Chapter 05: Analysis of altered cell cycle kinetics during reprogramming utilising the inducible OctER
5.1 Introduction

The inducible OctER would make a useful probe to study the changes that occur in the early stages of reprogramming by creating a synchronised reprogramming system. The synchronised system would enable studies in to the mechanism of the cell cycle switch that occurs during the initiation phase. Further, these studies could give us insight into the role of two of the core pluripotency factors in maintaining the rapid cell cycle seen in embryonic stem cells.

In this chapter I present results of reprogramming using OctER. Prior to testing this inducible system, I standardised all the reagents and protocols required for robust reprogramming. I first derived MEFs from an Oct-eGFP mouse that would enable easy visualisation of successful reprogramming. I successfully reprogram the Oct-eGFP MEFs to iPSC which I then show are pluripotent using various assays. I study the effect of the Yamanaka factors on MEFs cell proliferation and show a correlation with Cyclin A activity. Finally via gene expression studies, I show that Oct-3/4 and Klf4 along with initiating the cell cycle switch during the initial stages of reprogramming, also initiate the Mesenchymal to Epithelial transition (MET).

5.2 Results

5.2.1 Derivation of Oct-eGFP MEFs (OGM)

During reprogramming of MEFs to iPSC, many cells acquire a partially reprogrammed state that are morphologically similar to mES cells yet do not express any of the pluripotency factors such as endogenous Nanog or Oct-3/4 (Takahashi and Yamanaka, 2006; Mikkelsen et al., 2008). Visual differentiation between partially reprogrammed and completely reprogrammed cells is therefore difficult. Methods to identify successfully reprogrammed cells include the use of drug selection as was used in the original Yamanaka protocol or live staining for cell surface markers such as SSEA-1 which are costly (Takahashi and Yamanaka, 2006).

One method to overcome this issue is the use of MEFs containing pluripotency reporter lines such as Oct-eGFP or Nanog-eGFP (Szabo et al., 2002; Brambrink et al., 2008). These lines express eGFP only when the endogenous Oct-3/4 or Nanog loci are activated, a read-
out of successful reprogramming. In this study, MEFs derived from an Oct-eGFP transgenic mouse were used. The transgene contained the Oct-3/4 distal enhancer and promoter driving eGFP expression (Szabo et al., 2002).

Oct-eGFP mice were originally generated by Hans Schöler and kindly provided by Richard Harvey, VVCRI Australia, under permission from Hans Schöler.

All mice experiments were approved by the inStem/NCBS animal ethics committee and were performed under justified guidelines for animal use.

A male C57BL/6 heterozygous for Oct-eGFP was crossed with a super ovulated female 129X1/SvJ/NCBS (an inbred strain of 129X1/Sv) maintained at the NCBS animal facility. 13.5 days post coitum, the female was sacrificed by cervical dislocation and the embryos isolated from the uterine horn. As the cross was between a heterozygous mouse with a wild type mouse, each of the embryos were processed separately for tissue homogenisation. The cell suspension was then plated at the ratio of 1 embryo to two 15 cm dishes and cultured for five days after which they were cryopreserved. A small aliquot of cells were kept for genotyping.

![Figure 5.1 Genotyping of Oct-eGFP MEFs](image)

**Figure 5.1 Genotyping of Oct-eGFP MEFs**

Well numbers are indicated on the top of each lane. Gel loading order, embryo 1 to 8, 100bp ladder, embryos 9 to 15, eGFP positive control. Expected band size for embryos positive for eGFP was 187bp and are highlighted with *. The 100bp and 200bp bands of the DNA ladder are marked.
Genotyping was performed using a modified protocol as described in (Laird et al., 1991). 6 out of the 15 embryos tested positive for eGFP (Figure 5.1). MEFs derived from eGFP positive embryos were used for reprogramming while MEFs derived from eGFP negative embryos were used for all the other studies to ensure that the effect of different genetic backgrounds did not influence the results.

5.2.2 Standardisation of viral production

Primary cell cultures such as MEFs are difficult to transfect with plasmid DNA, exhibiting very low transfection efficiencies (Lee et al., 2017). To overcome the low transfection efficiency, viral transductions were used as a gene delivery system. Along with high transduction efficiencies, viral transductions have the added advantage of stable genome integration (Kim and Eberwine, 2010), and the ability to modulate the multiplicity of infection (MOI), restricting the number of copies per genome.

5.2.2.1 Standardisation of plasmid transfection

To transfec 293LX for lentiviral packaging or Plat-E for retroviral packaging, three methods were tested

1) Calcium phosphate based transfection method using 2X BES (Sigma, cat#14280) (Sambrook et al., 1989; Kingston et al., 2003)
2) Lipofectamine LTX (Invitrogen) based transfection method
3) Polyethylenimine (PEI) (Polysciences Inc) based transfection modified from (Boussif et al., 1995; Reed et al., 2006; Longo et al., 2013)

1 μg of FUGW (Addgene plasmid# 4883), a vector containing a constitutively expressed eGFP was transfected per 10 cm dish (~2 x 10⁷ cells) using the three transfections methods. 24 hours post transfection, the cells were imaged to visualize eGFP expression. Calcium phosphate based transfection displayed the lowest transfection efficiency with < 10% of the cells expressing eGFP (Figure 5.2). Both Lipofectamine LTX and PEI displayed similar transfection efficiencies with the cells expressing >50% efficiency (Figure 5.2). Due to the cost effective nature of PEI and similar transfection efficiencies, PEI was used for all subsequent transfections.
Figure 5.2 Transfection efficiencies using different transfection protocols
Representative images displaying transfection efficiencies using different transfection methods. Viral packaging cells were transfected with FUGW using three different transfection methods. Calcium phosphate based transfection displayed the lowest transfection efficiency with < 10%, while Lipofectamine LTX and PEI displayed similar transfection efficiencies of >50% efficiency. Scale bar is 20 µm.

