CHAPTER 2 : MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Embryonic Stem Cells

2.1.1.1 AB2.2

AB2.2 was derived from a 129S6 blastocyst (McMahon and Bradley, 1990). ESCs were maintained in Knockout^{TM} DMEM (Invitrogen), supplemented with 15% serum, 1x Penicillin-L-glutamine-Streptomycin, 100 μM β-mercaptoethanol and 100 U/ml leukaemia inhibitory factor (LIF) (Millipore) (M15-LIF medium) on a layer of irradiated SNL76/7 feeder fibroblasts as previously described (Ramirez-Solis et al., 1993). For the purpose of maintenance, medium was changed daily. To perform routine passaging, cells were washed once with phosphate buffered saline (PBS) before being treated with 0.1% trypsin-EDTA in PBS at 37°C for 5 minutes. Trypsin activity was quenched with an equal volume of M15-LIF medium and clumps were mechanically disrupted by pipetting before centrifugation. Supernatant is aspirated and the resuspended pellet is transferred at desired proportions into a fresh plate pre-fed with M15-LIF.

2.1.1.2 ZHBTc4 ESCs

ZHBTc4 ESCs were derived from CGR8 ESCs, where genetic manipulations (i) ablated the remaining endogenous copy of Pou5f1 (Niwa et al., 2000) and (ii) inserted a transgene expressing Oct4 under a tetracycline repressible system. These cells were a kind gift from Dr. Austin Smith (Centre for Stem Cell Research, Cambridge). These ESCs were maintained in GMEM (Sigma), supplemented with 10% serum (Hyclone), 1x Penicillin-L-glutamine-Streptomycin (Invitrogen), 1x non-essential amino acids (Invitrogen), 1x sodium pyruvate (Invitrogen), 100 μM β-mercaptoethanol (Sigma) and 100 U/ml LIF (Millipore), on tissue culture plates coated with 0.1% Gelatin (Sigma). Cells were maintained in a similar manner to AB2.2 ESCs.

2.1.2 Mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were maintained in Knockout^{TM} DMEM (Invitrogen), supplemented with 10% serum, 1x Penicillin-L-glutamine-Streptomycin and 1x non-essential amino acids (M10). To obtain MEFs, mice of desired genotypes were set as mating pairs. Females were inspected for the formation of plugs daily. Upon the observation of a plug, the pregnant female was sacrificed 13 days later, such that the embryos have reached developmental stage E13.5. The embryos were separated from the female and placed into cold PBS. On a Petri dish, the embryos were dissected to remove the surrounding membranes and placenta. The head and visceral organs were discarded and the remaining tissues were washed with fresh PBS. If genotyping is required, a small portion of the tail is extracted for DNA. Using a pair of sterile scissors, the tissues were finely minced and transferred into 3ml of 0.1% trypsin-EDTA in PBS at 37°C for 20 minutes. An equal volume of trypsin was added and incubated at 37°C for another 20 minutes. To neutralise trypsin, 6ml of M10 was added to the mixture and centrifuged. The cell pellet obtained from one embryo was distributed into one 15-cm 0.1% gelatin coated plate. After 3 days, the cells had reached confluency and were dissociated using trypsin treatment. The cells were counted and frozen into vials at a density of 5 million cells.

2.1.3 iPSCs

2.1.3.1 Generation of iPSCs

To generate iPSCs, frozen MEFs were thawed and 5 million cells were seeded into three 15-cm gelatin-coated plates. After 4 days or when the cells attain 70% confluency, the cells were washed once with PBS and dissociated in 4ml of trypsin-EDTA at 37°C for 5 minutes. An equal volume of M10 was added to neutralise trypsin and the cell clumps were disrupted mechanically through the use of pipettes. 10μl of the cell suspension was used for counting, while the remaining cells were collected and centrifuged at 1000rpm for 3 minutes. The cell pellet was resuspended in OptiMEM (Invitrogen) at a density of 5 million cells/ml. Plasmids used for reprogramming were aliquoted at appropriate amounts (**Table 1**) into 1.5ml eppendorf tubes and mixed with 200μl of cell suspension and transferred into an Amaxa nucleofection cuvette. The cuvette was inserted into the Amaxa

nucleofector and electroporation was initiated using program A-023. Once electroporation occurred, the cuvette was quickly removed and 1ml of M10 was added to resuscitate the cells. The cell mixture was then transferred into a fresh 10-cm plate pre-fed with M15-LIF. To increase reprogramming efficiency, irradiated SNL76/7 feeder fibroblasts were employed to support the transfected cells. In the event that the doxycycline inducible platform was applied, 1.0μg/ml doxycycline (Clontech) was introduced immediately after transfection. Media for these transfected cells were changed regularly and the plates were maintained over a span of three weeks to determine reprogramming efficiencies.

PLASMID	AMOUNT TRANSFECTED
piggyBac transposase (PL623)	2μg
PB-CAG-cDNA	1μg
PB-CAG-rtTA	1μg
PB-TRE-cDNA	1μg

Table 2.1 Amounts of plasmid DNA transfected into MEFs to obtain iPSCs In a typical reprogramming experiment, 1 x 10^6 MEFs were transfected with 2μg of PL623 and 1μg of each transposon construct consisting of reprogramming factors, unless otherwise stated. If doxycycline inducible platform was employed, 1μg of PB-CAG-rtTA was co-introduced into the cells.

