Functional analysis of SOX3 binding at the *Dbx1* locus

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Declaration

I declare that this thesis does not incorporate, without my acknowledgment, any material previously submitted for a degree or diploma in any other university. To the best of my knowledge this thesis does not contain any material written or published by any other person, except where due reference is made.

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ABSTRACT

Sox3 a members of the SOX transcription factor family, is essential for normal brain development and required for growth of pituitary and hypothalamus. Sox3, as well as Sox2 which is another member in SOXB1 subfamily are widely expressed in neural progenitor cells and show functional redundancy. ChIP-seq data by Bergsland et al, 2011 has identified five putative SOX3 binding sites near/at the *Dbx1* locus. Microarray data from the lab (N. Rogers, unpublished data) has identified *Dbx1* as downregulated in *Sox3* null neural progenitor cells. Together these data suggest that *Dbx1* may be regulated directly by SOX3.

To investigate the possibility that SOX3 regulates the *Dbx1* locus *in vitro*, we performed gel shift assays and Luciferase Reporter Assays to see if SOX3 binds any of the five *Dbx1* regulatory sites. Due to time constraints we were not able to optimize the gel shift assays to obtain any informative results. Secondly, we optimized Luciferase Reporter Assays providing preliminary data suggesting SOX3 may bind at one of the tested *Dbx1* sites. To study the redundancy between *Sox2* and *Sox3*, *Sox2* was also tested in the Luciferase Reporter Assays indicating Sox2 may also regulate the same site as *Sox3* . Due to time constraints, the other three binding sites remain to be analyzed in the future.

The function of Dbx1 is best characterized in the context of the developing neural tube (also known as the spinal cord). To identify how other neural tube marker genes are regulated by *Sox3*, qPCR was performed with some marker genes in Sox3 null E9.5 mouse embryos compared with WT embryos. *Dbx1*, *Pax6*, *Ngn2* and *Olig2* all showed significant decrease in *Sox3* null. Further study of these genes will be required to assess the significant outcome of their down regulation in an *in vivo* context.

Chapter 1. Introduction

1.1 *Sox3* is a member of the Sox B1 subfamily belong to Sox family

The SRY related HGM box containing (Sox) family of transcription factors was first described in 1990 with the Y chromosome-linked sex-determing region Y(*SRY*) gene as the founding member (Gubbay, Collignon et al. 1990). *SRY* is widely considered as the main initiator of male sex determination and encodes a transcription factor containing a highly conserved High Mobility Group box (HMG box), a DNA binding domain 79 amino acids in length (Foster and Graves 1994). The HMG box superfamily of transcription factors are divided into two subfamilies: one is the HMG/UBF group which have a duplicated HMG domain that lack sequence-specific binding activity, while the members in the TCF/SOX/MATA group generally have single, sequence-specific HMG box binding domains (Bowles, Schepers et al. 2000). Members of the Sox family share greater than 50% or higher amino acid identity with *SRY* in the conserved HMG box (Bowles, Schepers et al. 2000).

Analysis of the whole genome in 2002 identified 20 Sox genes in mouse and humans (Schepers, Teasdale et al. 2002). Comparison of different domains (transactivation, transrepression and dimerization domains) in SOX proteins have further categorized the genes into 8 groups, A to H, with additional sub groups within the Sox B genes, Sox B1 and Sox B2 (Lefebvre, Dumitriu et al. 2007). The members of the Sox B1 subgroup, *Sox1*, *Sox2* and *Sox3*, generally act as transcriptional activators while the Sox B2 genes, *Sox14* and *Sox21*, generally repress transcription (Lefebvre, Dumitriu et al. 2007). This is supported by experiments using obligatory activator (SOX3 HMG-VP16) and repressor (SOX3 HMG-EnR) fusion proteins-overexpression of the former inhibits neurodifferentiation (as does WT SOX) while the latter promotes neurodifferentiation (Bylund, Andersson et al. 2003).

Sox3 is a single exon gene located on the X chromosome. Of the 20 SOX proteins in mammals, SOX3 shows both highest percentage of amino acid identity (67%) to SRY within the HMG domain. Based on sequence alignments and gain of function studies, it is thought that *Sry* evolved from *Sox3*, athough *Sox3* is not required sex determination (Sutton, Hughes et al. 2011). *Sox3* is widely expressed in the CNS and

is also essential for normal mammalian brain development (Rizzoti, Brunelli et al. 2004; Woods, Cundall et al. 2005; Sutton, Hughes et al. 2011). Furthermore, it is required for the formation of both hypothalamus and pituitary in mice as well as associated with human hypopituitarism and mental retardation (Rizzoti, Brunelli et al. 2004; Woods, Cundall et al. 2005)

Sox3 is first expressed between 5.5 to 6.5 dpc in the epiblast and extraembryonic ectoderm of mouse embryos and it is upregulated in the developing neural plate (Wood and Episkopou 1999). Between 8.0 to 9.0 dpc (the beginning of neuralation), *Sox3* is expressed in the neuroectoderm (Wood and Episkopou 1999). Sagittal section of 10.5 dpc wild-type embryo has shown that *Sox3* is present in the neuroprogenitor cells throughout the CNS (Rizzoti, Brunelli et al. 2004; Rogers, Cheah et al. 2013). Importantly, Sox3 expression is downregulated in the vast majority of neurons during embryonic brain development. As such, *Sox3* is expressed after birth in the residual population of stem/progenitor cells and a small subset of neuron of indeterminate phenotype (Rogers, Cheah et al. 2013). SOX3 is also express in the spermatogonial progenitor cells in the testis and is required for early spermatogenesis (Collignon, Sockanathan et al. 1996; Rizzoti, Brunelli et al. 2004).

1.2 *Sox3* is required for brain development

Sox3 null mice have been generated using gene targeting in embryonic stem cells (Rizzoti, Brunelli et al. 2004). They exhibit variable phenotypes with the more seriously affected mice dying before weaning (3 weeks). The pituitary growth hormone level in *Sox3* null mice is approximately one third that of wild type mice resulting in dwarfism in some mutants. Other anterior pituitary hormones are also generally lower. The pituitary develops from the infundibulum in the ventral diencephalon and the Rathke's pouch and the secretory is regulated by the hypothalamus. In the *Sox3* null mutant mice, pituitary dysgenesis is first observed in the 11.5-d.p.c. mutant brains (see Figure 1.1). Furthermore, an extra cleft appears between the anterior lobe and intermediate lobe resulting in a smaller anterior and the mixture of growth hormone-positive anterior lobe cells and intermediate cells (see Figure 1.2) (Rizzoti, Brunelli et al. 2004). Other CNS defects include agenesis of the corpus callosum and absence of the dorsal hippocampus. *Sox3* mutants also exhibit variable craniofacial defects including the pinna and teeth as well as the defects in

both testes and ovaries. Together these studies show that Sox3 is required for normal development of the brain, craniofacial region and gonads.

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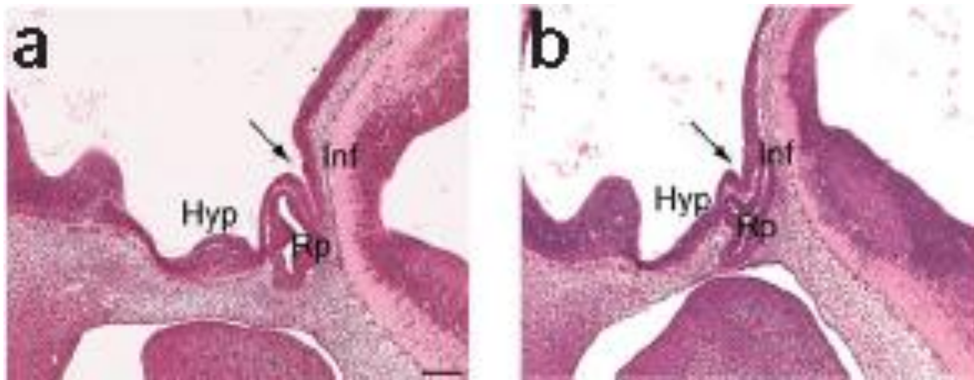


Figure 1.1 . Sagittal sections through brains of the 11.5dpc mice (**a**) wild type (**b**)*Sox3* null embryos, dorsally expanded Rathke's pouch and the less obvious evagination of the infundibulum.(Rizzoti, Brunelli et al. 2004)

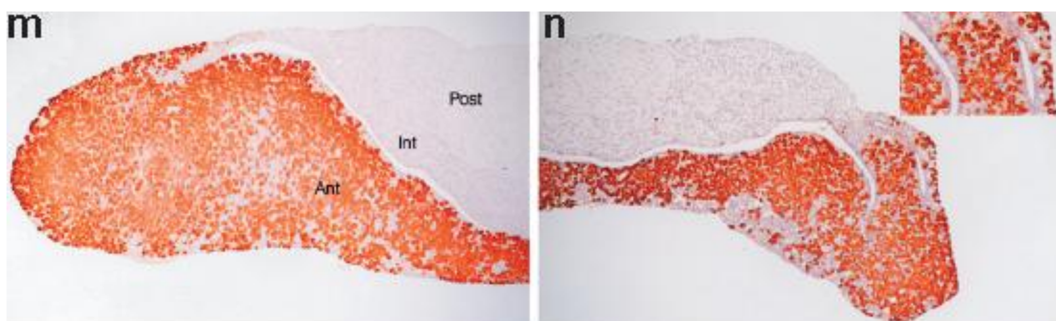


Figure 1.2 .immunohistochemistry of 6-weeks wild type and *Sox3* null mice growth hormone on pituitary sections .(**m)** The pituitary can be divided clearly by three parts :anterior lobe (Ant); intermediate lobe(Int); posterior lobe(Post). The heavy staining section is the growth hormone-generation somatotrophs (**n**) The inset figure show the extra cleft in the mutant.(Rizzoti, Brunelli et al. 2004)

In humans, the previous studies of *SOX3* duplications (at Xq26-27 (3.9Mb)) indicated this chromosomal abnormality is associated with mental retardation, growth hormone deficiency (X-linked Hypopituitarism) presumably due to overexpression of SOX3 (Solomon, Ross et al. 2004). Polyalanine tract expansion (+11 Ala) in SOX3 is also associated with mental retardation and short stature due to growth hormone (GH) deficiency (Laumonnier, Ronce et al. 2002). Furthermore, a recent study

demonstrated that both overdosage and underdosage of *Sox3* resulting from duplications of Xq26-27 (685.6kb) and an expansion of a polyalanine tract (by 7 alanines), respectively, leads to Infundibular Hypoplasia and Hypopituitarism. For the patients with duplication of *SOX3,* they had short stature due to GH deficiency and one of them also had a cyst in the corpus callosum. For the patients with underdosage of SOX3, they were characterized as having short stature and GH deficiency but not mental retardation (Woods, Cundall et al. 2005). A recently published knock-in mouse model from the Thomas laboratory has shown that the (+11Ala) expansion allele functions as a partial loss-of-function mutation (Hughes, Piltz et al. 2013).

1.3 SoxB1 functional redundancy in NPCs

Despite the widespread expression of *Sox3* in the developing nervous system, the CNS phenotype of *Sox3* null mice is relatively mild, suggesting that other genes may compensate for the loss of *Sox3* function. Given their similarity in sequence, expression and function, several lines of evidence suggest that the two other members of the SOXB1 subgroup*, Sox1 and Sox2,* are functionally redundant with *Sox3*, as outlined below.

1. *Sox1*, *Sox2* and *Sox3* have overlapping expression at most stages of the neural development. Overlapping expression is mainly observed in the developing CNS of the embryo, as indicated by in situ hybridization (see Figure 1.3). Both *Sox2* and *Sox3* are expressed in the neuroectoderm from the onset of somitogenesis between 8 and 9 dpc. From the 6 somite stage, *Sox2* transcripts are present in ectoderm adjacent to the hindbrain as well as *Sox3*. At the 8-10 somite stage, both of them are expressed in the ectoderm overlying the second branchial arch, *Sox2* and *Sox3* are sequentially expressed in the nasal placode . Last but not least, *Sox2* and *Sox3* are detected in the gut endoderm and the posterior region of the foregut respectively. However, it should be noted that there are subtle differences of expression between *Sox2* and *Sox3*. For example, at 4-6 somite stage, *Sox2* is downregulated in rhombomere (r) 5 and 6 while *Sox3* becomes upregulated in r5 (Wood and Episkopou 1999).

2. Overexpression experiments in chick embryos indicate that all SOXB1 members have the ability to inhibit neuroprogenitor differentiation (Bylund, Andersson et al. 2003).

3. It has been demonstrated *in vitro* that both SOX2 and SOX3 bind to the motif AACAAT, the consensus binding sequence of the SRY protein (Collignon, Sockanathan et al. 1996). Although SOX3 has greatest sequence homology to SRY within the HMG box, SOX2 exhibits similar affinities to SOX3 compared to SRY (Collignon, Sockanathan et al. 1996).

4. A recent ChIP-seq study in NPC on *Sox2* and *Sox3* binding sites has identified 1160 overlapping sites from the ChIP-seq data (Bergsland, Ramskold et al. 2011).

Given the strong evidence for SOXB1 functional redundancy outlined above, it is interesting to consider the primary cause of the defects in the *Sox3* null mice. One possibility is that these defects arise from as yet undefined embryonic CNS regions that express *Sox3* but not *Sox2* or *Sox1*. An alternative possibility is that the defects reflect regions that are particularly sensitive to the SOXB1 dosage, such that the loss of *Sox3* cannot be fully compensated by *Sox2* and/or *Sox1*. To begin to address this issue, it is essential to define the direct target genes that are regulated by *Sox3* during CNS development.

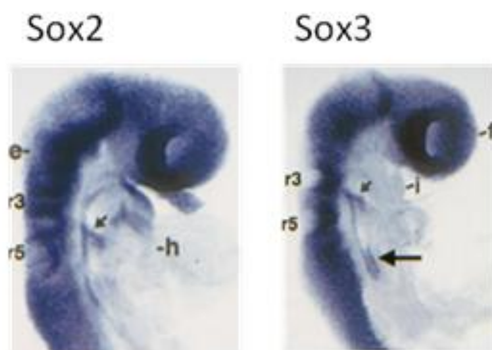


Figure 1.3, Expressions of *Sox2* and *Sox3* in the mouse embryos at 9 dpc.It shows the *Sox2* and *Sox3* overlapping expression in the CNS of mouse embryo. Figure modified from Wood et al. 1999 (Wood and Episkopou 1999).

1.4 SOX3 target gene regulation

SOX3 acts by binding specific DNA sequences, as such it is important to know where SOX3 binds within the genome. A recent study by Bergsland et al. 2011 identified 9720 unique SOX3 binding sites in NPCs by ChIP-Seq. Analysis of the binding sites identified a SOX3 DNA binding motif (tCYTTTSTyyk) (Figure 1.4) (Bergsland, Ramskold et al. 2011). Additionally, the same experiment was performed with SOX2 in NPCs, identifying 1388 SOX2 biding sites over 90% of which were also bound by SOX3.

To identify CNS genes that require *Sox3* for their expression, our lab performed genome wide expression profiling comparing WT and *Sox3* null day 4 NPCs (N. Rogers, unpublished data). The microarray data revealed that *Dbx1* was significantly downregulated, which was subsequently validated on independent NPCs by qRT-PCR (see Figure 1.5). Notably, *Dbx1* expression is still down 2-fold in NP cells in culture at day 6 (N. Rogers, Unpublished Data). Using the ChIP-seq data published by Bergland et al, we identified 5 SOX3 binding sites at or near the *Dbx1* locus in regions of high mammalian conservation (Bergsland, Ramskold et al. 2011) (Figure 1.6). Interestingly, only 3 of the 5 SOX3 binding sites near *Dbx1* also bound *Sox2* in NPCs suggesting the possibility of SOX3-specific regulation(Figure 1.6).