5.2.2.2 Optimisation of viral titre
To enable high transduction efficiencies in MEFs would require the use of high viral titres. For this, the amount of DNA used for viral packaging was adapted after considering several protocols for optimised viral production (Sena-Esteves et al., 2004; Mitta et al., 2005; Segura et al., 2006; Takahashi et al., 2007a). For retroviral packaging, 30 µg of DNA was transfected per 15 cm dish and for lentiviral packaging, 44 µg total DNA was transfected per 15 cm dish. A ratio of 2:1:1 for lentiviral expression vector (~24 µg) to packaging vector (~12 µg) to envelope vector (~8 µg) was maintained. To further ensure higher viral titres, the virus-containing supernatant was concentrated using Amicon Ultra-15 centrifugal filter units, 100MWCO (Millipore, cat# UFC910024) (Figure 5.3).
Viral titres were calculated using serial dilution of concentrated viral supernatant on MEFs. The cells were imaged 48 hours later and viral titre calculated. A general yield of $10^6$ Transducing Units (TU)/ml per 15cm dish was achieved. With this, an optimised viral production method for MEF transduction was standardised.

![Figure 5.3 Transduction efficiency before and after viral supernatant concentration](image)

MEFs were transduced with equal volumes of viral supernatant containing eGFP viruses before and after concentration. 48 hours later, the MEFs were imaged for eGFP. Scale bar is 20 µm

### 5.2.3 Standardisation of LIF concentrations to maintain mES

mES cells require the cytokine Leukaemia Inhibitory Factor (LIF) to prevent differentiation and maintain pluripotency (Smith et al., 1988; Williams et al., 1988). LIF functions by activating the JAK-STAT signalling cascade which leads to the activation of STAT3. STAT3 is translocated to the nucleus where it activates a network of genes required to maintain the undifferentiated pluripotent state (Matsuda et al., 1999).

LIF was produced in-house by transfecting Cos7 cells with a LIF expressing plasmid (Smith, 1991). LIF secreted into the conditioned media by the cells was harvested daily
for a week. The LIF-containing supernatant was centrifuged, filtered through a 0.1 μm PVDF filter (Nalgene) to remove cell debris, aliquoted and stored at -20°C.

To test the amount of LIF supernatant require to maintain mES in an undifferentiated state, 10,000 E14 cells (a feeder free mES cell line) were seeded per well in a 6 well dish that was coated with 0.1% gelatin (Hooper et al., 1987). The cells were grown in media containing different dilutions of LIF supernatant. The media was changed daily for a week after which cell morphology was examined. The dilution which maintained the mES cells with the least differentiation as judged by visual examination was noted.

Pluripotency was further confirmed by staining for alkaline phosphatase (AP) (Figure 5.4). Colonies that appeared compact and had strong AP staining were considered positive (Figure 5.4). Colonies that appeared diffuse and lightly stained were considered negative (Figure 5.4). The dilution of LIF containing media which gave the least differentiation was considered to contribute most LIF and was used for all subsequent experiments.
Figure 5.4 Alkaline phosphatase staining to determine pluripotency in different dilutions of LIF

E14 were seeded in a 6 well dish and cultured in media containing different dilutions of LIF containing supernatant. Media was changed daily and the cells were stained for alkaline phosphatase (AP) after a week. Positive colonies (inset A) and negative colonies (inset B) were counted. The dilution which gave the least number of negative colonies was used for subsequent experiments.

5.2.4 Standardisation of reprogramming

The protocol to reprogram Oct-eGFP MEFs (OGM) to iPS was adapted from the original Yamanaka protocol (Takahashi et al., 2007a). Equal volumes of concentrated viral supernatant containing Oct-3/4, Klf4, Sox2 and c-Myc were used. Viral titres of $10^5$ TU/ml were used and a multiplicity of infection (MOI) of 1 was maintained for each factor. 48 hours post transduction, the cells were trypsinised and replated onto 0.1% gelatin coated plates containing mitotically inactivated feeders. The cells were maintained in MEF media (DF10) for the first 2 days after which media was switched to ES media (ESM). Daily media changes were performed for 21 days after which the cells were fixed and stained for AP.
**Figure 5.5 Inefficient reprogramming of Oct-eGFP MEFs to iPSC**

AP staining of untransduced Oct-eGFP and OKSM transduced Oct-eGFP. Arrow A & C indicate partially reprogrammed colonies, arrow B indicates a successfully reprogrammed colony. Inset D is a magnification of a partially reprogrammed colony, inset E is magnification of a partially reprogrammed and successfully reprogrammed. OGM – Oct-eGFP MEFs, OKSM – Oct-3/4, Klf4, Sox2, c-Myc

Initial reprogramming experiments yielded mostly partially reprogrammed cells and very few completely reprogrammed cells as indicated by diffuse AP staining (Figure 5.5). mES cells display a compact and intense AP stain (Figure 5.4). To reduce the frequency of partially reprogrammed cells, the media composition was modified. KnockOut serum replacement (Invitrogen, cat# 10828028) was used instead of FBS. KnockOut serum replacement (KSR) is a proprietary serum replacement specifically designed for culturing embryonic stem cells (https://www.thermofisher.com/order/catalog/product/10828028). Use of KSR during reprogramming increases reprogramming efficiency along with reducing partially reprogrammed cells (Bleloch et al., 2007; Okada et al., 2010). Unfortunately, MEFs cannot tolerate serum free KSR containing media for long durations, resulting in cell death. MEFs are generally maintained in media containing 10% FBS, so a compromise was reached by reducing the concentration of FBS to 5% in the reprogramming media. DMEM/F12 was used instead of KnockOut DMEM as it was more cost effective and could maintain mES cells equally well. The modified composition of reprogramming media (DFKSR) was DMEM/F12 + 15% KSR + 5% FBS + Penicillin/Streptomycin + Glutamax + NEAA + LIF + β-Mercaptoethanol.