2.1.3.2 Establishment of iPSC colonies

Upon transfection of MEFs, the cells are maintained for 14 days or until the colonies are visible by eye. Prior to the picking of colonies, 50µl of trypsin-EDTA was added into each well of a 96-well round bottom plate. The reprogrammed cells were washed once with PBS and 10ml of fresh PBS was added into each 10-cm plate. Viewed under the microscope, colonies were picked and transferred into 50µl of trypsin-EDTA. After picking, an equal volume of M15-LIF was added and the colony was disrupted by pipetting the mixture approximately 10-30 times, depending on the compactness of the colony. The cell suspension was then transferred into a 96-well feeder coated plate pre-fed with fresh M15-LIF. The cells are maintained by a change of fresh media daily. Once confluent, the cells were dissociated using trypsin-EDTA and the cell pellet obtained after neutralisation and centrifugation was seeded onto a feeder coated 24-well plate.

To obtain a homogenous population of ground state naïve iPSCs, the colonies are maintained in NDiff N_2B_{27} (Stem Cells, Inc) supplemented with 1µM PD 0325901 (Axon Medchem), 3µM CHIR 99021 (Axon Medchem) and 100 U/ml leukaemia inhibitory factor (LIF) (Millipore) (2i-LIF).

2.1.3.1 4F-iPSCs and 6F-iPSCs

To generate 4F iPSCs, 2µg PL623, 1µg PB-TRE-OCKS and 1µg PB-CAG-rtTA were introduced into 1 x 10^6 Rex1::EGFP-IRES-Puro MEFs. 6F iPSCs were obtained using 2µg PL623, 1µg PB-TRE-OCKS, 1µg PB-TRE-RL and 1µg PB-CAG-rtTA. Primary colonies in both conditions were picked and maintained in 2i-LIF on gelatin coated plates.

2.2 Genetic Screen

2.2.1 Cloning of PB-CAG-CKS-CAG-SD

A transposon cassette containing CAG promoter and the four murine factors (mFx), namely OCT4, C-MYC, KLF4 and SOX2, was obtained from Wei Wang, Wellcome Trust Sanger Institute. To generate PB-CAG-C-MYC-KLF4-SOX2, OCT4 was excised using EcoRI and SbfI. As C-MYC contains an internal SbfI recognition site, PB-CAG-C-MYC (also obtained from Wei Wang) was simulataneously digested with EcoRI and SbfI (New England Biolabs) and ligated to the products obtained from the pre-described restriction digestion step. To generate PB-CAG-C-MYC-KLF4-SOX2-CAG-SD, PB-CAG-C-MYC-KLF4-SOX2 and PB-CAG-SD were both digested with XhoI and NheI (New England Biolabs) and ligated using T4 ligase (New England Biolabs).

2.2.2 Transduction of PB-CAG-CKS-CAG-SD into MEFs

As previously described, MEFs at 70% confluency were treated with 0.05% trypsin (Invitrogen), pelleted and resuspended in OptiMEM. 10μg of PB-CAG-CKS-CAG-SD was mixed with 1 x 10^6 cells. 10 x 10^6 cells were used in ten separate electroporations. Amaxa Nucleofector program A-023 was used to electroporate the mixtures. Modified cells from each electroporation were transferred to 10-cm feeder coated plates and maintained regularly in M15-LIF. Colonies were observed after 20 days and 2μg/mL puromycin was introduced to positively select for fully reprogrammed colonies.

2.2.3 Analysis of integration sites

2.2.3.1 Extraction of DNA

10cm plates of iPS colonies were treated with 0.05% trypsin (Invitrogen) and dislodged cells were pelleted and washed with PBS. The cells were pelleted by centrifugation and resuspended in 1mL of lysis buffer (50mM Tris-HCl pH7.5, 50mM EDTA pH8.0, 100mM NaCl, 1% SDS, 1mg/mL proteinase K) The cells were incubated overnight in 65°C and double the amount of 100% ethanol was added. Precipitation was allowed to occur at room

temperature for 2 hours and DNA was pelleted by centrifuging at 1000rpm for 10 minutes. Supernatant was aspirated and 70% ethanol was used to wash the DNA pellet twice. After another round of centrifugation, the precipitate was resuspended in Tris-EDTA buffer overnight at 65°C.

2.2.3.2 Splinkerette PCR and cloning

4μg of DNA from each 10cm plate was digested with BfuCI (New England Biolabs) at 37°C for 3 hours and ligated with splinkerettes (**Table 2.2**) at 16°C for 15 hours. Upon ligation, splinkerette PCR was conducted using primers against the splinkerette and the left or right transposon arms. A small aliquot of the PCR products were separated on 1.5% agarose to visualise a ladder of various band sizes. The remaining PCR products were purified using the QIAGEN PCR Purification Kit and eluted in 30μL of the provided elution buffer. 3μL of the purified PCR products was cloned into pGEMT-EASY (Promega). Subsequently, 96 colonies were picked for DNA obtained from each 10cm plate and grown in LB with 100μg/mL ampicillin. The resultant bacterial cultures were sequenced using capilliary sequencing. Sequences were analysed with the help of Stephen Rice (WTSI) and categorised according to the exact integration site, query coverage and quality, neighbouring annotated genes, percentage of match, and the orientation of the integration.