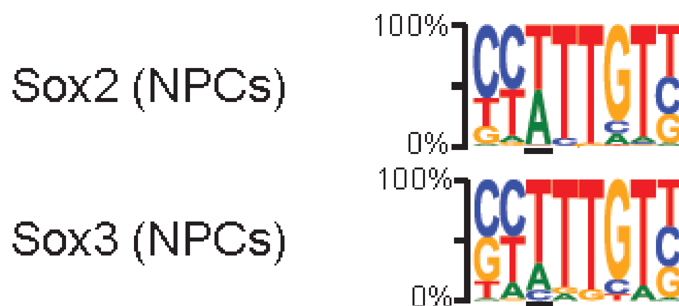


Figure 1.4 . Sequence binding preference of *Sox2* and *Sox3* in NPCs.(Bergsland, Ramskold et al. 2011)

Gene Symbol	p Value	FC (KO vs. WT)	Sox3 BS
Sox3	0.01	-4.10	✔
Raver2	0.02	1.26	✖
Tmem123	0.02	1.32	✔
St6galnac5	0.02	1.34	✔
Cspg5	0.02	1.49	✔
Tagln3	0.04	1.54	✔
Slit1	0.04	1.87	✔
Tmem163	0.04	-1.45	✔
1200009O22Rik	0.04	1.41	✔
Olfm2	0.04	1.36	✔
Slc44a5	0.05	1.44	✔
Fezf2	0.05	-1.61	✔
Kif26b	0.05	1.29	✔
Rtl1	0.05	-1.78	✖
Dbx1	0.05	-2.35	✔
Cx3cl1	0.05	1.26	✖
Dtx4	0.05	1.30	✔
Npnt	0.05	1.32	✔
Ctgf	0.05	1.45	✔
Ednrb	0.05	1.53	✔
Gpr56	0.05	1.56	✖
Efnb3	0.05	1.59	✔
Fgfr3	0.05	1.65	✖
Cp	0.05	2.04	✔

Figure 1.5 . Microarray results of *Sox3* null versus WT cells at day 4(Nicholas Rogers, Unpublished Data). Sox3BS: Sox3 binding sites; FC(KO vs. WT): fold change (Knock out vs. Wild type).

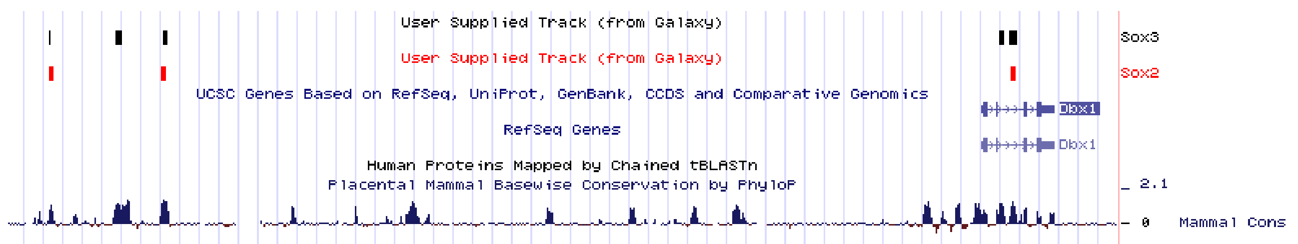


Figure 1.6. Five potential binding sites of SOX3 in/near *Dbx1* From Dale and (Bergsland, Ramskold et al. 2011).

1.5 Function of the *Dbx1* gene in mammalian development

The *Dbx1* gene encodes the developing brain homeobox protein 1 (DBX1) and is expressed as early as 8.25 dpc within five discrete subsets: EM (early midbrain), vDi (ventral diencephalon), dDM (dorso-lateral diencephalon and mesencephalon), ANR (anterior neural ridge) and FE (facial ectoderm) of cells in the cephalic region in the developing mouse. Genetic ablation of all *Dbx1*-expressing cells gives rise to the absence of dorsal neural tube closure at midbrain level, a severe reduction in the size

of the forebrain as well as serious defects of craniofacial structure (Causeret, Ensini et al. 2011)(Figure 1.7). Comparison of *Sox3* expression (figure 1.7) with *Dbx1* by situ hybridization at 12ss (approximately 9.5 dpc) (Causeret, Ensini et al. 2011), reveals regions of overlapping expression in the midbrain, forebrain and spinal cord, consistent with the notion that *Dbx1* is a direct target of SOX3.

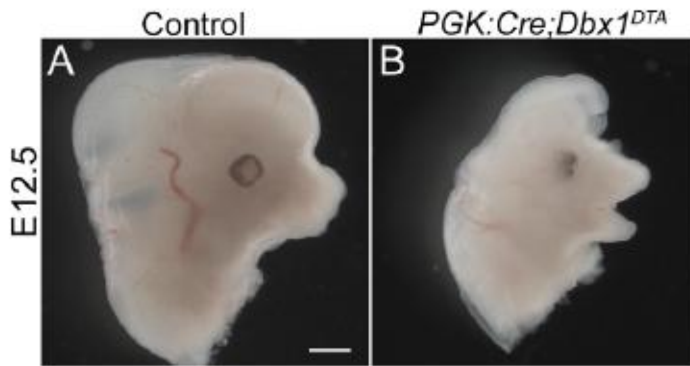


Figure 1.7. Heads of wild-type(A) and PGK:Cre;Dbx^{DTA} (B) E12.5 mouse embryos. Mutants show severe neural and craniofacial defects(Causeret, Ensini et al. 2011)

1.6 The spinal cord development with SoxB1

The function of *Dbx1* is best characterized in the context of the developing neural tube (also known as the spinal cord). During the development of neural tube, morphogens Sonic hedgehog (Shh), retinoic acid (RA) and bone morphogenetic proteins (BMPs) “pattern” the NPCs by restricted activity at ventral, intermediate and dorsal region of the neural tube, respectively (Oosterveen, Kurdija et al. 2013). Shh expression leads to different classes of positionally defined neurons in the ventral neural tube (Briscoe, Pierani et al. 2000). Activation or repression by Shh give rise to 2 classes of homeodomain TFs expressed by NPCs which act as intermediaries to coordinate neural patterning (Oosterveen, Kurdija et al. 2012)(figure 1.8). Furthermore, class I and II TFs redefine domain boundaries through cross-repression interaction (Jessell 2000). It is obvious that Sox3 also expressed in the spinal cord (see figure 1.3) and Dbx1 as well which was mentioned above. Furthermore, Dbx1 is one of the class I genes expressing in the neural tube (Oosterveen, Kurdija et al. 2012).

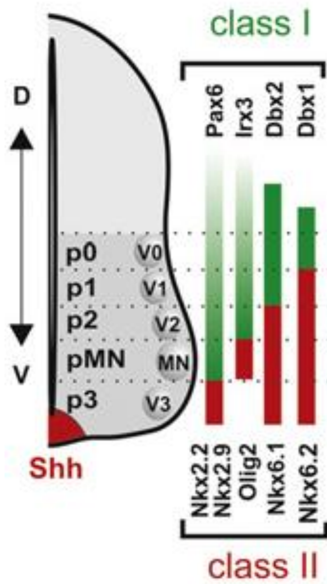


Figure 1.8. Schematic illustrating the expression domains of class I and II TFs in ventral neural tube. class I TFs are repressed by shh; class II TFs are induced by shh. Expression of class I and II TFs within an individual progenitor region controls neuronal fate.(Oosterveen, Kurdija et al. 2012)

1.7 Aims and Objectives

In summary, *Sox3* is expressed throughout the central nervous system (CNS) in the developing mouse embryo (Collignon, Sockanathan et al. 1996; Wood and Episkopou 1999; Archer, Jin et al. 2011). *Sox3* has been shown to maintain cells as neural progenitor cells (NPC), both preventing differentiation as well as stimulating cell proliferation (Guth and Wegner 2008). What is more, microarray data from our lab has identified *Dbx1* as the top downregulated gene in the *Sox3* null NPCs and five putative SOX3 binding sites at *Dbx1* locus have been identified by ChIP-seq from Bergsland et al. Together, these data strongly suggest that *Dbx1* is a **directly** regulated by SOX3. However, additional evidence of SOX3 binding at the *Dbx1* locus is required to definitively show that Dbx1 is a direct target of SOX3. Therefore, the first aim is to investigate SOX3's ability to bind specific DNA sequences at the Dbx1 locus using in vitro assays.

Specifically, my aim was to:

1. Design probes based on SOX3 binding sites identified by ChIP-seq.
2. Optimization transient SOX3 over-expression in 293T cells.
3. Optimize and perform a gel shift assay.
4. Validate SOX3 binding with a luciferase reporter assay.

The second aim of this project is to investigate potential functional compensation by SOX2 at the Dbx1 locus.

Specifically, I aimed to

1. Generate a *Sox2* expression plasmid.
2. Repeat Aim 1 with the *Sox2* expression plasmid.

Chapter 2.
Materials and Methods

2.1 Abbreviations

Amp	Ampicillin
APS	Ammoniumpersulfate
bp	base pair
BSA	Bovine Serum Albumin
^{o}C	Degree Celsius
cDNA	Complimentary deoxyribonucleic acid
CO2	Carbon dioxide
Cy3	Cyanine 3
DAPI	4’,6-diamidino-2phenylindole
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
Dpc	Day post coitum
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EtBr	Ethidium Bromide
EtOH	Ethanol
FCS	Foetal Calf Serum
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HS	Horse Serum
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRES	Internal ribosome entry site
Kan	kanamycin
kb	kilo base pairs

L	Litre
LB	Luria Broth
M	Molar
mA	milliampere
mg	Milligram
min	Minutes
mL	Millilitre
mm	millimeter
mM	Millimolar
MQ	Milli-Q
ng	nanogram
P1000	1000 μ L pippete
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PMSF	phenylmethanesulfonylfluoride
polydIdC	Poly(deoxyinosinic-deoxycytidylic) acid sodium salt
qPCR	Quantitative polymerase chain reaction
RO	Reverse osmosis
rpm	Revolutions Per Minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
sec	Seconds
TBE	Tris/Borate/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)-aminomethane
Triton X-100	Octylphenol ethylene oxide condensate
Tween-20	Polyoxyethylene-sorbitan Monolaurate
V	Volts
v/v	Volume per Volume
x-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
μ g	Microgram

μL	Microlitre
μM	Micromolar

2.2 Materials

2.2.1 Chemicals and Reagents

DMEM	Gibco
FCS	Life Technologies
Trypsin	Gibco
Fugene Transfection Reagent	Promega
SOC media	CSU
BSA	New England Biolabs
Bradford Reagent	Bio-Rad
Skim Milk Powder	Diploma
Isopropanol	EMSURE
Prestained Protein Marker	Bio-Rad
1kb Plus DNA Ladder	Life Technologies
dNTP Mix	Roche
BigDye Version3.1	Amersham
Tween-20	Fisher Biotech
DAPI	Life Technologies
EtBr	Sigma Aldrich
Triton X-100	Sigma
Klenow	New England Biolabs
DMSO	Sigma Aldrich
Phosphorus-32 Radionuclide(α-^{32}P-dATP)	PerkinElmer
PFA	Sigma Aldrich
HS	Life Technologies
Protease Inhibitor Cocktail	Roche

2.2.2 Enzymes

Enzyme	Manufacturer
Restriction Enzymes	New England Biolabs
T4 Ligase	New England Biolabs
Calf Intestinal Phosphatase(CIP)	FINNZYMES
Shrimp Alkaline phosphatase(SAP)	Roche

2.2.3 Antibodies

Antibody	Dilution	Species	Manufacturer
Anti-Sox3	1:1000	Goat	R&D Systems
Anti-Sox2	1:2000	Rabbit	Millipore
Anti-Histone H3	1:2000	Rabbit	Abcam
Anti-Beta-tubulin	1:1000	Rabbit	Cell signaling
Anti-Goat HRP	1:5000	Donkey	Rockland
Anti-Rabbit HRP	1:5000	Donkey	Rockland
Anti-Rabbit Cy3	1:500	Donkey	Rockland

2.2.4 Solutions

2.2.4.1 Buffers for EMSA

Hypotonic Buffer:10mM Tris pH 7.9, 1.5mM $MgCl_2$, 10mM NaCl, 10% Glycerol

Added fresh

1mM DTT, 1mM PMSF, 1x Protease Inhibitor Cocktail

Hypertonic Buffer:10mM Tris pH 7.9, 1.5mM $MgCl_2$, 420mM NaCl, 10% Glycerol

Added fresh

1mM DTT, 1mM PMSF, 1x Protease Inhibitor Cocktail

4x Gel Shift buffer

40mM Hepes pH 7.9,40% Glycerol, 0.4mM EDTA, 2mM DTT, 12mM $MgCl_2$, 16mM Spermadine

5x TGE

250mM Tris pH 8.5, 1.9M Glycine, 1mM EDTA

10x Klenow buffer(TM)

100mM Tris pH 7.5, 50mM $MgCl_2$, 75mM DTT

2.2.4.2 Buffers and Solutions for Western Blots

1x GTS:192mM Glycine, 25mM Tris-HCl, 0.1% SDS

Western Blot transfer buffer : 192mM Glycine, 25mM Tris-HCl, 15% Methanol

PBS: 30mM NaCl, 2.5mM KCl, 10mM Na_2HPO_4, 30mM NaH_2PO_4, HCl to pH 7.4

PBST:0.1%(v/v)Tween-20 in PBS

Buffers for Protein expression

Whole cell extract lysis buffer: 420mM NaCl, 25% glycerol, 0.5% NP-40, 1.5mM $MgCl_2$, 20mM Hepes(pH7.5) and fresh Protease Cocktail Inhibitors

2.2.5 Bacterial Strains

E.Coli DH5α: host for recombinant plasmids.

Tissue culture cell lines

HEK293T : human embryonic kidney 293 cells stably expressing the SV40 large T-antigen

Cos-7 Cells: Monkey kidney cells, expressing SV40 large T-antigen

2.2.6 Primers and Oligos

2.2.6.1 Oligonucleotide designed for EMSA and Luciferase

NAME	Oligonucleotide(5'-3')
Dbx1(site1)Forward	AATTAGGCCACATTCAATTACTCTTAAGACAATTATATG
Dbx1(site1)Reverse	AATTCATATAATTGTCTTAAGAGTAATTGAATGTGGCCT
Dbx1(site2)Forward	AATTCCATCTAGGCTCCATTCAGCCCCCTAGACACC
Dbx1(site2)Reverse	AATTGGTGTCTAGGGGGCTGAATGGAGCCTAGATGG
Dbx1(site3)Forward	AATTCTGCTAAGAGGCTCATTCAGTGCCCAGGG
Dbx1(site3)Reverse	AATTCCCTGGGCACTGAATGAGCCTCTTAGCAG
Dbx1(site4)Forward	AATTAAAAAAGCAGCCCTGAATGAATGTTAACAG
Dbx1(site4)Reverse	AATTCTGTTAACATTCATTCAGGGCTGCTTTTTT
Dbx1(site5)Forward	AATTTTGTCGGCAAAAGCCTGAATGGTGGTGGAA
Dbx1(site5)Reverse	AATTTTCCACCACCATTCAGGCTTTTGCCGACAA
Dbx1(Negative)Forward	AATTTAACCTTTGGTCTCCACAAGCTTTCTCCCCTTAAC
Dbx1(Negative)Reverse	AATTGTTAAGGGGAGAAAGCTTGTGGAGACCAAAGGTTA
Dbx1(Positive)Forward	AATTTCGAGGAAGAGAAACACCAACAAAGGAGGAGAAAGGCC
Dbx1(Positive)Reverse	GGCCTTTCTCCTCCTTTGTTGGTGTTTCTCTTCCTCGAAATT

2.2.6.2 Oligonucleotide primers used for qPCR

Gene	5'-3'Forwad	5'-3'Reverse
β-actin	CTGCCTGACGGCCAGG	GATTCCATACCCAAGAAGGAAGG
Sox1	GACTTGCAGGCTATGTACAACATC	CCTCTCAGACGGTGGAGTTATATT
Sox2	ACCAGCTCGCAGACCTACAT	TCGGACTTGACCACAGAGC
Sox3	GAACGCATCAGGTGAGAGAAG	GTCGGAGTGGTGCTCAGG
Dbx1	CGTTCGCCTTTCCATACTTC	GAGAAGGTCCCAGGGATAGG
Pax6	GCACATGCAAACACACATGA	ACTTGGACGGGAACTGACAC
Ngn2	AACTCCACGTCCCCATACAG	GAGGCGCATAACGATGCTTCT
Neurod4	CCAGAGACTGTGGTACTGAAAGA	GGATTGTGTGTTGACCAGCTC
Oligo2	GCAGCGAGCACCTCAAATCTA	GGCCCCAGGGATGATCTAAG
Nkx2.9	AACAGACGTGCGTTCCACA	GAGTCTGCAGGGCTTGTCTC
Nkx6.1	AGAGCACGCTTGGCCTATTC	TGCGTGCTTCTTTCTCCACTT
Nkx6.2	CATGACCGAGAGCCAAGTGAA	CGGCATCCGAGTCTTGCTT

2.2.7 Commercially Available kits

SUPPLIER	KIT NAME	CATALOGUE
Qiagen	QIAquick Gel Extraction kit	28706
Qiagen	QIAfilter Plasmid Midi kit	12243
Thermo Scientific	SuperSignal West Pico Stable Peroxide Solution	1856135
Thermo Scientific	SuperSignal West Pico Luminol/Enhancer Solution	1856136
Promega	Dual-Luciferase Reporter Assay System 10-Pack	289339
Applied Biosystems	Fast SYBR Green Master Mix	1205065

2.3 METHODS

2.3.1 Agarose gel electrophoresis (AGE)

AGE was used to measure and separate DNA fragments in different sizes. 1% v/w agarose powder was dissolved in an appropriate volume of 1x TBE, EtBr was added to a final concentration of 1/50,000(v/v) and poured into appropriate gel casting trays. Samples were mixed with 6x DNA loading buffer and run about 100V in 1x TBE running buffer. Electrophoresed gels were observed using a UV transilluminator(UVP BioDoc-It® Imaging System).

