT AND LEADS TO AN INCREASE IN OCT4A 30

FIGURE 5. INCREASED CJUN AND OCT4A PROTEIN LEVELS CORRELATE WITH AN INCREASE IN GATA4 35

FIGURE 6. INCREASED CJUN AND GATA4 CORRELATE WITH INCREASED BEATING CARDIOMYOCYTES 40

FIGURE 7. DRUG-INDUCED MODULATED CJUN EXPRESSION ALTERS EXPRESSION OF KEY POTENCY MARKERS 43

FIGURE 8. DRUG MODULATION SHOWS CO-LOCALIZATION OF CJUN AND OCT4A AT 2 AND 4 HOURS POST-TREATMENT 49

FIGURE 9. INCREASED CJUN EXPRESSION BY ANISOMYCIN AND NOCODAZOLE IMPEDE EMBRYOID BODY FORMATION 50

LIST OF APPENDICES

Appendix A

SUPPLEMENTAL FIGURE 1. TRANSFECTION EFFICIENCY WITH NEW OCT4 CLONE THAT ENCOMPASSES COMPLETE PROMOTER SEQUENCE (-3066- +1) 73

SUPPLEMENTAL FIGURE 2. POSITIVE CONTROL PLASMID TRANSFECTION EFFICIENCY 74

SUPPLEMENTAL FIGURE 3. J1 MESCS ARE NOT EFFICIENTLY TRANSFECTED COMPARED TO 293T 75

SUPPLEMENTAL FIGURE 4. EXPRESSION OF CJUN CONSTRUCT CAN BE DETECTED BY WESTERN BLOT 76

SUPPLEMENTAL FIGURE 5. DIFFERENTIATION OF MESC INTO EMBRYOID BODIES 77

SUPPLEMENTAL FIGURE 6. GFP PLASMID EXPRESSION IS STILL DETECTED 4 AND 6 DAYS AFTER LOW ADHERENT CULTURE 78

SUPPLEMENTAL FIGURE 7. REPLICATED WESTERN BLOTS SHOW AN INCREASE IN GATA4 WHEN CJUN IS OVER-EXPRESSED 79

SUPPLEMENTAL FIGURE 8. OCT4A EXPRESSION IS NOT SIGNIFICANTLY ALTERED AFTER TWO HOURS 80

SUPPLEMENTAL FIGURE 9. THERE IS VARIATION IN POTENCY MARKER EXPRESSION UNDER DRUG MODULATION 81

Appendix B

SUPPLEMENTAL TABLE 1. ANTIBODIES USED FOR IMMUNOBLOT AND IMMUNOCYTOCHEMISTRY 83

INTRODUCTION

OCT4 as a Key Developmental Transcription Factor

OCT4 is a member of the POU transcription factor family homeodomain. It is highly expressed during the early stages of mouse development and has been shown to be essential in the formation of the pluripotent inner cell mass (ICM) (Scholer et al. 1990; Nichols et al. 1998). Without *Oct4*, the cells of the ICM are unable to give rise to the three germ layers, and are therefore developmentally restricted to yielding only the trophectoderm (TE) lineage (Nichols et al. 1998; Velkey and O'Shea 2003). Induced deletion of *Oct4* in embryos as they transition from the morula to blastocyst stage causes a deficiency in lineage determination; the cells of the ICM cannot differentiate toward primitive endoderm and the embryo does not progress from blastocyst to epiblast (Le Bin et al. 2014). Reduced OCT4 concentration in mESCs through heterozygosity maintains pluripotency, but causes a delayed response to differentiation cues that ultimately affect the critical timing of development (Karwacki-Neisius et al. 2013).

Previous work has established OCT4 as an essential regulatory protein that determines lineage commitment (Niwa et al. 2000; Zeineddine et al. 2006; Rodriguez et al. 2007; Le Bin et al. 2014). The transcription factor is expressed at levels comparable to the ICM in migrating endoderm cells, which come into contact with the TE as they differentiate during the blastocyst stage (Palmieri et al. 1994). A less than two-fold increase in *Oct4* expression can lead to mesoderm and primitive endoderm formation,

while a less than two-fold decrease causes embryonic stem cell differentiation to TE, suggesting that there is a critical, highly specific, level of *Oct4* necessary to maintain pluripotency; it's not simply on or off when regulated (Niwa et al. 2000). Further analyses revealed that increased *Oct4* expression can result in the establishment of the cardiac lineage population in both mESCs and embryos entering the early stages of differentiation (Zeineddine et al. 2006; Rodriguez et al. 2007).

There is significant evidence that *Oct4* is also critical to the regulation of later stages of development. Boyer et al. (2005) discovered that a group of genes regulated by OCT4, NANOG and SOX2 are key developmental players. RNAi studies have shown that reduced expression of OCT4 and NANOG in mES cells affects the gene expression of their targets, ultimately changing cell fate (Loh et al. 2006). OCT4 binds to low-accessible genomic regions, activating enhancers that would otherwise remain unresponsive to specific differentiation cues (Simandi et al. 2016). Frum et al. (2013) found that OCT4 is required for NANOG mediated Fgf4/MAPK signaling as the primitive endoderm (PrE) is specified, since exogenous treatment of Fgf4 was not sufficient to induce expression of Sox17 and other PrE markers in *Oct4* null E2.75-E4.5 mouse embryos. As cells are guided toward endoderm differentiation, OCT4 alternates binding the enhancer regions of the *Sox2/Sox17* loci between *Sox2* and *Sox17*. This has been shown to be an essential step in the formation of mesendoderm and mesoderm (Aksoy et al. 2013; Abboud et al. 2015).

Coordinated regulation of *Oct4* gene expression is critical to achieving proper embryonic development. Therefore, a significant amount of work has been performed to

better understand the regulation of *Oct4* transcription and post-transcriptional mRNA processing. *Oct4* gene expression is conserved across many mammalian species. Gene transcription is initiated by a GC-rich region of the TATA-less promoter (Reviewed in Jerabek et al. 2014). Upstream of the transcription start site, the *Oct4* promoter region possesses two highly conserved enhancer elements which cumulatively span a region of approximately 3,000 base pairs. The distal (-3.2 kb-1.39 kb) and proximal (-1.39 kp-0) enhancers are both tissue-specific and developmentally restricted (Yeom et al. 1996; Reviewed in Jerabek et al. 2014; Choi et al. 2016). The distal enhancer is the primary source of regulation preimplantation (Choi et al. 2016). Upon implantation, the proximal enhancer plays a key role in driving transcription within the epiblast and the distal enhancer is restricted to the germ cell lineage (Reviewed in Jerabek et al. 2014). The promoter/enhancer region of *Oct4* is hypomethylated in the pluripotent cells of the ICM; however, it is hypermethylated in the cells that ultimately give rise to the trophectoderm, further highlighting the critical developmental timing of *Oct4* expression (Hattori et al. 2004).

Many transcription factors have been shown to bind target sequences within the *Oct4* promoter. One of which is OCT4. OCT4 heterodimerizes with SOX2 and binds an OCT-SOX regulatory element in the distal enhancer to increase transcription (Chew et al. 2005). Another transcription factor complex that binds *Oct4* is LEF/TCF4. Three LEF/TCF4 binding sites have been identified in the *Oct4* promoter, one in the proximal region (-881/875) and two in the distal (-1661/1665 and -2032/2026). Constitutively active Beta-catenin can increase activity off of these sites, suggesting regulation of *Oct4*

expression is one mechanism by which the Wnt signaling cascade regulates pluripotency (Wei et al. 2012). As the developing embryo next exits gastrulation, the *Oct4* gene is directly bound by germ cell nuclear factor (GCNF) which leads to the subsequent recruitment of a *de novo* methyltransferase; and ultimately, expression is silenced in all cells except those of the germ line (Fuhrmann et al. 2001; Wang et al. 2016).

Alternative splicing and translation are other mechanisms used to regulate OCT4 activity. The murine *Oct4* gene generates four isoforms: OCT4A 352aa, OCT4B-247aa, OCT4B-190aa and OCT4B-164aa (**Figure 1**). The OCT4A isoform is the only one to contain the first exon and therefore has an unique N-terminus. Additionally, this alternative splicing leads to the exclusion of exon 2a, which becomes the distinct N-terminus of the OCT4B isoforms. As in humans, the OCT4A isoform is regarded as a keeper of pluripotency. Until recently, the OCT4B isoforms remained elusive in function and sequence. Of the identified OCT4B isoforms, OCT4B-247aa, containing the full intact N-terminus, and OCT4B-164aa were localized in the nuclei of non-pluripotent cells while OCT4B-190aa was mainly found in the cytoplasm, suggesting these different isoforms may have different functions due to their subcellular localization (Guo et al. 2012). Furthermore, during somatic cell reprogramming, OCT4B isoforms were unable to replace OCT4A thereby offering additional evidence that OCT4A, specifically, is essential for pluripotency (Guo et al. 2012). Because many IRES containing mRNAs are involved in stress response, Guo et al. (2012) examined the OCT4B isoforms under hyperpyrexia and hyperxia conditions, in mES cells. Interestingly, they found that

OCT4B-190aa, which was mainly found in the cytoplasm, was upregulated over time in response to stress.

While there is still a great deal left unknown about the mouse *Oct4* gene and the variants it encodes, more information is emerging regarding the corresponding human OCT4 isoforms. Analogously, the human *OCT4* gene generates three known, stable isoforms through alternative splicing or alternative translation: OCT4A, OCT4B and OCT4B1. Over the last several years, the differential expression patterns of the various isoforms have been studied more actively, noting that the OCT4A variant is highly expressed, and restricted, to the nuclei of pluripotent human ES (hES) cells and embryonal carcinoma (hEC) cells (Atlasi et al. 2008; Reviewed in Wang and Dai 2010). OCT4A has been a known transcription factor and key regulator of pluripotency in stem cells while OCT4B, which is typically located in the cytoplasm of non-pluripotent cell types, has more recently been implicated in stress resistance (Atlasi et al. 2008; Wang et al. 2009). OCT4B1, interestingly, has a nearly identical amino acid sequence (residues 1-80 are the same) compared with OCT4B, however it is highly expressed in pluripotent hES and hEC cells and downregulated during differentiation (Atlasi et al. 2008). The OCT4A variant has been shown to be essential in maintaining stemness during embryogenesis while OCT4B, was proven insufficient for self-renewal and maintaining pluripotency (Reviewed in Wang and Dai 2010). Taken together these data suggest that the OCT4A isoform will play a much greater role in the development and differentiation of an organism.

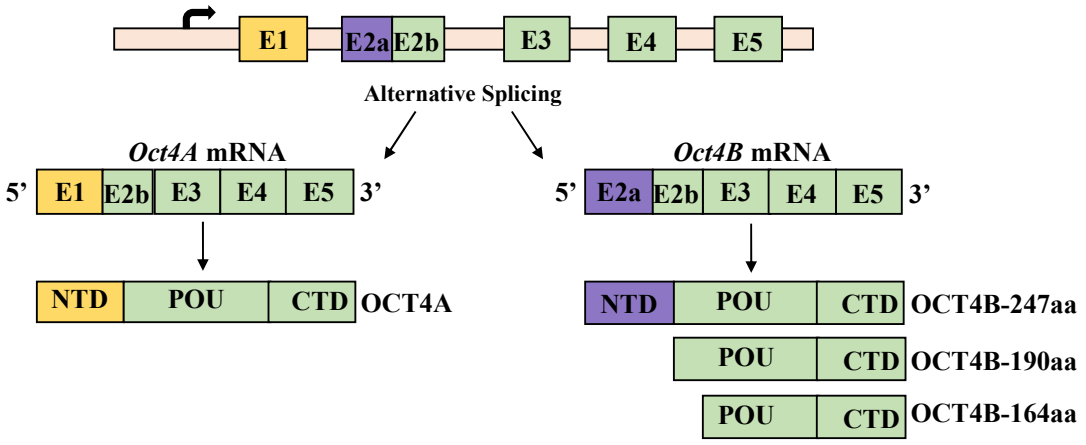


Figure 1. Alternative Splicing and IRES in mouse Oct4 Gene Leads to Multiple Isoforms

(top) The mouse *Oct4* gene contains five exons and four introns following the transcription start site (black arrow). Through alternative splicing, two different mRNA transcripts are produced: Oct4A (left, middle) and Oct4B (right, middle). The Oct4A transcript is unique in that it's the only one that contains the first exon, which then becomes the unique N-terminus of the subsequent OCT4A protein (left, bottom). The Oct4B transcript contains an IRES which gives rise to three unique isoforms of varying length (right, bottom) (Guo et al 2012).

The Regulatory Potential of cJun on *Oct4*

cJun is a transcription factor belonging to the AP-1 family. It is implicated in a myriad of cellular processes including cellular division, cellular migration, and apoptosis (Wilkinson et al. 1989; Eferl et al. 1999; Behrens et al. 2002). Its activity is regulated by many processes including gene expression, alternative translation and post-translational modifications. cJun has been shown to regulate its own transcription through a positive feedback loop in response to specific stimuli (Angel et al. 1988) however, perhaps the best characterized example of its regulation is post-translational: the direct phosphorylation of cJun by c-Jun N-terminal kinases (JNKs). JNKs are a subfamily of

mitogen-activated protein kinases (MAPKs) discovered by their ability to bind and phosphorylate cJun. Active JNK binds c-Jun at leucines L40/42 to phosphorylate serines 63 and 73. Phosphorylation of these residues is necessary for transcriptional activity of consensus AP-1 sites (Smeal et al. 1992; Czaja 2003; Sprowles and Wisdom 2003). If phosphorylated cJun dimerizes with a dominant negative mutant, the DNA binding activity may be reduced (Smeal et al. 1992).

cJun can also be regulated at the translational level. A cap-independent translation mechanism for cJun was first proposed in chicken cells (Sehgal et al. 2000). Others have now corroborated this report in various cell lines including HeLa, 293T, NIH3T3 and patient derived glioblastoma cell lines (Polak et al. 2006; Blau et al. 2012). They found that upon de-polymerization of actin filaments and microtubules, there was an increase in cJun protein but not mRNA. Furthermore, the protein half-life was comparable to the controls suggesting that the increase was not simply due to an accumulation of protein over time. MAPKs, particularly JNKs, are activated in response to cytoskeletal stress and are known to regulate cJun; however, increased translation via the virus-like IRES domain in the 5'UTR (untranslated region) occurs independent of MAPK activity (Polak et al. 2006; Spangler et al. 2010; Blau et al. 2012). Increased cJun by cytoskeletal disruption has also been associated with increased malignant behaviors of glioblastoma lines (Blau et al. 2012).

cJun plays a critical role not only in disease, but in embryonic development. A null mutation in the cJun locus results in embryonic lethality at 11-12 days post coitus in mice (Johnson et al. 1993). cJun-/- mice die during mid- to late-gestation and show

impaired development of some endoderm-derived organs (Hilberg et al. 1993). The ability to form fibroblasts with proper morphology and liver cells in culture were adversely affected by the loss of cJun suggesting a greater role in specific cell types, including those involved in cardiac formation (Johnson et al. 1993; Eferl et al. 1999). cJun is also consistently expressed during neural development in normal embryogenesis (Raivich and Behrens 2006). Exogenous cJun expression in p19 cells leads to endoderm- and mesoderm-like differentiation and has been shown to be essential for cardiomyocyte differentiation (Groot et al. 1990; Eriksson and Leppa 2002).

More recently, the role for cJun in ICM potency and cell fate has been described in murine embryonic stem cells. cJun forces mESCs out of the ground state of pluripotency and on to a somatic fate with restricted proliferation (Liu et al. 2015). Cells overexpressing cJun have increased mRNA of early developmental genes including *Gata4* and *Sox17*, while the expression of pluripotent genes, including *Oct4*, *Nanog* and *Sall4*, critical to stem cell function, become inhibited (Liu et al. 2015). The work performed by Liu et al. caused them to propose a model wherein cJun serves as a master regulatory function to drive the differentiation of the inner cell mass within the developmental context.

Our lab discovered a putative AP-1 binding site within the promoter region of the murine *Oct4* promoter **(Figure 2)**. Furthermore, we have demonstrated that when cJun is phosphorylated at S63, the OCT4A isoform is overexpressed in mESCs (Hosawi et al. *in prep*). Transient expression of cJun constructs has demonstrated a decrease in differentiation capacity of mESCs to pancreatic islet-like clusters (Hosawi et al. *in prep*).

Taken together these data suggest that JNK signaling and cJun affect cell fate during differentiation by altering the potency of mESCs (Hosawi et al. *in prep*). Based on our work and that of Liu et al. (2015), we hypothesize these changes would be best characterized as the ICM cells move out of pluripotency and into early germ layer development.

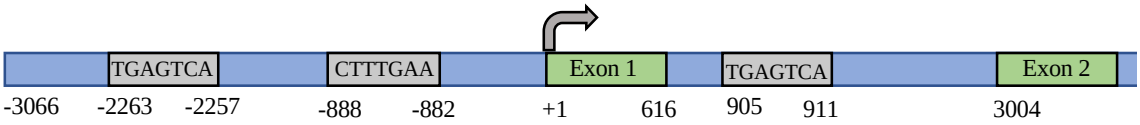


Figure 2. Identification of AP-1 Binding Sites in the *Oct4* Promoter of Mus musculus

Utilizing the NCBI database (Accession number NT_039662), a putative AP-1 binding site was found in the distal promoter of *Oct4* approximately 3,000 base pairs (bp) upstream of the transcription initiation site. Three TCF/LEF sites 2kb (not shown), 1.6kb (not shown) and 800bp (shown at -888 bp to -882 bp) upstream of the initiation site have been shown to regulate Oct4 expression by others (Li et al 2012). The location and proximity of these two binding sites suggest they may be involved in transcriptional regulation of the Oct4 gene.

Embryoid Bodies as a Model for Murine Development

Embryoid bodies (EBs) are one system used to study the cellular and genetic changes that occur as mES cells exit the pluripotent state. These small, cellular structures form due to spontaneous differentiation of mESCs when grown in suspension culture in the absence of a feeder layer (Reviewed in Keller 1995). They have been used to study the specific onset of developmental programming in human ES cells (Itskovitz-Eldor et al. 2000) and mouse ES cells (Reviewed in Desbaillets et al. 2001). EBs offer an advantage as a developmental model because they maintain the integrity of cell-cell contacts, which may offer critical developmental cues (Reviewed in Keller 2005). EBs

offer structural complexity which mimics *in vivo* events, as shown by Wnt-mediated axis formation (Berge et al. 2008). The expression patterns of primary germ layer markers such as Sox2, Brachyury, and Gata4, as well as markers of hematopoietic lineage determination suggest that EBs accurately reflect *in vivo* murine germ layer formation spatially and temporally (Leahy et al. 1999; Reviewed in Keller 1995, 2005). Due to their accurate temporal and spatial modeling, they have been used to specifically model cardiac (Doetschman et al. 1985; Sanchez et al. 1991), hepatocyte (Chinzei et al. 2002) and, neural (Reviewed in Keller 1995, 2005) specification, each of which arise from a different germ layer. They have also been used as a model to study epigenetic regulation and modification in developing embryos (Kremenskoy et al. 2003). Based on the well-established literature regarding EBs as an accurate model for germ layer formation, we selected this system to carry out our studies.