PRIMER/OLIGO NAME	SEQUENCE
Splinkerette 1	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGTGTCGACACTAGTGG
Splinkerette 2	GATCCCACTAGTGTCGACACCAGTCTCTAATTTTTTTTTTCAAAAAAA
Splinkerette primer 1	CGAAGAGTAACCGTTGCTAGGAGAGACC
Splinkerette primer 2	GTGGCTGAATGAGACTGGTGTCGAC
piggyBac left primer 1	CAGTGACACTTACCGCATTGACAAGCACGC
piggyBac left primer 2	GAGAGAGCAATATTTCAAGAATGCATGCGT
piggyBac right primer 1	CCTCGATATACAGACCGATAAAACACATGC
piggyBac right primer 2	ACGCATGATTATCTTTAACGTACGTCACAA

Table 2.2 Sequences of splinkerettes and primers used during splinkerette PCR

2.2.3.3 Validation of integration sites within BNC2

Collection of DNA from pools of iPSCs obtained from the genetic screen was described in 2.2.3.1. 1µl of DNA was used to amplify BNC2 genomic regions comprising of transposon integrations. Primers employed are listed in **Table 2.3** and Extensor Hi-Fidelity PCR master mix 2 (ABgene) was used to perform PCR using the following settings: 94°C for 4 min, followed by 30 cycles of 94°C for 10 sec, 55°C for 30 sec and 68°C for 30 sec. Final incubation was at 68°C for 10 min.

Name	Primer Sequence	Position of integration	Position of Primer	Distance from Integration Site
Forward-1	CAGGCAATCACGGCTATTTT	84147715-84147827	84147534	-181
Reverse-1	GGGAGGCCACACACATAATC	84147715-84147827	84148031	316
Forward-2	TGATTCAGAAAGCCCAACCT	84304000-84304026	84303769	-231
Reverse-2	CCCACATCACAACCAAAACA	84304000-84304026	84304183	183
Forward-3	AAAGGGTGAACAAGCAAAGC	84193364-84193694	84193024	-340
Reverse-3	CGCTTGCTCATCTTTTCTCA	84193364-84193694	84193781	417
Forward-4	CAGCCACAGTCCTCTGACAA	84195187-84195313	84194988	-199
Reverse-4	GGTGCCTTGGTTCTGTTGTT	84195187-84195313	84195515	328

Table 2.3 Sequences of primers which correspond to the integrations within BNC2 observed in the genetic screen

2.3 Constructs generated for validation

2.3.1 PB-CAG-BNC2

A transposon cassette containing CAG promoter and RARγ and LRH1, was obtained from Wei Wang, Wellcome Trust Sanger Institute (Wang et al., 2011c). Using EcoRI and NotI, cDNAs encoding RARγ and LRH1 were excised. BNC2 cDNA was obtained from GeneService and primers flanking the cDNA were designed. The forward and reverse primers were tethered to EcoRI and NotI recognition sites respectively (**Table 2.4**). BNC2 cDNA was amplified using Pwo Master (Roche) according to the manufacturer's instructions. A portion of the PCR product was visualised on an agarose gel to determine its purity and size. The remaining PCR product was purified using QIAGEN PCR Purification Kit and digested using NotI (New England Biolabs) at 37°C for an hour. As there are internal EcoRI restriction sites within the coding sequence of BNC2, 0.05µl of EcoRI (New England Biolabs) was added to the digested mixture and incubated at 37°C for 15 minutes. The digestion mixture was immediately run on an agarose gel. The partial digestion gave rise to a series of bands and the one of correct size was extracted and purified according to the manufacturer's instructions (QIAGEN Gel Purification Kit). The purified fragments were ligated to a pre-digested PB-CAG-bpA plasmid backbone described earlier, using T4 Ligase (New England Biolabs) in a 10µl reaction at room temperature for 1 hour. 1.5µl of the ligation mix was introduced to a vial of MegaX DH10β electrocompetent cells (Invitrogen) and transferred to an electroporation cuvette. The cuvette was placed into the BioRad GenePulser X cell and electroporation took place at a setting of 1800V, 25µF, 200Ω. Immediately after electroporation, 300µl of Lysogeny Broth (LB) was added to the bacterial culture to resuscitate the cells and the entire mixture was subsequently incubated at 37°C, 200rpm, for 1 hour. The cells were then plated onto a pre-warmed LB Agar plate supplemented with 100µg/mL ampicillin and incubated overnight at 37°C. Bacterial colonies were picked the next day and grown in LB supplemented with 100µg/mL ampicillin and incubated for 15 hours at 37°C. Following the manufacturer's protocol, the cloned plasmids were retrieved from the bacterial cultures using QIAGEN Miniprep Kit.

PRIMER NAME	RESTRICTION SITE	SEQUENCE
mBNC2 Forward	EcoRI	AATTGAATTCTGACAACTCCATGCAGTTCG
mBNC2 Reverse	NotI	AATTGCGGCCGCCTAATCTATTGAAGTGAAGGGA
mRARβ Forward	EcoRI	AATTGAATTCAGGATAAGCACTTTTGCAGAG
mRARβ Reverse	NotI	AATTGCGGCCGCCTGCAGCAGTGGTGACTGA
mRORα Forward	BamHI	AATTAAGCTTCGCGGCGTAAAGGATGTATTT
mRORα Reverse	XbaI	AATTCTAGATTACCCATCGATTTGCATGGC

Table 2.4 Sequences of primers used for cloning (Chapters 2.3.1- 2.3.6)

2.3.2 PB-CAG-RARβ

A similar approach was taken to generate PB-CAG-RARβ. cDNA corresponding to full length RARβ was obtained from GeneService and primers flanking the cDNA were designed. EcoRI and NotI sites were included in the forward and reverse primers respectively (**Table 2.4**). RARβ cDNA was amplified using Pwo Master (Roche) according to the manufacturer's instructions and purified. As RARβ contains internal EcoRI recognition sites, the PCR product was first digested using NotI and partially digested by EcoRI. The correct product size was identified and purified, before ligating to a pre-digested PB-CAG-bpA plasmid backbone.