2.3.2 Transformation of Bacteria

5µL ligation reactions or 10ng plasmid DNA was mixed with 50µL chemically competent *E.coli* DH5α cells. Cells were incubated on ice for 30 mins and then heat-shocked at 42ºC for 90 secs. After being returned to ice for 5 mins, 250µL SOC medium was added into the mixture and incubated in 37ºC with shaking for 45 mins. Reactions was spread on LB agar plate containing appropriate antibiotics (Amp/Kan) for selection and incubated at 37ºC overnight.

2.3.3 Restriction digestion

This method was used for cloning and checking insertion. The reaction was incubated at least for 60 mins at 37ºC.

COMPONENT	**VOLUME**(µL)
10x buffer	1.5
10x BSA(if required)	1.5
Restriction Enzyme	0.5
DNA plasmid	5
MQH_2O	6.5(if BSA added) or 7(if not)
Total	15

2.3.4 Ligations

The reaction of ligations were performed as below:

COMPONENT	VOLUME(μL)
Insertion of the plasmid	3(1)
Vector	0.5
10x ligation Buffer	1
T4 ligase	0.5
MQH_2O	5(7)
Total	10

Reaction was incubated at 4℃ overnight or at RT for 2 hours. Backbone only ligations were performed as a negative control.

2.3.5 Dephosphorylation

To increase the efficiency of ligation, CIP or SAP were used to remove the 5' phosphate of the digested vector to prevent self-ligation.

COMPONENT	VOLUME(μL)
SAP	1
10x SAP buffer	1.5
Vector	12.5
Total	15

The reaction were incubated for 60 mins at 37℃ followed by 15 mins at 65℃. For the CIP, 1μL CIP was added to 15μL cut vector followed by incubation at RT for 1 hour.

2.3.6 Polymerase chain reaction (PCR)

PCR reaction system:

COMPONENT	VOLUME(μL)
Eppicenter Buffer B	7.5
Forward primers (100ng)	1
Reverse primers (100ng)	1
DNA template(100ng)	1
Pfu	0.5
H_2O	4
Total	15

Reactions were incubated at 95ºC for 2 mins, followed by 40 cycles of 95ºC for 30 secs, 50ºC for 30 secs and 68ºC for 3 mins, followed by final extention at 68ºC for 5 mins.

2.3.7 Gel purification

To extract the DNA fragments in Agarose gel, Qiagen QIAquick Gel Extraction kit was used to purified them according to manufacturers instructions.

2.3.8 Blue-white selection

40µL 100mM IPTG and 40µL x-gal was spread on agar plates prior to plating transformed DH5αbacteria.

2.3.9 Plasmid extraction

Single transformed colonies was picked and cultured overnight at 37ºC in LB medium containing antibiotic(100µg/mL Amp or 50µg/mL Kan) with shaking at 250rpm. 2mLs of culture was grown for plasmid mini-preps and 50 mLs for Midipreps. For plasmid mini-preps, cultures were transferred to 1.5 mL tubes and centrifuged for 2 mins at 14,000rpm. The supernatant were then removed and pellets were resuspended in P1.P2 was added and tubes were inverted 4-6 times followed by incubation at RT for 5 mins. P3 buffer was added and the mixture was inverted 5 times with incubation on ice for 5 mins. Samples were centrifuged for 2 mins at 14,000rpm and the supernatant was transferred to new tubes and precipitated with isopropanol by centrifuging for 15 mins at 14,000rpm. Plasmids were precipitated with 70% ethanol and centrifuged for 2 mins at 14,000 rpm. For large scale plasmid preparation the Qiagene QIAfilter Plasmid Midi kit was used as per manufacturers instructions.

2.3.10 DNA sequencing

COMPONENT	VOLUME(μL)
H_2O	11.5
Template	1
Primer(50ng/μL)	1
4x Better buffer	5
Big Dye V3.1	1.5
Total	20

Samples were thermocycled at 96℃ for 3 mins; 35 cycles of 96℃ for 10 secs,50℃ for 10 secs and 60℃ for 30 secs. Sequencing products were precipitated with 60% isopropanol. Samples were mixed and incubated at RT for 15 mins, followed by centrifugation for 15 mins at 14,000rpm. The pellet was washed with 200μL 75% isopropanol with spinning for another 5 mins. Clean sequencing reactions were analysed by the Australian Genome Research Facility, Adelaide.

2.3.11 293T cells culture, passage and transfection

293T cells were cultured in 37℃, 5% CO_2, 10 mL DMEM (4,500mg/L D-glucose, L-glutamine, 25 mM HEPES buffer) with 10% FCS in 100mm cell culture dish.

Cells were passaged every 2-3 days when the cells reached 90% confluence. Old media was aspirated and 1 mL trypsin was added and incubated at RT for 5 minutes. Trypsin was neutralised with 1 mL of 10% FCS DMEM and cells were triturated to form a single cell suspension. Cells were washed twice and $1/10^{th}$ were replated.

10^5 cells were plated in each well of a 24 well plate or 100mm dish for transfection. Cells were then cultured in the same condition mentioned above overnight. For different plasmids, there were different ratios between Fugene and plasmids(For EMSA, Western blot and Immunofluorescence , Fugene:plasmid=7μL:2μg; for Luciferase assay, Fugene: plasmid=3μL:1μg). Appropriate volume of Fugene was added into plasmids diluted in MQH_2O and made the total volume to 20μL. The mixture was incubated at RT for 15 mins and was then added to the cells. Assays could be performed after 24-48 hours culturing in 37℃, 5% CO_2.

2.3.12 Western Blots

2.3.12.1 Protein concentration quantification

Bio-rad Protein Assay Dye Reagent Concentrate was diluted 1:4 and 200uL of the diluted Bradford solution was added into each diluted samples and BSA standards (0.1 to 0.5mg/mL). The concentration of protein samples was calculated from the line of best fit from the BSA standards using Excel.

2.3.12.2 SDS-PAGE

SDS-PAGE gel was composed of 10% or 12% resolving gel and 5% stacking gel. Protein samples were denatured with 6x loading buffer and incubated at 95ºC for 5 mins before loading. Gels were run at 100V in 1x GTS until the dye reached the bottom of the gel.

2.3.12.3 Transfer, blocking and detection

After running, the proteins were transferred to nitrocellulose membrane in Western blots transfer buffer at 250mA for 1.5 hour. After transfer, the membrane was blocked with 5% skim milk PBST at RT for 1-2 hours or at 4ºC overnight. After twice PBST wash, the membrane was incubated on nutator at 4ºC with primary antibody diluted in 1% skim milk PBST. After triple 5mins PBST wash, the membrane was then incubated at 4ºC with 1% skim milk PBST diluted secondary antibody for 1 hour in RT or at 4ºCovernight. After this, the membrane was washed 20 mins in PBST three times and developed in 500μL SuperSignal West Pico Stable Peroxide Solution and 500μL SuperSignal West Pico Luminol/Enhancer Solution reagent for 5 mins.

2.3.13 EMSA Methods

2.3.13.1 Annealing of probe

Lyophilized stocks of primers was dissolved in sterile 1x TE. Complementary primers was then combined to a final concentration of 100μM with 50mM NaCl and incubated in 95ºC heating block for 5 mins. The heating block was taken out and left

to cool to room temperature on the bench. 1μM annealed probes was made up and stored at -20ºC.

2.3.13.2Klenow labelling of probe

As annealed probes had EcoRI overhangs(5'aatt3') and it can be labelled with α-^{32}P-dATP.added as the following order:

7μL	Sterile MQ H_2O
2μL	10xTM buffer
1μL	Annealed probe at 25ng/μL or approximately 1μM
2μL	2mM dNTP's (dCTP,dGTP,dTTP)
2μL	100mM DTT
4μL	α-^{32}P-dATP(40μCi)
2μL	Klenow

Total 20μL

Fresh Klenow and DTT was added.

The mixture was incubated at 30ºC for 20 mins before 30μL 1x TE were added to the reaction. The whole reaction was transferred to the centre of prespun Amersham Probequant G-50 micro column with spinning at 3000rpm for 2 mins.150μL 1x TE then added into the mixture to make the final volume to 200μL.

2.3.13.3Nuclear and cytosolic extracts for EMSA

293T cells were cultured for 24 hours after transfection in a 6 well tray. Cells were removed from the culture dish, transferred to a 1.5mL tube and pelleted by centrifuging at 4ºC for 5 mins at 1,200 rpm. Cells were lysed in 50μL of hypotonic buffer, leaving the nuclei intact. Samples were incubated on nutator at 4ºC for 30 mins and then snap froze in EtOH/dry ice. Samples were thawed on ice and centrifuged at 14,000 rpm for 30 mins at 4ºC.Supernatant(cytosolic fraction) were kept and nuclei pellets were resuspended in 30μL cold hypertonic buffer with incubating on nutator at 4ºC for 30 mins. Solutions were Centrifuged at 14,000 rpm and the supernatant(nuclear extract) was stored at -80ºC.

2.3.13.4 Gel preparation, running and exposure

The gel was performed in large Owl electrophoresis apparatus.

For the 5.5%/1x TGE gel:

5.5mL	30% Acrylamide:bis-acrlamide
8mL	5x TGE
26.5mL	MQ H_2O
40μL	TEMED
240μL	10% APS

Pre-electrophoresis was performed in 1x TGE at 30 mA for 30 mins. Binding reactions were loaded as well as free probe containing visible loading buffer. Electrophoresis was performed at 35mA until free probe was 1/2 cm from the bottom and plates were separated. Gel stuck to one of the plates and a piece of whatman was used to cover the gel and remove it from plate. The gel was dried by the gel dryer and leaved in a cassette to expose for 2 days and visualised by Typhoon scanner.

2.3.14 Immunofluorescence

Cells were transfected in 35mm dish and grown for 48 hours. Media was removed gently and cells were washed once with PBS. The PBS was aspirated and 1mL PFA was added with incubation at RT for 15 mins folloed by 3 PBS washes.1% TritonX-100 PBS was added for another 15 mins incubation. 10% HS was diluted in 0.25% TritonX-100 PBS for blocking and incubated at RT for 30 mins. Primary antibody(Sox2 antibody 1 in 700 dilution) was diluted in 0.25% TritonX-100 PBS as well and incubated in the fridge overnight. Cells were washed 3 times with PBS and incubated with the secondary antibody (Cy3 anti-rabbit 1 in 500 dilution) diluted in 0.25% TritonX-100 PBS. The solution was incubated at RT for 1 hour and washed with PBS 3 times, covered in DAPI and coverslipped. Fluorescence images were obtained using Zeiss Axioplan 2 at 20x, 63x and 100x magnification. Oil immersion was added when using the 63x and 100x objective lens.

2.3.15 Dual-Luciferase Reporter Assay

10^5 cells per 500μL 293T cells were cultured in 24-well trays in 10% FCS DMEM for the Luciferase Assay overnight. Appropriate amounts of plasmids (expression plasmid, PGL3-promotor vector and pRL-CMV vector) were mixed with suitable ratio of Fugene HD for 15 mins. The mixture was then added equally to each well and cultured in 37℃, 5% CO_2 for 24 hours.

Culture medium was aspirated and 1x PBS was added gently to wash the cells. 100μL 1x passive lysis buffer was added to each well and rotated for 15 mins. Suspended cells were transferred to new eppendorf tubes and were centrifuged at 14,000 rpm for 30 secs. 10μL of the supernatant was taken from each well and added to 96-well plate. GloMax™ 96 Microplate Luminometer and GloMax software were used to do this assay. For measurement, 1 sec delay and 10 sec read time was determined; 75μL of LARII was dispensed to measure luciferase activity and 75μL of Stop & Glo® Reagent was dispensed to measure the *Renilla* luciferase activity.

2.3.16 qPCR

15μL qPCR reactions in 96 wells consisted of 50% Fast SYBR Green, approximately 5 ng template cDNA, 200nM forward and reverse primers concentration and MQ H_2O. Each samples were done in triplicate. Quantitative reaction were proceeded using an Applied Biosystems Step one Plus thermo cycler, with the parameters: Enzyme activation at 95℃ for 20 secs; denature at 95℃ for 3 secs, anneal and extend at 60℃ for 30 secs for 39 cycles. Using the prism 6 to deal with the data after analysis.

Chapter 3.
Results

To analyze the SOX3 TF binding at the *Dbx1* locus *in vitro*, probes containing the putative binding sequences were designed for both EMSA and Dual-Luciferase Reporter Assays. Based on the ChIP-seq data combining the mouse genome sequence (see Figure 1.6), it is obvious there are 5 possible specific binding sites in/near the *Dbx1* gene. According to the consensus binding sequence of *Sox3* in NPCs (see Figure 1.4) and the 5 potential SOX3 binding sites in/near *Dbx1* identified by ChIP-seq (see Figure 1.6), 5 probes containing the potential binding site (Figure 3.1) were designed as well as a positive control and a negative control. The negative control was a scrambled sequence not containing any SOX binding motifs.

3.1 Optimization of the SOX3 TF expression

To perform gel shift analysis of the putative SOX3 binding sites, a source of SOX3 protein is required. Human Embryonic Kidney 293T cells are a common cell type for overexpressing high levels of protein *in vitro*. Three *Sox3* overexpression plasmids previously generated in the Thomas lab were assessed for SOX3 expression levels; p*Sox3*-Myc, p*Sox3*-IRES2-eGFP and p*Sox3*-flag. Each construct was transfected into 293T cells with FugeneHD, at a 3:2 ratio FugeneHD:DNA, pN1-IRES-EGFP was also transfected into a separate poplutaion of cells as a transfection efficiency control. Cells were collected 48 hours post transfection followed by nuclear protein fractionation. Protein lysates were run on 4-12% SDS PAGE followed by Western blot analysis using anti-SOX3 antibody. p*Sox3*-IRES2-EGFP and *Sox3*-myc expression plasmid generated the highest levels of SOX3 expression, with a band at approximately 42kDa (figure 3.2A). Western blot was repeated with only p*Sox3*-IRES2-EGFP and *Sox3*-myc transfected cells and Anti-H3 antibody was used as a loading control (figure 3.2B).