Statement of Aims

The overall goal of this project was to further define the role of cJun in the regulation of Oct4 and embryonic stem cell potency. Previous evidence in our laboratory suggested that modulation of cJun/JNK signaling affected mESC potency, and that this could be through upregulation of OCT4A expression. To address this question, we proposed the following two aims:

1. **Address the potential of cJun to transcriptionally regulate Oct4.** Utilizing bioinformatics, our lab has identified a putative AP-1 binding site in the *Oct4* distal enhancer region. The presence of this site suggested that AP-1 family members, including cJun, may participate in the regulation of the *Oct4* gene. To address this, luciferase assays were conducted in 293T cells with a reporter construct containing the candidate AP-1 binding sequence of the *Oct4* proximal promoter (bp -2263-2257). The construct, -3066 +1, contains approximately 3 kb that precede the translation start site of OCT4A (aa. 1, exon 1). The promoter sequence was cloned into the pTA Luc reporter plasmid. Reporter activity was measured in the presence of GFP, GFP cJun, and a GFP cJun mutant that has been shown to be transcriptionally inactive (GFP cJun L40/42A). Additionally, we utilized active Beta-catenin, a known activator of canonical Wnt targets alone or co-transfected with each of the cJun constructs. Thirty-six to forty-eight hours post transfection, cells were imaged for GFP expression to confirm successful transient transfection, then the cells were analyzed with the Promega Dual Glo Stop and Glo Kit to assess luciferase and Renilla production. Data was collected

on the SpectraMax5L plate reader by Molecular Devices and analyzed by a one-way ANOVA followed by Tukey's Multiple Comparisons.

2. **Determine the role of cJun in cell fate determination of pluripotent, murine embryonic stem cells.** Previous work demonstrated that modulation of cJun affects the potency of mESCs and murine embryonic carcinoma cells (mECCs) (Groot et al. 1990; Liu et al. 2015; Veluscek et al. 2016; Hosawi et al. *in prep*). To better characterize the role of cJun and JNK signaling on mESC potency and cell fate decision making, cJun/JNK signaling was modified through transient transfection of J1 embryonic stem cells with ef1-GFP, ef1-GFP cJun, the transcriptionally inactive cJun mutant ef1-GFP L40/42A, or treatment with chemicals that modify the JNK signaling pathway (anisomycin, a JNK activator, and the JNK inhibitor, SP600125). Treated stem cells were harvested 36 hours post-transfection, or two and four hours post-drug treatment, as well as differentiated into embryoid bodies (EBs). A fraction of these samples harvested prior to differentiation were utilized for immunocytochemistry(ICC) and western blot analysis to assess early changes in potency markers, including OCT4A. The remainder of the samples were used to generate embryoid bodies (EBs), an *in vitro* model of early gastrulation, which display markers indicative of the three primary germ layers, thus allowing us to study the influence of cJun on development. It was expected that because modulated *Oct4* can select toward either the mesoderm or endoderm (Rodriguez et al. 2007), the regulatory effect of cJun on *Oct4* will play a crucial role in the early steps of lineage commitment.

The EB samples generated from J1 mESCs were utilized for western blot analysis to detect changes in markers for the different germ layers. Based on these results transfected cells were differentiated into beating cardiomyocytes, through a hanging drop intermediate, to further assess differentiation potential.

MATERIALS AND METHODS

Cell Culture and Maintenance of 293T Cells

Lenti-X™ 293T cells were obtained from Clontech (Mountain View, CA). They were grown on adherent tissue culture grade dishes at 35,000 cells/cm^2 and grown at 37℃ in 5% CO_2 in 1X DMEM from Gibco (Ref. 11995-065) and 10% Hyclone® Fetal Bovine Serum (FBS) (cat. SH30070.03). Cells were subcultured at approximately 90%-95% confluency by washing with 1X Sterile PBS (Gibco® Life Technologies, Grand Island, New York, ref. 14190-235) and treating with 0.05% Trypsin-EDTA (Gibco® Life Technologies, Grand Island, New York, ref. 25300-054). Cells were spun down at 1400 x g for 5 minutes at room temperature, resuspended in 1-3ml of prewarmed media and tested for viability using a Trypan Blue Assay (Gibco® cat#15250-061).

Basic Cell Culture of J1 Murine Embryonic Stem Cells

Murine Embryonic cell line, J1, was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown on a layer of murine embryonic fibroblast (MEF) cells treated with mitomycin C to inhibit cell division and optimize growth factor secretion. MEFs were plated at 50,000 cells/cm^2. The mESCs were grown in ES-DMEM media containing DMEM base, 2.0 mM L-alanyl-L-glutamine, 1X nonessential amino acids, 0.1 mM 2-mercaptoethanol, 50 units/ml Lif and 15% fetal bovine serum (FBS). The MEFs were plated prior to passaging the mESCs, in 1X DMEM base from Gibco® with 10% Hyclone® FBS. For each passage cells were washed with sterile PBS, treated with 0.05% trypsin, spun down at 700 x g for 5 minutes at room

temperature and resuspended in warmed fresh mESC media. A trypan blue assay was performed to assess viability and all cells were plated at 30,000 cells/cm^2 and kept at 37℃ and 5% CO_2.

Transient Transfection of 293T cells and J1 Murine Embryonic Stem Cells

Prior to transfection, tissue culture dishes were coated with 0.1% gelatin in water from Stem Cell Technologies (catalog # 07903) and incubated at room temperature for 30 minutes. Transfection cocktails were prepared following manufacturer's' instructions for the Lipofectamine® 3000 kit (Invitrogen Thermo Fisher Scientific cat#. L3000-015) using Gibco® OPTI-MEM® I(1X) (Grand Island, New York, cat # 31985-062). Each transfection cocktail contained one of the following plasmids: pLVX-EF1a-AcGFP1-cJun, pLVX-EF1a-AcGFP1-L40/42A or pLVX-EF1a-AcGFP (total of 2.5 µg DNA per 1 x 10^6 cells). Cells were harvested as described above and 1x10^6 cells were added to each cocktail with additional cells for "no treatment" controls. The reaction was optimized using 2.5 µg total DNA per 1 x 10^6 cells, 3.75 µl of Lipofectamine 3000 and 5 µl of P3000 per cocktail. The DNA-cell cocktails were then spun at 37℃ at 700 x g for one hour. Cells were then gently resuspended and plated on to the gelatin coated dishes or coverslips for ICC, incubated for 36 hours, then fixed or harvested for protein and/or embryoid body formation.

Luciferase Assays in 293T Cells and J1 Murine Embryonic Stem Cells

Human embryonic kidney 293T cells or J1 mES cells were cultured as described above. 293T cells were plated at 30,000-35,000 cells/cm^2, grown to 90% confluency and

harvested as described above. Cocktails were then set up containing the appropriate Oct4-Luciferase reporter construct, a Renilla control plasmid and one of the following GFP expression constructs: pLVX-EF1a-AcGFP1-cJun, pLVX-EF1a-AcGFP1-L40/42A or pLVX-EF1a-AcGFP. Each GFP expression construct was also tested in the presence of an additional Beta-catenin plasmid or a filler DNA plasmid (pcDNAgfp). The lentiviral vector utilized to clone each of the cJun variants was developed by Clontech and is expressed in many cell lines including most murine embryonic lines. Positive control wells were transfected with the pTALucSUPER TOP plasmid +/- Beta-catenin to ensure that the luciferase reagents were working properly. Additionally, three wells of cells without any transfected DNA were processed through the luciferase assay to determine biological background signal. The above transfection protocol was used, however the cJun mutants/Beta-catenin plus filler DNA or cJun construct with Beta-catenin only comprised 20% of the total DNA (split evenly 10%cJun, 10% filler/Beta-catenin). The pTALucOct4 plasmid was generated by cloning 3000 bp (the entire proximal promoter region (0 to -1.39 kb) as well as a portion of the distal promoter (-1.4--3.0 kb) of the *Oct4* promoter sequence into the pTALuc plasmid, which contains a putative AP-1 binding site at bp-2257 identified by our laboratory through bioinformatic analysis, and three TCF/LEF binding sites previously demonstrated to be responsive to active Beta-catenin (Li et al. 2012). This construct comprised 78% of total DNA and a Renilla reporter driven by the constitutive SV40 promoter (pRL-SV40, Sherf et al. 1996) was 2.7% of total DNA and utilized as an internal control. Plasmid DNA was prepared as needed following manufacturer's instructions from the Clontech NucleoBond® Xtra Midi

Plus EF kit (ref. 740422.10). Cells were incubated for 36-48 hours post-transfection and imaged with the Ziess Axio Observer Z1 Fluorescent Microscope at 20X total magnification to detect GFP expression. Promega's Dual-Glo® Luciferase Assay System (ref. E2940) was used as instructed by the manufacturer to carry out the Luciferase Assay. Data was collected using a SpectraMaxL plate reader, from Molecular Devices, with the following parameters: 1-minute dark adapt, 5 second shake before reading and 10 second total well scan per well.

Embryoid Body Formation

cJun/JNK signaling was modified in mESCs through either 1.) transient transfection with pLVX-EF1a-AcGFP1-cJun, pLVX-EF1a-AcGFP1-cJun-L4042A or the vector alone or 2.) treatment with anisomycin, SP600125 or DMSO. Thirty-six hours post transfection or two to four hours post drug treatment the cells were harvested from gelatin coated dishes for embryoid body formation. 1 x 10^6 cells per treatment, per time point were resuspended in differentiation medium containing 15% Hyclone® FBS (catalog # SH30070.03), 0.1mM MEM Non-Essential Amino Acids (catalog # 07600), 2.0mM L-Glutamine 200 (catalog # 07100), 1.0mM MTG (1:10 Dilution in DMEM) (Sigma #M-6145) and 1X DMEM to final volume. The cells were then plated to ultra-low adherent tissue culture dishes, fed every other day and harvested either 4 days (96 hours) or 6 days (144 hours) post-plating for protein extraction and western blot analysis. For the drug treated cells, the appropriate concentration of drug was added to the fresh

media each time the cells were fed. Low adherent cultures were incubated at 37℃ with 5% CO_2.

Nuclear Protein Extraction, BCA Protein Assay and Immunoblot Blot

Nuclear extractions were performed on transiently transfected (36 hours post-treatment) or chemically treated (2-4 hours post-treatment) J1 mESCs and embryoid bodies harvested from ultra-low adherent tissue culture dishes on days four and six. All samples were harvested according to the protocol listed in cell maintenance. The NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit by Thermo Scientific (Prod # 78835, Rockford, Il) was used according the manufacturer's instructions.

A BCA protein concentration assay was conducted on all extract samples to ensure proper concentration and loading for westerns. The assay was carried out according the manufacturer's instructions for the Pierce BCA Protein Assay Kit by Thermo Scientific (Ref 23225, Rockford, Il).

6X SDS sample buffer was added (to a final 1X concentration) to each sample and heated at 95℃ for 5 minutes. For nuclear extracts, an average of 4-5 µg of protein was loaded per sample. For cytoplasmic extracts, approximately 10 µg of protein was loaded per sample. 6 µl of EZ-Run™ Pre-Stained Rec Protein Ladder was also loaded (Ref. BP3603-500). Novex™ 10-20% Tris-Glycine Gel (EC6135BOX, Rockford, Il) gradient precast gels was run at 110V for approximately 2 hours at room temperature in 1X SDS running buffer. A Millipore PVDF membrane (cat. IPVH00010) was used for the transfer. Membranes were activated for 2-3 minutes in 100% methanol. Both the gel

and the membrane were allowed to equilibrate for several minutes, without contact, in cold transfer buffer (1X Tris-Gly Buffer, 20% Methanol and ddH_2O to volume) while the transfer apparatus was prepared. The transfers were run at 20V, for two and a half hours at 4℃. Membranes were placed in blocking buffer (1X TBST, 1% BSA and 0.1% nonfat milk) for 15 minutes on a rocker. Primary antibodies including: cJun, phosphorylated cJun, eGFP, JNK, phosphorylated JNK, OCT4, OCT4A, GATA4, Brachyury, SOX1, Nanog, SOX2 and GAPDH were prepared at appropriate dilutions in block and incubated overnight at 4℃ (**Supplemental Table 1**). The membranes were washed three times with 1X TBST for 5 minutes each on a rocker. Secondary antibody was added at a 1:2000 dilution in blocking solution and incubated for 1 hour at room temperature. Three additional 7-minute washes with 1X TBST were done prior to development. WesternSure Premium Chemiluminescent Substrate (cat. C50528-02) was prepared at a 1:1 ratio immediately prior to imaging with the Li-Cor-C-Digital machine on high sensitivity. To obtain a GAPDH loading control for each gel run, membranes were gently stripped for 5-10 minutes after imaging with medium stripping buffer (Thermo Fisher Scientific, Waltham, MA, cat. 21059). They were washed three times for 5 minutes each with 1X TBST, blocked for 15 minutes and put into GAPDH primary antibody overnight at 4℃. Secondary antibody and imaging steps remained the same. Densitometry was performed on each blot to assess the fold change in protein relative the GAPDH.

Immunocytochemistry

Coverslips were coated with 2 μg/ml fibronectin (STEMCELL Technologies Inc, Vancouver, BC, Canada) and incubated at 37℃ for 60 minutes. MEFs were then plated to the coverslips and allowed to adhere for 24 hours. Next, mES cells were plated to coverslips and fixed 36 hours post-plating with 4% paraformaldehyde in PBS for 5 minutes. Cells were washed with PBS twice for five minutes per wash. Nonidet™ P 40 Substitute (Sigma- Aldrich®, Louis, MO) was diluted in PBS to a working concentration of 0.1% and used to permeabilize cells for 30 minutes at room temperature. Cells were washed twice with PBS for 2 minutes and incubated for 30 minutes in blocking buffer that consisted of 0.1% Triton™ X-100 (Sigma- Aldrich®, Louis, MO) and 1% goat serum (Thermo Fisher Scientific, Waltham, MA) in PBS. Cells were incubated in primary antibody including: cJun, OCT4A and OCT4 diluted in blocking buffer for one hour and washed for five minutes in PBS three times (**Supplemental Table 1**). Secondary was diluted in block and incubated at room temperature for one hour. Coverslips were mounted using ProLong® Gold antifade reagents with DAPI (Life Technologies, Grand Island, NY) to stain the nuclei, and allowed to dry at room temperature overnight prior to viewing. For analysis, the Zeiss microscope and software were used (Carl Zeiss Inc, Thornwood, NY). To detect DAPI the 350 nm excitation and 450 nm emission filters were used. Texas Red was used to offset for dual stains and detected using 595 nm excitation and 615 nm emission filters whereas green fluorescence (Alexa Fluor 488 or plasmid generated eGFP) was detected using 490 nm excitation and

520nm emission filters. In all cases samples without primary antibody were generated to account for background during processing of images.

Hanging Drop Embryoid Body Formation and Directed Differentiation to Cardiomyocytes

After transfection, cells for each treatment were harvested from 0.1% gelatin (Stem Cell Technologies, catalog # 07903), counted using a Trypan Blue Assay and then an aliquot of 40,000 cells was removed. Bacterial grade dishes (Fisher Scientific, Waltham, MA, cat. S35839) were used to create hanging drops. The bottoms were filled with 10 ml of sterile PBS and 20 µl drops of cell suspension were placed on the lid to cover the surface while maintaining separation between drops. Dishes were then closed and incubated for 48 hours at 37℃ in 5% CO_2 with the cell droplets inverted. After 48 hours hanging drop EBs were harvested by slowly adding media to pool the drops then placing the suspension into a 15 ml conical tubes. Cells were allowed to settle for approximately 5 minutes at room temperature then the media was aspirated off. Cells were gently resuspended in differentiation media (see: *Embryoid Body Formation* above for recipe) and plated to low adherent dishes. EBs were kept in ULA dishes for four and six days before being plated back to adherent dishes for 10-15 days. EBs were fed every other day as described above and checked daily to monitor for beating sites.

Data Analysis

Luciferase Assay: due to concerns regarding the variation of raw Renilla values, an ANOVA was carried out, prior to normalizing the luciferase data, which showed that

there was no significant difference following removal of outliers (data not shown). Next, Luciferase signal was normalized with Renilla to generate F/R ratios per treatment, per run which is represented as Relative Light Units (RLU). The F/R ratios were averaged and a one-way ANOVA was used to determine significance. Tukey's multiple comparisons was used as post-hoc analysis to compare specifically between different transfection treatments ($n = 12$). Significance was defined as a p-value of $p < 0.05$. Data are shown as the mean Fold Change in normalized Luciferase Signal ± Standard Error of the mean (SEM).

Immunoblot: protein expression was quantified by analyzing western blots using densitometry software for the LiCor C-Digit scanner to assess fold changes in expression relative to the GAPDH housekeeping gene. These fold changes were then compared using a 2-way ANOVA or 3-way ANOVA (depending on how many variables were being included in the analysis) followed by Tukey's multiple comparison with significance defined as $p < 0.05$. ($n = 3$ blots). Data are shown as Mean ± SEM.

Immunocytochemistry: For transfected cells, ICC was quantified by counting the number of GFP positive cells, the number of OCT4A positive cells, and the number of cells co-expressing GFP and OCT4A ($n = 6$ slides per treatment). A ratio of co-expressing cells:GFP positive cells was generated for each treatment and analyzed with a non-parametric Friedman test. eF1-GFP treated cells were considered as the baseline to compare cJun and L4042A using Dunn's multiple comparison. Significance was reported as $p < 0.05$. Data are shown as Mean ± SEM.

Differentiation: Beating cardiomyocytes were analyzed using a mixed effects model in R to account for EBs as a random factor and the number of EBs per treatment, as it varied by experiment (though the initial number of cells per treatment was the same prior to EB formation) with beating as the dependent variable. Based on the resulting model, the data were graphed and significance was determined using a Satterthwaite Approximation and $p < 0.05$. ($n = 3$ wells per treatment).