2.3.3 PB-CAG-RORα

An identical approach was taken to generate PB-CAG-RORα. cDNA corresponding to full length PB-CAG-RORα was obtained from GeneService and primers flanking the cDNA were designed. BamHI and XbaI sites were included in the forward and reverse primers respectively (**Table 2.4**). PB-CAG-RORα cDNA was amplified using Pwo Master (Roche) according to the manufacturer's instructions and purified. The correct product size was identified and purified, before ligating to a pre-digested PB-CAG-bpA plasmid backbone.

2.3.4 PB-TRE-BNC2

A similar approach was taken to generate PB-TRE-BNC2. PB-TRE-bpA plasmid backbone was achieved through the digestion of PB-TRE-RARγ-2A-LRH1 using EcoRI and NotI (**Table 2.4**). This plasmid was obtained from Wei Wang, Wellcome Trust Sanger Institute (Wang et al., 2011c). Using PCR products of BNC2 which have been digested using EcoRI and NotI, as described above, both the insert and plasmid backbone were ligated and transformed into MegaX DH10β electro-competent cells.

2.3.5 PB-TRE-RARβ

A similar approach was taken to generate PB-TRE-RARβ. PB-TRE-bpA plasmid backbone was achieved through the digestion of PB-TRE-RL using EcoRI and NotI (**Table 2.4**). Using PCR products of RARβ which have been digested using EcoRI and NotI, as described above, both the insert and plasmid backbone were ligated and transformed into MegaX DH10β electro-competent cells.

2.3.6 PB-TRE-RORα

A similar approach was taken to generate PB-TRE-RORα. PB-TRE-bpA plasmid backbone was achieved through the digestion of PB-TRE-RL using BamHI and XbaI (**Table 2.4**). Using PCR products of RORα which have been digested using BamHI and XbaI, as described above, both the insert and plasmid backbone were ligated and transformed into MegaX DH10β electro-competent cells.

2.3.7 PB-TRE-CKS

A similar approach was taken to generate PB-TRE-CKS. PB-TRE-bpA plasmid backbone was achieved through the digestion of PB-TRE-RL using EcoRI and NotI. PB-CAG-CKS (as described in 2.2.1) was digested using EcoRI and NotI to excise c-Myc-2A-Klf4-2A-Sox2. This fragment was ligated with a pre-digested plasmid backbone containing PB-TRE-bpA and transformed into MegaX DH10β electro-competent cells.

2.3.8 Other plasmids used in the study

PB-CAG-rtTA, PB-CAG-LRH1, PB-CAG-RARγ, PB-CAG-RARα, PB-CAG-OCKS, PB-CAG-RL, PB-TRE-LRH1, PB-TRE-RARγ, PB-TRE-RARα, PB-TRE–OCKS and PB-TRE-RL were obtained from Wei Wang, Wellcome Trust Sanger Institute (Wang et al., 2011c).

2.4 Analysis of iPSC colonies

2.4.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was first extracted using RNeasy Mini Kit (Qiagen) following manufacturer's guidelines. Subsequently, reverse transcription reactions were performed using QuantiTect Reverse Transcription Kit (Qiagen). PCR was then carried out using primers against pluripotency marker genes (Sigma) (**Table 2.5**) and an Extensor Hi-Fidelity PCR master mix 2 (ABgene). PCR was performed using the following settings: 94°C for 4 min, followed by 30 cycles of 94°C for 10 sec, 55°C for 30 sec and 68°C for 30 sec. Final incubation was at 68°C for 10 min.

PRIMER NAME	SEQUENCE
mOct4-RT-En-Forward	TCTTTCCACCAGGCCCCCGGCTC
mOct4-RT-En-Reverse	TGCGGGCGGACATGGGGAGATCC
mSox2-RT-En-Forward	TTGCCTTAAACAAGACCACGAAA
mSox2-RT-En-Reverse	TAGAGCTAGACTCCGGGCGATGA
mKlf4-RT-En-F orward	GCGAACTCACACAGGCGAGAAACC
mKlf4-RT-En-Reverse	TCGCTTCCTCTTCCTCCGACACA
mNanog-RT-En-F orward	CAGGTGTTTGAGGGTAGCTC
mNanog-RT-En-Reverse	CGGTTCATCATGGTACAGTC
mRex1-RT-F orward	ACGAGTGGCAGTTTCTTCTTGGGA
mRex1-RT-Reverse	TATGACTCACTTCCAGGGGGCACT
mEsg1-RT-F orward	GAAGTCTGGTTCCTTGGCAGGATG
mEsg1-RT-Reverse	ACTCGATACACTGGCCTAGC

mFgf4-RT-Forward	CGTGGTGAGCATCTTCGGAGTGG
mFgf4-RT-Reverse	CCTTCTTGGTCCGCCCGTTCTTA
mDax1-RT-Forward	TGCTGCGGTCCAGGCCATCAAGAG
mDax1-RT-Reverse	GGGCACTGTTCAGTTCAGCGGATC
mGdf3-RT-F orward	GTTCCAACCTGTGCCTCGCGTCTT
mGdf3-RT-Reverse	AGCGAGGCATGGAGAGAGCGGAGCAG
mUtf1-RT-F orward	GGATGTCCCGGTGACTACGTCTG
mUtf1-RT-Reverse	GGCGGATCTGGTTATCGAAGGGT
mBactin-RT-Forward	GTTTGAGACCTTCAACACCCC
mBactin-RT-Reverse	GTGGCCATCTCCTGCTCGAAGTC

Table 2.5 Sequences of primers used for RT-PCR of pluripotency markers

2.4.2 Quantitative real time PCR

In a probe-based assay (**Table 2.6**), quantitative real time PCR was performed using Absolute Fast qPCR Master Mix (ABgene) according to manufacturer's instructions. Data were normalised with GAPDH. The assay was performed using the following settings: 95°C for 5 min, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec.