The MOI was also increased to improve reprogramming. Increasing expression of the Yamanaka factors in partially reprogrammed cells leads to increased reprogramming to iPSC (Polo et al., 2012). In initial experiments a MOI of 1 for each factor was maintained was increased to MOI of 10.

Reprogramming was repeated with the modifications (Figure 5.6). OGM were transduced with equal volumes of concentrated viral supernatant containing Oct-3/4, Klf4, Sox2 and
c-Myc. Higher viral titres of $10^6$ TU/ml were used and a MOI of 10 for each factor was maintained. 48 hours later, the cells were trypsinised and replated onto 0.1% gelatin coated plates containing mitotically inactivated feeders. The cells were maintained in MEF media (DF10) for 2 days after which it was switched to the modified reprogramming media DFKSR. Daily media changes were performed for 21 days after which the cells were fixed and stained for alkaline phosphatase. Reprogramming efficiency increased 100 fold from 0.00001% to 0.001% which was in line with the original Yamanaka protocol (Takahashi et al., 2007a) (Figure 5.7). There was a marked increase in the number of successfully reprogrammed cells as represented by iPS cells expressing eGFP, which also exhibited intense AP staining (Figure 5.8) and a general reduction in the number of partially reprogrammed cells with diffuse AP staining (Figure 5.7). With this, an optimised reprogramming protocol was standardised which allowed for successful reprogramming.

Transduce Oct-eGFP with equal volumes of concentrated viral supernatant (MOI of 10) containing Oct-3/4, Klf4, Sox2, c-Myc

48 hours later trypsinise cells, seed onto 0.1% gelatin coated plates with feeders

Maintain cells in MEF media for two days

Change media to DFKSR

Daily media change for 21 days

Stain for Alkaline phosphatase

**Figure 5.6 Flowchart depicting optimised reprogramming protocol**
Figure 5.7 Successful reprogramming of Oct-eGFP to iPS
AP staining of OKSM transduced Oct-eGFP successfully reprogrammed using the modified reprogramming protocol. Arrow A indicates successfully reprogrammed colony, arrow B a partially reprogrammed colony. Inset is a magnification of the image.

Figure 5.8 AP positive iPS colonies express eGFP
AP positive iPS reprogrammed using OKSM expressed eGFP, a marker of successful activation of endogenous Oct-3/4. Scale bar is 0.2 mm.
5.2.5 Reprogramming OGM using OctER

To reprogram Oct-eGFP MEFs (OGM), equal proportions of concentrated viral supernatant containing a combination of OctER, Klf4, Sox2 & c-Myc (OE+KSM) or Oct-3/4 Klf4, Sox2 & c-Myc (OKSM) with a MOI of 10 was used (Figure 5.9). 48 hours later, the cells were trypsinised and replated onto 0.1% gelatin coated plates containing mitotically inactivated feeders. A small aliquot of cells was also seeded onto coverslips to test for transduction efficiency. ~90% transduction efficiency was achieved as determined by staining for OctER or Oct-3/4 (Figure 5.10).

Transduce Oct-eGFP with equal volumes of concentrated viral supernatant (MOI of 10) containing

Oct-3/4, Klf4, Sox2, c-Myc or OctER, Klf4, Sox2, c-Myc

48 hours later trypsinise cells, seed onto 0.1% gelatin coated plates with feeders

Pulse cells daily with either 100 nM OHT (induced) or ethanol (uninduced/control)

Maintain cells in MEF media (DF10) for two days

Change media to DFKSR

Daily media change for 21 days

Stain for Alkaline phosphatase

Figure 5.9 Flowchart depicting OctER reprogramming protocol used
Figure 5.10 Transduction efficiency of ~90% were achieved for OctER & Oct-3/4

10,000 transduced Oct-eGFPs were seeded onto 13mm No. coverslips. The cells were pulsed with either 100 nM OHT (induced) or ethanol (control). 24 hours later, cells were fixed and immunostained for an antibody against Oct-3/4. 90% cells were positive for OctER or Oct-3/4. Scale bar is 20 μm.

The cells were maintained in MEF media (DF10) for 2 days after which media was switched to the modified reprogramming media DFKSR. Daily media changes were performed for 21 days after which the cells were fixed and stained for alkaline phosphatase. The cells were pulsed daily with either 100 nM OHT or ethanol (vehicle control). 100 nM OHT was used for induction as it displayed maximal activity in the Nanog promoter based luciferase reporter assay (Figure 3.18).

Morphological changes in cells were seen from day 5 onwards (Figure 5.11) and cells expressing eGFP started appearing from 10 day onwards. The plates were processed for AP staining 21 days later after which AP positive colonies were counted. Untransduced OGM were negative for AP positive colonies, while OE+KSM uninduced displayed partially reprogrammed AP positive colonies (Figure 5.12). Both O+KSM and OE+KSM induced had successfully reprogrammed OGM to iPS though OE+KSM reprogrammed at a lower efficiency as compared to O+KSM (Figure 5.13). OE+KSM-iPS colonies that were
positive for AP staining were also confirmed for eGFP expression indicating successful reprogramming (Figure 5.14).

**Figure 5.11 Morphological changes seen during early stages of reprogramming**

During the early stages of reprogramming, fibroblasts morphology changed. There was a compaction in the cell size, reduction of the cytoplasm volume and the nuclei was more prominent. Normal fibroblasts as can be seen in the foreground displayed large cytoplasm and generally spread out cell morphology. Scale bar is 20 μm.
Figure 5.12 OctER successfully reprograms Oct-eGFP to iPS

AP staining was used to test for successful reprogramming. (A) Untransduced Oct-eGFP did not display an AP positive colonies. (B) OE+KSM uninduced displayed a few partially reprogrammed colonies as depicted by arrow F. (C) & (D) O+KSM and OE+KSM induced successfully reprogrammed Oct-eGFP to iPS as indicted by AP staining (arrow G & H indicate completely reprogrammed iPS).