RESULTS

A Regulatory Role for cJun in the Activation of the *Oct4* Promoter

Bioinformatic analyses conducted by others in our laboratory identified an AP-1 binding site 2257bp upstream of the murine *Oct4* transcription start site, suggesting cJun might regulate *Oct4* expression. To test this, we created a luciferase reporter construct containing bases +1 to -3000 of the *Oct4* promoter region. In addition to the putative cJun binding site, this region also includes the entire *Oct4* proximal promoter (+1 to -1.39 kb), a portion of the *Oct4* distal promoter (-1.4 to -3.2 kb), and three TCF/LEF binding sites previously demonstrated to be responsive to active Beta-catenin: -2032/2026; -1661/1655; and -881/875 (Li et al 2012). 293T cells were transfected with 1. eF1-GFP, eF1-GFP –cJun (referred to as GFP cJun), eF1-GFP cJun L40/42A (referred to as L4042A); 2. either pcDNA3-GFP or pcDNA3-MT BCA (activated Beta-catenin); 3. pTALucOct4, and 4. a Renilla plasmid driven by the SV40 promoter (pRL- SV40) (**Figure 3A, Supplemental Figure 1A-B**). Additional wells of 293T cells were transfected with TOP FLASH with and without activated Beta-catenin as a positive control for the assay. Luciferase assays were performed forty-eight hours post transfection after GFP imaging confirmed high transfection efficiency (85%) (**Figure 3B, Supplemental Figure 2A-B**).

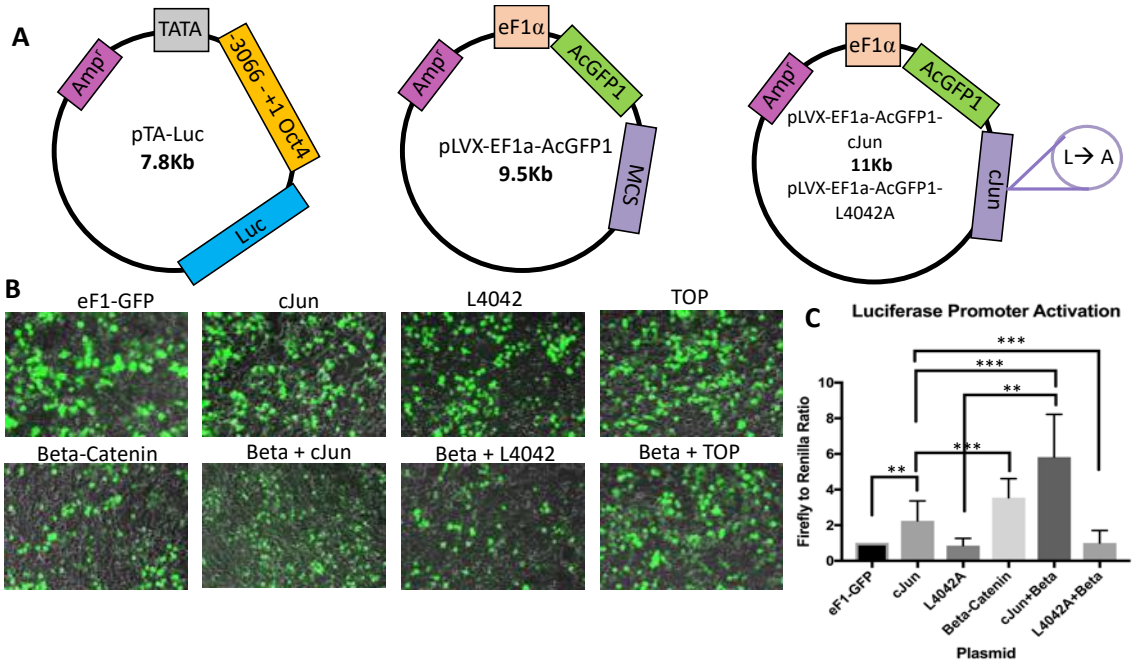


Figure 3. cJun Binds and Activates the Oct4 Promoter in 293T Cells

A. Diagrammatic representation of the plasmid vectors used to clone the Oct4 reporter construct (left) the eF1-GFP vector (center) and the GFP-cJun constructs (right). **B.** Transfection efficiency of 293T cells 48 hours post-transfection was approximately 85% or greater. Cells were imaged at 10X with the Zeiss Axio Observer and identified by GFP expression prior to luciferase assays. Representative images are shown. **C.** Luciferase data are represented as the average Luciferase to Renilla ratio to represent the normalized luminescence of the reporter. A One-Way ANOVA followed by Tukey's HSD was used to compare promoter activation across different transfected samples (n = 12). Activated Beta-catenin significantly increased promoter activity compared to GFP cJun validating a role for Wnt signaling in the regulation of this *Oct4* promoter construct($p<0.001$***). GFP cJun significantly increased promoter activity compared to the empty eF1-GFP vector ($p<0.01$**) suggesting that it too can affect *Oct4* promoter activity. The transcriptionally inactive mutant construct, GFP L4042A, was not significantly different from the control vector. When GFP cJun and Beta-catenin were co-transfected with the *Oct4* promoter construct, they synergized and significantly increased the reporter activity compared to GFP cJun alone ($p<0.001$***), demonstrating a cooperative role for cJun and canonical Wnt signaling. The addition of Beta-catenin to the L4042A mutant did not increase activity, but rather showed a significant reduction in activity compared to GFP cJun ($p<0.001$***), demonstrating the importance of transcriptionally active cJun to Bet-catenin activation of the *Oct4* promoter.

Transient expression of GFP, GFP cJun, and GFP L4042A from the plasmids was confirmed by cJun and GFP immunoblot, as previously described (Hosawi et al *in prep.*) (**Supplemental Figure 4**). Tukey's HSD was used as post-hoc analysis to compare promoter activation across different transfected samples (n=12) and data are shown as Mean ± SEM (**Figure 3C**).

Our results showed that like Li et al. (2012), transfection of active Beta-catenin significantly increased promoter activation by an average of nearly four-fold (3.6 ± 0.62), validating this plasmid as a reliable measure of *Oct4* promoter activity. Transfection of GFP cJun increased the *Oct4* reporter construct activity by approximately two- fold (2.2 ± 0.65), a significant increase when compared to our vector control, eF1-GFP ($p<0.001$***). The cJun mutant GFP L4042A did not increase luciferase expression. Rather, it slightly decreased the activity of the reporter construct (0.8 ± 0.23). When cJun and Beta-catenin were transfected together, there was a striking increase in promoter activation (5.8 ± 1.38) significantly greater than that of GFP cJun alone ($p<0.001$***). In contrast, when the L4042A mutant was co-transfected with Beta-catenin the average activation of the reporter construct was roughly equal to that of the eF1-GFP vector (1.0 ± 0.4). That we see a significant increase in *Oct4* promoter activity with the combination of cJun and active Beta-catenin, which is diminished upon use of L4042A instead of wild-type cJun, suggests that cJun is able to affect Wnt activation of the *Oct4* promoter.

Having demonstrated the regulation of the Oct4 luciferase construct by cJun in 293T cells, we were interested to see if the results of the assay would be changed

if performed in murine embryonic stem cells. Knowing transient transfection is less efficient in this cell type, we first transfected J1 mESCs with our robust TOP FLASH reporter construct in the presence of Beta-catenin. Unfortunately, while we were able to achieve approximately 350-fold-increase in TOP FLASH reporter activity with 293T cells, the mESCs did not show increased plasmid activity. We suspect that the lower transfection efficiency (estimated as mESCs = 30%, 293T = 90%) contributed largely to these results and did not pursue the analysis of cJun regulation of the Oct4 luciferase construct in mES cells any further (**Supplemental Figure 3**).

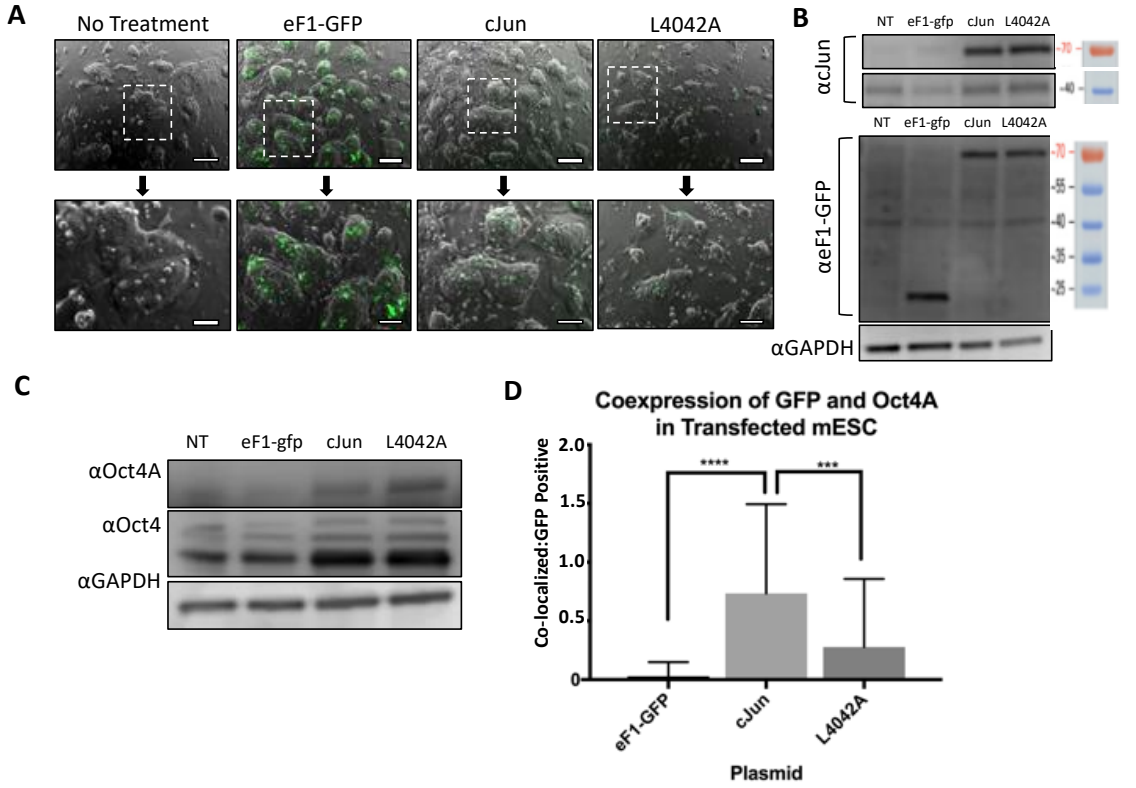
Transient cJun Specifically Increases OCT4A Expression in J1 mESC

The luciferase assay results suggested cJun could increase the expression of Oct4 mRNA. To test this hypothesis, J1 mESCs were transfected with GFP, GFP cJun, or GFP L4042A. Thirty-six hours later, transfection efficiency was evaluated by GFP imaging. Although transfection efficiency was quite low (approximately 30%), the cells were harvested for protein analysis (**Figure 4A)**. Expression of the GFP constructs was verified by immunoblot with antibodies for murine cJun and GFP. All samples expressed endogenous cJun (37 kDa), with levels slightly higher in those transfected with GFP cJun and GFP L4042A. This was expected, as cJun binds its own promoter via a consensus AP-1 site to regulate activity. Both antibodies also revealed a band at approximately 70 kDa, the size of the GFP cJun and GFP L4042A fusion proteins. The protein lysates from cells transfected with

GFP had a 27 kDa protein appear with the GFP antibody only, which is the expected size of eGFP. (**Figure 4B**).

Having confirmed the expression of the GFP and GFP cJun proteins, we performed an immunoblot to evaluate OCT4 expression. Two different OCT4 antibodies were used: one that recognizes all isoforms of the protein (noted Oct4all in figures) and one that only binds to the first exon of the protein, which is only included in the OCT4A isoform. Murine embryonic stem cells transiently expressing either GFP cJun or GFP L4042A samples showed a modest increase in OCT4A protein signal relative to GAPDH while eF1-GFP and untreated mESCs showed much less OCT4A (**Figure 4C**). The signal was measured using densitometry, which demonstrated that the GFP cJun construct did increase OCT4A expression more than the L4042A mutant, however the difference did not yield statistical significance.

To confirm the cells which successfully expressed the cJun plasmid were in fact the same cells over-expressing OCT4A, we performed an immunocytochemistry experiment. We identified transfected cells using the FITC channel since our cJun plasmids were cloned into a vector with a GFP-fused promoter. We then stained for either OCT4A or OCT4all (Texas Red). The results showed that the cells expressing GFP cJun demonstrated increased expression of OCT4A in the nucleus, while cells transfected with eF1-GFP did not show over-expression of OCT4A above background (**Figure 4E**).


A
No Treatment
eF1-GFP
cJun
L4042A
B
NT eF1-gfp cJun L4042A
αcJun
αeF1-GFP
αGAPDH
C
NT eF1-gfp cJun L4042A
αOct4A
αOct4
αGAPDH
D
Coexpression of GFP and Oct4A in Transfected mESC
Co-localized:GFP Positive
2.0
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eF1-GFP
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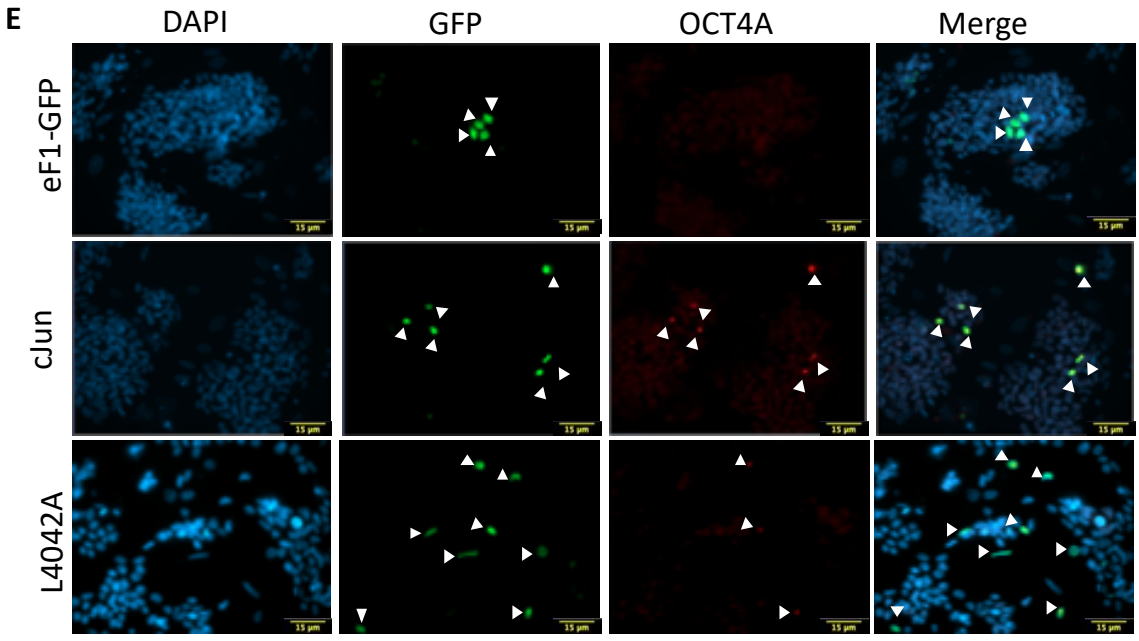

E
DAPI
GFP
OCT4A
Merge
eF1-GFP
cJun
L4042A

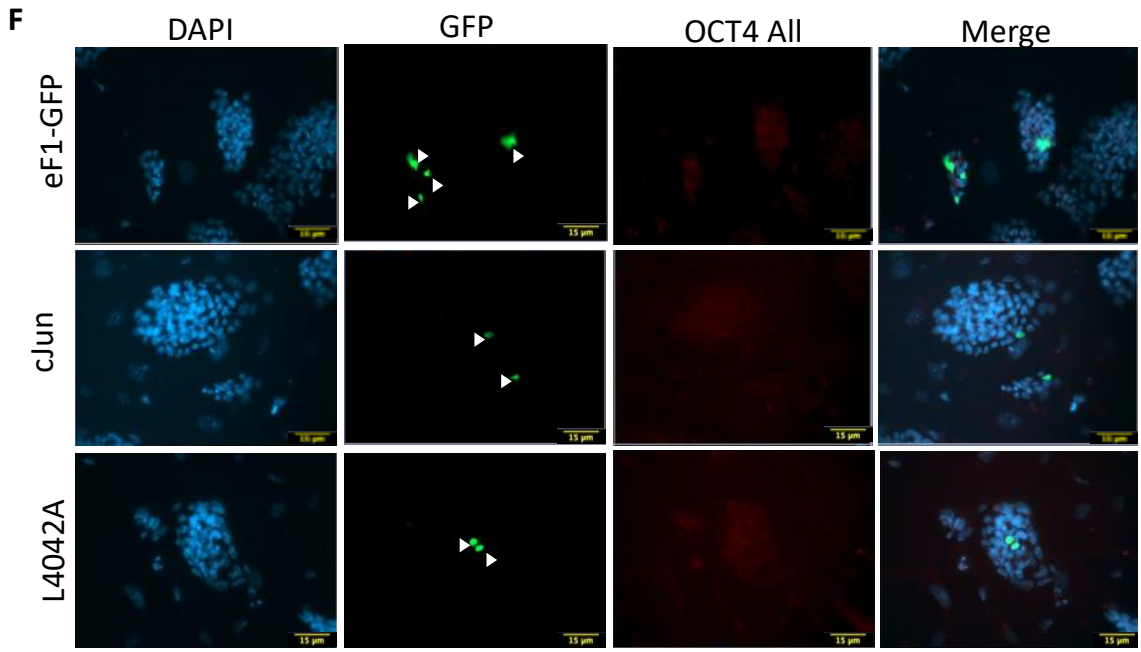


Figure 4. Transfected cJun is Detected by Western Blot and Leads to an Increase in OCT4A

A. J1 mESCs were transiently transfected with GFP, GFP- cJun, or GFP L4042A and imaged for GFP expression 36 hours later. Cells were imaged using brightfield and the FITC channels. (scale bar = 25µm, bottom row) (scale bar = 50µm, top row). eF1-GFP appears the brightest, and most efficiently transfected, as it is a cytoplasmic signal. Both cJun and L4042A localize to the nuclei and therefore are harder to detect within the colonies. **B.** Transfected mES cells were harvested for protein analysis to confirm successful over-expression of plasmids. The results show GFP (27 kDA) and GFP cJun (70 kDA) in cells transfected with EF1-GFP or EF1 GFP- cJun/L4042A, respectively. All samples express endogenous cJun (39 kDA) and GAPDH **C.** cJun and L4042A transfected samples showed a modest increase in OCT4A protein expression relative to eF1-GFP and untreated controls. Overall OCT4 levels did not appear to vary across samples. GAPDH was used as a loading control (n = 3). **D.** Co-expression of OCT4A expression with GFP, GFP cJun or GFP L40/42A was quantified by counting the number of GFP positive cells (marked by white arrowheads) that displayed OCT4 expression above the levels expressed in untransfected cells. Data are represented as the ratio of co- localized cells to GFP positive cells per treatment. A Freidmann Test was used followed by Dunn's Multiple Comparisons which revealed a significant increase in co-localization of cJun treated cells with OCT4A compared to GFP ($p < 0.0001$****) and GFP L4042A ($p < 0.001$***) (n = 6). **E.** Immunocytochemistry demonstrates increased OCT4 protein in cells expressing GFP cJun. Transfected cells (GFP) and OCT4A overexpression (Texas Red) are marked by white arrowheads. Levels of

OCT4A protein above those in non-transfected cells were only documented in cells expressing GFP cJun or GFPL4042A, however not all GFP-L4042A expressing cells showed co-localization of OCT4A. (n = 6). **F.** Immunocytochemistry was carried out with OCT4all (Texas Red) and transfected cells were identified by GFP expression. GFP positive cells are identified by white arrowheads. Total OCT4 expression did not appear to be greatly altered across samples (n = 6).