ASSAY ID	TARGET	APPLIED BIOSCIENCE GENE NAME
Mm00658129_gH	mOct4	POU domain, class 5, homeobox 1
Mm00516104_m1	mKlf4	Kruppel-like factor 4
Mm02384862_g1	mNanog	Nanog homeobox
Mm03053975_g1	mRex1	Zinc finger protein 42
4352341E	β-actin	Mouse ACTB Endogenous Control

Table 2.6 Applied Bioscience pre-designed TaqMan probes to detect quantitative levels of pluripotency markers

Using a primer-based approach, RT^2 qPCR SYBR Green/ROX mastermix (Qiagen) was applied following the manufacturer's instructions. PCR was performed at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. Data was normalised to GAPDH.

2.4.3 Immunostaining

iPSCs were pre-cultured in gelatin coated 24-well plates and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilised with 0.3% Triton X100 in PBS. The cells were then blocked with blocking buffer (1% BSA, 3% goat serum in 0.3% Triton in PBS) for 10 min at room temperature. Primary antibodies were made up using the blocking buffer with the following dilutions: Nanog (1:150, Rabbit

polyclonal; ab21603, Abcam), SSEA1 (1:10, mouse monoclonal, gift from Sheffield University) and Oct4 (1:150, Mouse monoclonal; sc-5279, Santa Cruz). After overnight incubation at 4°C, cells were washed 4 times with 0.3% Triton X100 in PBS and labelled with Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen). After a final wash with 0.3% Triton X100 in PBS, the cells were incubated with DAPI and visualised using Olympus IX81.

2.4.4 Bisulfite Sequencing

DNA from iPSCs was extracted as described above. Bisulphite conversion was performed using EpiTect Bisulfite Kit (Qiagen), according to manufacturer's recommendations. Approximately 400bp of the promoter regions of Rex1, Oct4 and Nanog was amplified using the following primers:

The PCR fragments were cloned into pGEMT-EASY (Promega) and sequenced. The sequences obtained were aligned to the following templates and CpG dinucleotides which were analysed are underlined.

Oct4

GGTTTTTTAGAGGATGGTTGAGTGGGTTGTAAGGATAGGTCGAGAGGGTGTAG
TGTTAATAGGTTTTGTGGTGCGATGGGGTATTCGAGTAATTGGTTTGTGAGGTG
TTCGGTGATTTAAGGTAGGGGTGAGAGGATTTTGAAGGTTGAAAATGAAGGTT
TTTTGGGGTTTCGTTTTAAGGGTTGTTTTGTTTAGACGTTTTTAATTTTCGTTTG
GAAGATATAGGTAGATAGCGTTCGTTTTAGTTTTTTTTATTTTTATAGTTTTGTT
TTTTTATTTATTTAGGGGGCGGGGTTAGAGGTTAAGGTTAGAGGGTGGGATTG
GGGAGGGAGAGGTGAAATCGTTTTTAGGTGAGTCGTTTTTTTATTAGGTTTTCG
GTTCGGGGTGTTTATTTTTTTTATGGTTGGATATTTGGTTTT

Nanog

GATTTTGTAGGTGGGATTAATTGTGAATTTATAGGGTTGGTGGGGCGTGGGTG
TCGTTTGGGTGTTTGGGAGAATAGGGGGTGGGTAGGGTAGGAGGTTTGAGGG
GGGAGGAGTAGGATTTATTTTTTAAATTTATCGTTTTGAGTCGTTGGTTTTTAG

ATAGGTTGATTTGGTTGGTGTTTTGTTTTTTTTGTGGGAAGGTTGCGGTTTATTT
TTTTTTGATTTTTTGATAATTTTGTATTAGATATTTAATTTTTTTTTTTTATGATTT
TTTTTTTTAGATATTGAGTTTTTTGGTTGTTGTTTAAAATTTTTTTAGAAATTTTT
TTTTTCGTTATTATATTGATATGAGTGTGGGTTTTTTTGGT

Rex1

TATATTAATGTTGGAAAAAGTTTAGGTAATTAGTGTATTTTGTAGCGGGGTTCG
GGAGAGGTTGGGGTTTAGAGTGGCGATGGGACGAAAGTGTAAAGAAAGTTTT
CGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTAGGTAGGTAG
GTAGAAATTGTATTTTTTGTTTGTGTAAATTCGGTTATAAGTTTGTAATAGAGG
TATTGAGATGTGATTGAGTTTTAAGGTTAGGCGATCGGGATTTTGAAGAGGTA
TTTGTATAATTGAGTAAGAGTTTTTGTTTTATTTTTTTACGTCGATTTAAGATAA
GGGGATAGTTTTGGGTGGGTTATTTGAAGGGTTAGGGGTTCGTTTATATTTTCG
TTTATATTTATTTTGAGCGTTTTTTATTGGGTATTTTAGTTTATTTAGTTCGGAG
TTAGTTATTTCGTAAGTGTGGTCGGAATAGAGTTCGTTTATTTAAAAAGGGAG
GGGTTTAAGGAGTT