As EMSA requires high transfection efficiency, optimization of transfection was performed with the p*Sox3*-IRES2-EGFP and *Sox3*-myc expression constructs using varying Fugene to DNA ratios (3:2, 4:2, 5:2, 6:2, 7:2 and 8:2). Transfection efficiency was observed by fluorescent microscopy (Nikon TE300) at excitation wavelength of 558nm to assess eGFP levels with only p*Sox3*-IRES2-EGFP transfected cells as *Sox3*-myc won't express GFP. Visualisation of GFP positive cells by flouescent microscope showed ratios 5:2 and 7:2 had the highest GFP expression (figure 3.3A, figure 3.3B); and the Western blot indicated that the 7:2 ratio gave the highest expression of SOX3

Dbx1-1	5′-AATTAGGCCA**CATTCAA**TTACTCTTAAGACAATTATATG-3′ (35bp)
	5′-AATTCATATAATTGTCTTAAGAGTAA**TTGAATG**TGGCCT-3′
Dbx1-2	5′-AATTCCATCTAGGCTC**CATTCAG**CCCCCTAGACACC-3′ (32bp)
	5′-AATTGGTGTCTAGGGGG**CTGAATG**GAGCCTAGATGG-3′
Dbx1-3	5′-AATTCTGCTAAGAGGCT**CATTCAG**TGCCCAGGG-3′ (29bp)
	5′-AATTCCCTGGGCA**CTGAATG**AGCCTCTTAGCAG-3′
Dbx1-4	5′-AATTAAAAAAGCAGCC**CTGAATG**AATGTTAACAG-3′ (30bp)
	5′-AATTCTGTTAACATT**CATTCAG**GCTGCTTTTTT-3′
Dbx1-5	5′-AATTTTGTCGGCAAAAGC**CTGAATG**GTGGTGGAA-3′ (30bp)
	5′-AATTTTCCACCAC**CATTCAG**GCTTTTGCCGACAA-3′

Figure 3.1: 5 pairs of probes containing the five potential SOX3 preference binding motif highlighted in red, each have EcoR I overhangs(5’aatt3’).

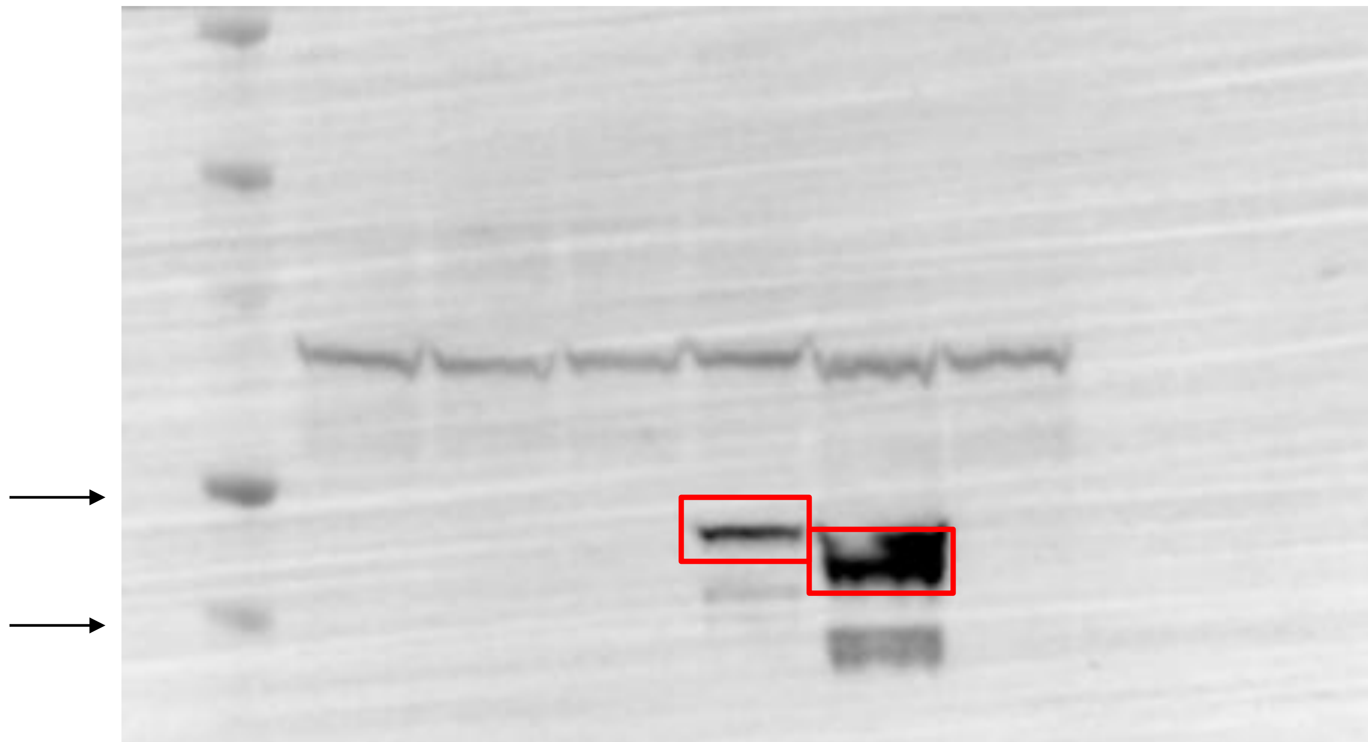


Figure 3.2A: Western blot of the SOX3 protein in 293T cells transfected by different Sox3 expression plasmids. Lane 1:Protein marker; lane 2 to 3 were nontransfected; lane4 to7 were cells transfected by N1-IRES-EGFP, Sox3-Myc, Sox3-IRES-eGFP and Sox3-flag, respectively.Sox3-Myc and Sox3-IRES-eGFP showed expression in 293T cells. Upper arrowhead is 50 kD; lower arrowhead is 37 kD. This is an anti-SOX3 antibody.

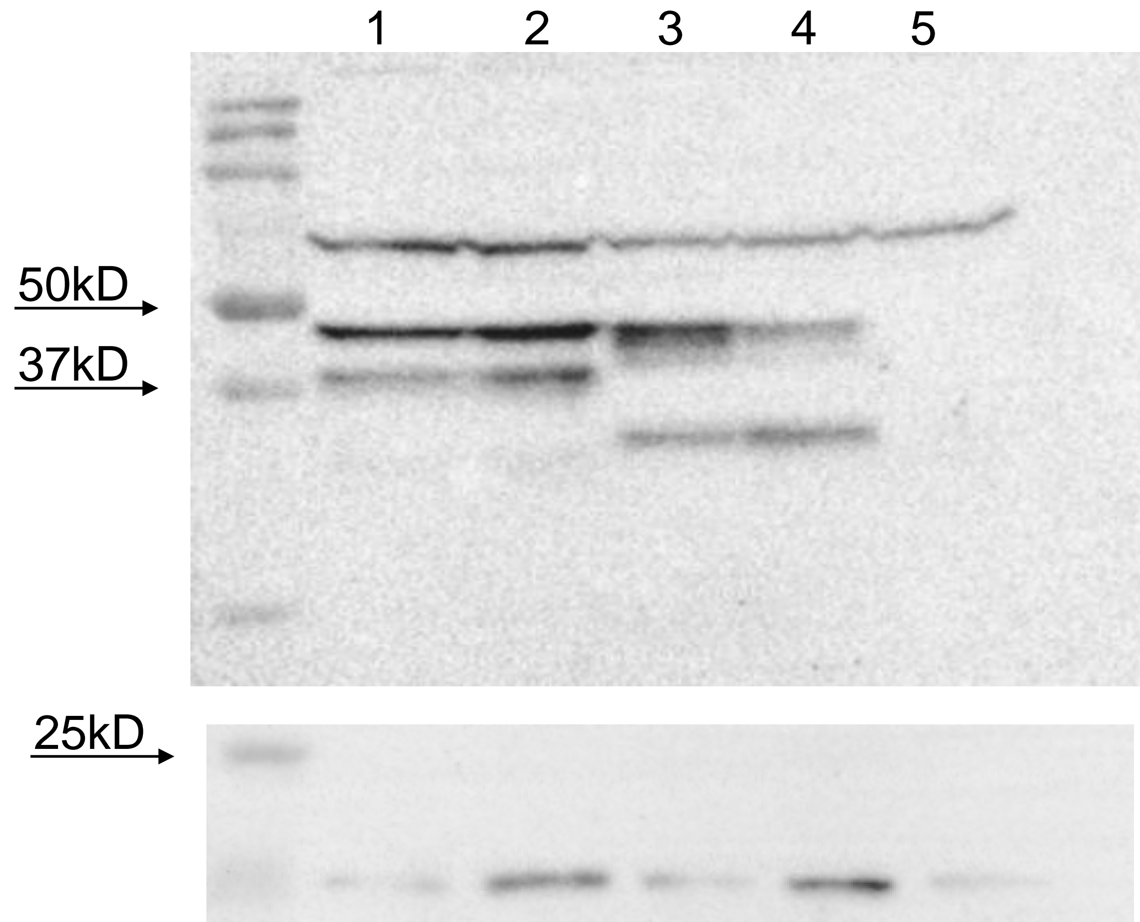
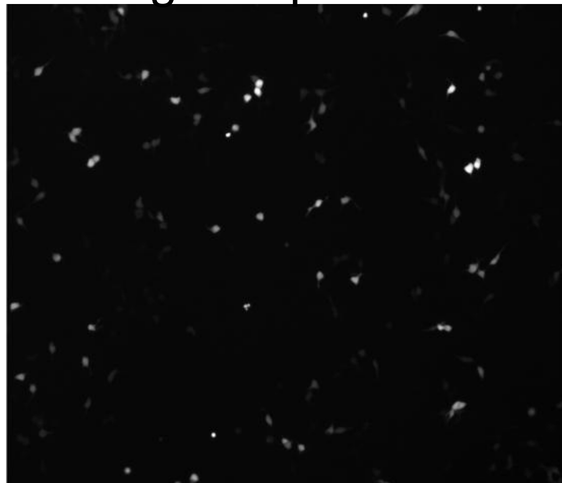
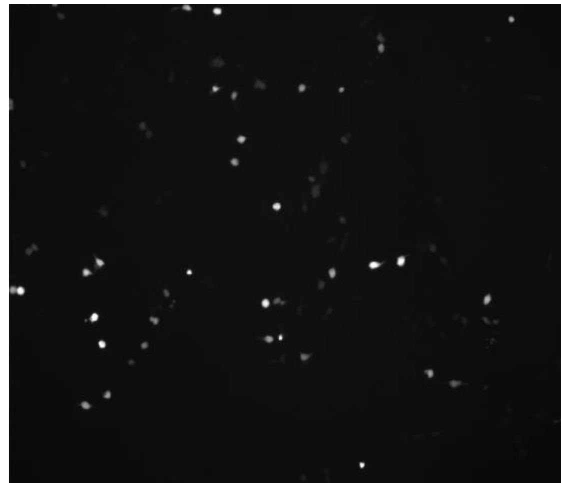


Figure 3.2B: 1 and 2 were pSox3-Myc transfected cell nuclear extract; 3 and 4 were pSox3-IRES2-eGFP transfected cell nucear extract; 5 was untansfected cell nuclear extract. Sox3-Myc and Sox3-IRES2-eGFP showed SOX3 expression in 293T cells and size was approximately 42 kDa. H3 is 15kDa.

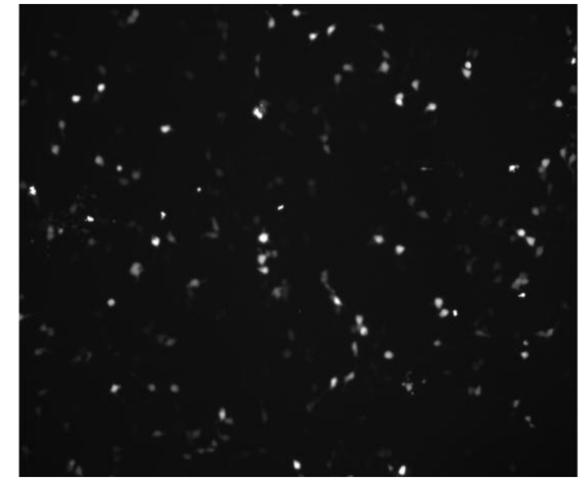
Fugene :plasmid=3:2



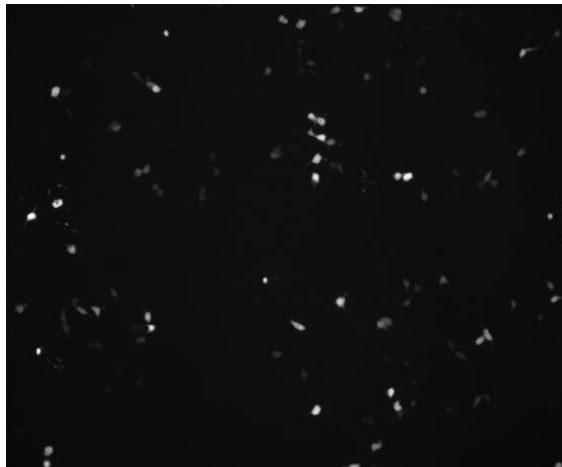
4:2



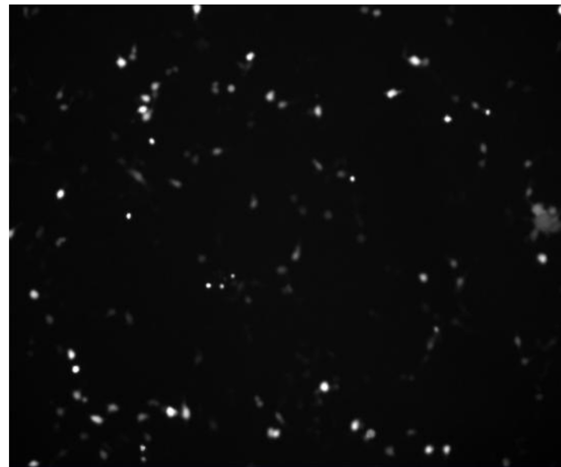
5:2



6:2



7:2



8:2

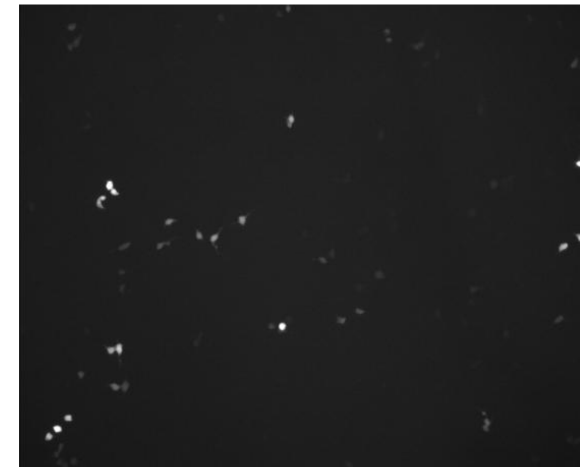
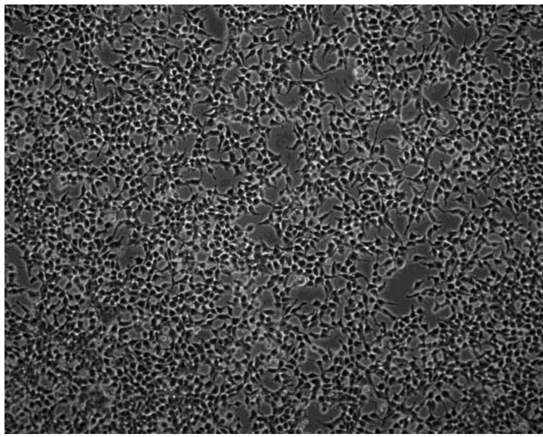
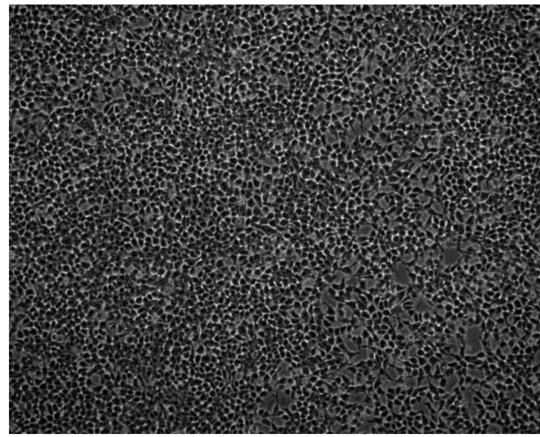


Figure 3.3A: Different ratio of Fugene to plasmid Sox3-IRES-eGFP in transfection. It was photographed by the UV microscope with eGFP filter.5:2 and 7:2 showed better eGFP expression.