Cells expressing the GFP-L4042A construct did show some co-localization, however not all GFP positive cells over-expressed OCT4A. We then quantified the number of cells which were GFP positive (successfully transfected), had OCT4A expression above endogenous levels, and the number of cells which co-expressed OCT4A and GFP. Our results showed cells expressing GFP cJun had significantly increased nuclear expression of OCT4A compared to either eF1-GFP ($p<0.0001$****) or L4042A ($p<0.001$***) cells (**Figure 4D)**. This was not the case in the immunocytochemistry for OCT4, which had no notable difference in localization and expression beyond background levels across all of the transfected samples (**Figure 4F**).

Transient cJun Expression is Associated with an Increase in GATA4

OCT4A is the isoform classically responsible for regulating pluripotency (Nichols et al. 1998, Niwa et al. 2000, Velkey and O'Shea 2003). To see if the increases in OCT4A we observed in GFP cJun transfected cells could affect the developmental fate of mES cells, we evaluated expression of germ layer markers in embryoid bodies (EBs) derived from GFP, GFP cJun or GFP L4042A transfected mESCs (**Supplemental Figure 5**). Day four and day six EBs were imaged and harvested for protein to analyze expression of GATA4 (endoderm), Brachyury T (mesoderm) and Sox1 (ectoderm) (**Figure 5A**). Though there was no selection to induce the incorporation of these expression constructs into the genome; modest GFP expression was still detectable at both time points (**Supplemental Figure 6**). We

analyzed GATA4 expression, specifically, and found that it was significantly upregulated in the Day 6 EBs, regardless of treatment, compared to Day 4 ($p < 0.05$*) (**Figure 5B**). Individual germ layer marker expression was evaluated based on expression relative to GAPDH by immunoblot followed by densitometry and normalized values were shown as the Mean ± SEM (**Figure 5C-D**). For EBs cultured in low adherent conditions for four days (referred to as Day 4 EBs), Brachyury expression levels were relatively constant across treatments (**Supplemental Figure 7C**). Sox1 and GATA4 were highest in untreated cells (Sox1=0.070±0.054, GATA4= 0.085±0.048). GATA4 expression was relatively similar in eF1- GFP (0.048±0.011) and cJun (0.050±0.032) samples, with L4042A just slightly lower (0.036±0.013). Interestingly, Sox1 expression was decreased in cJun samples (0.044±0.016) and L4042A (0.037±0.005) compared to eF1-GFP (0.076±0.035).

Day 6 untreated EBs expressed 1.77±0.137 GATA4, 0.139±0.033 Brachyury and 0.035±0.010 Sox1 (**Supplemental Figure 7D**). Corresponding GFP samples produced slightly elevated germ marker expression compared to untreated cells (GATA4=0.161±0.124, BRY= 0.161±0.070, Sox1=0.050±0.0009), however the standard error was still quite large. Samples expressing GFP cJun appeared to have the greatest mean expression of all three germ layer markers after six days in low adherent culture. Specifically, GATA4 expression (0.257±0.150) showed a noteworthy increase of approximately 1.5 times greater than untreated EBs. Brachyury expression appeared constant across all treated samples, with untreated cells

expressing the lowest amount. Sox1 expression followed a similar trend wherein cJun samples expressed 0.068±0.036 compared to untreated and GFP samples.

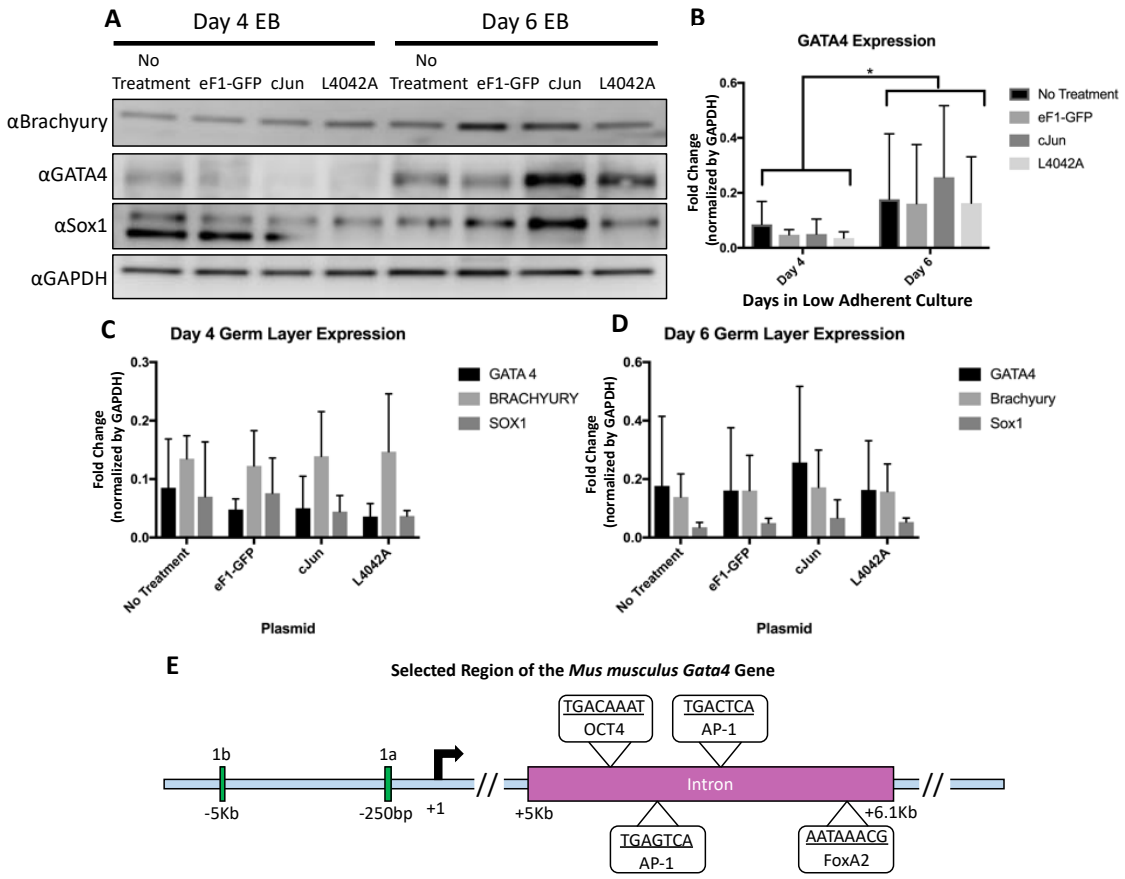


Figure 5. Increased cJun and OCT4A Protein Levels Correlate with an Increase in GATA4

A. Western blot analysis revealed a striking increase in GATA4 expression when cells were transfected with cJun prior to embryoid body differentiation. Brachyury levels appeared relatively constant while Sox1 appeared increased in both the no treatment and eF1-GFP samples after four days of differentiation. Additionally, Sox1 appeared to be increased in the Day 6 cJun sample. Both Sox1 and Brachyury had variable expression patterns across replicates. GAPDH was used as a loading control. (n = 3) **B.** Densitometry was utilized to quantify expression of GATA4, Brachyury and Sox1 relative to GAPDH expression (shown as ratio of GAPDH: protein of interest). A three-way ANOVA was utilized to compared germ layer expression per treatment per days differentiated (Day 4 versus Day 6) followed by Tukey's Multiple Comparison which showed a significant increase in all germ layer markers in the Day 6 samples relative to the Day 4 samples ($p < 0.05$*) (n = 3). **C.** Two-way ANOVA followed by Tukey's Multiple Comparisons was used to examine germ layer expression per treatment after four days of differentiation. (n = 3) **D.** **T**wo-way ANOVA followed by Tukey's Multiple Comparisons was used to examine

germ layer expression per treatment after six days of differentiation. While GATA4 after cJun treatment appeared higher, there was a great deal of variation within samples which likely contributed to a lack of statistical significance. (n = 3). **E**. Schematic representation of selected annotated regions of *Gata4* from *Mus musculus*. (NCBI Accession NC_000080.6:c63271715-63198914). The upstream promoter has two well- characterized enhancer regions 1b and 1a (green) at -5 Kb and -250 bp respectively relative to the transcription start site (black arrow). The second intronic region, which begins approximately 5 kb after the transcription start site and spans a 1.1 kb region, contains an OCT4 consensus binding site (sequence shown top left), two AP-1 binding sites (bottom left and top right), and a FoxA2 binding site (bottom right). This enhancer region has been shown to be conserved for human and mouse (Rojas et al. 2010).

L4042A expressed nearly identical levels of GATA4 (0.163±0.097), Brachyury (0.157±0.055) and Sox1 (0.053±0.0008) relative to the eF1-GFP samples. This suggests that cJun is able to modify the differentiation of mES cells.

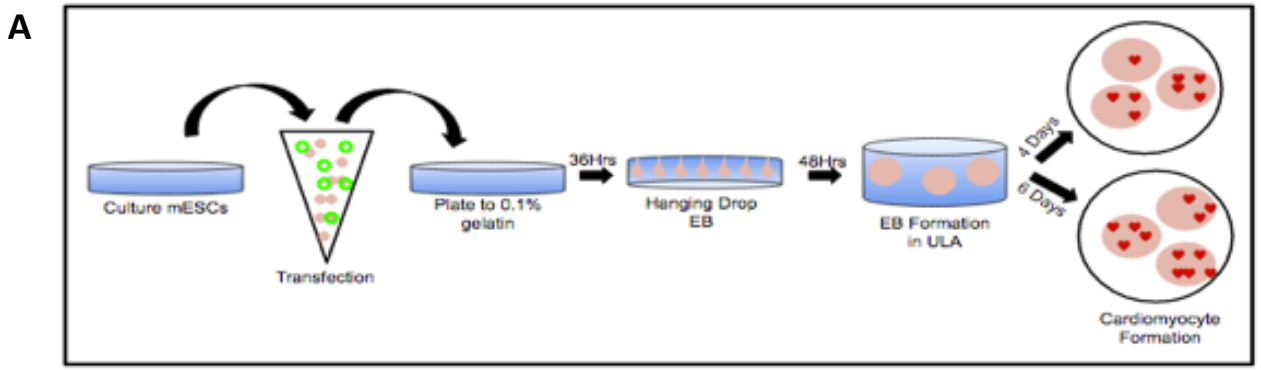
To begin elucidating a molecular link between OCT4, cJun and GATA4 we carried out a preliminary bioinformatics analysis. Within the second intronic region of *gata4*, which spans 1.1 kb area between the second and third exons, there is a highly conserved enhancer sequence which contains a FoxA2 binding site to specify the formation of foregut endoderm (Rojas et al. 2010). Approximately 1 kb upstream of the FoxA2 binding site, we found several AP-1 binding sites and one OCT4 consensus binding site, however there were no sites present in the promoter (**Figure 5E**). The presence of these binding sites offer evidence that OCT4 and cJun can interact directly with *gata4* to alter its expression and subsequent cell fate.

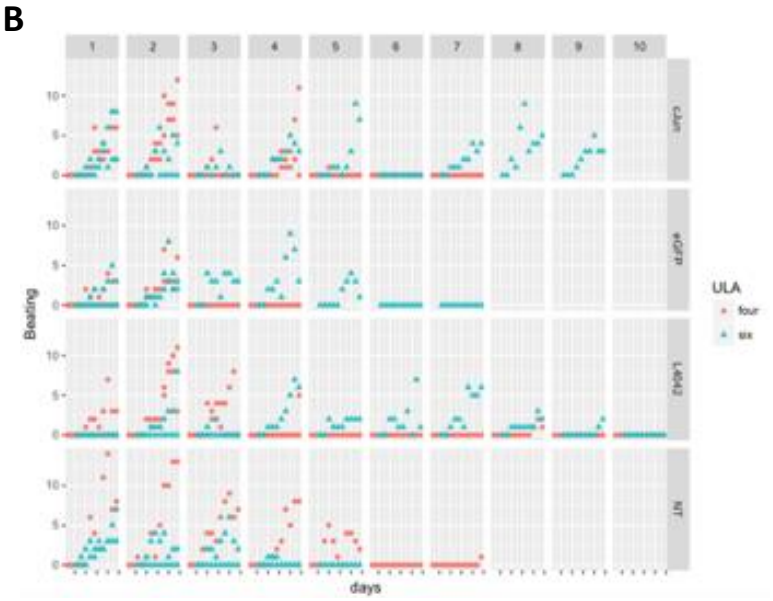
Transient cJun Expression Increases Cardiomyocyte Formation

GATA4 expression is a marker for endoderm formation, but is also essential for the formation of cardiac mesoderm (Narita et al. 1997, Holtzinger et al. 2009). These data coupled with the knowledge that slight changes in OCT4 levels have the ability to increase primitive endoderm and mesoderm formation suggested we might analyze differentiation to the myocardial lineage. Embryoid bodies derived from hanging drops are an established model for differentiation to cardiomyocyte formation, so we examined cardiomyocyte formation of EBs formed by hanging drop with mES cells transfected with GFP, GFP cJun or GFP L40/42A (**Figure 6A**). The

cells were the monitored for beating clump formation over a 10-day period (**Figure 6B**). Cells that received no plasmid DNA (untreated) and were kept in low adherent conditions for four days were utilized as the baseline to compare the mean number of beating sites over the 10-day period (n = 3) (**Figure 6C**).

After four days in low adherent culture, cJun treated samples had an average of 5.00 ± 0.25 (Mean ± SEM) beating sites, which was greater than the average number of beating colonies compared to the GFP vector control sample (2.77 ± 0.34) ($p < 0.001$***). Untreated cells also had a greater average number of beating sites (3.57 ± 0.42) compared to the GFP control ($p < 0.05$*). Day 6 cJun samples had a greater mean number of beating sites (3.3 ± 0.05) compared to untreated samples (0.6 ± 0.34) ($p < 0.01$**), GFP samples (2.24±0.36) ($p<0.05$*) and L4042A samples (2.84 ± 0.28) ($p < 0.05$*). Beating cardiomyocytes derived from the GFP cJun expressing EBs appeared to sustain their beating colonies beyond day 7 of monitoring, when both the untreated and GFP samples had ceased (**Figure 6B**). L4042A day 6 samples, unlike the other treatments, did not begin beating on day one of monitoring, suggesting a possible delay in differentiation. L4042A samples did, however, continue beating after the untreated and GFP samples stopped, albeit at lower numbers than cJun treated samples. Taken together, these data demonstrate that an increase in cJun expression can potentially increase cardiomyocyte beating.


A
Culture mESCs
Transfection
Plate to 0.1% gelatin
36Hrs
Hanging Drop EB
48Hrs
EB Formation in ULA
4 Days
6 Days
Cardiomyocyte Formation


B
Beating
days
ULA
four
six

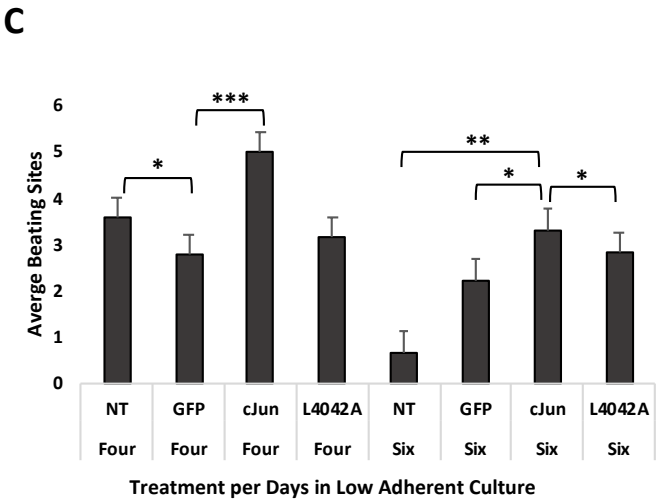

C
Averge Beating Sites
NT Four
GFP Four
cJun Four
L4042A Four
NT Six
GFP Six
cJun Six
L4042A Six
Treatment per Days in Low Adherent Culture

Figure 6. Increased cJun and GATA4 Correlate with Increased Beating Cardiomyocytes

A. Schematic of the experimental approach for cardiomyocyte differentiation. **B.** After four (pink circle) or six (blue triangle) days in ultra-low adherent culture conditions, cells were plated and maintained in cardiomyocyte differentiation media for 10 days (x-axis). Beating clusters were counted and monitored daily (left y-axis) for each transfected sample (right y- axis). As represented in this graph, untreated cells and cells transfected with cJun produced more beating colonies on Day 1 than eF1-GFP or L4042A samples. Over 10 days cJun treated cardiomyocytes appeared to maintain the greatest number of beating colonies, specifically from the group kept in low adherent conditions for six days. **C.** A lmer model was fit and followed with a Satterthwaite Approximation to assess the average beating per treatment, per days in low adherent culture, per number of EBs plated for cardiomyocyte formation. After four days of low adherent conditions cJun samples had the greatest mean beating colonies compared to eF1-GFP ($p < 0.001$***). There was no significance between cJun and either L4042A or untreated samples after four days in low adherent culture. The untreated cells (shown as NT) also had significantly more beating colonies than the eF1-GFP sample ($p < 0.05$*). After six days in low adherent conditions, cJun still had the greatest number of beating colonies relative to all other treatments (NT = $p < 0.01$**; eF1-GFP = $p < 0.05$*; L4042A = $p < 0.05$*) suggesting that overexpression of cJun plays a role in enhancing the production of beating cardiomyocytes.