PRIMER NAME	SEQUENCE
Oct4-DMR-Forward	GGCCCCAGAAATAATTGG
Oct4-DMR-Reverse	TTTTGGTTTATAGGTTGG
Rex1-DMR-Forward	CACATTAATGCTGGAAAAAGTTCAGGCAA
Rex1-DMR-Reverse	TTGAGGAATTTGGGGAGGGAAAAATTTATT
Nanog-DMR-Forward	GACCCTGCAGGTGGGATTAACTGTGAATTC
Nanog-DMR-Reverse	TGGTTTTTTTGGGTGTGAGTATAGTTATAT

Table 2.7 Sequences of primers used to amplify promoter regions of pluripotency markers for bisulfite sequencing

2.4.5 *In vitro* differentiation

For differentiation into the ectoderm lineage, iPSCs were seeded at a density of $2x10^5$ cells / well in a 6-well plate. The cells were cultured in NDiff® N2B27 (Stem Cells, Inc).

For differentiation into the endoderm and mesoderm lineage, iPSCs were trypsinised and seeded onto an uncoated 6-cm dish to form embryoid bodies. After 3 days, the embryoid bodies were seeded onto a gelatin coated 6-cm dish and media was switched to M10 for 3 days before staining.

2.4.6 Teratoma Formation

iPSCs were resuspended at a concentration of 5×10^6 cells/ml in PBS. 200µl of the cell suspension was administered subcutaneously into the dorsal flank. 3 to 5 weeks after the injection, tumours were observed and surgically dissected from the mice. Samples were fixed in 10% formalin, and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin.

2.4.7 Chimera Production

iPSCs were injected into blastocysts of C57BL/6 (albino) and implanted to C57BL/6 (albino) foster females. Chimeric males were chosen, based on iPSC coat colour contribution, to breed with C57BL/6 (albino) females in order to test for germline transmission.

2.4.8 Alkaline Phosphatase Staining

Staining was performed according to manufacturer's instructions (Alkaline Phosphatase Kit, Sigma). iPSCs were washed once with fresh PBS and incubated in 4% PFA in PBS at room temperature for 20 minutes. The cells were then washed once with PBS before

freshly prepared alkaline-dye solution was added. The plates were incubated in the dark for 20 minutes before they were rinsed with distilled water and left to dry.

2.4.9 Karyotyping

Cells were treated with 0.1 µg/ml colcemid for 50 minutes before trypsin treatment for collection. 10ml of 75mM KCl was added in a dropwise fashion using a Pasteur pipette. The solution was incubated at room temperature for 10 minutes before centrifugation at 1600rpm for 8 minutes. 1ml of fixing solution which consists of Glacial Acetic Acid and Methanol at a ratio of 3:1 was added to the disturbed cell pellet and centrifuged at 400g for 8 minutes. The pelleted cells were then resuspended in 2ml of fixing solution.

To prepare slides, they were sonicated and soaked in 96% ethanol before drying. 20µl of cell suspension obtained in the previous step was dropped onto the slide and placed in a 50°C water bath for 1 minute. The spread of the chromosomes were inspected under the microscope.

After aging the slides overnight, the slides were placed in 10mM HCl with 0.01% pepsin for 3 minutes. The slides were then washed twice with 2xSSC for 5 minutes each. For dehydration, the slide was passed through an ethanol dehydration series: 70%, 90% and 100% ethanol for 5 min each. Denaturation of DNA was next performed by incubating the slides in 70% formamide and 30% 2xSSC and the slides were put through another series of dehydration as previously described. The slides were air-dried and baked at 65°C for 1 hour.

Formamide was added to the slides at 61°C for 1.5 minutes before quenching in ice cold 70% ethanol. The slides then had to undergo dehydration from 70%, 90% to 100%. The slides were then air-dried and pre-aliquoted probes were added before coverslips were placed.

For detection, the slides were placed into a 43°C water bath for 5 minutes to remove the coverslip. Probes were washed using 50% Formamide and 50% 2xSSC twice for 5 minutes each. Pre-aliquoted antibodies were added onto the slides and incubated at 37°C. The slides were then visualised under the microscope.

2.4.10 Analysis of transgene expression

RNA was first extracted using RNeasy Mini Kit (Qiagen) following manufacturer's guidelines. Subsequently, reverse transcription reactions were performed using QuantiTect Reverse Transcription Kit (Qiagen). PCR was then carried out using primers against junctions between transgenes (Sigma) (**Table 2.8**) and an Extensor Hi-Fidelity PCR master mix 2 (ABgene). PCR was performed using the following settings: 94°C for 4 min, followed by 30 cycles of 94°C for 10 sec, 55°C for 30 sec and 68°C for 30 sec. Final incubation was at 68°C for 10 min.