Figure 5.13 Comparing reprogramming efficiencies of OctER to Oct-3/4

50,000 Oct-eGFP were transduced with a viral cocktail containing either OctER, Klf4, Sox2, c-Myc (OE+KSM) or Oct-3/4, Klf4, Sox2, c-Myc (O+KSM). The media was changed daily and the cells were pulsed with 100 nM OHT (OE+KSM induced) or ethanol (OE+KSM uninduced/O+KSM). 21 days later, the plates were stained for AP. Only those compact, intensely stained AP colonies were considered positive. Values represent mean ± SEM, N=2.

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Figure 5.14 AP positive OE+KSM-iPS colonies express eGFP
AP positive iPS reprogrammed using OE+KSM expressed eGFP, a marker of successful activation of endogenous Oct-3/4. Scale bar is 0.2 mm.

5.2.6 Characterisation of OE+KSM-iPS
3 OE+KSM-iPS colonies were selected at random and processed for characterisation. They were passaged 10 times in the absence of OHT on feeders to determine if they were truly reprogrammed. After 10 passages, all the colonies exhibited eGFP indicating that they were now OHT independent and completely reprogrammed (Figure 5.15). These independently derived colonies of iPS were then used for characterisation as described.

Figure 5.15 OE+KSM-iPS are OHT independent and truly reprogrammed
OE+KSM-iPS were eGFP positive even after 10 passages in the absence of OHT. Scale bar is 20 μm
5.2.6.1 Gene expression

OE+KSM-ips cells were converted to feeder-free cultures by passaging them without feeders for 3 passages. They were positive for eGFP and a visual check confirmed the absence of residual feeder cells. RNA was isolated from the cells, cDNA synthesised and qRT-PCR performed for somatic and pluripotency markers. As a control, E14 feeder-free mES cells were used. All gene expression data was normalised to the expression values in OGM control cells and fold change \(2^{-\Delta\Delta C_t}\) calculated.

OE+KSM-ips tested negative for fibroblast markers N-cadherin, Snail, Slug and Thy1 (Figure 5.16). They were positive for pluripotency markers E-cadherin, Dppa3, Zfp42, Nanog (endogenous), Oct-3/4 (endogenous) and Sox2 (endogenous) (Figure 5.17 & Figure 5.18).

![Gene expression graph](image)

**Figure 5.16 OE+KSM-ips are negative for somatic cell markers**

Gene expression analysis was performed on feeder free OE+KSM-ips colonies. Fold change \(2^{-\Delta\Delta C_t}\) was calculated with respect to Oct-eGFP. E14, a feeder free mES cell line was used as a positive control. Values represent mean ± SEM, N=3, * p < 0.05
Figure 5.17 OE+KSM-iPS are positive for key pluripotency markers
Gene expression analysis was performed on feeder free OE+KSM-iPS colonies. Fold change \((2^{-\Delta\Delta CT})\) was calculated with respect to OGM. E14, a feeder free mES cell line was used as a positive control. Values represent mean ± SEM, N=3, * p < 0.05

Figure 5.18 OE+KSM-iPS are positive for key pluripotency markers
Gene expression analysis was performed on feeder free OE+KSM-iPS colonies. Fold change \((2^{-\Delta\Delta CT})\) was calculated with respect to OGM. E14, a feeder free mES cell line was used as a positive control. Values represent mean ± SEM, N=3, * p < 0.05
5.2.6.2 Immunostaining

OE+KSM-iPS were seeded onto 0.1% gelatin coated coverslips containing feeders. 24 hours later, they were fixed and stained for the pluripotency marker Nanog and ES cell surface marker SSEA-1. OE+KSM-iPS stained positive for both (Figure 5.19).

![Immunostaining Images]

**Figure 5.19 OE+KSM-iPS are positive for Nanog and SSEA-1**

OE+KSM-iPS were seeded onto 0.1% gelatin coated coverslips containing feeders. 24 hours later, they were fixed and stained for the pluripotency marker Nanog and ES cell surface marker SSEA-1. OE+KSM-iPS stained positive for Nanog and SSEA-1. The mitotically inactivated MEF feeders were used as a negative control. Scale bar is 20 μm.

5.2.6.3 Teratoma assay for differentiation into three germ layers

All mice experiments were approved by the inStem/NCBS animal ethics committee and were performed under justified guidelines for animal use.

OE+KSM-iPS cells were converted to feeder-free cultures by passaging them without feeders for 3 passages. They were positive for eGFP and a visual check confirmed the absence of residual feeder cells. The cells were then trypsinised, washed in DPBS twice, resuspended in DPBS and a viable cell count was performed. 5x10^6 viable OE+KSM-iPS cells were used for the teratoma assay. The cells were centrifuged and resuspended in 200μl DPBS after which they aspirated into insulin syringes.
Two month old Nude mice were used for the teratoma assay. The mice were certified healthy by the institute veterinarian before proceeding with the injections. Animal house staff handled the mice at all times ensuring sterile conditions.

The cell suspension was injected sub cutaneous between the hind limb and fore limb and the mice marked with ear clippings for identification. A small bump was seen on the mice two weeks post injection which was monitored for another two weeks. On the fourth week, the teratomas had an average measurement of $\sim 11$ mm x $\sim 9$ mm as measured using a Vernier calliper. The mouse was sacrificed by cervical dislocation and the teratoma surgically excised (Figure 5.20).

All teratomas showed clear vascularisation and were solid. The teratomas were cut in to four parts and each part was fixed in 4% PFA overnight at 4°C. One part from each of the teratomas was paraffin embedded, 5 µm thick sections were cut from the paraffin embedded teratoma block using a Leica microtome and stained for standard histological examination using Haematoxylin & Eosin (H & E).

The stained sections were analysed for tissue morphology and structures related to the three germ layers were identified in consultation with a mouse pathologist. Each of the teratomas assayed displayed endodermal, mesodermal and ectodermal structures (Figure 5.21).