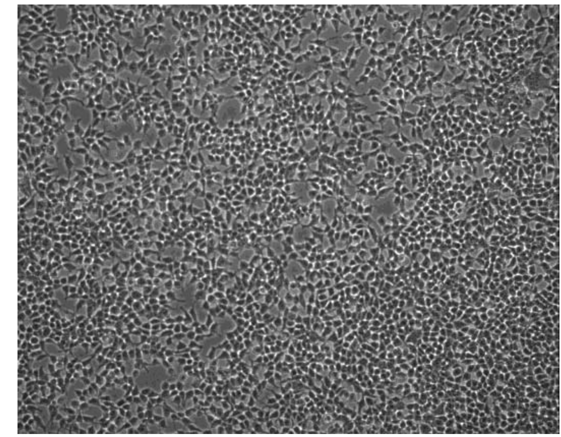
Fugene :plasmid=3:2



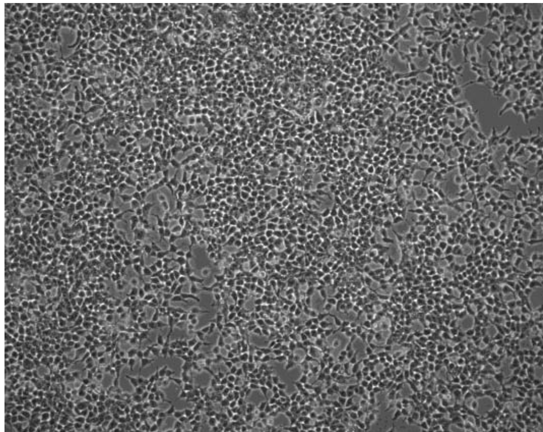
4:2



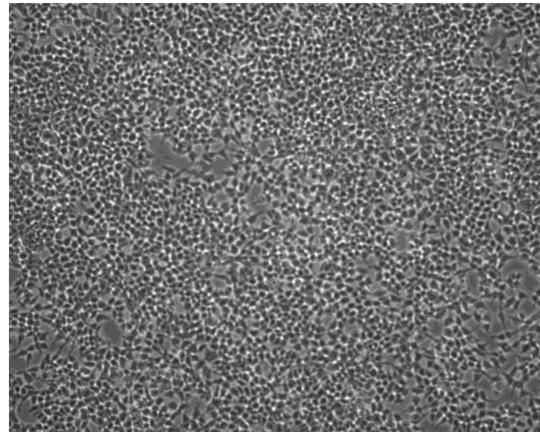
5:2



6:2



7:2



8:2

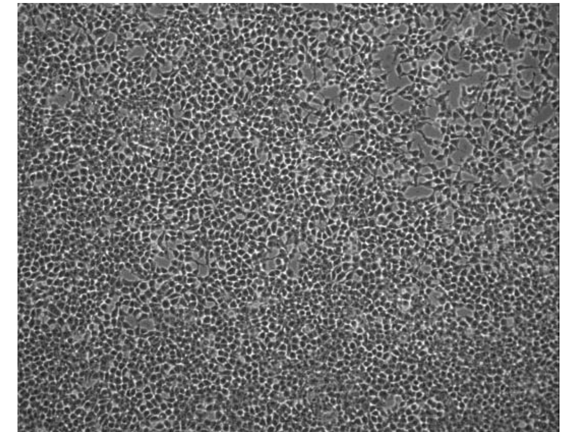


Figure 3.3B: phase contrast images of different ratio of Fugene to plasmid Sox3-IRES-eGFP in transfection. Total number of cells in each dish is about the same.

(figure 3.4). The ratio of 7:2 with plasmid *Sox3*-pIRES2-EGFP was selected for the EMSA.

3.2 Assessing the DNA binding properties of SOX3 at putative *Dbx1* binding sites by EMSA

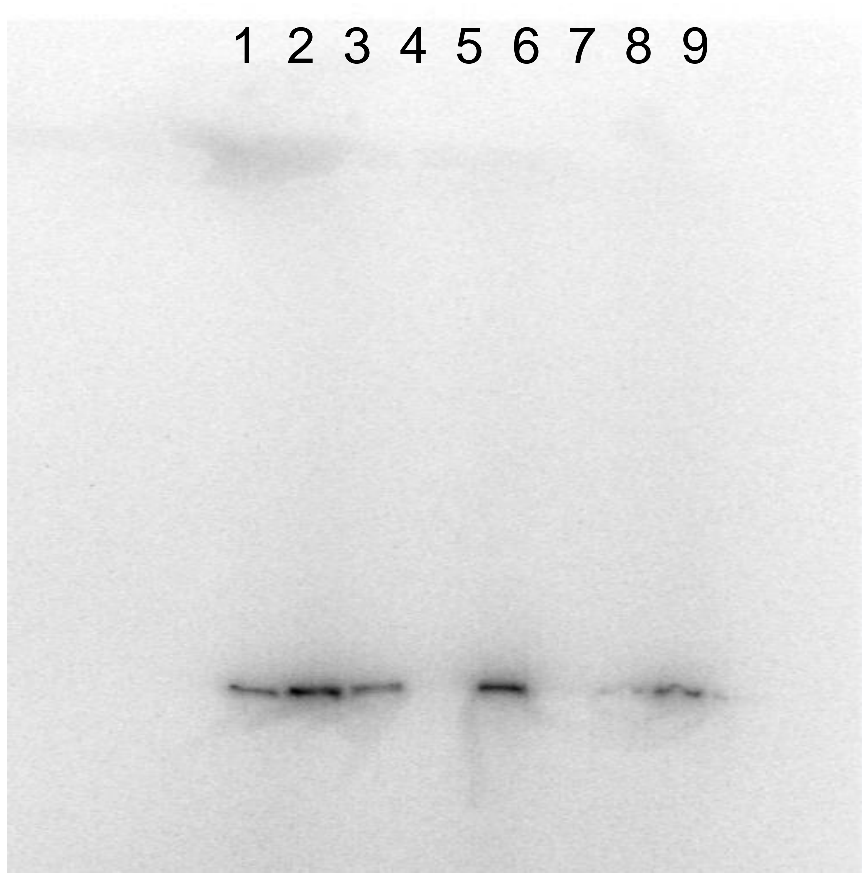
The Electrophoretic Mobility Shift Assay (EMSA), also known as the gel shift assay, is commonly used to measure the specific DNA binding activity of a protein of interest that has been purified or generated in cultured cells through transfection of an expression construct (Hellman and Fried 2007). If a protein:DNA interaction occurs, the radiolabelled (^{32}P) probe containing the putative binding site will migrate more slowly than unbound probe. Accordingly, this sensitive and simple method was used to assess SOX3 binding to the *Dbx1* sequences.

Initial EMSA experiments tested three probes, Dbx1-1, Dbx1-neg and Dbx1-pos, for interactions with SOX3, using 12ug of nuclear extract which came from *Sox3*-transfected and non transfected cells. No bands were observed on the gel after 48 hours exposure, nor was any free probe observed.

Then, to optimize the amount of nuclear extract, as the protocol (Hapgood, Cuthill et al. 1989; 2012) indicated that 8-16µg of nuclear extract was needed in each reaction, 12µg *Sox3*-transfected nuclear extract and double amount (24µg) as well as 12µg untransfected 293T cell nuclear extract were used in conjunction with the Dbx1-1, Dbx-neg and non probes. These samples generated a very faint band without any different between the Dbx1-1 and Dbx-neg (figure 3.5A).

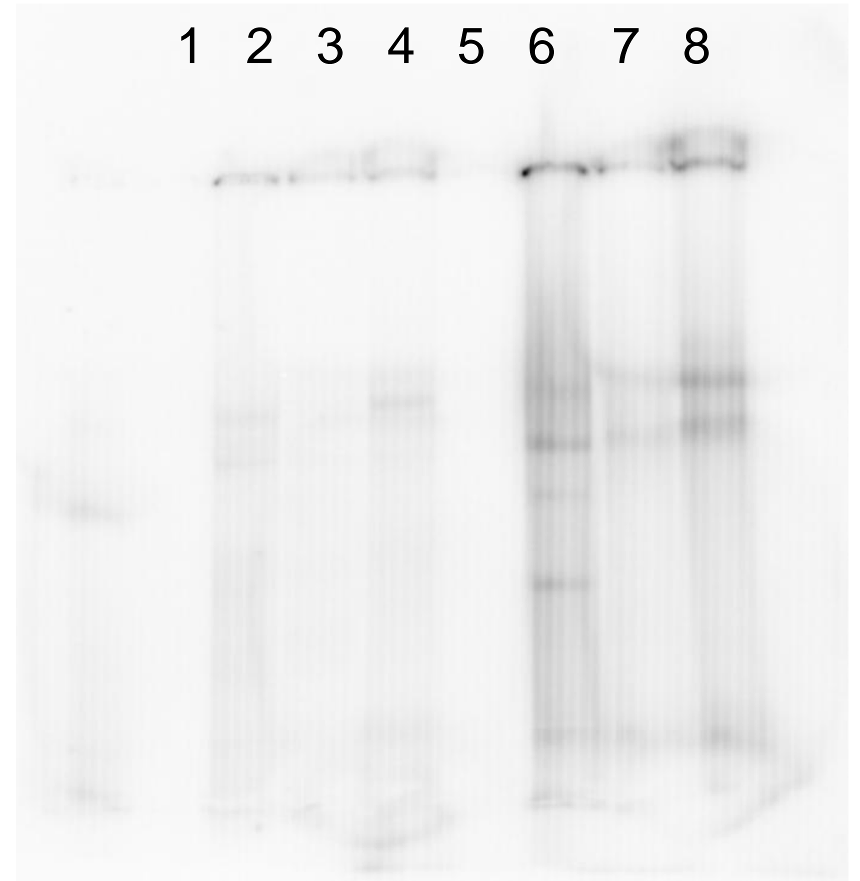
Subsequently, to confirm whether this assay can work, a positive control containing the SOX motif (5-agctcaaacaaata*aacaat*taactc-3)_previously shown to bind SOX3 (Woods et al, 2005) was used, while keeping the lysate constant at 24µg. *Sox3* transfected samples displayed darker bands than the untransfected but still without significant difference between both Dbx-pos and Dbx-neg probes (figure 3.5B). As it was not possible to obtain consistent results with the gel shift assay, an alternative approach (the Dual luciferase reporter assay) was used (see Section 2.5).

A



	1	2	3	4	5	6	7	8	9
Neg	-	+	-	-	+	-	-	+	-
Dbx1-1	+	-	-	+	-	-	+	-	-
	2×Sox3			Sox3			control		

B



	1	2	3	4	5	6	7	8
Neg	-	+	-	-	-	+	-	-
Dbx1-1	-	-	+	-	-	-	+	-
Pos	-	-	-	+	-	-	-	+
	Control				Sox3			

Figure 3.5 Dbx1-1 did not display a binding specificity distinct from Dbx-neg and Dbx1-pos. (A)Sox3 gel shift assay. Low molecular weight nonspecific bands were observed in most lanes, including the untransfected lysate(lanes 7-9) (B) no difference between Dbx-pos (lane 8) and Dbx-neg (lane 6).

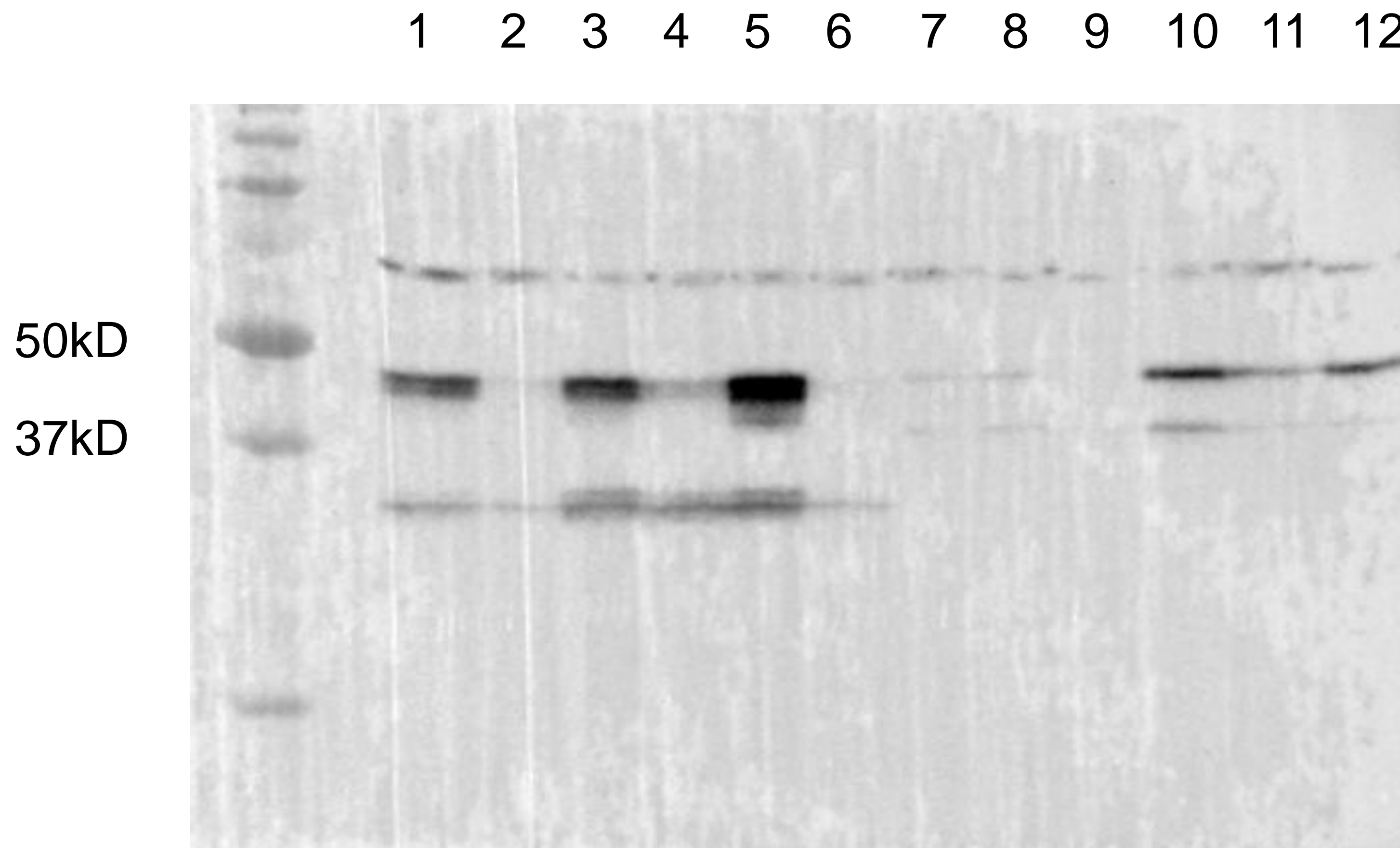


Figure 3.4: 293T transfection optimisation. Western blot of cell lysates transfected with varying Fugene:DNA ratios of 3:2, 4:2, 5:2, 6:2, 7:2, 8:2 for both Sox3-IRES-EGFP (lanes1-6) or Sox3-myc (lanes 7-12), respectively. Lane 5 (Fugne : Sox3-IRES-eGFP=7:2) expressed the highest SOX3 protein in 293T cells.

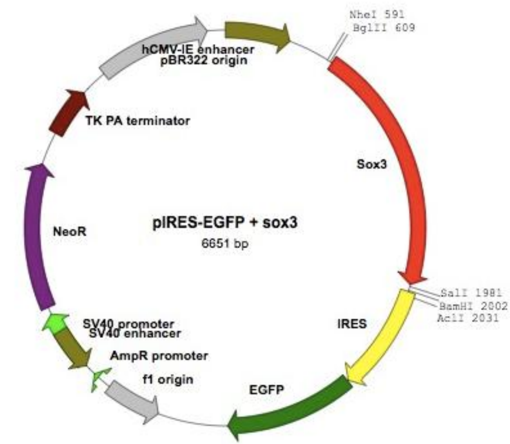
3.3 Construction and expression of *Sox2*-pIRES2-EGFP

To address the ability of SOX2 to bind the *Dbx1* regulatory regions we required a SOX2 expression vector. To generate the *Sox2* expression vector multiple methods were used. In an attempt to subclone the *Sox2* ORF from pcDNA3.1-myc-*Sox2*, the ORF was removed from this Sox2 plasmid (already available in the Thomas laboratory) with XhoI and SalI and gel purified. The purified product was then ligated into pIRES-EGFP, linearized with XhoI and SalI and transformed into DH5a cells. However, no colonies were observed.