PRIMER NAME	SEQUENCE	APPLICATION
Oct4-Forward	CAGAAGGGCAAAAGATCAAG	Amplification of Oct4-c-Myc junction
c-Myc-Reverse	TACGGAGTCGTAGTCGAGGT	
c-Myc-Forward	CGCCTACATCCTGTCCATTC	Amplification of c-Myc-Klf4 junction
Klf4-Reverse	GCCGGGCCGGACGCGAACGT	
LRH1-Forward	ATCCGGGCAATCAGCAAGCA	Amplification of LRH1-bpA junction
bpA-Reverse	GGCACAGTCGAGGCTGATCAGC	
rtTA-Forward	GGACGAGCTCCACTTAGACG	Amplification of rtTA
rtTA-Reverse	AGGGCATCGGTAAACATCTG	

Table 2.8 Sequences of primers used to detect the presence of reprogramming transgenes

2.5 Chromatin Immunoprecipitation (ChIP)

2.5.1 RARβ-directed ChIP in MEFs

Frozen MEFs (as described earlier) derived from wildtype C57BL/6J mice were thawed and 5 million cells were seeded into three 15-cm gelatin-coated plates. After one week, the cells attained 100% confluency. Harvest of the cells was conducted using ChIP-IT Express (Active Motif), according to the manufacturer's instructions. Media was aspirated and 20ml of fresh serum-free media was added. 0.54ml of 37% Paraformaldehyde was added to the media and the plate was rocked gently at room temperature for 20 minutes. The cells were washed once with ice cold PBS and 10ml of Glycine was added to quench the action of formaldehyde. The mixture was rocked gently for 10 minutes at room temperature and washed with ice cold PBS. 2ml of fresh ice cold PBS supplemented with 0.5mM PMSF was added onto the plate for the scraping of cells. A cell scraper was used to collect the cells and the cells were pelleted through centrifugation at 4°C, 2500rpm for 10 minutes. The cell pellet was resuspended in 1ml of supplied lysis buffer supplemented with 1mM PMSF and protease inhibitor cocktail. Lysing of the cells took place on ice for an hour. Cells were pelleted at 5000rpm for 10 minutes and the pellet was resuspended in 600µl of the provided shearing buffer. The mixture was divided into two Lo-Bind eppendorf tubes (Eppendorf), placed into a BioRuptor (Diagenode) and pulsed with 15 cycles of 30 seconds sonication and 30 seconds rest. The resultant mixture was centrifuged at 13,500rpm for 20 minutes and 100µl of the supernatant was used for the execution of ChIP. 20µl of the lysate was used to examine the quality of shearing, as stated in the manufacturer's protocol.

To conduct ChIP, 100µl of the lysate was incubated with ChIP buffer 1, 3µg of RARβ (Santa Cruz) or IgG (Cell signalling) antibody, protease inhibitor cocktail and 25µl of provided magnetic Protein G beads in a total volume of 400µl. The tubes were placed on a rotator in 4°C overnight.

After an overnight incubation with the respective antibodies, the tubes were placed in a rack with a magnetic strip to pellet the beads. Supernatant was removed and 800µl of ChIP buffer 1 was used to wash the beads. The beads were subsequently washed twice with ChIP buffer 2. 50µl of elution buffer was added to the beads and rotated at room temperature for 20 minutes. 50µl of reverse cross-linking buffer was then added and the beads were pelleted. Supernatant was collected and purified using QIAGEN PCR Purification Kit. For quantitative analysis, 1µl of the eluate was used for real-time PCR (**Table 2.9**).

PRIMER NAME	SEQUENCE
8873bp Forward	AAGGTCAGATGAGGGCATTG
8873bp Reverse	CAGCACCTGAATGATGGATG
8244bp Forward	GGTGCTCTTACCCACTGAGC
8244bp Reverse	AGGCCAAACACTCCAATGAC
6932bp Forward	GGTCTTTTGAGCCACCAGAA
6932bp Reverse	GATTTGCCTCTCTGGGTCAG
4836bp Forward	AGCCAGGGCTACACAGAGAA
4836bp Reverse	GTGGGGAGACAGGAATGAGA
2610bp Forward	CCAAGTGCTGGGATTAAAGC
2610bp Reverse	TCTGCCCCCTTTAAGAGTCA
2170bp Forward	GGCTGCAGGCATACTTGAAC
2170bp Reverse	GCTACAACCTCCCCACACC
1940bp Forward	CTCTCGTCCTAGCCCTTCCT
1940bp Reverse	CCTCCACTCTGTCATGCTCA
1611bp Forward	TGGTGAAGTCGATGAAGCTG
1611bp Reverse	GAGCTGTTGGCTAGGGTCAG
1130bp Forward	TCCTCCTAATCCCGTCTCCT
1130bp Reverse	ATACCCTGCTTCCCTTCCTC
275bp Forward	AATTGGCACACGAACATTCA
275bp Reverse	GTCCTTACAGCCCACTCAGC
100bp Forward	CCTAAGGGTTGTCCTGTCCA
100bp Reverse	AGCGCTATCTGCCTGTGTCT

Table 2.9 Sequences of primers used for ChIP-qPCR of putative RAREs upstream of Oct4

2.5.1 H3K4me3-directed ChIP in differentiated iPSCs

4F and 6F iPSCs were maintained on 10-cm gelatin coated plates in 2i-LIF. When confluent, the cells were collected using trypsin and seeded onto three 15-cm gelatin coated plates. The cells were maintained in M10 supplemented with 0.1μM all-trans retinoic acid for 6 days to drive differentiation. After 6 days, the cells were treated with doxycycline as described and cross-linked and harvested as earlier described. After lysis and sonication, the lysate was incubated at 4°C overnight with 1μl of α-H3K4me3 antibody (Cell signalling). The pull down was conducted as earlier described.