These results indicate that the OE+KSM-iPS created using OctER were truly pluripotent.
Figure 5.20 OE+KSM-iPS can form teratomas in Nude mice
A four week old teratoma formed by OE+KSM-iPS cells was excised from a Nude mouse. Clear vascularisation was seen and the tumour was solid. Ruler depicts cm.

Figure 5.21 H & E staining of OE+KSM-iPS teratoma section
5 μm thick sections were cut from the paraffin embedded teratoma block and stained using H&E. The sections were analysed for tissue morphology and the different germ layers were identified. Representative image of sections depicting ectoderm (keratinocyte rosette), mesoderm (striated muscle) and endoderm (ciliated columnar epithelium). White arrows indicate defining feature identified for each germ layer.
5.2.7 Analysis of cell proliferation of MEFs after transduction of the Yamanaka factors

To explore the role of the Yamanaka factors in the cell cycle switch, their effect on MEF proliferation was tested using a Water Soluble Tetrazolium Salt (WST-1) based cell proliferation assay that measures the activity of mitochondrial dehydrogenases, an indicator of viable and metabolically active cells.

5.2.7.1 Effect of individual factors

MEFs were transduced with concentrated viral supernatant containing either of the Yamanaka factors or an empty vector (pLVX-puro) to serve as a control. 48 hours later, the MEFs were split and seeded in triplicate in a 96 well plate for four time points (day 0, 1, 3, & 5). During the reprogramming process, morphological changes were first noticed from day 5 onwards (Figure 5.11) indicating that the initiation stage which involves the mesenchymal to epithelial transition may have occurred during this period (Li et al., 2010; Samavarchi-Tehrani et al., 2010; Polo et al., 2012). Day 0 was considered 24 hours post seeding, and each subsequent time point was calculated with reference to this. The MEFs were maintained in MEF media (DF10) and the media was changed daily.

c-Myc, a known oncogene increased the MEF proliferation as has been reported (Bretones et al., 2015) (Figure 5.22). Oct-3/4 had a negative effect on MEF proliferation rate with a subsequent reduction in proliferation as has been reported (Hochedlinger et al., 2005) (Figure 5.22). Interestingly Sox2 also had a negative effect on MEF proliferation rate with a subsequent reduction which has not been reported so far (Figure 5.22). Klf4 effect on MEF proliferation was not as severe Oct-3/4 or Sox2 (Figure 5.22). The empty vector also reduced MEF proliferation which suggested that viral transductions in general affected MEF proliferation (Figure 5.22). Due to this, all subsequent proliferation assays were compared to empty vector.
Figure 5.22 Proliferation of MEFs while expressing various Yamanaka factors

MEFs transduced with c-Myc, a known oncogene showed the highest proliferation rates while MEFs transduced with either Oct-3/4 or Sox2 showed a drastic drop in proliferation. Klf4 transduced MEFs also showed a decrease in proliferation but not at the same levels of Oct-3/4 or Sox2. The empty vector also reduced proliferation but not to the same level as that of Oct-3/4 or Sox2. Values represent mean ± SEM, N=3

5.2.7.2 Effect of Oct-3/4 & Klf4

As somatic cells can be reprogrammed in the absence of c-Myc, it is thought that c-Myc is most likely not essential for the cell cycle switch (Nakagawa et al., 2008; Wernig et al., 2008). Sox2 which is mostly important for the maturation phase of iPS reprogramming had a negative effect on proliferation, hence the effect of Klf4 and Oct-3/4 were studied in detail.

The effect of combined expression of Oct-3/4 & Klf4 on MEF proliferation was subsequently tested using the same protocol as in (Figure 5.22). MEFs were transduced with equal volumes of Oct-3/4 & Klf4 containing concentrated viral supernatant and their proliferation potential studied. MEFs expressing both Oct-3/4 & Klf4 showed an increase in proliferation rate, in contrast to their inhibitory effect when expressed alone (Figure 5.23). This novel finding suggests that Oct-3/4 & Klf4 together either cooperate or network to induce early effects on rate of proliferation.
Figure 5.23 Proliferation of MEFs while expressing Oct-3/4 and Klf4
MEFs transduced with Oct-3/4 and Klf4 showed an increased rate of proliferation in contrast to their individual effects which inhibited proliferation. Values represent mean ± SEM, N=3.

5.2.7.3 Comparison of OctER to Oct-3/4
The effect of inducible OctER was compared to Oct-3/4 and MEF proliferation was subsequently tested using the same protocol as in (Figure 5.22). MEFs were transduced with equal volumes of OctER or Oct-3/4 containing concentrated viral supernatant and their proliferation potential studied. The time point Day 1 was 24 hours post induction and each subsequent time point was calculated using this as reference. OctER affected MEF proliferation in an inducible manner (Figure 5.24). Uninduced OctER displayed a proliferation rate similar to empty vector control. On induction with 100 nM OHT, the inducible OctER reduced MEF proliferation to the same extent as Oct-3/4 (Figure 5.24). The OctER functioned similar to Oct-3/4, validating the use of OctER as a substitute for Oct-3/4.
Figure 5.24 Comparing MEF proliferation while expressing OctER and Oct-3/4

MEFs were transduced with either Oct-3/4 or OctER. OctER affected MEF proliferation in an inducible manner with uninduced OctER displaying a proliferation rate similar to Empty. On induction with 100 nM OHT, the inducible OctER reduced MEF proliferation in a similar manner to Oct-3/4. Values represent mean ± SEM, N=3

5.2.7.4 Klf4 and OctER affect MEF proliferation in an inducible manner

The combined effect of Klf4 and inducible OctER on MEF proliferation was subsequently tested using the protocol as in (Figure 5.22). MEFs were transduced with equal volumes of Klf4 and OctER containing concentrated viral supernatant and their proliferation potential studied. The time point Day 1 was 24 hours post induction and each subsequent time point was calculated using this as reference. Klf4 and OctER combined affected MEF proliferation in an inducible manner (Figure 5.25). In the uninduced state, there was a reduction in proliferation which even lower than empty vector (Figure 5.25). This affect was reversed with increasing doses of OHT, which showed a subsequent increase in the MEF proliferation rate. These results were in concurrence to the effect of Oct-3/4 & Klf4 combined as seen in (Figure 5.23) which suggests that Oct-3/4 & Klf4 have a synergistic effect on MEF proliferation.