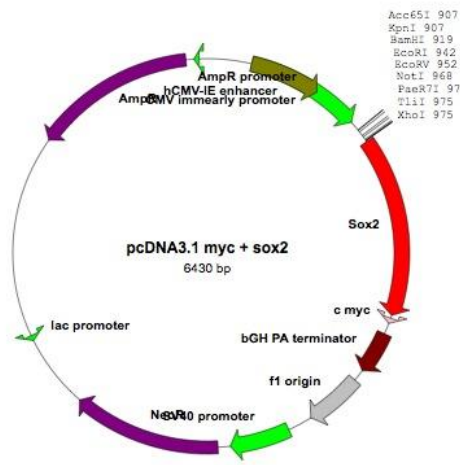
Next, a *Sox2* ORF PCR product was produced from pCl-neo-*Sox2* with Pfu DNA polymerase and ligated into EcoRI-digested pBluescript vector. Through Blue-white selection, the recombinant vector was generated. Sequencing the vectors highlighted several mutations within each vector. The PCR was repeated several times with either Taq polymerase, giving no PCR product or Pfu polymerse, however all clones contained error according to the sequencing.

Finally, pcDNA3.1 myc-*Sox2* was digested with AgeIand XhoI. pIRES2-EGFP was linearised with XhoI and XmaI (AgeI(A/CCGGT) and XmaI(C/CCGGG) would produce the compatible sticky ends) (see figure3.6). The recombinant vector was digested and checked by XhoIand PstI(see figure 3.7) and validated by Sanger sequencing.

To confirm the expression of SOX2 from the *Sox2*-pIRES2-EGFP expression construct, it was transfected in 293T cells as described above for the *Sox3* expression experiments. Protein lysates from the transfected cells were run on a SDS-PAGE, transferred to nitrocellulose membrane and analyzed by western blot with anti-SOX2 antibody (figure 3.8A) and repeated western blot with anti-beta-tubulin-antibody as a loading control (figure 3.9B). A band was observed at ~35kDa (the expected size) in the transfected lane and not in the untransfected control (figure 3.8). In addition to the western blot, immunofluorescence was performed using an anti-*Sox2* antibody to test the expression and localisation in cells. SOX2 expression was observed in the nucleus of *Sox2*-pIRES2-EGFP transfected cells, while no expression was observed in pIRES2-EGFP transfected cells (see figure 3.9).



Digested with the Xho1 and Age1



Digested with the Xho1 and Xma1

Ligation

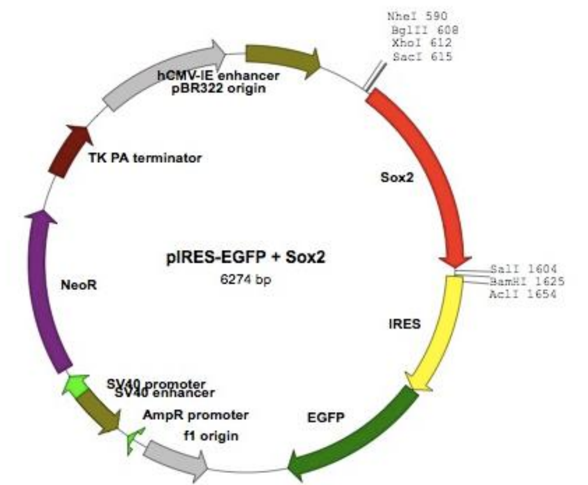


Figure 3.6: Schematic diagram of the construction of the Sox2-IRES-eGFP vector

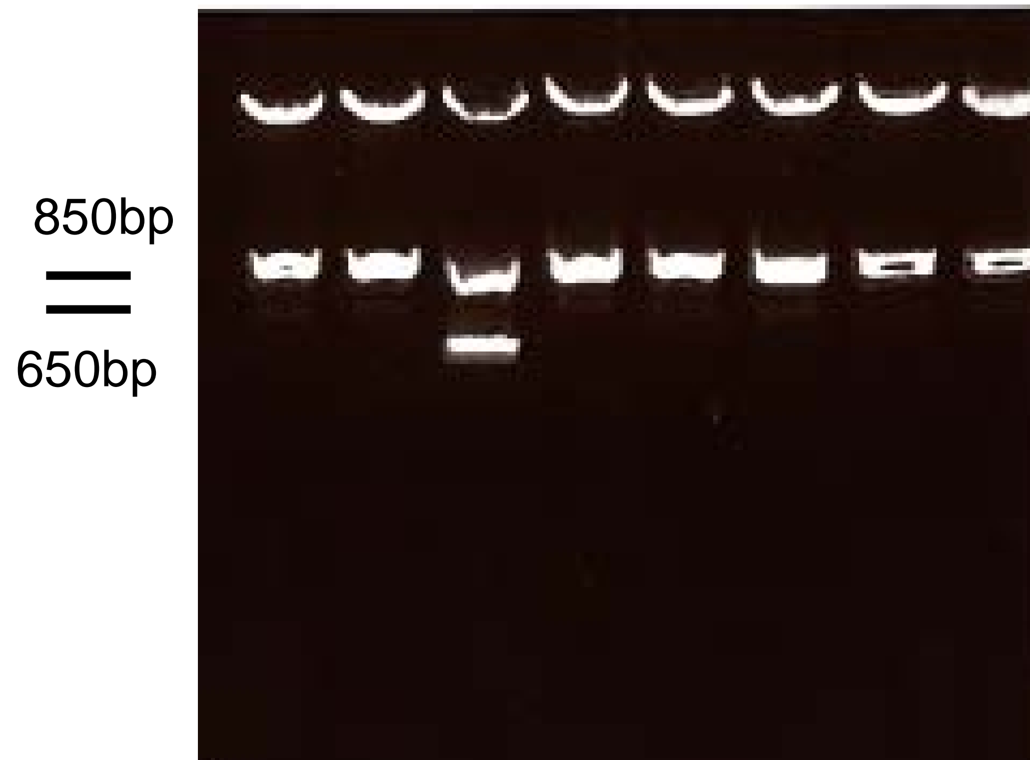
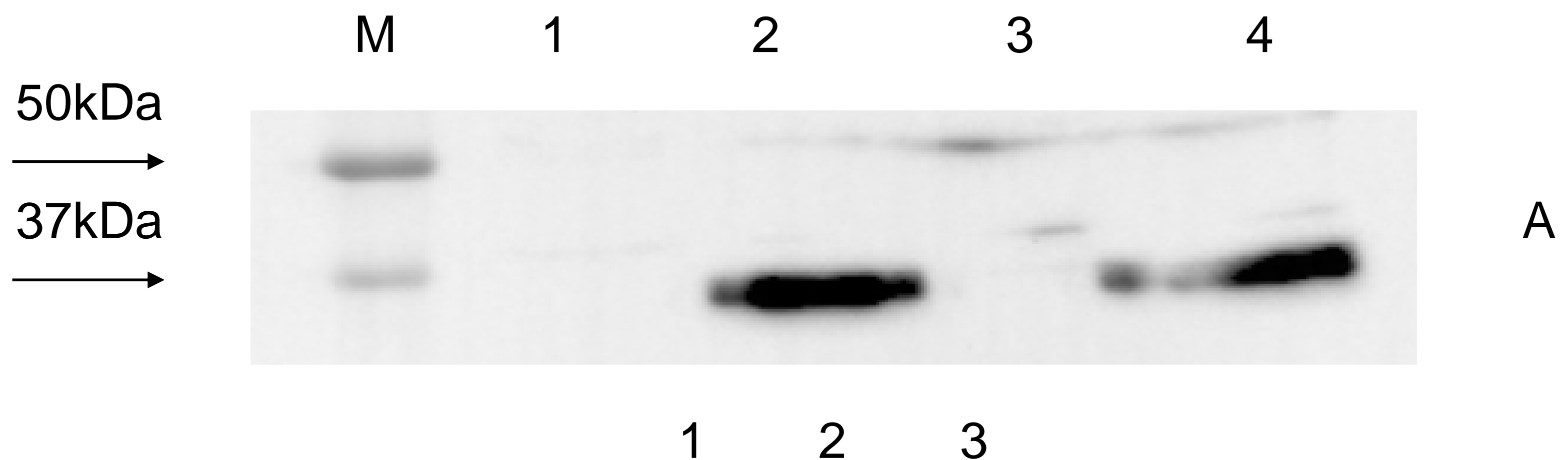


Figure3.7 Check digest of the Sox2-IRES-eGFP vector by Xho1 and Pst1. Three bands were expected of sizes: 793bp, 897bp, 5.4kb respectively.Lane 1-2, 4-8 showed correct banding pattern. Lane 3 did not have correct bands.



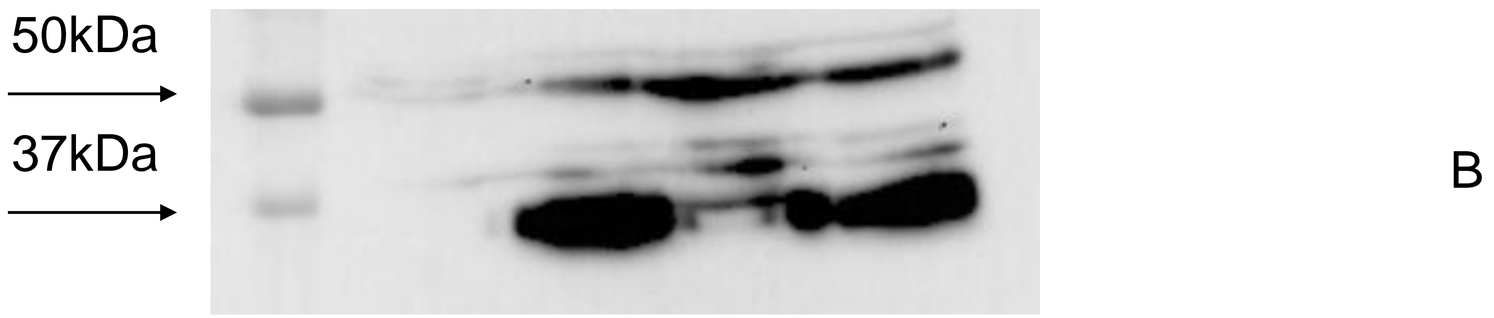


Figure3.8 A). Lane 1and 3 were untransfected 293T cells lysates as negative control; lane 2 and 4 were SOX2 transfected samples. B). 1 and 3 showed nuclear extract from *Sox2*-pIRES2-EGFP transfected cells, 2 was untransfected nuclear extract. The size of SOX2 TF was approximately 35kD and beta-tublin was 50kD as loading control.

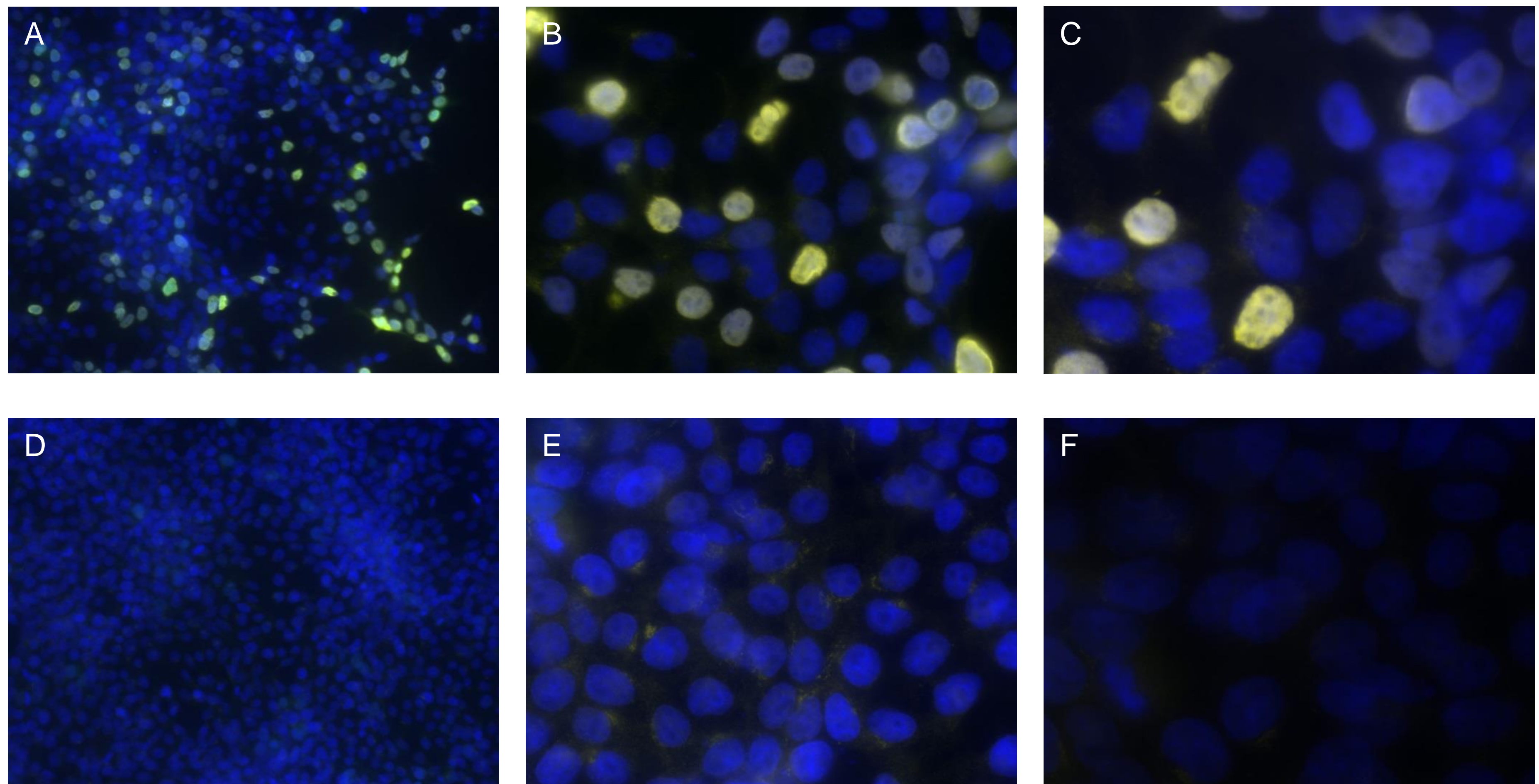


Figure 3.9 Sox2-pIRES2-EGFP expression in 293Ts 48h post transfection. Overexpression of SOX2 in 293T cells detected by SOX2 specific antibody. A: 20x, B: 63x, C: 100x magnification. D, E, F show transfection with empty vector. B,C show nuclear localisation of SOX2 in transfected cells

3.4 Dual-Luciferase Reporter Assay

Dual-Luciferase Reporter Assay is another common technique to study the transcriptional activity of putative regulatory regions in cells. The putative regulatory region is cloned before a luciferase reporter. When the transcription factor binds at this region, it can activate the expression of the reporter. Upon addition of appropriate substrate, the signal is amplified providing a highly sensitive method for assessing transcriptional activity. This assay was used as an alternative method to study the binding activity of SOX3 at specific sequences in/near *Dbx1*.