Chemical Modulation of JNK Signaling Affects Potency, cJun Expression and OCT4

Jun-N-Terminal Kinase (JNK) phosphorylates cJun at two different serine residues (S63 and S73) to increase cJun transcriptional activity (Smeal et al. 1992; Czaja 2003). This phosphorylation requires the physical association of JNK with cJun at leucine residues 40 and 42 (Sprowles et al. 2003). That the cJun mutant L4042A impairs transcriptional activation of the *Oct4* luciferase promoter construct, reduces the effect of GFP cJun on the levels of GATA4 and SOX1 expressed in EBs, and the differentiation of mES cells to cardiomyocytes, suggests JNK signaling might be an upstream regulator of cJun in these processes. To test this hypothesis, two different chemical modulators of JNK and cJun activity were tested to see how they might affect the potency of mECSs. SP600125, a chemical inhibitor of JNK kinase activity (Bennett et al. 2001), was utilized to downregulate activity while anisomycin (fungal-derived) was used to activate the kinase (Miroslav et al. 1999). To see if JNK activity was necessary for cJun's effects on embryonic stem cells, the cytoskeletal destabilizer nocodazole was selected, as it has been shown to increase cJun expression independent of increased cJun transcription or MAPK activity (Polak et al. 2006, Blau et al. 2012).

Murine embryonic stem cells were plated to gelatin in mESC media and treated with one of the following: 10 µM SP600125, 10 ng/ml anisomycin, or 5 µg/ml nocodazole. Both a no treatment and a DMSO-only control were included, as DMSO is the solvent used for all three chemicals, and DMSO has been shown to alter the transcription and differentiation of some cells (Friend et al. 1971; Nomura and Oishi

1983; Juang and Liu 1987; Iwatani et al. 2006). Two hours post treatment the cells were harvested for protein and analyzed by immunoblot.

Although the expected changes in cJun and JNK were detected, the effects on OCT4 expression were modest and inconsistent across replicates (n = 3) (**Supplemental Figure 8**). In light of this, we added an additional four-hour time point and additional drug concentrations (50 ng/ml anisomycin) and again looked at phosphorylated cJun (p-cJun), phosphorylated JNK (p-JNK), the overall endogenous cJun and JNK content, as well as potency markers OCT4A and SOX2 (n = 4) (**Figure 7**). As expected, anisomycin increased the phosphorylation state of both JNK and cJun, while nocodazole treatment increased the amount of phosphorylated cJun without affecting JNK. The JNK inhibitor, SP600125, successfully decreased p-cJun and p-JNK by western blot. These decreases also coincided with potency expression similar to the untreated controls and DMSO controls.

Although there was still variation between replicates, all chemical treatments affected OCT4 expression, but in different ways. At two hours Anisomycin showed a decrease in OCT4A, while Nocodazole treatment increased OCT4A expression at four hours. As seen by cJun transfection, neither drug treatment had a noticeable effect on the overall levels of OCT4 in the cell.

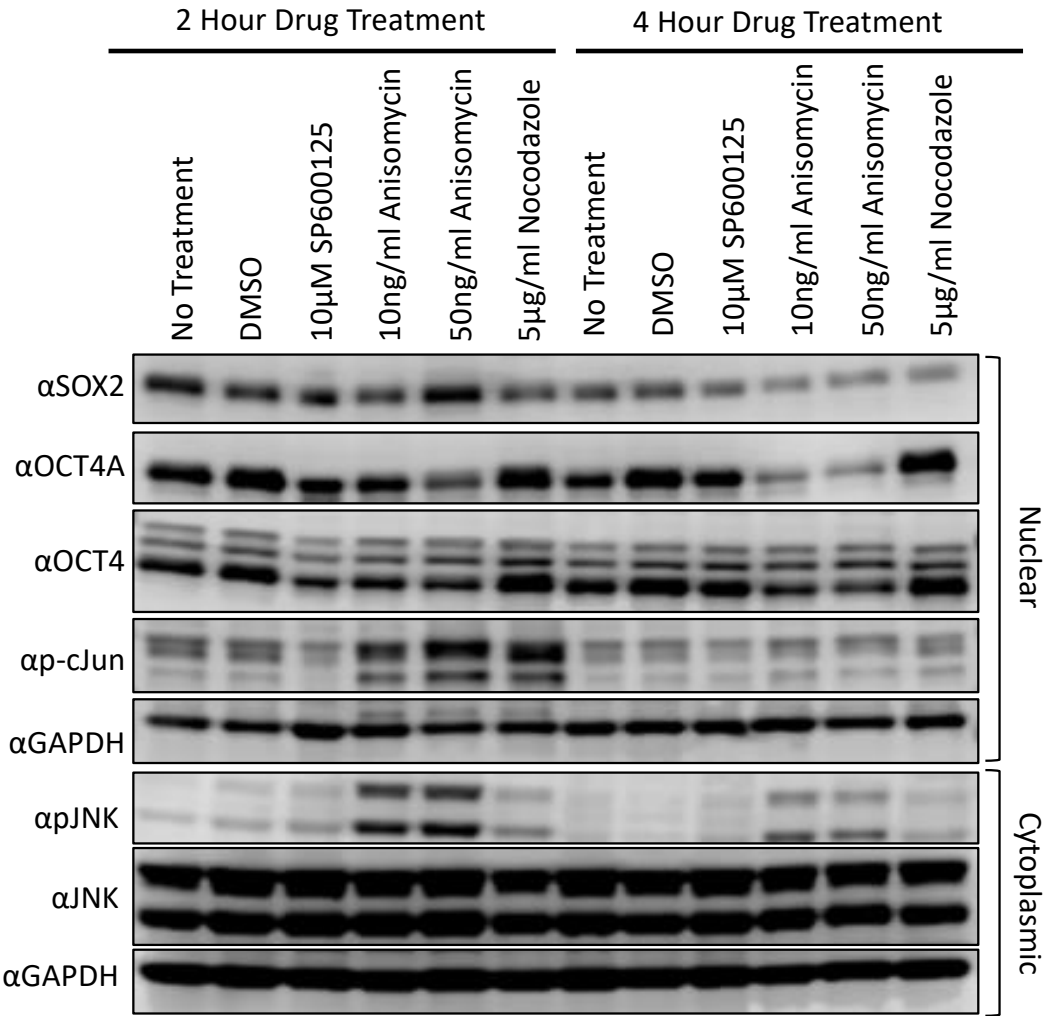


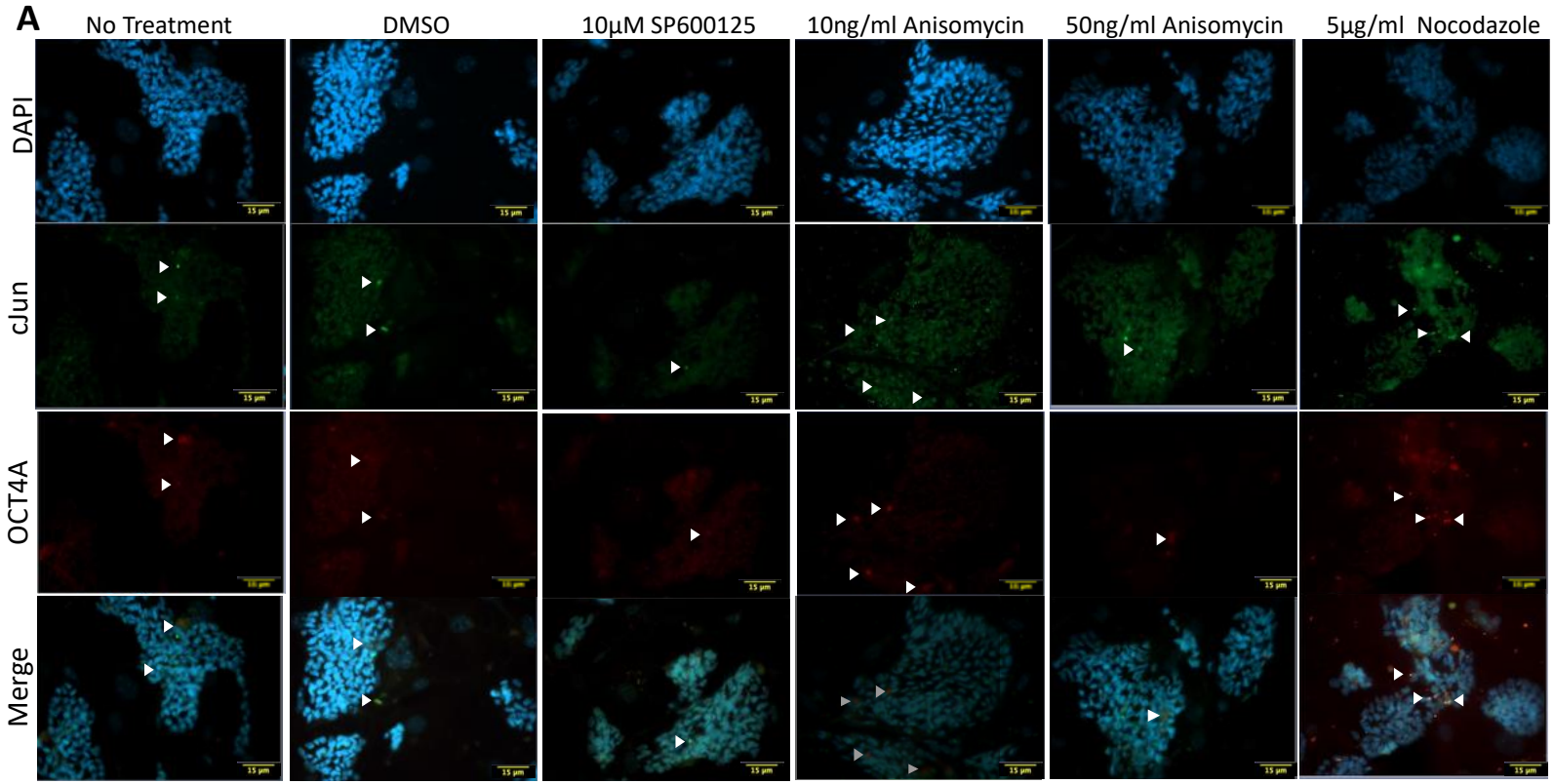
Figure 7. Drug-Induced Modulated cJun Expression Alters Expression of Key Potency Markers

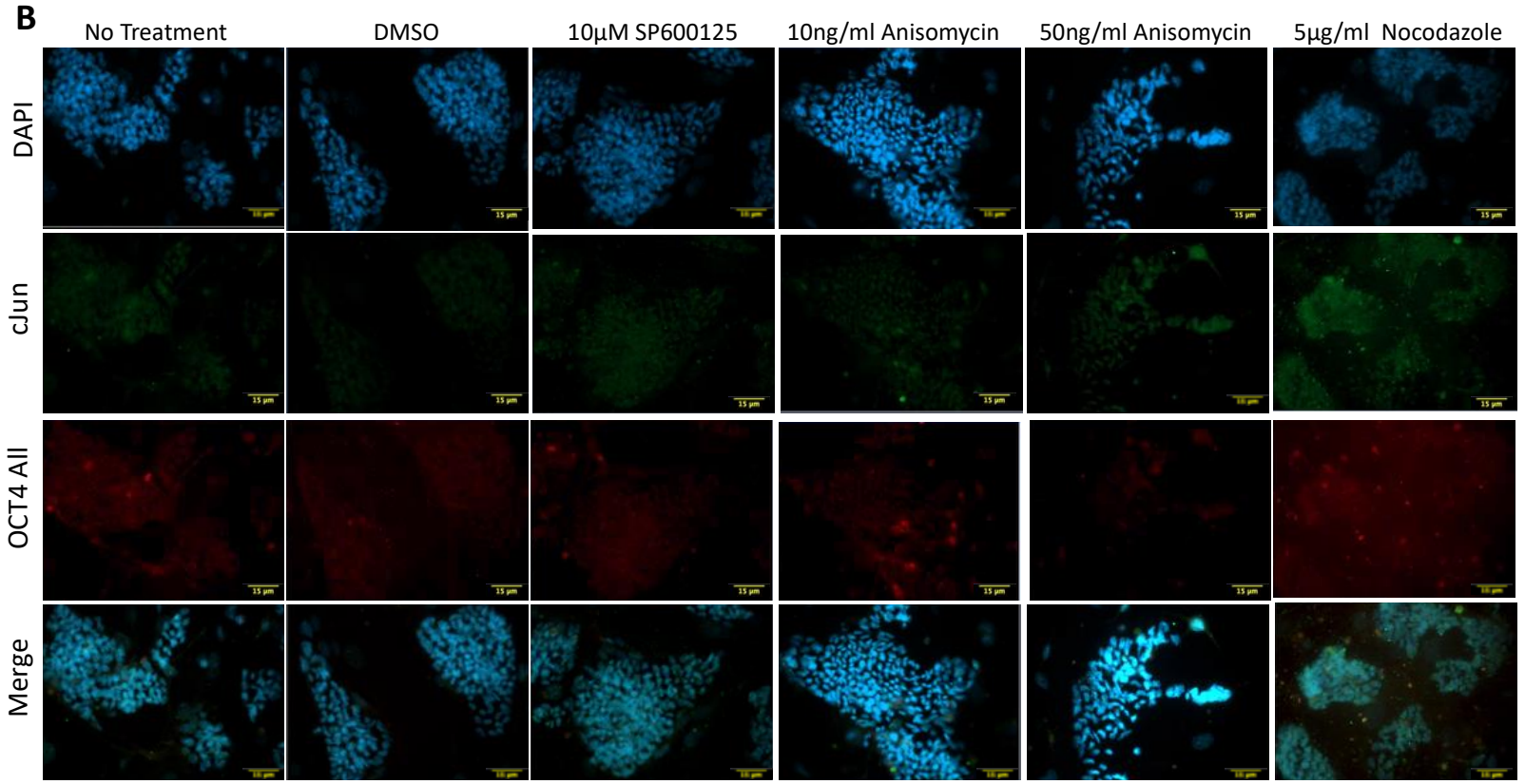
Two or four hours post-drug treatment cells were harvested for nuclear and cytoplasmic protein extraction. Western blot analysis revealed a decrease in OCT4A expression when cells were treated for four hours with a high dose of anisomycin. Nocodazole increased OCT4A expression after four hours, while overall OCT4 expression (OCT4) did not appear altered. SOX2 expression appeared altered across treatments after two hours, however by four hours expression levels were relatively similar. Phosphorylated cJun (p-cJun) and JNK (p-JNK) confirm that inhibition with SP600125 or stimulation with anisomycin was successful within the two-hour incubation period. GAPDH was utilized as a loading control. (n = 4).

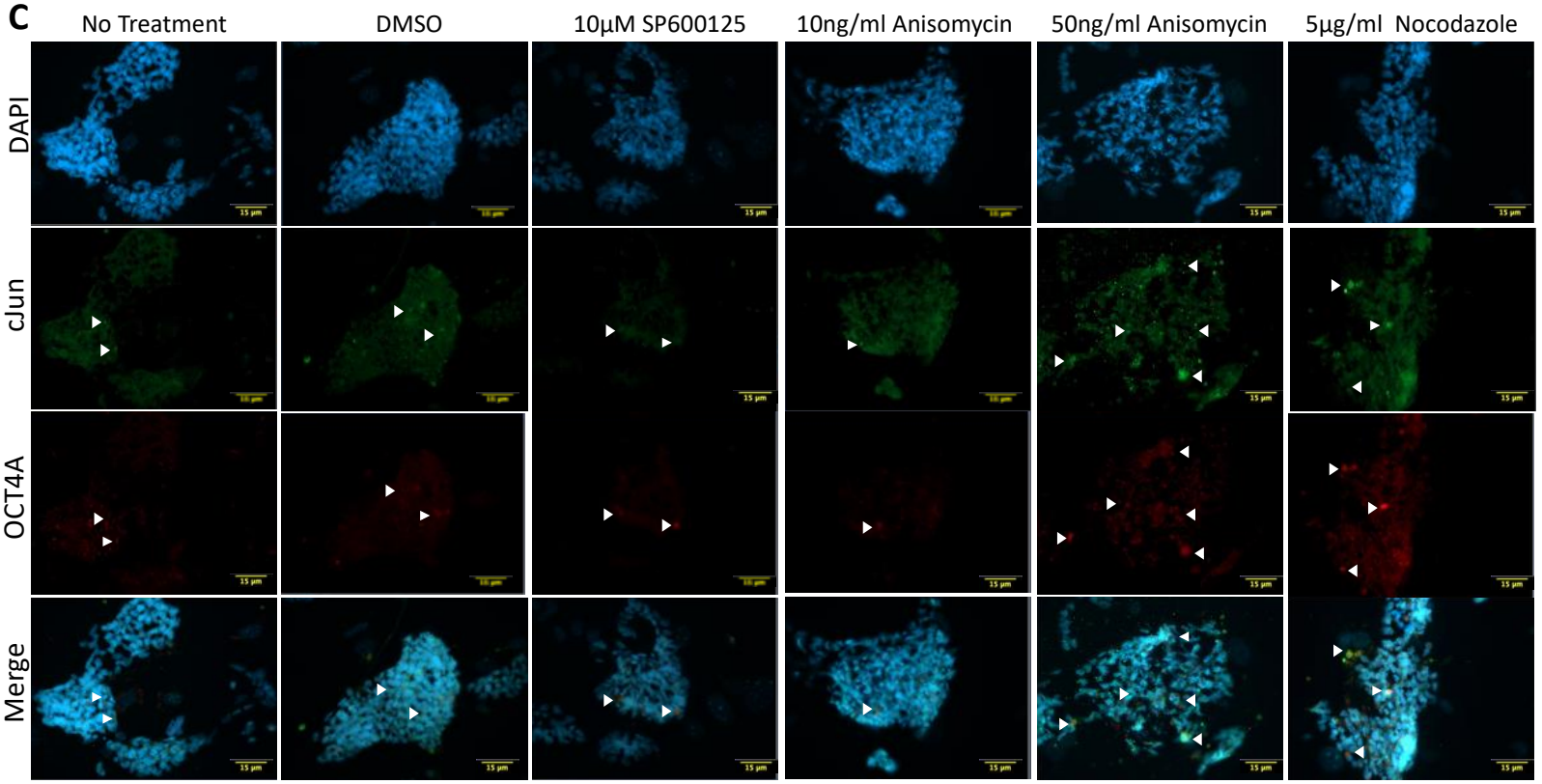
Immunocytochemistry was also carried out following two and four-hour incubations with drug treatments as described previously. The cJun antibody revealed increased cJun expression in both Anisomycin and Nocodazole treated cells two hours post-treatment (**Figure 8A**). There was minimal change in OCT4 all expression across treatments but a noticeable increase in OCT4A, suggesting that OCT4A is specifically being upregulated while the overall OCT4 protein content remains consistent (**Figure 8B**).