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**Figure 5.25 Klf4 and OctER effect MEF proliferation in an inducible manner**

MEFs were transduced with Klf4 and OctER which affected their proliferation in an inducible amount. With increasing doses of OHT, there was a subsequent increase in proliferation. Values represent mean ± SEM, N=3

**5.2.8 Effect of Yamanaka factors on Cyclin A**

To investigate the role of the individual Yamanaka factors in regulating MEF proliferation, the effect of the Yamanaka factors was tested on expression of Cyclin A. Cyclin A, a positive cell cycle regulator is highly expressed in mES and is also crucial in maintaining the rapid proliferation seen in mES (Stead et al., 2002; Kalaszczyńska et al., 2009). Its expression is controlled by a Cell Cycle Responsive Element (CCRE) present at its promoter (Huet et al., 1996). The CCRE is a negative element that leads to repression of Cyclin A expression in G1 and derepression at G2/S. Further, the bioinformatics analysis done in this thesis indicated that Cyclin A is a direct target of Oct-3/4 & Klf4.

The CCRE region (-500 bp to +209bp) was cloned from the mouse genome and ligated to a luciferase reporter construct (Figure 5.26). Cos-7 cells were used for the assay, and equimolar quantities of either Oct-3/4, Klf4, Sox2 or c-Myc along with a fixed amount of the CCRE luciferase reporter and renilla plasmid were transfected into the cells. 24 hours post transfection, the cells were processed using the Dual-Luciferase Reporter Kit
according to the manufacturer's protocol and luminescence was measured using a multimode plate reader (Perkin Elmer).

c-Myc showed the highest activity while Oct-3/4 strongly suppressed CCRE luciferase activity (Figure 5.27). Both Klf4 & Sox2 also showed very low CCRE luciferase activity, but greater than Oct-3/4 (Figure 5.27). These results are consistent with the WST-1 cell proliferation assays where c-Myc induces greater proliferation, while Oct-3/4, Klf4 & Sox2 inhibit proliferation (Figure 5.27). In addition, these results validate the bioinformatics analysis performed in this study (Figure 4.2 & Figure 4.3) showing that Cyclin A is indeed a target Oct-3/4, which has not been previously reported.

**Figure 5.26 Schematic of CCRE-Luciferase reporter**

-500 bp to +209bp was cloned from the mouse genome and ligated to a luciferase reporter construct. This region contains the CCRE which regulates expression of Cyclin A. Restriction sites used for cloning are specified. Potential binding sites as based on bioinformatics analysis are shown for Oct-3/4 (red) and Klf4 (purple). Image not drawn to scale.
Figure 5.27 Effect of the Yamanaka factors on Cyclin A expression

c-Myc displayed the highest CCRE luciferase activity with Oct-3/4 heavily suppressing CCRE luciferase activity. Both Klf4 and Sox2 showed negligible activity. -500 bp to +209bp was cloned from the mouse genome and ligated to a luciferase reporter construct. This region contains the CCRE which regulates expression of Cyclin A2. Restriction sites used for cloning are specified. Values represent mean ± SEM, N=2

5.2.9 Effect of OctER & Klf4 on Cyclin A

To investigate the combined effect of Oct-3/4 and Klf4 on Cyclin A expression, OctER and Klf4 were co-expressed in the presence of the CCRE luciferase construct. In the uninduced state, there was a decrease in luciferase activity, suggesting repression of Cyclin A (Figure 5.28) which was similar to the individual effect observed for Klf4 (Figure 5.27). On addition of OHT, there was an increase in luciferase activity which was directly proportional to the amount of OHT added (Figure 5.28). The luciferase activity increases till 250 nM OHT after which it plateaus (Figure 5.28). These results indicate that, Oct-3/4 and Klf4 individually inhibit Cyclin A expression via the CCRE and when combined, enhance Cyclin A expression in a synergistic manner.
**Figure 5.28 Effect of the OctER & Klf4 on Cyclin A expression**

OctER & Klf4 were transfected in Cos7 cells along with the CCRE luciferase construct. 24 hours post transfection, the cells were pulsed with various doses of OHT or ethanol (uninduced/vector alone). Twenty four hours post induction, luciferase activity was measured. In the uninduced state, there was a repression of luciferase activity which was similar to the effect of Klf4 alone. On OHT induction, there is a gradual increase in luciferase activity which plateaus at 250 nM. These results suggest that Oct-3/4 and Klf4 regulate the expression of Cyclin A via the CCRE. Values represent mean ± SD, N=3, * p < 0.05

### 5.2.10 Gene expression analysis during the initiation stage of reprogramming

To further explore the potential role of the Yamanaka factors in converting the cell cycle from a slower somatic state to the faster embryonic state, expression of selected genes based on the bioinformatics analysis were analysed by qRT-PCR. MEFs were transduced with equal volumes of OctER or Klf + OctER (KOE) or Klf4 as described. 48 hours post transduction, the cells were equally split into 3 batches and seeded onto 6 well dishes. Each batch corresponded to a time point of Day 1, 3, & 5. Day 1 was considered 24 hours post induction and each subsequent time point was calculated with reference to this. The cells were pulsed daily with either 2.5 nM, 10 nM or 100 nM OHT to induce OctER which corresponds to low, medium and high activity of OctER as previously determined by the Nanog promoter based luciferase assay (Figure 3.18). For uninduced OctER and Klf4, the
cells were pulsed daily with ethanol. The MEFs were maintained in MEF media (DF10) and media was changed daily.

