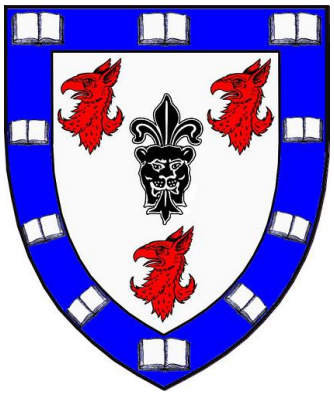
The Role of Retinoic Acid Receptors in the Replacement of Oct4 during the Generation of Induced Pluripotent Stem Cells

Ooi Yu Zhu, Jolene



This dissertation is submitted for the degree of Doctor of Philosophy

Homerton College, University of Cambridge

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DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text and acknowledgements.

This thesis does not exceed the word limit of 60,000 as set by the Degree Committee for the Faculty of Biology.

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ABSTRACT

Reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) has been accomplished through the delivery of four transcription factors, Oct4, c-Myc, Klf4 and Sox2. Although the reprogramming capacity of the four genes has been recapitulated across cell types and species, the molecular intricacies involved in the reprogramming process are not fully delineated. To address this, genetic analyses have been conducted to identify factors that can replace or enhance the defined set of four genes. Of the reprogramming cocktail, Oct4 has been most recalcitrant to replacement, as suggested by the inability of its family members, Oct1 and Oct6, to act as substitutes. The importance of Oct4 is highlighted in its obligatory need during development and embryonic lethality by ablation of Oct4. To decipher the role of Oct4 during the establishment of pluripotency, this project aims to identify genetic replacements of exogenous Oct4 during the generation of iPSCs using a genome-wide piggyBac transposon-assisted mutagenesis approach.

Transposons are mobile genetic elements that integrate into the host genome in a random fashion. The consequent disruption of genes can facilitate the identification of critical genes in a process-of-interest, and acts as the basis to my project. Mouse fibroblasts were mutagenised and assessed for their ability to generate iPSCs in the presence of ectopic c-Myc, Klf4 and Sox2. Basonuclin-2 (BNC2) and Retinoic Acid Receptor beta (RARβ) were identified to be novel candidate genes involved in reprogramming. Both genes were validated and able to generate iPSCs from mouse fibroblasts in cooperation with c-Myc, Klf4 and Sox2, hence abolishing the need for the ectopic expression of Oct4. To demonstrate the pluripotent potential of these iPSCs, I showed that these iPSCs resembled ESCs, and displayed the capacity of contributing to both somatic lineages and the germline, when introduced into mouse blastocysts.

As the retinoic acid pathway was recently implicated in the reprogramming process, the molecular mechanism behind the action of RARβ was pursued. Bioinformatics and experimental data have described several Retinoic Acid Response Elements (RAREs) in the

enhancer and promoter regions of Oct4. Given that RAREs are motifs recognisable by Retinoic Acid Receptors, I demonstrated that RARβ binds to the RARE within the distal enhancer of Oct4 and activates Oct4 expression. I also examined RARγ, another RAR family member, which has been described to promote reprogramming speed and efficiencies in synergy with LRH1. Similar to RARβ, RARγ also had the capacity to positively regulate Oct4 levels through the Oct4 distal enhancer. Moreover, a combination of RARγ, LRH1, Oct4, c-Myc, Klf4 and Sox2 (6F) represents the most efficient was to activate Oct4 expression in the luciferase assay.

As the 6F reprogramming cocktail (RARγ, LRH1, Oct4, c-Myc, Klf4, Sox2) improves the speed, quality, and efficiency of reprogramming, I next investigated if these properties were partly attributed to rapid epigenetic changes at the Oct4 distal enhancer. By examining this enhancer locus over the first three days of reprogramming, I demonstrated that the region was associated to activating histone marks within 24 hours of the ectopic expression of 6F, drawing a parallel to rapid reprogramming. In contrast, the Oct4 distal enhancer remained silent for the first 3 days when only four reprogramming factors (Oct4, c-Myc, Klf4, Sox2) were used. These findings represent a unique approach to dissect the kinetics of reprogramming through the observation of epigenomic changes at the Oct4 locus, culminating in the better understanding of molecular events during nuclear reprogramming.