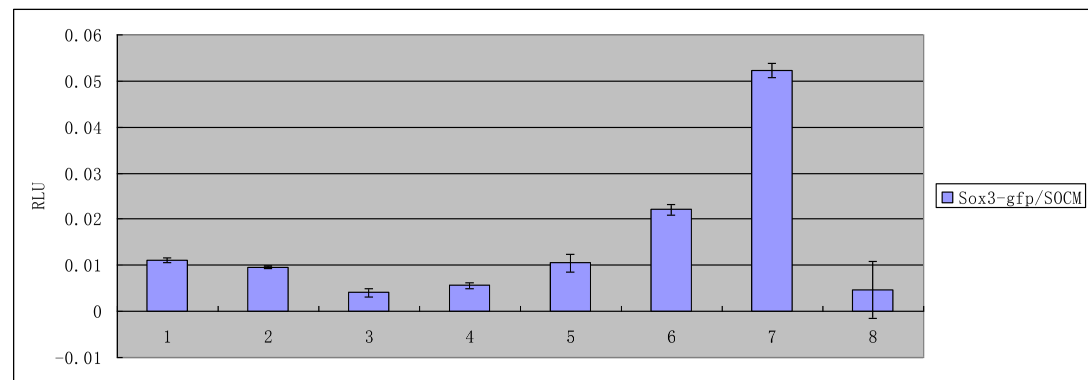
Initially, expression plasmids were transfected into 293T cells with the following Fugene:DNA ratios, *Sox3*-pIRES2-EGFP /pIRES2-EGFP: pGL3-SOCM/pGL3-empty: pRL-CMV=110ng: 80ng: 10ng with 0.6μL Fugene HD. Unfortunately, there was no significant difference between either the negative control or the (data not shown).

To reduce the background level as much as possible and to optimize the amount of signal generated from the assay, the ratio of either Fugene:DNA or Reporter:Sox3 plasmids was altered (see Figure 3.10A). Increasing the Fugene:DNA ratio did not change the transcriptional reporter levels. Adjusting the ratio of SOX3:reporter did vary the transcriptional output. From the data (see Figure3.10B), the ratio of *Sox3*-pIRES2-EGFP:pGL3-SOCM:pRL-CMV=100ng: 280ng: 10ng with 1.8μL Fugene showed the best activity.

To demonstrate that SOX3 overexpression was responsible for the observed reporter activity, the amount of SOX3 expression plasmid was increased relative to a constant amount of reporter plasmid (figure 3.10C). By increasing the amount of SOX3 expression plasmid from 0ng to 1000ng, we observed an increase in reporter activity relative to the amount of expression plasmid (figure 3.10D), pcDNA3.1 was added to maintain total DNA amounts. *Sox3*-pIRES2-EGFP:pGL3-SOCM:pRL-CMV=1000/3ng: 280ng: 10ng with 1.2μL Fugene showed the highest RLU activity, and was used for the remaining reporter assays.

	Sox3-gfp(ng)	SOCM(ng)	Fugene(uL)
1	110	80	0.5
2	110	80	0.7
3	110	80	1.4
4	140	50	0.6
5	50	140	0.6
6	280	100	1.2
7	100	280	1.2
8	0	0	0

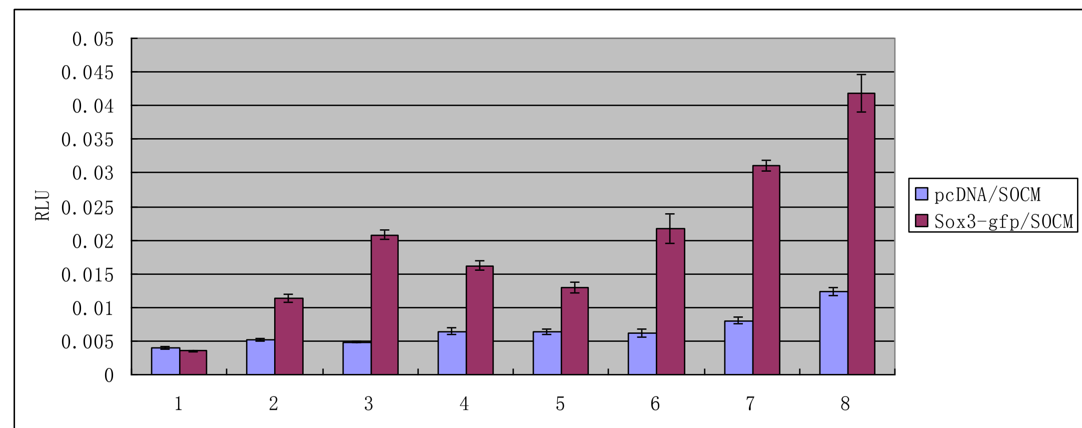
A



B

	PGL3-SOCM	IRES-EGFP(ng)	pcDNA/Sox3-gfp(ng)
1	140	1000/3	0
2	140	900/3	100/3
3	140	500/3	500/3
4	140	0	1000/3
5	280	1000/3	0
6	280	900/3	100/3
7	280	500/3	500/3
8	280	0	1000/3

C



D

Figure 3.10 Optimization of the Dual-Luciferase Reporter Assay. (A) Amounts of DNA added in each well in triplicate; (B) no difference was observed for transfections 1-5. The optimal plasmid:Fugene ratio was determined to be #7 ; (C) the amount of plasmid added in each well in triplicate; (D) Dual-Luciferase Reporter Assays results of (C), (blue) pcDNA, (red) Sox3-pIRES2-EGFP. 5-8 demonstrated dose dependent response. The optimal plasmid:Fugene ratio was determined to be #8. RLU: relative luciferase units. Error bars represent standard deviation.

To begin to assess Sox3 binding to the *Dbx1* sites, *Dbx1*-1-pGL3 and *Dbx1*-4-pGL3 reporter vectors were generated through two steps: ligating dsOligos into pBluescript and subcloning them into the pGL3-basic vectors. As the annealed *Dbx1* probes contained EcoRI overhangs they were ligated into EcoRIdigested pBluescript. Successful transformants were selected by the means of white-blue colour selection, followed by restriction enzyme digestion (figure 3.10E), and sequencing data. *Dbx1*-1-pGL3, *Dbx1*-4-pGL3 were successfully constructed.

Finally, using the optimized luciferase assay (figure 3.10C and D), the transcriptional activity of SOX2 and SOX3 was tested separately for activity on the *Dbx1*-1-pGL3 and *Dbx1*-4-pGL3 reporter vectors. No significant difference was observed between the positive control and negative control for both *Sox2* and *Sox3* expression plasmids. The *Dbx1*-4-pGL3 transfected cell lysate were lower than the negative control, however the *Dbx1*-1-pGL3 reporter showed statistically higher activity compared with either the empty reporter plasmid or the SOCM reporter for both SOX2 and SOX3 (figure 3.11).

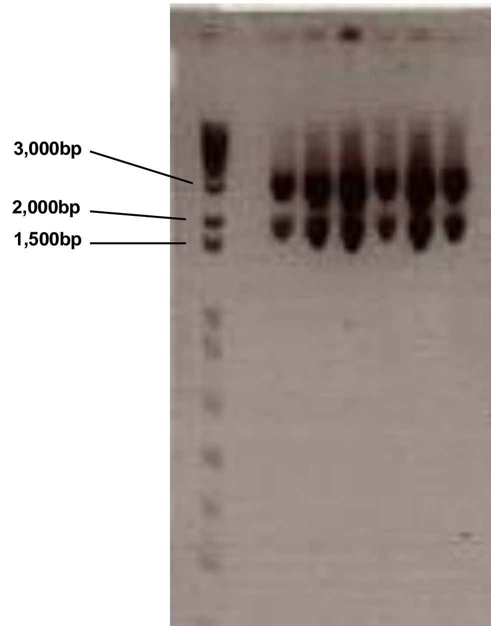
3.5 real-time PCR analysis of *Sox3*-null mouse at E9.5

Real-time PCR is a technique to infer the relative amount of a gene transcript in a sample. qRT-PCR provides relative differences in gene expression compared to a known constant within each sample, a reference gene, in this case *ActB*. Considering *Dbx1* expression is reduced in the developing spinal cord (N.Rogers, unpublished data), we decided to check the expresson levels of other spinal cord development marker genes in Sox3 null embryos (Pierani et al. 2001, and Oosterveen et al.2013).

For the purpose of comparing ventral neural tube marker genes' expression between WT and *Sox3* KO samples, qPCR was performed using cDNA samples produced from RNA extracted from whole embryos of 4 WT and 3 KO E9.5 mouse embryos. Expression of *Sox3*, *Sox1*, *Sox2*, *Dbx1*, *Pax6*, *Ngn2*, *Neurod4*, *Olig2*, *Nkx2.9*, *Nkx6.1* and *Nkx6.2* was measured by qPCR. *Sox3*, *Dbx1*, *Pax6*, *Ngn2* and *Olig2* all showed significant decrease in *Sox3* null mice compared to WT. Conversely, *Sox1*, *Sox2*, *Nkx2.9*, *Nkx6.1* and *Nkx6.2* revealed no changes. *Neurod4* was also decreased in the KO, however this was not statistically significant (p=0.0674) (see figure 3.12 and

3.13). Together, these data show for the first time that, in addition to *Dbx1*, several genes that have important roles in neural development are abnormally expressed in Sox3 KO embryos.

A



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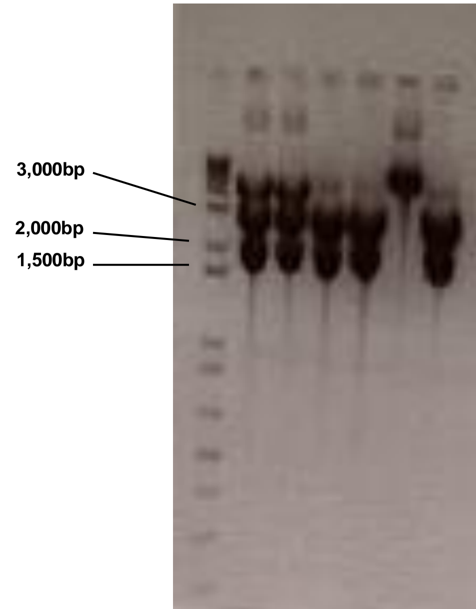

figure 3.10E: digestion and checking Dbx1-1-PGL3(A) and Dbx1-4-PGL3.
(A) digested by Afl2 and XbaI, and expected to be digested to~1900bp and ~3100bp; (B) digested by XbaI and expected to be ~2000bp and ~3000bp

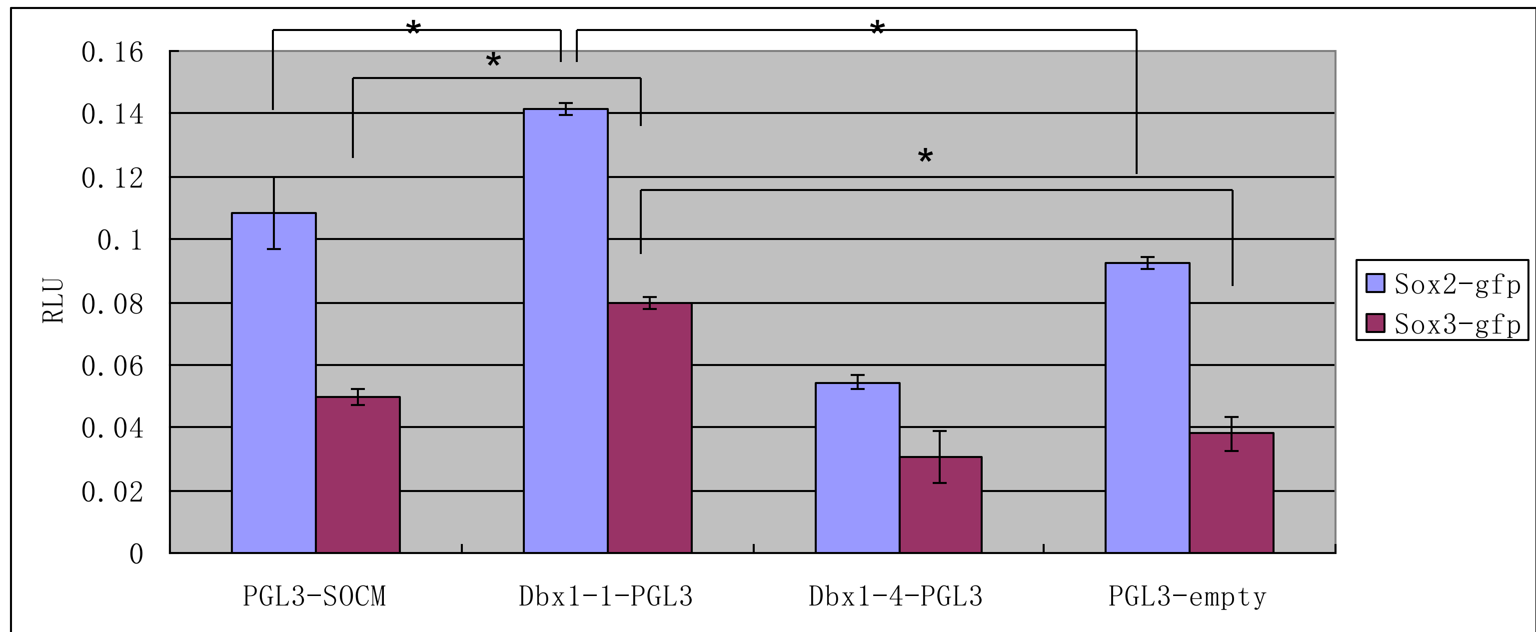

Figure3.11 Luciferase reporter assays comparing SOX2/3 activity on Dbx1-1/Dbx1-4 reporter constructs. The amounts of these two plasmids came from the previous optimization. Positive control (PGL3-SOCM) and Negtive(PGL3-empty) did no difference; Dbx1-4-PGL3 demonstrated lower RLU than PGL3-emty with both Sox2 and Sox3 expression vectors; however, Dbx1-1 showed higher activity than Dbx1-4. RLU: relative luciferase units. Error bars represent standard deviation.

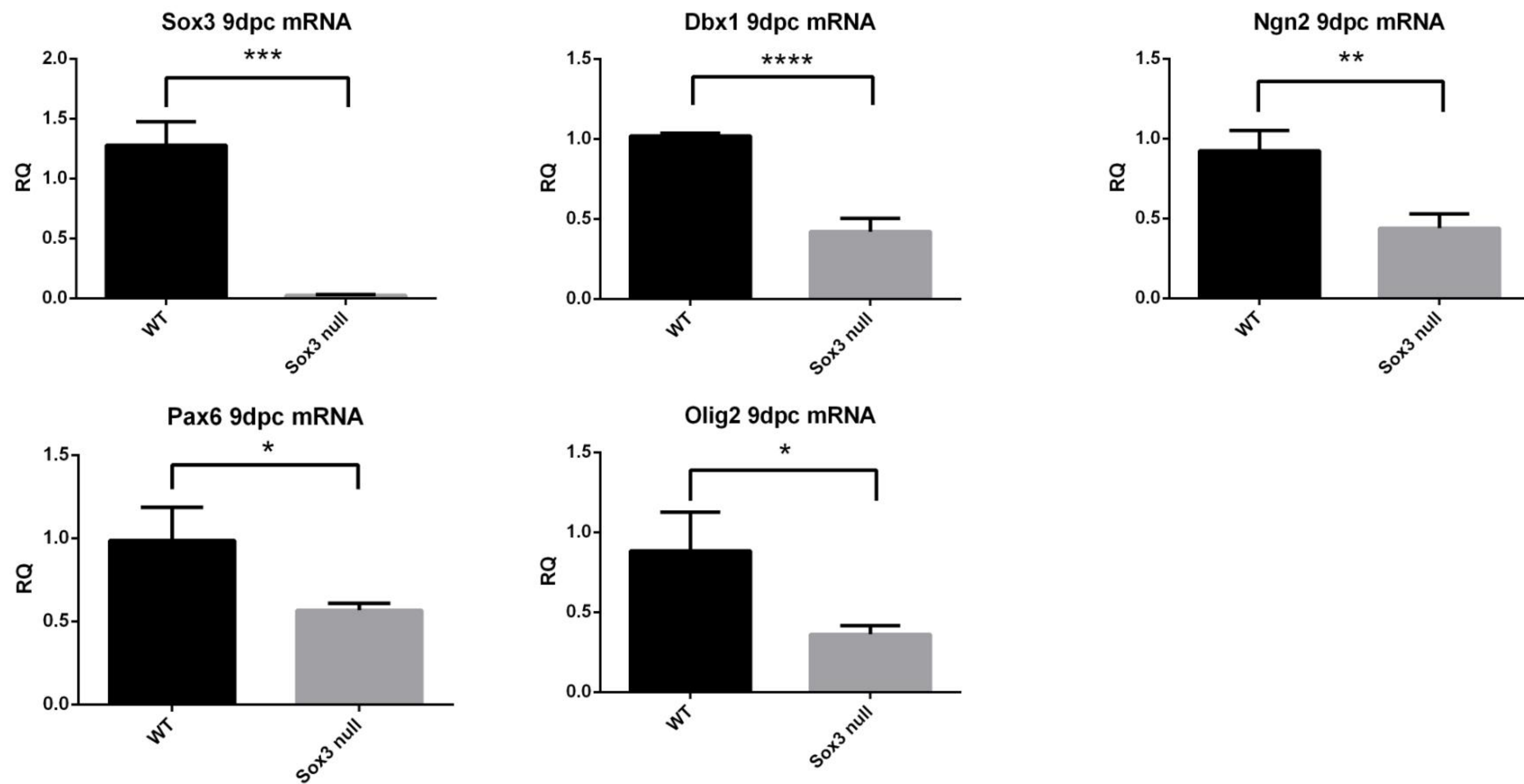

Figure3.12 Comparative mRNA expression of *Sox3*, *Dbx1*, *Ngn2*, *Pax6* and *Olig2* relative to β-actin. A significant decrease in gene expression is observed by qPCR between WT and *Sox3* null embryos for all genes. n≥2, error bars represent standard deviation. *: P≤0.05, **: P ≤ 0.01, ***: P ≤ 0.001, ****: P ≤ 0.0001.