The observed increase in OCT4A expression was seen only in the cells overexpressing endogenous cJun in response to Anisomycin or Nocodazole (n = 3) (**Figure 8C**). The increase in OCT4A seen with Nocodazole is consistent with what was detected by western blot (**Figure 7**). OCT4 all expression, again remained relatively unchanged across all samples (**Figure 8D**)

Taken together, these data suggest that the increase in OCT4A and cJun may occur in a JNK-independent manner, however, JNK signaling does affect potency. To look at the effect of cJun on OCT4 expression, via JNK- dependent and independent expression, on cell fate we cultured these cells after two or four hours of exposure in ultra-low adherent conditions (n = 4). We found that EBs did not form in the presence of anisomycin or nocodazole, however SP600125 had limited effect on EB formation (**Figure 9**). Because Nocodazole destabilizes the cytoskeleton, it is possible that cells were unable to form critical cell-cell adhesions and therefore were unable to form EBs.


A
No Treatment
DMSO
10µM SP600125
10ng/ml Anisomycin
50ng/ml Anisomycin
5µg/ml Nocodazole
DAPI
cJun
OCT4A
Merge


B
No Treatment
DMSO
10µM SP600125
10ng/ml Anisomycin
50ng/ml Anisomycin
5µg/ml Nocodazole
DAPI
cJun
OCT4 All
Merge


C
No Treatment
DMSO
10µM SP600125
10ng/ml Anisomycin
50ng/ml Anisomycin
5µg/ml Nocodazole
DAPI
cJun
OCT4A
Merge

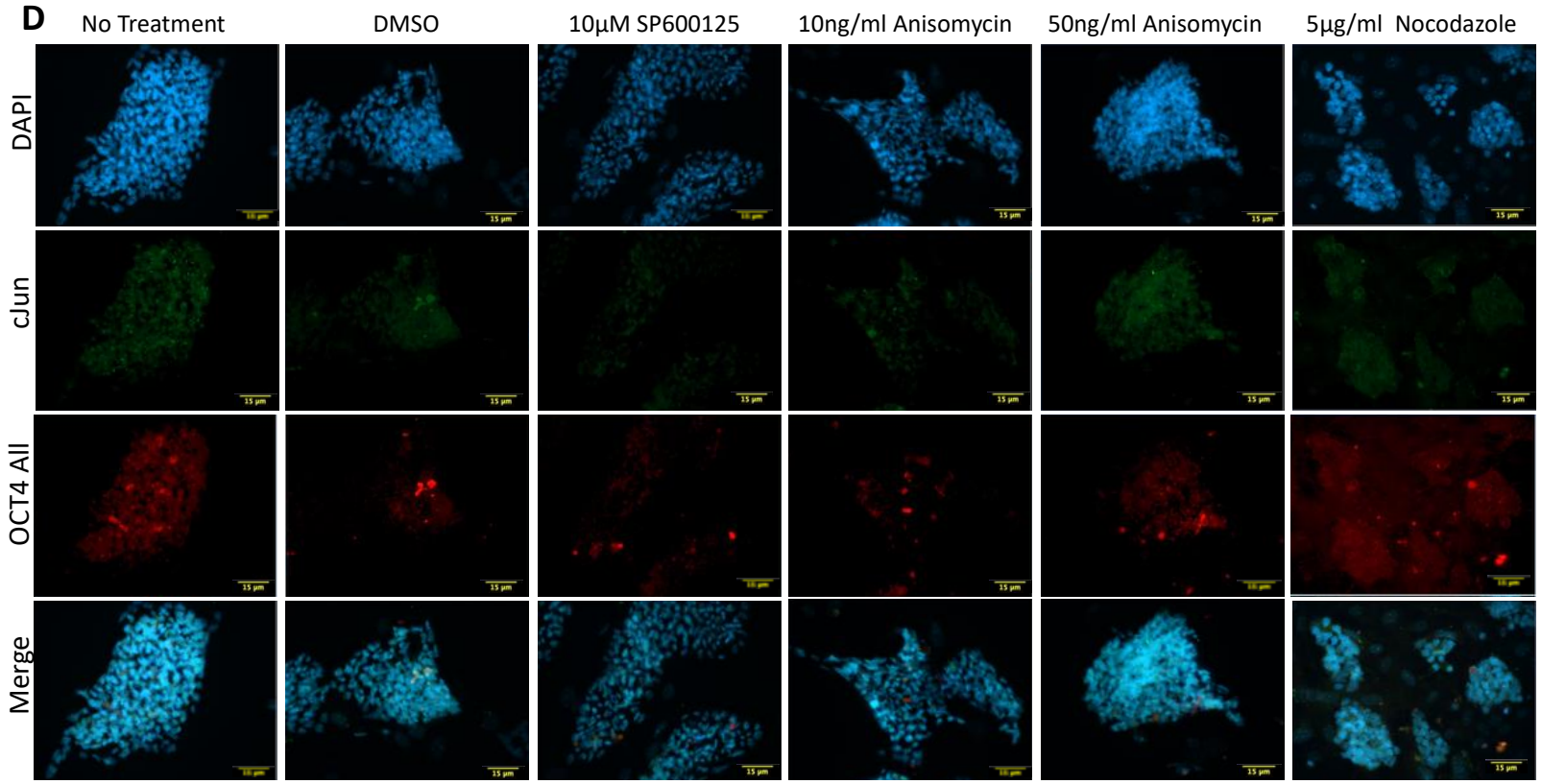

D
No Treatment
DMSO
10µM SP600125
10ng/ml Anisomycin
50ng/ml Anisomycin
5µg/ml Nocodazole
DAPI
cJun
OCT4 All
Merge

Figure 8. Drug Modulation Shows Co-localization of cJun and OCT4A at 2 and 4 Hours Post-Treatment

A. Two hours post drug-treatment cells were fixed and stained for cJun (FITC), OCT4A (Texas Red) and DAPI (blue) to detect co-localization within the nuclei (indicated by white arrowheads). As expected cJun expression was lower in the SP600125 treated cells, however it was dramatically increased under Anisomycin and Nocodazole conditions. Scale Bar=15 μm (n = 3 replicates) **B.** Cells were stained for cJun (FITC), DAPI (blue) and OCT4 all (Texas Red) after two hours of drug- treatment. Both the SP600125 and no treatment samples had slightly elevated levels of OCT4 all compared to the DMSO cells. Nocodazole, however showed a striking increase in overall OCT4 levels compared to all of the other samples. Scale Bar = 15 μm (n = 3 replicates) **C.** Immunocytochemistry was also carried out four hours post-treatment for cJun (FITC), DAPI (blue) and OCT4A (Texas Red). Co-localization is indicated by white arrowheads. This a striking increase in OCT4A expression following nocodazole treatment. Similarly, the higher dose of anisomycin showed an increase in OCT4A. Scale Bar = 15 μm (n = 3 replicates) **D.** OCT4 all expression (Texas Red) was examined four hours post-treatment and did not appear to change despite variation in cJun expression (FITC) across samples. Scale Bar = 15 μm (n = 3).

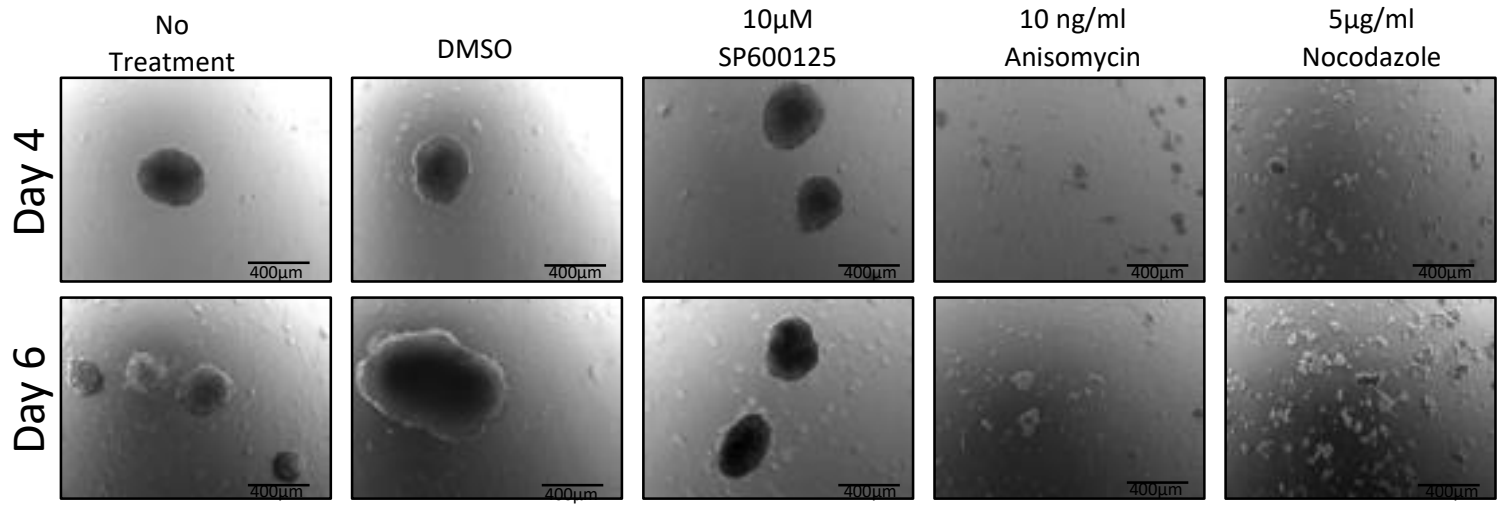


Figure 9. Increased cJun Expression by Anisomycin and Nocodazole Impede Embryoid Body Formation

Cells incubated for two hours with No treatment, DMSO, a JNK inhibitor (SP600125), a JNK activator (anisomycin) or an anti-cancer drug (nocodazole) were differentiated for four days in low-adherent conditions. While the JNK inhibitor samples formed embryoid bodies of comparable size to the controls (no treatment and DMSO), both anisomycin and nocodazole only formed cell clumps of 30 cells or less **(top**). Scale Bar = 400µm. This experiment was carried allowing the cells to differentiate for six days, however additional time in culture yielded similar results **(bottom)**.

DISCUSSION

The goal of this study was to determine the ability of cJun to regulate the expression of *Oct4*. Additionally, we sought to characterize the developmental consequences of this relationship and how cJun transcriptional activity would affect cell fate. Our results indicate that increased cJun can lead to an increase in OCT4 protein expression, transcriptional activity and a subsequent increase in a GATA4+ population, which is associated with increased formation of cardiomyocytes. That overexpression of the cJun L40/42A mutant reduced the transcriptional and developmental effects of wild type cJun overexpression suggests that the phosphorylation of cJun at serine 63 is necessary for these results, even though modulation of the JNK signaling pathway did not yield clear results. We believe this work will provide a necessary foundation for better understanding the role of cJun during early embryonic development, specifically endoderm and cardiac mesoderm formation.

cJun Increases Oct4 Promoter Activity

Increased expression of both *Oct4* and cJun have been shown to reduce potency in murine embryonic stem cells. Niwa et al. (2000) demonstrated that a less than two-fold increase in *Oct4* expression directs mES cells toward differentiation to primitive endoderm and mesoderm and overexpression of cJun has also been shown to induce differentiation of mES cells (Abujarour et al. 2010, Liu et al. 2015). Our data demonstrating the transcriptional activity of our *Oct4*-luciferase reporter construct is

significantly altered by the biochemical state of cJun in 293T cells support a model that these two transcription factors might work together towards this end. Expression of GFP cJun increased reporter activity by approximately two-fold compared to the eF1-GFP control, while the transcriptionally inactive GFP L4042A mutant construct reduced promoter activity of the *Oct4*-luciferase reporter construct compared to the eF1-GFP vector alone, demonstrating the importance of phosphorylation of cJun, on basal levels of promoter activity. This supports our previous bioinformatics analysis that identified an AP-1 consensus site in the *Oct4* distal promoter region, suggesting cJun may directly increase *Oct4* expression by binding the enhancer to increase mRNA production.

Though the effects of GFP cJun overexpression are somewhat modest, they are within the range of the changes reported by Niwa et al. that affect mES potency. Furthermore, that co-transfection of GFP cJun with active Beta-catenin increases reporter activity more so than Beta-catenin alone, suggests a broader role for cJun in *Oct4* gene regulation and may be essential in further understanding the molecular regulation of critical potency genes. Wnt signaling is a well-established and highly conserved regulator of ICM potency and Toualbi et al. (2007) demonstrated the synergism of Beta-catenin and AP-1 members, including cJun, when cyclin-D1 transcription is activated. Overall, the binding partners of cJun appear to be critical in determining its activity.

Over-Expressed cJun Leads to Increased Expression of the OCT4A Isoform in Murine Embryonic Stem Cells

Previous work has demonstrated increases in cJun expression in ground state mES cells reduce their potency, as evidence by reduced mRNA levels of pluripotency genes including *Oct4* (Liu et al 2015). Our luciferase assay results showed the opposite; however, they were performed in 293T cells, which are derived from human embryonic kidney cells immortalized with large T antigen. Therefore, we decided to evaluate overexpression of cJun on *Oct4* expression in mES cells cultured under standard mES cell conditions. Unfortunately, our attempts to perform luciferase assays in mES cells were unsuccessful likely due to low transfection efficiency. Despite significant effort, we were not able to achieve transfection efficiencies high enough for our positive control to provide a signal above background (<20% of mES cells vs. > 90% of 293T ells). (**Supplementary Figure 3B**). Therefore, we decided to evaluate OCT4 expression levels in the presence of the GFP cJun or GFP L4042A construct. Though transfection efficiency of the mES cells was significantly lower than that of 293T cells (**Supplemental Figure 3A**), the expression levels of eF1-GFP, GFP-cJun, and GFP-L40/42A were sufficient to detect by immunoblot, as was OCT4A; when GFP cJun is over-expressed in mES cells, the overall OCT4 levels remain similar compared to eF1-GFP treated and untreated samples, however OCT4A protein levels appear modestly increased. This suggests a potential connection between the transcription of the OCT4A isoform and cJun. This result was confirmed when mES cells transfected with cJun were analyzed for OCT4 expression by immunocytochemistry: only cells overexpressing the GFP-cJun construct had significantly increased levels of OCT4A. Furthermore, WT-cJun

and endogenous OCT4A were co-localized in the nuclei of the same mES cells, which would be necessary if cJun is increasing OCT4A expression directly.

When evaluated by immunoblot, OCT4A expression was also increased in the presence of the GFP cJun L40/42A construct, but the immunocytochemistry analysis showed a significant reduction when compared to GFP cJun, which does support the luciferase assay data. Phosphorylation of L40/42A by JNK is known to be required for transcriptional activity off of AP-1 sites associated with cellular proliferation (Jun promoter), but it is not required by all promoters (e.g., MMP1) (Blau et al. 2012). Liu et al (2015) reported that the L40/42 sites are not required for inhibition of iPSC generation by cJun, but that the bZIP domain is. The full length cJun protein is known to have an inhibitory function by dimerizing the inhibitor JDP2, which subsequently enhances iPSC formation and also requires the bZIP domain (Liu et al. 2015). This raises the possibility that, congruent with what Liu et al. discovered, *Oct4* expression is regulated primarily based on the binding partner utilizing the bZIP domain. Ultimately, this suggests the regulation of *Oct4* expression may involve the recruitment of partners that inhibit transcription. A more thorough explanation of the role of JDP2 in this process and JNK activity will be required to fully understand the regulatory function of cJun.

Overexpression of cJun results in increased GATA4 Expression

Overexpression of cJun in ground state mES cells has previously been shown to affect germ layer marker expression (Liu et al. 2015; Veluscek et al. 2016). Because the

liver and pancreas are derived from the endoderm and increased *Oct4* has been linked to mesoderm/endoderm differentiation, we examined the effect of over-expressed cJun on the formation of the primary germ layers during gastrulation using an *in vitro* embryoid body model.

One of the earliest choices made as cells move from the blastocyst through gastrulation is the formation of the epiblast and primitive endoderm (PrE). It has been shown that the transcription factor GATA6 controls the speed and proportion of PrE-fate commitment, relative to epiblast formation, by mediating FGF/ERK signaling (Schrode et al. 2014). Erk signaling, specifically, plays a crucial role in endodermal differentiation by suppressing PrE (Hamilton and Brickman 2014) and therefore maintaining an intricate balance between differentiation cues. As cells become committed to the epiblast, GATA6 expression is altered and markers including GATA4, Brachyury (BRY) and SOX1 emerge to indicate germ layer formation.

SOX1 is one of the earliest spatial/temporal indicators of induction toward neural ectoderm (Penvy et al. 1998). While we did not detect striking changes in SOX1 expression, we did note that it was decreased in EBs transfected with GFP cJun expression. We have previously seen that EBs formed from mES cells transfected with GFP cJun does impede differentiation toward an ectoderm derived lineage (Hosawi et al. *in prep*). Overexpression of the full length cJun protein has also been shown to be sufficient for inducing apoptosis of sympathetic neurons (Ham et al. 1995; Ham et al. 2000), which could explain the lack of neuronal clusters originally observed by Hosawi et al. (*in prep*). Brachyury expression, however, remained relatively constant regardless of

treatment with cJun, suggesting perhaps cJun does not play an integral role in directly specifying this lineage.

We did observe a striking increase in GATA4 protein in day 6 embryoid bodies formed from mESCs transfected with WT-cJun prior to differentiation. GATA4 was also increased in the EBs transfected with GFP L40/42A cells, but at a reduced level, again suggesting phosphorylation at these sites increases cJun activity. Although the densitometry values showed it was not significant, cJun treated samples did have the greatest mean GATA4 expression following six days of low-adherent differentiation. These data agree with a study performed by Liu et al. (2015) who showed that expression of cJun in ground state mES cells increases GATA4 expression 24 hours post-induction. A more sensitive approach, such as quantitative PCR (qPCR) may be beneficial in elucidating a significant trend whereby GATA4 is clearly upregulated in cells over-expressing cJun at the mRNA level.