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ABBREVIATIONS

2i	2 inhibitors (CHIR99021 and PD0325901)
4F	Oct4, c-Myc, Klf4, Sox3
6F	Oct4, c-Myc, Klf4, Sox2, RARγ, LRH1
Ac	Activator
ADH	Alcohol Dehydrogenase
AID	Activation-Induced cytidine Deaminase
AP	Alkaline Phosphatase
ATRA	All-Trans Retinoic Acid
AZA	5-aza-cytidine
BAF	Brahma-related gene 1-Associated Factors
BMP	Bone Morphogenetic Protein
BNC1	Basonuclin 1
BNC2	Basonuclin 2
bp	base pairs
CAG	Composite CMV early enhancer/chicken beta actin promoter
Cald1	Caldesmon 1
CAT	Chloramphenicol AcetylTransferase
cDNA	complementary Deoxyribonucleic Acid
ChIP	Chromatin Immunoprecipitation
CKS	c-Myc, Klf4, Sox2

CKS-B	c-Myc, Klf4, Sox2, BNC2
CKS-Rβ	c-Myc, Klf4, Sox2, RARβ
cM	centimorgan
CMV	Cytomegalovirus
CR	Conserved Region
CRABP	Cellular Retinoic Acid Binding Protein
CRBP	Cellular Retinol Binding Protein
DBD	DNA Binding Domain
DCAF5	DDB1-CUL4A-associated factor 5
DE	Distal Enhancer
DNA	Deoxyribonucleic Acid
DOXYCYCLINE	Doxycycline
dpc	days post coitum
DR	Direct Repetition
Ds	Dissociator
EC cells	Embryonic Carcinoma cells
EG cells	Embryonic Germ cells
EGFP	Enhanced Green Fluorescence
ENU	N-ethyl-N-nitrosourea
EpiSCs	Epiblast Stem cells
ER	Estrogen Receptor

ESCs	Embryonic Stem cells
ESRRβ	Estrogen Receptor Related Receptor-beta
FA	Fanconi's Anemia
FGF	Fibroblast Growth Factor
FX	Fragile X
Gb	Gigabases
H3K4me1	monomethylated lysine 4 of histone 3
H3K4me2	dimethylated lysine 4 of histone 3
H3K4me3	trimethylated lysine 4 of histone 3
HBV	Hepatitis B Virus
HSV	Herpes Simplex Virus
Id	Inhibitor of differentiation
iPSCs	Induced Pluripotent Stem cells
IRES	Internal Ribosomal Entry Site
kb	kilobases
Klf4	Kruppel-Like Factor 4
LBD	Ligand Binding Domain
LIF	Leukemia Inhibitory Factor
lincRNA	large intergenic non-coding Ribonucleic Acid
LRH1	Liver Receptor Homologue
MAPK	Mitogen Associated Protein Kinase

Mb	Megabases
MEF	Mouse Embryonic Fibroblasts
MET	Mesenchymal-to-Epithelial Transition
mFx	murine factors
MHC	Major Histocompatibility Complex
minP	minimal promoter
mRNA	messenger Ribonucleic Acid
MSCV	Murine Stem Cell Virus
OCKS	Oct4, c-Myc, Klf4, Sox2
Oct4	Octamer-binding transcription factor 4
PB	piggyBac
PBase	piggyBac transposase
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PE	Proximal Enhancer
PGCs	Primordial Germ Cells
PGK	Phosphoglycerate Kinase
PI3K	Phosphoinositide 3-Kinase
POU	Pit-Oct-Unc
PP	Proximal Promoter
RA	Retinoic Acid

RALDH	Retinaldehyde Dehydrogenase
RARα	Retinoic Acid Receptor alpha
RARβ	Retinoic Acid Receptor beta
RARE	Retinoic Acid Response Element
RARγ	Retinoic Acid Receptor gamma
RBP4	Retinol Binding Protein 4
RDH	Retinol Dehydrogenase
RNA	Ribonucleic Acid
RORα	Retinoic Acid Receptor-related Orphan Receptor alpha
RT	Reverse Transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
rtTA	Reverse Tetracycyline Transactivator
RXR	Retinoic X Receptor
SA	Splice Acceptor
SCNT	Somatic Cell Nuclear Transfer
SD	Splice Donor
SF	Steel Factor
SF1	Steroidogenic Factor 1
Sox2	SRY (sex determining region Y)-box 2
TAF	TBP Associated Factors
TALEs	transcription activator-like effectors

TBP	TATA Binding Proteins
Tc	Tetracycline
tetR	tet Repressor
TGFβR	Transforming Growth Factor beta Receptor
TRE	Tetracycline Response Element
TSS	Transcriptional Start Site
tTA	Tetracycline Transactivator
UTR	Untranslated Regions
VPA	Valproic Acid