RNA was isolated at the indicated time points, cDNA synthesised and gene expression quantified using qRT-PCR. The fold change \(2^{\Delta\Delta CT}\) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For KOE induced, the fold change was calculated with respect to KOE uninduced. Results are summarised in Table 5.1.

**Table 5.1 Summary of gene expression analysis**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>OctER</th>
<th>Klf4</th>
<th>Klf4 + OctER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin A</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Cyclin B</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Upregulated</td>
<td>Upregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>No change</td>
<td>Downregulated</td>
<td>No change</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Cdk1</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Cdk2</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Cdk4</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>No change</td>
</tr>
<tr>
<td>Cdk6</td>
<td>Upregulated</td>
<td>Upregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>p21</td>
<td>Upregulated</td>
<td>Upregulated</td>
<td>Downregulated</td>
</tr>
<tr>
<td>p27</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>No change</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>No expression</td>
<td>Upregulated</td>
<td>Upregulated</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Downregulated</td>
<td>No change</td>
<td>Downregulated</td>
</tr>
<tr>
<td>Snail</td>
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<td>Downregulated</td>
<td>Downregulated</td>
</tr>
<tr>
<td>Slug</td>
<td>Downregulated</td>
<td>Downregulated</td>
<td>Downregulated</td>
</tr>
<tr>
<td>Thy1</td>
<td>Upregulated</td>
<td>No change</td>
<td>Upregulated</td>
</tr>
</tbody>
</table>
Table 5.1 Summary of gene expression analysis (continued)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>OctER</th>
<th>Klf4</th>
<th>Klf4 + OctER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-3/4 endogenous</td>
<td>No expression</td>
<td>No expression</td>
<td>No expression</td>
</tr>
<tr>
<td>Sox2 endogenous</td>
<td>No expression</td>
<td>No expression</td>
<td>No expression</td>
</tr>
<tr>
<td>Nanog endogenous</td>
<td>No expression</td>
<td>No expression</td>
<td>No expression</td>
</tr>
</tbody>
</table>

5.2.10.1 Positive cell cycle regulators

Overexpression of Klf4 in MEFs led to a downregulation of the positive cell cycle regulators Cyclin A, Cyclin E and their corresponding Cdk partner Cdk2 (Figure 5.29). There was also a downregulation of Cyclin B1 and its Cdk partner Cdk1 (Figure 5.30). OctER activity in MEFs also showed a similar result (Figure 5.29 & Figure 5.30). When KOE was active in MEFs, this trend was reversed, with an upregulation Cyclin A, Cyclin E and Cdk2 along with an upregulation of Cyclin B and Cdk1 (Figure 5.29 & Figure 5.30). Further, these positive cell cycle regulators showed an OHT dependant switch, with increasing doses of OHT leading to increased gene expression (Figure 5.31 & Figure 5.32).

Cyclin D1 was upregulated in all the three conditions, with Klf4 & OctER individually displaying greater than 2 fold increase in expression levels (Figure 5.33). Cyclin D2 did not show any noticeable differences in all the three conditions while Cyclin D3 was downregulated only in Klf4 (Figure 5.33). Cdk4 was downregulated in Klf4 & OctER but showed no change in KOE (Figure 5.34). Cdk6 on the other hand showed an upregulation in Klf4, OctER & 100 nM KOE (Figure 5.34).
Figure 5.29 Gene expression changes seen in the positive cell cycle regulators Cyclin A, Cyclin E and their corresponding Cdk, Cdk2

The fold change \(2^{\Delta \Delta Ct}\) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced. OE – OctER induced, KOE is Klf4 + OctER.
Figure 5.30 Gene expression changes seen in the positive cell cycle regulator Cyclin B and its corresponding Cdk, Cdk1

The fold change ($2^{\Delta \Delta C_t}$) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced. OE = OctER induced, KOE is Klf4 + OctER.
Figure 5.31 Gene expression changes seen in the positive cell cycle regulators Cyclin A, Cyclin E and their corresponding Cdk, Cdk2 are OHT dependant
The fold change ($2^{-\Delta\Delta C_T}$) was calculated and plotted. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced.
Figure 5.32 Gene expression changes seen in the positive cell cycle regulator Cyclin B its corresponding Cdk, Cdk1 are OHT dependant

The fold change \(2^{\Delta C_t}\) was calculated and plotted. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced.
Figure 5.33 Gene expression changes seen in the three D-type cyclins

The fold change ($2^{-\Delta \Delta C_t}$) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced. OE – OctER induced, KOE is Klf4 + OctER.
Figure 5.34 Gene expression changes seen in Cdk4 and Cdk6
The fold change (2−ΔΔCt) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced. OE – OctER induced, KOE is Klf4 + OctER.

5.2.10.2 Negative cell cycle regulators
p21 was highly upregulated in Klf4 & OctER and this trend was reversed in KOE in an OHT dose dependant manner (Figure 5.35 & Figure 5.36). p27 on the other hand was downregulated in Klf4 and OctER and showed no change in levels in KOE (Figure 5.35).

In summary, these gene expression results suggest that when Klf4 is overexpressed or OctER is active in MEFs, it leads to a general downregulation of the positive cell cycle regulators and an upregulation of the negative cell cycle regulators which would essentially lead to a slowdown in proliferation. When KOE was active in MEFs, this trend was reversed, there was an upregulation of the positive cell cycle regulators and a
downregulation of the negative cell cycle regulators which would lead to an increase in proliferation. These results were consistent with the WST-1 based cell proliferation assay where Klf4 or OctER individually showed reduced proliferation while KOE showed the opposite.

![Graphs showing fold change of p21 and p27](image)

**Figure 5.35 Gene expression changes seen in the cell cycle inhibitors p21 & p27**

The fold change ($2^{-\Delta \Delta C_t}$) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced. OE – OctER induced, KOE is Klf4 + OctER.
**Figure 5.36 Gene expression changes seen in the cell cycle inhibitors p21 is OHT dependant**

The fold change ($2^{\Delta\Delta Ct}$) was calculated and plotted. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced.