Overexpression of cJun During Embryoid Body Formation Increases Differentiation to Cardiomyocytes

The increase in GATA4 expression we characterized in the GFP cJun EBs suggested the EB differentiation potential could be affected. Previously in our lab we saw that an increase in cJun caused impaired differentiation into insulin-secreting pancreatic islet-like cells (Hosawi et al. *in prep*). Specifically, the neuronal projections between clusters, which are derived from the ectoderm, exhibited inappropriate morphology and reduced overall formation. The endoderm-derived portions of the cluster, however, were able to form in the presence of over-expressed cJun. Conversely, when cells were

transfected with the L4042A construct, the clusters were larger and displayed appropriate neuronal morphology. These data coupled with the knowledge that slight changes in *Oct4* levels have the ability to increase primitive endoderm and mesoderm formation suggested we might analyze differentiation to the myocardial lineage. GATA4 has been known to contribute to the formation of cardiac mesoderm, so we examined cardiomyocyte formation *in vitro* wherein cells were transfected, placed in hanging drops and subsequently differentiated in low adherent conditions prior to plating for cardiomyocyte formation.

Our data show that increased cJun through transfection ultimately led to an increase in GATA4 and finally an increase in the number of beating colonies. Others have shown that GATA4+ expression in embryoid bodies is sufficient to significantly increase cardiomyocyte formation (Holtzinger et al. 2009). Interestingly, cardiac mesoderm forms due to the production and secretion of cardiac-inducing factors from endoderm-derived GATA4+ cells, rather than GATA4 expression within the mesoderm cells themselves (Narita et al. 1997; Holtzinger et al. 2009). Moreover, GATA4 levels are altered by BMP expression as cells of the foregut endoderm are guided toward a hepatic fate while simultaneously selecting against a pancreatic fate (Rossi et al. 2001). BMP4-deficient embryos generated through homologous recombination show that it is required for mesoderm formation (Winnier et al. 1995). BMPs work in parallel with FGF signaling from the cardiac mesoderm, which maintains Brachyury expression through gastrulation (Schulte-Merker and Smith 1995), to guide endoderm derived foregut tissue toward a hepatic fate **(**Rossi et al. 2001). In response, the GATA4+ endoderm population

produces cardiac-inducing factors and ultimately are able to contribute to the temporal and spatial generation of cardiomyocyte progenitors from associated mesoderm (Narita et al 1997, Holtzinger et al. 2009). Taken together, these data present a model wherein over-expressed cJun binds to the *Oct4* promoter to increase transcription of the OCT4A isoform and ultimately drive cells toward a specific endodermal fate.

Simandi et al. (2016) have shown that *Oct4* is upregulated in response to differentiation cues due to its ability to bind to low-accessible genomic regions and activate enhancers which would otherwise remain unresponsive. Therefore, it is important to confirm that the increase in *Oct4* is essential for increased cardiomyocyte formation, and not simply a byproduct of *in vitro* differentiation cues. Furthermore, it is crucial to confirm the necessity of increased *Oct4* preceding differentiation. This can be done through co-transfection of our WT-cJun construct and a RNAi construct designed to target *Oct4* transcripts. Previous reports have also shown that ablation of Beta-catenin in embryonic endoderm causes a distinct shift toward pre-cardiac mesoderm while cJun-/- mice have impaired cardiac development (Eferl et al. 1999; Lickert et al. 2002). It would be of interest to examine the effect of co-transfection with WT-cJun and Beta-catenin on cell fate, or treatment with GSK3β, to further understand the role of Wnt signaling particularly, in regards to cardiac specification. Additionally, since germ cell nuclear factor (GCNF) functions as a repressor of *Oct4* through direct binding of the proximal promoter in non-germ line cells and subsequently silencing it during gastrulation (Fuhrmann et al. 2001), it would be interesting to investigate the effect of over-expressed cJun on GCNF. Overall, there is a great deal of cross-talk between major signaling

pathways as the embryo undergoes gastrulation and proceeds with development that remain to be studied.

Upstream Modulation of the JNK/cJun Signaling Pathway

Building on our previous data, we treated mES cells with chemicals to modulate the JNK pathway in hopes of elucidating the molecular mechanism through which cJun is activated. Unfortunately, these data were met with mixed results. Similar to the results seen with the transcriptionally inactive L4042A construct, samples treated with low doses of SP600125 showed minimal difference in OCT4 expression compared to untreated controls. It has previously been shown that cells derived from either JNK1-/- or JNK2-/- embryos were able to undergo self-renewal, however, their ability to undergo lineage specification was severely impaired (Xu and Davis 2010) demonstrating that ultimately JNK does play a critical role if inhibition is aggressive enough. Samples treated with anisomycin did show a promising increase in endogenous phosphorylated cJun; however, the expression of OCT4A was highly variable and cells did not efficiently form embryoid bodies to assess the germ layer potential. It is possible that the discrepancies in OCT4A expression were due to different pathways converging on cJun to activate it. Hosawi et al. showed that differentiation of anisomycin treated mESCs resulted in impaired pancreatic islet-like clusters, similar to cells transfected with WT-cJun (*in prep*). This striking parallel in results suggests that while JNK may not be the only pathway active, it does play a role. Because both JNK and p38 are able to phosphorylate cJun through JNK/MAPK and mTOR signaling respectively, it would be of interest to inhibit them separately. This could elucidate a better understanding of which protein is necessary, and

perhaps solely sufficient, to drive the context-specific increase in OCT4A and endoderm formation.

Modulation of cJun Expression by Increased Translation Also Increases OCT4A Expression

Preliminary results conducted using the anti-cancer drug nocodazole were also variable. Nocodazole functions by disrupting the cytoskeleton to prevent spindle formation, however previous work has shown that it actually causes an increase in cJun protein, but not mRNA (Polack et al. 2006; Blau et al. 2012). Given that cJun is heavily implicated in the oncogenesis of cancers, including hepatocarcinomas and glioblastomas, the dramatic increase resulting from an anticancer drug is worthy of further investigation. Particularly since cJun is highly necessary for liver formation during embryonic development. Here we have shown that increased cJun does appear to increase OCT4A protein in some instances following low-dose treatments with nocodazole. Immunocytochemistry showed a striking increase in overall OCT4A expression as well as areas of co-localization with cJun in the nuclei, further supporting our conclusion that increased cJun expression increases OCT4A, but not the overall levels of OCT4 in the cell. It is possible that there is a complimentary decrease in the other OCT4 isoforms which prevents the overall levels from changing. Alternatively, it is possible that the Oct4 all antibody is also identifying phosphorylated versions of OCT4. Each of these possibilities warrant further investigation.

Unfortunately, we were not able to characterize the effect of nocodazole on differentiation as nocodazole-treated cells were unable to form proper embryoid bodies

under standard low-adherent conditions. Nocodazole causes the de-polymerization of microtubules, which could affect the formation of cell-cell adhesions. These adhesions are extremely important in determining germ layer formation and organization. As gastrulation occurs, the cells that become the endoderm must undergo an epithelial to mesenchymal transition (EMT) as they travel from the primitive streak to the underlying hypoblast, where they will incorporate themselves. Upon cJun induction, cells begin to rapidly upregulate genes involved in this EMT (Liu et al. 2015), which is consistent with the established role of cJun in migration and invasion during the onset of both cancer (Polak et al. 2006; Blau et al. 2012) and development (Veluscek et al. 2016). One mechanism by which cJun contributes to this is direct binding of the *Fn1* (Fibronectin) locus, as epiblast-derived stem cells exit pluripotency, which results in subsequent altered expression of the adhesion molecule N-cadherin (Veluscek et al. 2016). Together, this could further implicate cJun, in part, as a key regulatory component during gastrulation. Furthermore, it is possible that the cells undergo apoptosis prior to forming aggregates due to a known role for cJun in cell death.

Conclusions

cJun is required for many cellular processes including proliferation and is implicated in a variety of cancers. Here we have established a link between *Oct4*, the classic potency marker, and cJun through which cell fate can be regulated. When the WT-cJun construct is overexpressed, OCT4A is also increasingly expressed, but the overall levels of OCT4 in the cells do not seem to be affected. Considering that as little as a two-fold change in

Oct4 transcriptional activity can alter cell fate toward mesoderm and endoderm or trophoblast, it is possible that cJun regulation is an initial step in lineage commitment. Upon transfection with cJun, increased OCT4 expression and differentiation into embryoid bodies, there is a detectable increase in GATA4 protein. Based on previous literature, GATA4 is required for specification of cardiac mesoderm as well as the formation of liver buds from the foregut endoderm (Rossi et al. 2001). cJun is a necessary component of this lineage commitment because cJun-/- embryos display cardiac deformations and die by mid- to late-gestation (Eferl et al. 1999). Considering the increase in cardiomyocyte activity when cells are transfected with cJun, it is imperative that the link between cJun, *Oct4* and cardiac fate be further explored. Additionally, these data provide an interesting model which implicates cJun as a regulator of *Oct4* in a developmental context. Given the known role of cJun in hepatogenesis and hepatocyte maintenance (Eferl et al. 1999), as well as the positive feedback loop Oct4 initiates with cJun in hepatocarcinomas (Kuo et al. 2016), it would be interesting to compare the diseased state with this developmental model.

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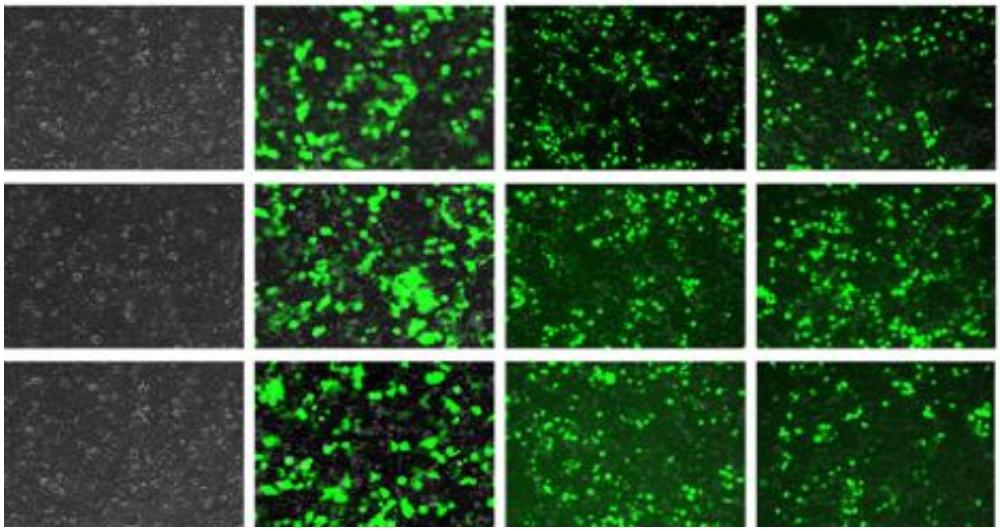
APPENDIX A

Optimization of Luciferase Assays

A



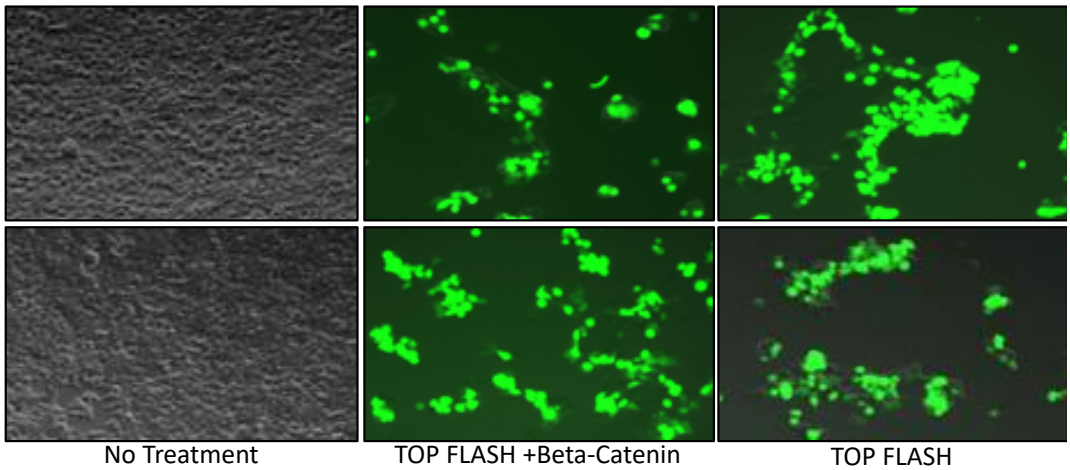
B

Sample	Luciferase	Luc- Avg NT	Renilla	Renilla-Avg NT	F/R Ratio	Avg F/R Ratio	Fold Change
NT	71.21		62.89				
NT	57.95		60.12				
NT	64.7		50.48				
egfp+Oct4	32816.31	32751.69	23468.16	23410.33	1.399027267		
egfp+Oct4	43963.83	43899.21	56158.84	56101.01	0.782503024		
egfp+Oct4	24248.69	24184.07	30539.9	30482.07	0.793386735	0.991639009	1
cJun + Oct4	21011.83	20947.21	9416.98	9359.15	2.238153037		
cJun + Oct4	24780.26	24715.64	13506.19	13448.36	1.837818143		
cJun + Oct4	19110	19045.38	18101.77	18043.94	1.055500074	1.710490418	1.724912395
Beta + Oct4	65667.5	65602.88	13250.02	13192.19	4.972857425		
Beta + Oct4	65595.37	65530.75	15643.98	15586.15	4.204421875		
Beta + Oct4	61385.89	61321.27	7043.16	6985.33	8.778578822	5.985286041	6.0357509
Top only	11750.95	11686.33	745805.73	745747.9	0.015670617		
Top + Beta	17861746	17861681.38	760479.82	760421.99	23.48916998		1498.930732

Supplemental Figure 1. Transfection Efficiency with New Oct4 Clone that Encompasses Complete Promoter Sequence (-3066- +1)

A. Phase contrast images taken at 200X total magnification show 293T cells have been successfully transfected with the various GFP-cJun plasmids as shown by positive GFP signal. **B.** Subsequent luciferase assay data is shown as raw readouts from the SpectraMaxL5. When first normalized firefly to Renilla signal and then to eF1-GFP signal, both cJun and Beta-catenin alone appear to activate the Oct4 reporter by 1.7 and 6.0-fold change respectively.

A

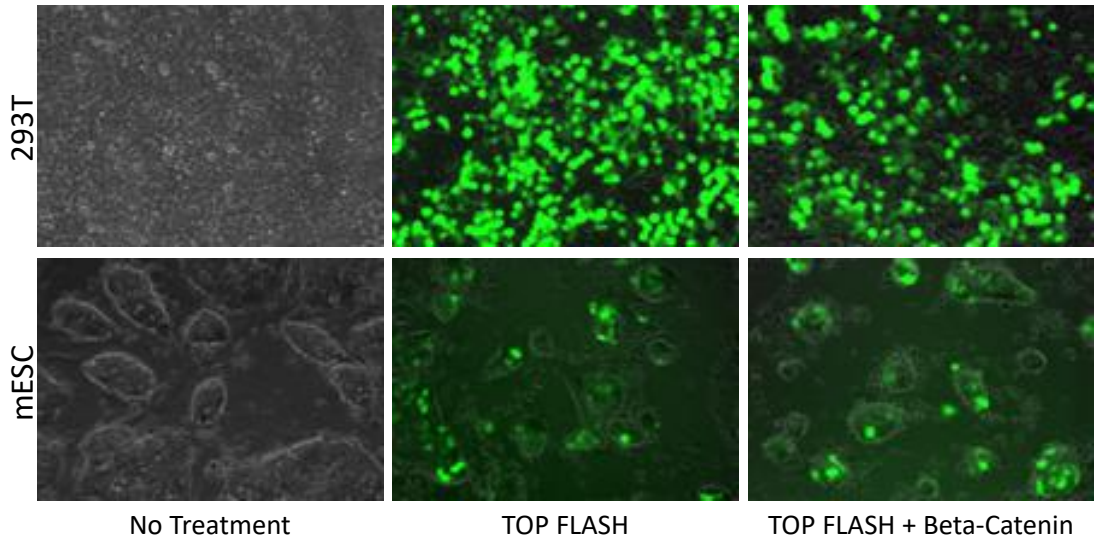


B

Sample	Luciferase	Luc-Avg. NT	Renilla	Renilla-Avg. NT	F/R Ratio	Avg F/R	Fold Change
NT	52.411538		76.88097				
NT	51.447225		76.6398				
NT	53.255311		87.24733				
TOP	46587.90608	46535.53473	321203.5	321123.244	0.144914875		
TOP	46601.05012	46548.67876	236737.4	236657.144	0.196692472		
TOP	49612.64122	49560.26987	293445.8	293365.544	0.168936915	0.170181	
TOP + Beta	10718253	10718200.63	72760.63389	72680.37786	147.4703482		
TOP + Beta	9553551.4	9553499.029	95580.6484	95500.39237	100.036228		
TOP + Beta	10431421.2	10431368.83	715164.16	715083.904	14.5876152	87.36473	
							513.362329

Supplemental Figure 2. Positive Control Plasmid Transfection Efficiency

A. Phase contrast with fluorescent FITC filter identify GFP positive cells, which have been successfully transfect with the TOP FLASH plasmid +/- Beta-catenin with a pcDNA-GFP plasmid. **B.** Data table demonstrating raw values and the preliminary calculations done to find the F/R ratio in relative light units (RLU) and ultimately the fold change in TOP FLASH activation, which is approximately 513-fold change greater when Beta-catenin is added. Based on this result, we moved forward using TOP FLASH with Beta-catenin as a positive control for all subsequent transfections.