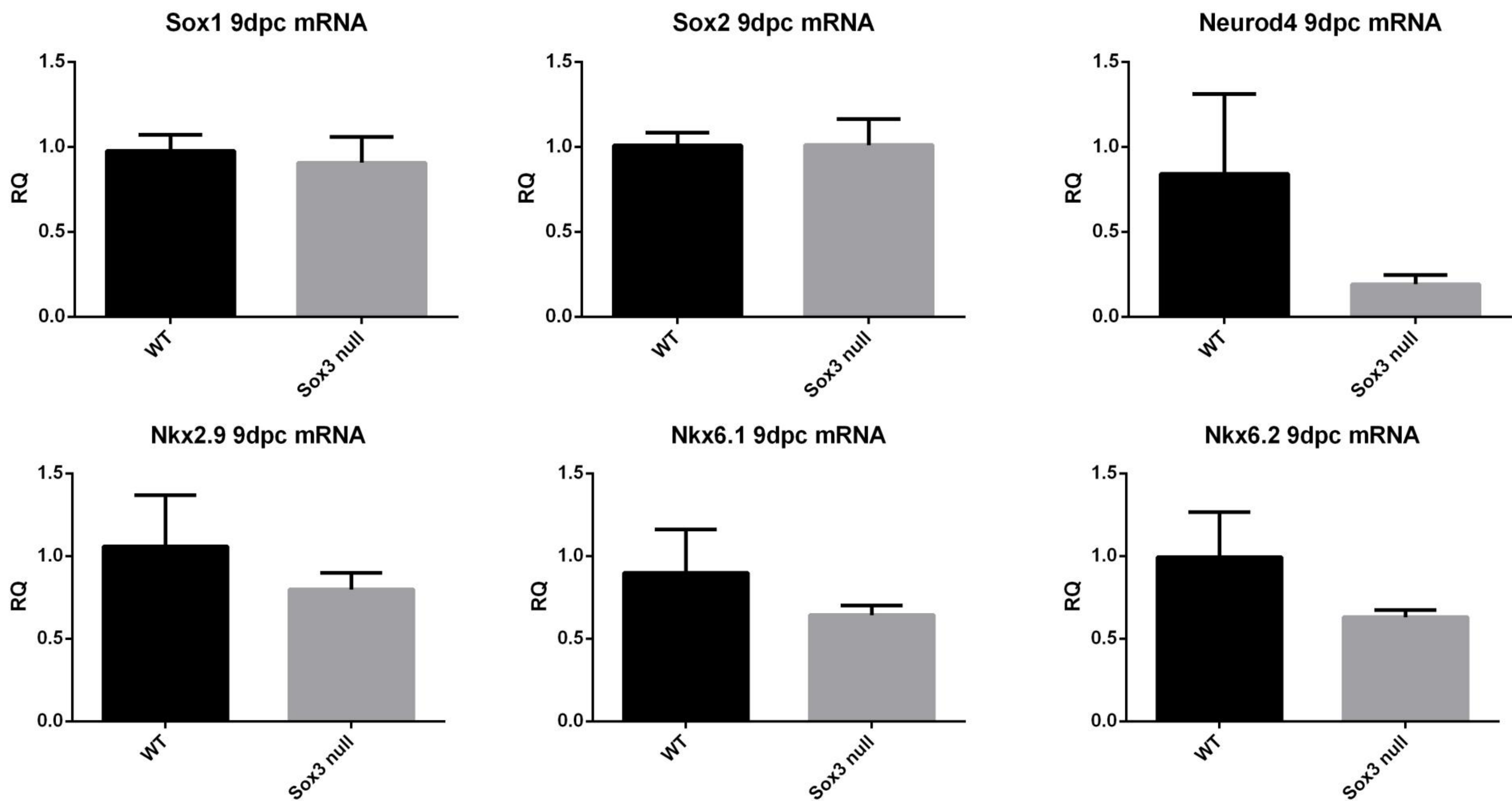

Figure 3.13 Gene expression of *Sox1*, *Sox2*, *Neurod4*, *Nkx2.9*, *Nkx6.1* and *Nkx6.2* relative to β-actin. The results show no significant change in gene expression between WT and *Sox3* null embryos. P value's for *Neurod4* and *Nkx6.2* were 0.0674 and 0.0765 respectively, both of which were not quite significant. n≥2, error bars represent standard deviation.

Chapter 4.
Discussion

Current data suggests Dbx1 may be directly regulated by SOX3; microarray and qRT-PCR evidence shows a 2-2.5 fold down regulation of Dbx1 in NPCs and embryos that lack Sox3 (N Rogers, unpublished data), and recently published ChIP-seq data indicates 5 SOX3 binding sites in or near the Dbx1 locus (Bergsland, Ramskold et al. 2011). The overall aim of this project was to confirm the regulation of Dbx1 by SOX3 in vitro.

Initially, EMSA was selected as a method to assess SOX3 binding to Dbx1 sites. This method can be performed using lysates derived from cells transfected with an expression construct encoding the transcription factor of interest. Therefore, SOX3 expression plasmids were assessed for their level of expression by western blot analysis. It was identified that transfecting Sox3-pIRES2-EGFP with Fugene at a 7:2 ratio into 293T cells provided the best transfection efficiency by both Western blot analysis for SOX3 (Figure 3.4) and EGFP positive cell count (Figure 3.3). EMSA requires high levels of SOX3 expression, therefore optimizing transfection efficiency should aid in producing successful EMSA experiment.

The EMSA did not show any evidence of any bands (Figure 3.5A) and unlike published EMSA data, very little free probe was observed at the bottom of the gel (Woods, Cundall et al. 2005), even under conditions where no protein was added to the reaction (ie. the “probe-only” control). Therefore, it is possible that the Klenow radiolabelling reaction was not efficient, possibly due to the age of the enzyme stock used for these studies. The lack of a positive control probe also made it difficult to identify the source of the problem.

The EMSA was repeated using fresh Klenow as well as including a positive control probe that was previously shown to bind SOX3 (Woods et. al 2005). Several faint bands were present on this EMSA, however all bands were background bands present in both experimental and either non transfected or negative control probe lanes (Figure 3.5B).

There are several possible explanations as to why the EMSA experiments did not work. One possibility is the SOX3 expression level was not sufficient for EMSA. As transiently transfected cells were used the amount of expression can vary greatly and

is dependent of the efficiency of transfection. An alternative method would be to make stable cell lines overexpressing SOX3 as all cells would be overexpressing SOX3 giving much higher and consistent protein levels. Secondly, as mentioned above, there was never a strong free probe signal which could be the due to using old radioactive isotopes that may no longer give a strong signal due to relatively short half life. However, multiple batches were used, at the peak of their signal with no increase in strength of the free probe, as such it seems unlikely. Another possibility is the Klenow labeling was not efficient. Hence, Fluorescence-based EMSA techniques could be applied in the future as these can quantify the amount of protein and DNA rigorously which is better for analyzing the results (Jing, Agnew et al. 2003). Finally, the binding reaction of the DNA to protein may require further optimization by altering buffer conditions or cell concentration or incubation time. Unfortunately due to time constraints, I was not able to verify the binding activity of SOX3 to Dbx1 regulatory regions through EMSA.

To assess the potential binding of SOX2 to the Dbx1 sites, it was necessary to generate a SOX2 expression construct. Construction of the Sox2-pIRES2-EGFP vector by direct subcloning the Sox2 ORF from pCI-neo-Sox2 into p-IRES2-EGFP did not produce any clones containing the Sox2 ORF. The same restriction sites were used to both remove the ORF and linearise the donor vector, removing the possibility of incompatible ends. As an alternative method, PCR based amplification using Pfu polymerase and cloning was used to generate the expression vector. Although several positive clones were sequenced, mutations were identified within all clones that were analysed. This PCR was repeated several times however the number of mutations did not reduce. Taq polymerase was used as an alternative polymerase, even though it is not a proof reading enzyme. Unfortunately Taq produced no PCR products under a range of different buffer conditions. A final attempt to generate a Sox2 expression vector involved subcloning the Sox2 ORF from pcDNA3.1myc into pIRES2-EGFP. AgeI and XmaI produce compatible restriction sites, therefore these enzymes in conjunction with XhoI were used for subcloning. The Sox2 expression plasmid was successfully cloned and the sequence was verified by Sanger sequencing.

Expression of SOX2 from the expression plasmid was confirmed by both Western blot (figure 3.8) and immunofluorescence (figure 3.9). The Western blot showed

bands corresponding to the expected size of SOX2 in the transfected lanes and not in the negative control (figure 3.8). The expression construct was also tested by immunofluorescence. Cells transfected with either Sox2-pIRES2-EGFP or pIRES2-EGFP (negative control) were compared to one another thorough use of an α- Sox2 antibody. No staining was observed in the negative control while distinct staining was present in the nuclei of Sox2-pIRES2-EGFP transfected cells (figure 3.9). The Sox2 expression plasmid contains an IRES EGFP reporter cassette to aid in identifying successfully transfected cells. Among all the SOX2 positive cells not all expressed EGFP (data not shown). As EGFP is expressed from an IRES it is possible that not all cells express EGFP to the same level. Another possibility is that EGFP antibody was not as sensitive as the SOX2 antibody and therefore could not detect relatively low levels of EGFP in transfected cells.

Given the difficulty in optimizing the EMSA experiments, transactivation reporter assays were instead used to assess SOX3 binding at Dbx1-associated genomic sites. Initial luciferase reporter assays showed no difference between SOCM-pGL3 (positive control reporter) and pGL3-empty (negative control vector) activity in response to overexpression of SOX3 (data not shown). Therefore, in order to get a response from the SOCM-pGL3 reporter, the ratio of the SOCM-pGL3 with Sox3-pIRES2-EGFP as well as Fugene was optimized. Altering the Fugene to DNA ratio in experiment 1 to 3 did not change the level of activity of the SOCM reporter (Figure 3.10A&B). This suggests that the lack of response is not caused by transfection efficiency. Then, altering the relative amount of Sox3-pIRES2-EGFP and SOCM-pGL3 showed an increase in the activity where the best response over background is seen in experiment 7. This indicates that the expressing protein to reporter vector ratio is critical in generating a luciferase reporter response. Next, the amount of Sox3-pIRES2-EGFP was changed while the SOCM-pGL3 was constant. It is expected that by increasing the amount of SOX3 transfected into the reporter assay would give a dose dependant response (Wong, Farlie et al. 2007), however, when the amount of Sox3-pIRES2-EGFP was increased from 300ng to 1500ng no difference in transactivation was observed (data not shown). One explanation was that the total amount of plasmid was not equal in each transfection. Therefore, pIRES2-EGFP was used to keep the total amount of DNA transfected the same. Increasing the amount of

SOX3 gave a corresponding increase in activity, compared to the empty pcDNA negative control (figure 3.10D).

Using the conditions identified above, the amount of Sox3 plasmid was increased to see if there is a dose dependent response (figure 3.10C&D). The conditions identified to give the best response (840ng SOCM: 1000ng Sox3-pIRES2-EGFP : 5.4uL Fugene) were then used to analyse the response of Dbx1-1 and Dbx1-4 reporter constructs. Accordingly, optimized ratio of the Fugene to DNA referring to experiment 7&8 in figure3.10D were performed with SOCM-pGL3, Dbx1-1-pGL3, Dbx1-4-pGL3, empty-pGL3 and Sox3-pIRES2-EGFP. No significant difference was seen between positive and negative control while the negative control showed higher luciferase activity than Dbx1-4 (figure 3.11). However, Dbx1-1-pGL3 reporter showed statistically higher activity compared with either the negative control reporter or the SOCM reporter for both SOX3 and SOX2. Although the activation of the Dbx1-1 reporter was statistically higher than both the positive and negative controls, this is only one experiment and further repeats would be required to gain confidence in the results. This is consistent with what has been observed by ChIP-PCR (McAninch, D., Unpublished data) where Dbx1-1 showed stronger enrichment than Dbx1-4.

To determine whether the cellular context has an impact on SOX3-mediated activation of the Dbx1 sites, cotransfection reporter assays were also performed using Cos-7 cells. However, due to time constraints, this experiment was attempted only once and no activation was seen by SOX3 with SOCM compared to empty pGL3. One reason was that the signal from the positive control was not particularly high (compared to the negative control) even though the SOCM-pGL3 had 4 repeated binding sites. It might be necessary to clone the Dbx1 probes into pGL3 as concatamers to increase the response observed. Furthermore the optimization of the ratio of the three vectors was not sufficient; a greater response is required for the positive control above background. Another option to improve this assay would be to try mouse P19 cells as they may express cofactors that may be required for more efficient transactivation. It was observed in Oosterveen's recent study in 2013 (Oosterveen, Kurdija et al. 2013) that co-activators might also be needed to activate the binding interaction, such as P300. However, referring to Dale's ChIP-seq data, site

1 showed stronger binding activity than site 4, which is consistent with the existing data.

As Sox3 plays important roles in brain development, a lot of studies have been analyzed upon its function as well as expression. However, little is known about the direct targets of Sox3. Recently, a landmark study focusing on identification of SoxB1 binding in neuroprogenitor cells has been published which has provided valuable information on the target genes of Sox3 (Bergsland, Ramskold et al. 2011; Oosterveen, Kurdija et al. 2012; Oosterveen, Kurdija et al. 2013). Besides that, according to the expression of dorsoventral neural tube development marker genes in other articles, we determined to use the qPCR as an approach to analyze the expression difference of some of the marker genes between E9.5 Sox3 null and WT samples.

As expected, Sox3 was virtually undetectable in the Sox3 null embryos and Dbx1 was significantly downregulated (consistent with the unpublished results of Nicholas Rogers). Interestingly, Ngn2, Pax6 and Olig2 were all significant down-regulated. Pax6, Olig2 and Ngn2 are all regionally restricted marker genes in the neural tube which are likely coexpressed with Sox3, indicating that they may be direct or indirect targets of Sox3. While some of genes were not changed, like Sox1 and Sox2, which means no influence upon the other two SOXB1 family members in Sox3 null mice, as a consequence, they might compensate for some loss of function by the absence of Sox3. For the Neurod4 gene there was a trend towards lower expression in the KO embryos. Additional samples would be required to determine the significance of these data. Nkx2.9, Nkx6.1 and Nkx6.2 were not changed between the WT and KO. One explanation was that the changes were delayed (ie. not present at this timepoint) while another was that they were not the targets of Sox3 at all. For the future, given that Dbx1 is downregulated in the Sox3 null embryos and that DBX1 controls V0 neuron differentiation via Evx1 (Pierani, Moran-Rivard et al. 2001), it would be interesting to assess whether Evx1 expression is also decreased in the Sox3 null mutant embryos. Unfortunately, due to the primers not performing and time constraints, Evx1 expression could not be performed as part of this project.

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