2.6 Luciferase Assay

2.6.1 Cloning of luciferase reporter constructs

2.6.1.1 Constructs which test promoter function

To generate fragments consisting of various elements within the upstream region of Pou5f1, forward primers were designed at 2190bp, 1393bp, and 477bp upstream of the transcriptional start site (TSS). The reverse primer was designed 11bp before the TSS. The forward and reverse primers were flanked with XhoI and KpnI restriction sites respectively (**Table 2.10**).

PCR was conducted using mouse genomic DNA as a template. Pwo master mix (Roche) was employed to execute the PCR. An aliquot of the reaction mix was visualised on an agarose gel to ascertain the purity and size of PCR product. The remaining mixture was purified using QIAGEN PCR Purification Kit and digested using XhoI and KpnI (New England Biolabs) at 37°C for 1 hour. The digested products were purified using the QIAGEN PCR Purification Kit. pGL3-basic (Promega) was also digested using XhoI and KpnI at 37°C for 1 hour. The inserts and plasmid backbone were ligated using T4 ligase (New England Biolabs) and transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) following the manufacturer's instructions. Once recovered in LB, the

bacterial cells were plated onto LB agar plates supplemented with 100μg/ml ampicillin and picked the next day to inspect for correct insertions.

PRIMER NAME	RESTRICTION SITE	SEQUENCE
DE-PE-RAREoct Forward	XhoI	AATTCTCGAGAGGGCACATCTGTTTCAAGC
DE-PE-RAREoct Reverse	KpnI	GGTACCCTCTAGACGGGTGGGTAAGCAAGA ACTG
PE-RAREoct Forward	XhoI	AATTCTCGAGAAACACCATCCCTTGCAGAC
PE-RAREoct Reverse	KpnI	GGTACCCTCTAGACGGGTGGGTAAGCAAGA ACTG
RAREoct Forward	XhoI	CTCGAGCTCTAGACGGGTGGGTAAGCAAGA ACTG
RAREoct Reverse	KpnI	GGTACCCTCTAGACGGGTGGGTAAGCAAGA ACTG
DE Forward	XhoI	AATTCTCGAGAGGGCACATCTGTTTCAAGC
DE Reverse	KpnI	AATTGGTACCAGGAAGGGCTAGGACGAGAG
DE-PE Forward	XhoI	AATTCTCGAGAGGGCACATCTGTTTCAAGC
DE-PE Reverse	KpnI	AATTGGTACCGTCCTTACAGCCCACTCAGC

Table 2.10 Primers used for the cloning of Oct4 regulatory elements into luciferase reporter vectors

2.6.1.2 Constructs which test enhancer function

To generate fragments consisting of various elements within the upstream region of Pou5f1, a forward primer was designed at 2190bp upstream of the transcriptional start site (TSS). The reverse primers were designed at 1985bp, 383bp and 11bp before the TSS. The forward and reverse primers were flanked with XhoI and KpnI restriction sites respectively (**Table 2.10**).

The steps for cloning are similar to 2.6.1.1, except the plasmid employed was pGL4.23 (Promega) which comprised of a minimal promoter sequence before the luciferase reporter.

2.6.2 Transfection of constructs

To perform luciferase assays, MEFs were transfected with the described luciferase constructs. MEFs were thawed and expanded as previously described. When 70% confluent, the cells were trypsinised and collected as described and 1 million cells were used for each transfection. To explore the effect of cDNAs on luciferase activity, 10μg of each cDNA and 10μg of luciferase reporters were used for transfection. 1μg of TK-Renilla was co-introduced for the normalization of firefly luciferase readings. Transfection was performed as described earlier and resuscitated cells were transferred into a fresh 6-well gelatin coated plate pre-fed with M10. The experiment was conducted in triplicates.

2.6.3 Performing the assay

Luciferase assays were conducted according to manufacturer's instructions. Dual-Luciferase® Reporter Assay System (Promega) was used for this purpose. After 48 hours, unless otherwise specified, the cells were washed with PBS twice and lysed using the buffer provided. The cells were rocked at room temperature for 20 minutes and 20μl of the lysate was transferred to a 96-well luminometry plate. To obtain luciferase readings, 100μl of Luciferase Assay Reagent was added to each well and the plate was placed in

MicroLumat Plus LB 96V (Berthold Technologies). WinGlow was the program used to read the luciferase activity. After the firefly luciferase readings were taken, 100μl of Stop&Glo Reagent was added to quench the firefly luciferase activity and trigger Renilla activity. WinGlow was also used to determine the level of Renilla activity.

2.7 Manipulation of ZHBTc4 ESCs

2.7.1 Differentiation of ZHBTc4 ESCs

Differentiation of ZHBTc4 ESCs was performed under similar conditions to 4F and 6F iPSCs. ZHBTc4 ESCs were maintained in 6-well plates. Once confluent, the cells from one 6-well were trypsinised and seeded onto one gelatin coated 10-cm plate. The cells were differentiated in M10, supplemented with 0.1μM all-trans retinoic acid and 1μg/ml doxycycline, for 6 days.

2.7.2 Transfection of potential rescue constructs

After 6 days of differentiation, ZHBTc4 cells were trypsinised and collected as previously described. cDNAs were analysed for their ability to reprogram these differentiated cells in the presence or absence of doxycycline. To address this, cDNAs were introduced at 1μg per construct and appropriate cDNA combinations were transfected into 1 million ZHBTc4 cells. Transfected cells were divided into two 6-wells that were lined with SNL76/7 feeder cells. In one well, doxycycline was added at 1μg/ml, whereas the other well was left doxycycline-free.