293T Data

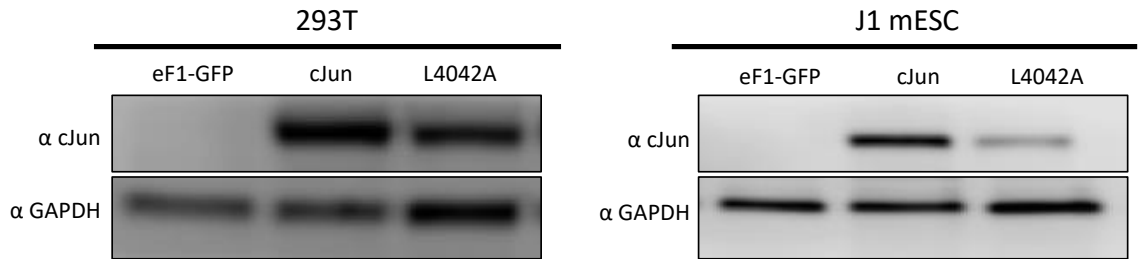
Sample	Luciferase Data	Luc-Avg NT	Renilla Data	Renilla- Avg NT	F/R Ratio	Avg F/R Ratio	Fold change
NT	72.903182		75.675582				
NT	72.541565		57.835796				
NT	72.541565		57.112562				
NT	70.251322		62.65736				
TOP	20410.59723	20338.53782	6080.606901	6017.286576	3.380018147		
TOP	19908.22377	19836.16436	2394.268346	2330.948021	8.509912781		
TOP	28537.60735	28537.60735	15810.47111	15747.15079	1.812239416		
TOP	24613.64481	24541.58541	8450.793697	8387.473372	2.925980724	2.706079429	
TOP + BETA	17292592.41	17292520.35	29812.86952	29749.5492	581.2699963		
TOP + BETA	17368849.88	17368777.82	22383.70305	22320.38273	778.1577062		
TOP + BETA	17155446.74	17155374.68	17074.26093	17010.94061	1008.490658		
TOP + BETA	18665461.62	18665389.56	16970.09992	16906.7796	1104.018034	963.5554659	356.0706517

mESC Data

Sample	Luciferase Data	Luc- Avg NT	Renilla Data	Renilla- Avg NT	F/R Ratio	Avg F/R Ratio	Fold Change
NT	107.738986		63.621672				
NT	125.337701		55.183937				
NT	111.596239		63.380594				
NT	82.66685		67.478923				
TOP	18105.26709	17998.43214	310.365445	247.9491635	77.40591001		
TOP	16201.91537	16095.08042	294.93641	232.5201285	69.22015967		
TOP	17959.87441	17853.03947	415.4758	353.0595185	50.5666567		
TOP	16953.4631	16846.62816	412.100696	349.6844145	48.1766629	61.34234732	
TOP + BETA	104591.9666	104485.1317	1539.993809	1477.577528	70.7138067		
TOP + BETA	111204.8622	111098.0273	3026.751377	2964.335096	37.47822823		
TOP + BETA	111360.3862	111253.5513	2739.379204	2676.962923	41.55961607		
TOP + BETA	109978.8963	109872.0613	568.561006	506.1447245	217.0763736	49.917217	0.813748074

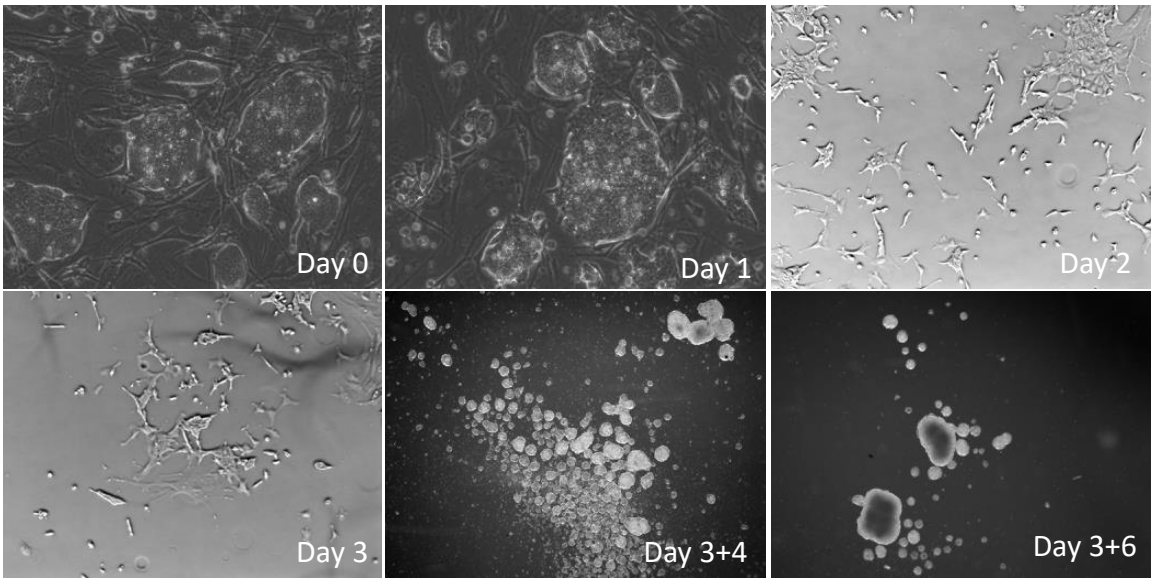
Supplemental Figure 3. J1 mESCs Are Not Efficiently Transfected Compared to 293T

A. Phase contrast images (total magnification of 200X) show varied degrees of transfection efficiency, with the 293T cells overall more successful based of GFP signal. **B.** Luciferase assay data shows that 293T cells are able to show a fold change with the positive control TOPFLASH plasmid greater than 300, while mESC show a decrease in plasmid activity. Based on these results we concluded that efficiency was too low in mESC to successfully carry out luciferase assays.



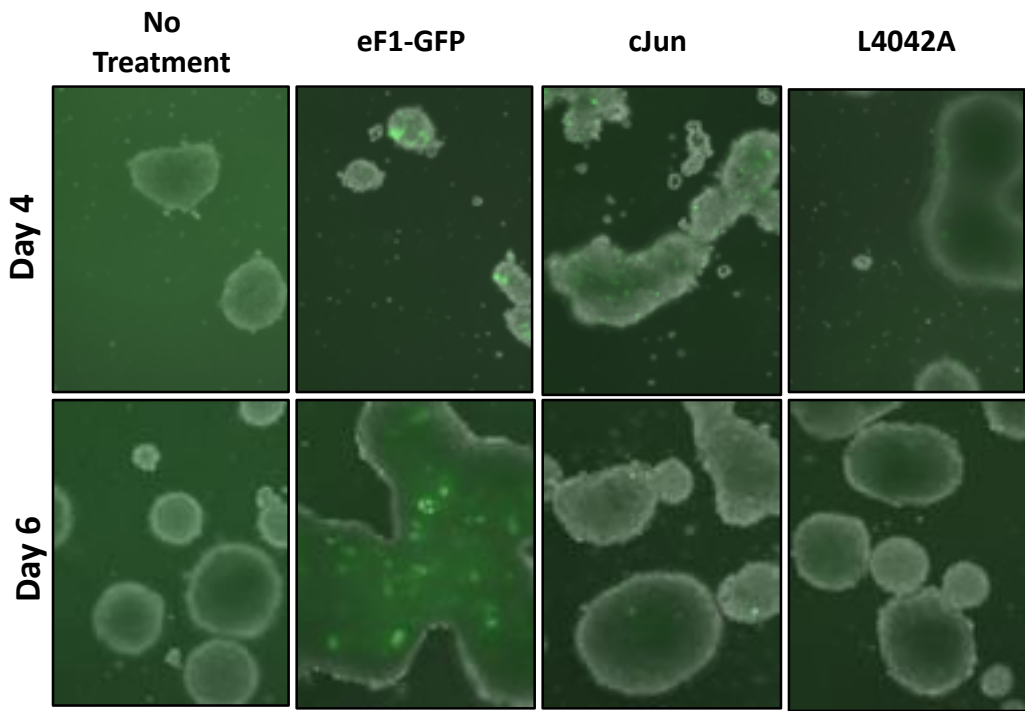
Supplemental Figure 4. Expression of cJun Construct can be Detected by Western Blot

293T (left) and J1 mES cells (right) were transiently transfected with the empty GFP vector, WT- cJun or the L4042A mutant and harvested for protein. Western blots were run using an anti- cJun antibody, and GAPDH as a loading control. Because efficiency was too low for Luciferase Assays, it was imperative to confirm the product of the cJun plasmids by western blot. Based on the above results, the cJun product from the plasmid was strongly detected in J1 mESCs, albeit at lower levels than 293Ts.



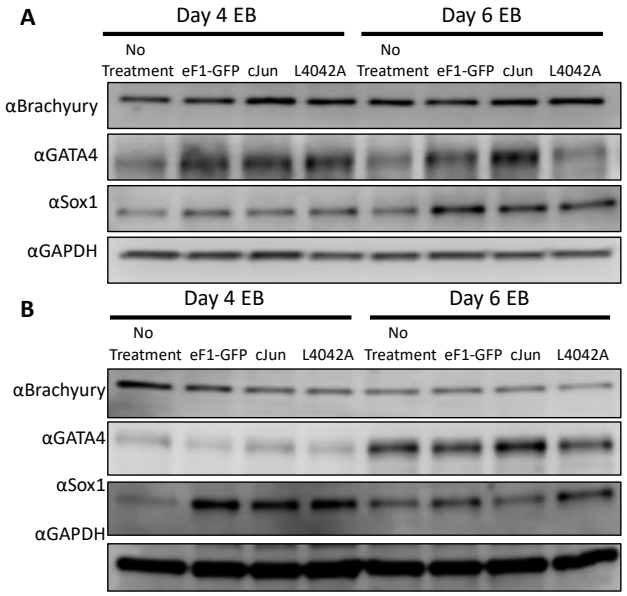
Supplemental Figure 5. Differentiation of mESC into Embryoid Bodies

Phase contrast images of J1 mES cells as they were differentiated from pluripotent colonies (Day 0 and Day 1) toEBs (Day 3+4 and Day 3+6). Cells were seeded at 30,000 cells/cm^2 and grown to 40% confluency. One million cells were lifted and plated to 0.1% gelatin for 48 hours (Day 2 and Day 3). Following gelatin, cels were lifted and maintained in ultra-low-adherent dishes for either four (Day 3+4) or six (Day 3+6) days prior to harvest for further analysis.



Supplemental Figure 6. GFP Plasmid Expression is Still Detected 4 and 6 Days After Low Adherent Culture

Embryoid bodies were imaged at 10X for GFP expression. eF1-GFP was still easily detectable in EBs following low adherent conditions, likely due to its cytoplasmic location. cJun and L4042A were not as visible due to their nuclear localization, however some faintly GFP positive cells were still detected. cJun appeared to have more GFP positive areas than L4042A, however efficiency with L4042A was lower initially.



C

Average Day 4 EB Densitometry Values Normalized to GAPDH

		NT	eF1-GFP	cJun	L4042A
GATA 4	Mean	0.085	0.048	0.050	0.036
	Standard Error	0.048	0.011	0.032	0.013
Sox1	Mean	0.070	0.076	0.044	0.037
	Standard Error	0.054	0.035	0.016	0.005
BRY	Mean	0.135	0.123	0.139	0.147
	Standard Error	0.023	0.035	0.044	0.057

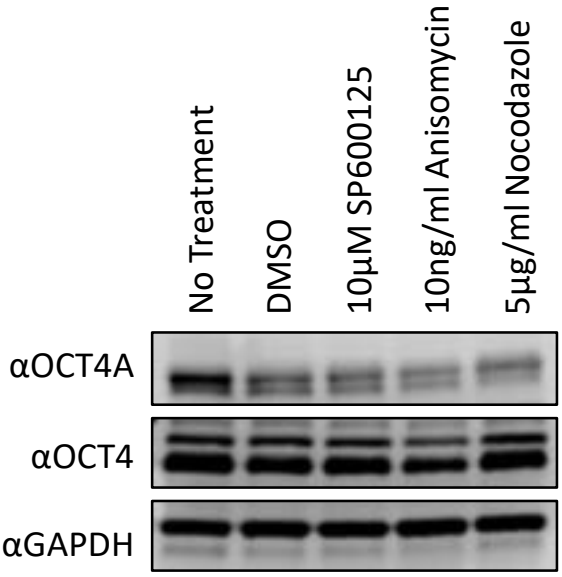
D

Average Day 6 EB Densitometry Values Normalized to GAPDH

		NT	ef1gfp	cJun	L4042A
GATA 4	Mean	0.177	0.161	0.257	0.163
	Standard Error	0.137	0.124	0.150	0.097
SOX1	Mean	0.035	0.050	0.068	0.053
	Standard Error	0.010	0.009	0.036	0.008
BRY	Mean	0.139	0.161	0.172	0.157
	Standard Error	0.033	0.070	0.073	0.055

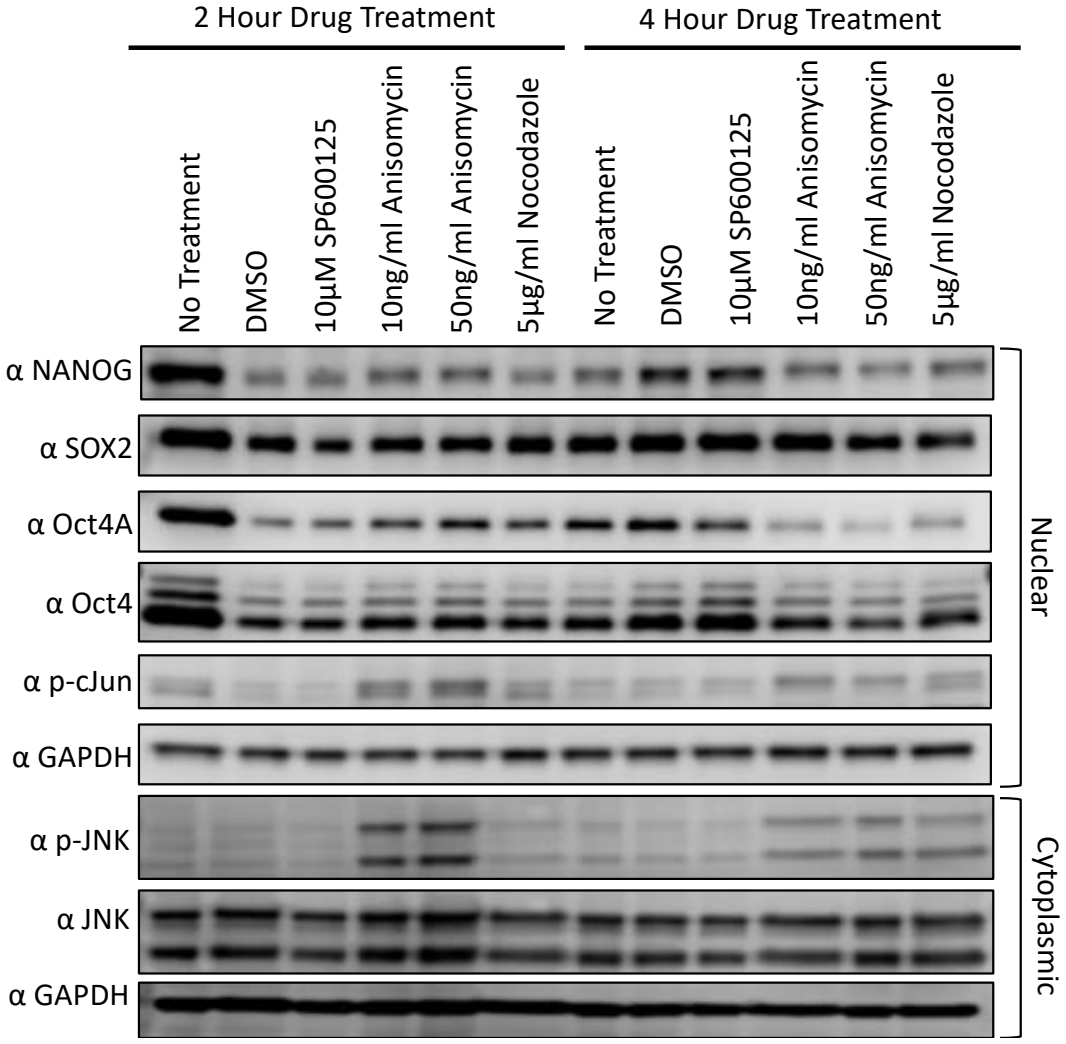
Supplemental Figure 7. Replicated Western Blots Show an Increase in GATA4 when cJun is Over-Expressed

A-B. mESCs were transfected and differentiated, as previously done. While experimental variables were kept constant, these replicates demonstrate variation in Sox1 and Brachyury expression. GATA4 expression patterns also showed minor variations. However, EBs grown in culture for 6 days after transfection with cJun do appear to maintain the trend of elevated GATA4 expression compared to the other samples. **C-D.** Tables with the mean germ layer expression, normalized to GAPDH and the standard error.



Supplemental Figure 8. OCT4A Expression is Not Significantly Altered After Two Hour Treatment

J1 mESCs were harvested two hours post-treatment with DMSO, a JNK inhibitor (SP600125), a JNK activator (Anisomycin) or a cytoskeletal disruptor (Nocodazole). Not in agreement with our expected results, OCT4A remained seemingly unchanged across treatments. Because of this, we included a four-hour time point for subsequent drug treatments.



Supplemental Figure 9. There is Variation in Potency Marker Expression Under Drug Modulation

While somewhat similar to the data in main figure 7, this replicate demonstrates variation in the expression of potency markers SOX2, NANOG and OCT4A. OCT4 all, however appears to remain relatively constant.

APPENDIX B

Antibody Information

Supplemental Table 1. Antibodies Used for Immunoblot and Immunocytochemistry

Target	Manufacturer &Catalog Number	Working Dilution
Western Blot Antibodies		
cJun	Abcam #ab32137	1/1000
Phospho-cJun S63	Abcam #ab32385	1/1000
Phospho-cJun S73	Cell Signaling #3270s	1/1000
JNK I/II/III	Abcam	1/1000
Phospho-JNK	Abcam #ab124956	1/1000
OCT4	Abcam #ab19857	1/1000
Oct4A	Cell Signaling #2840s	1/1000
SOX2	Cell Signaling #4195	1/500
NANOG	Santa Cruz Biotech #sc293121	1/500
eGFP	Clonetech #632381	1/1000
GATA4	Abcam #ab84593	1/500
BRACHYURY	Abcam #ab20680	1/500
SOX1	Abcam #ab87775	1/250
GADPH	Cell Signaling #97166	1/1000
(Secondary) Goat anti-Rabbit IgG (H+L)	Abcam #ab6721	1/2000
Immunocytochemistry Antibodies		
cJun	Abcam #ab32137	1/1000
OCT4	Abcam #ab19857	1/1000
OCT4A	Cell Signaling #2840s	1/1000
(Secondary) Texas Red Rabbit IgG	Life Technologies #A-6399	5μg/mL
(Secondary) Texas Red Mouse IgG	Life Technologies #T6390	5μg /mL
(Secondary) Goat anti Guinea Pig IgG H&L (FITC)	Abcam #ab69041	1/200