### 5.2.10.3 Mesenchymal to epithelial transition

During the initiation phase of reprogramming, along with the cell cycle switch, there is a mesenchymal to epithelial transition (MET) that also occurs (Li et al., 2010; Samavarchi-Tehrani et al., 2010; Polo et al., 2012). When Klf4 was overexpressed in MEFs, there was an upregulation of E-cadherin and when OctER was active there was a downregulation of N-cadherin (Figure 5.37). Interestingly, when KOE was active, there was a combinatorial effect of the individual factors, with upregulation of E-cadherin and a corresponding downregulation of N-cadherin which is a hallmark trait of MET (Figure 5.37). These results suggest that along with regulating the switch in the cell cycle, KOE also regulates MET.
Figure 5.37 Gene expression changes seen in the mesenchymal to epithelial transition markers, E-cadherin & N-cadherin

The fold change ($2^{-\Delta\Delta C_t}$) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced. OE – OctER induced, KOE is Klf4 + OctER.

5.2.10.4 Somatic cell markers

There was a general downregulation of Snail and Slug in all the three conditions (Figure 5.38). Thy1 on the other hand was highly expressed in OctER & KOE 100 nM and showed no change in the other conditions (Figure 5.38). Downregulation of Snail and Slug is in line with the reprogramming process as the somatic cell markers are generally suppressed in the initial stages of reprogramming (Brambrink et al., 2008; Stadtfeld et al., 2008; Polo et al., 2012). The significance of the upregulation of Thy1 in OctER & 100 nM KOE is not clear (Figure 5.38).
Figure 5.38 Gene expression changes seen in somatic cell markers Snail, Slug and Thy1

The fold change ($2^{-\Delta\Delta CT}$) was calculated and plotted. For OctER induced, the fold change was calculated with respect to OctER uninduced. For Klf4 alone, the fold change was calculated with respect to empty vector. For OctER + Klf4 induced, the fold change was calculated with respect to OctER + Klf4 uninduced. OE – OctER induced, KOE is Klf4 + OctER.
5.2.10.5 Pluripotency markers
The primers used for qRT-PCR were specific for expression of endogenous Oct-3/4, Sox2 & Nanog, none of which were expressed in any of the conditions. This is in line with current studies that show the endogenous pluripotency genes are generally upregulated only during the later stages of reprogramming (Brambrink et al., 2008; Stadtfeld et al., 2008; Polo et al., 2012).

5.3 Discussion
To easily identify successfully reprogrammed iPS colonies using a live cell marker, MEFs from an Oct-eGFP transgenic mouse were derived. The transgene contains the Oct-3/4 distal enhancer and promoter driving eGFP expression and would only express during activation of endogenous Oct-3/4, a mark of successful reprogramming (Szabo et al., 2002).

For reprogramming MEFs to iPS, a high levels of expression of the Yamanaka factors are required (Polo et al., 2012). To facilitate this, an optimised viral protocol was developed which achieved a 90% transduction efficiency. To reprogram MEFs to iPS, a modified protocol was developed that reduced the number of partially reprogrammed cells and increased the yield of successfully reprogrammed cells.

5.3.1 OctER can reprogram MEFs to a pluripotent state
The inducible OctER successfully reprogrammed OGM to iPS as indicated by the expression of eGFP in AP positive colonies. The reprogramming efficiency of OE+KSM was lower than O+KSM, possibly because the amount of OHT used for induction of reprogramming has an off target effect or alternatively the flexible linker used to build OctER is not long enough to completely separate the OHT binding domain from Oct-3/4, retaining residual steric hindrance. Further analysis is necessary to resolve these issues. However, the OctER was sufficiently active and inducible in a dose-dependent manner, fulfilling the basic criteria for synchronised reprogramming.

The OE+KSM-iPS produced were characterised for pluripotency by five assays, all of which indicated successful reprogramming to iPS state. (i) Gene expression analysis indicated that OE+KSM-iPS robustly expresses pluripotency markers E-cadherin, Nanog, Dppa3, Zfp42 at levels comparable to mES. (ii) They expressed endogenous Oct-3/4 and
Sox2 at levels comparable to mES. (iii) OE+KSM-iPS were negative for somatic markers Snail, Slug, Thy1 and N-cadherin. (iv) Immunostaining showed that OE+KSM-iPS express Nanog and embryonic stem cells surface marker SSEA-1. (v) OE+KSM-iPS could form teratomas in Nude mice. The teratomas were analysed for tissue structures representing the three germ layers. All the teratomas analysed showed the presence of ectoderm, mesoderm and endoderm, confirming OE+KSM-iPS are pluripotent.

5.3.2 Oct-3/4 and Klf4 combine to show a synergistic effect on MEF proliferation

Based on a WST-1 cell proliferation assay, it was seen that c-Myc on its own increased MEF due to its oncogenic nature (Cavaliere and Goldfarb, 1987). On their own, Oct-3/4, Klf4 & Sox2 each reduced proliferation in MEFs. These results suggest that the inherent somatic cell characteristics resists the effect of the pluripotency factors in trying to gain pluripotency. This effect is probably manifested in the subsequent upregulation of the Cdk inhibitor p21 and resulting slowdown of proliferation seen during the WST-1 assay.

When Oct-3/4 & Klf4 were transduced together in MEFs, there was a marked increase in proliferation. This was further confirmed by the use of Klf4 and OctER which showed an OHT dose dependant increase in proliferation. These results highlight two facts, first OctER can substitute for Oct-3/4 as they both display the same phenotype. Second, when Oct-3/4 or Klf4 are overexpressed in MEFs, they have an individual inhibitory effect on MEF cell proliferation which is reversed when they are expressed together suggesting a form of synergistic activity.

5.3.3 Oct-3/4 regulates Cyclin A expression along with Klf4 via the CCRE

The effect of the Yamanaka factors was also tested on Cyclin A expression. Cyclin A is constitutively expressed at high levels in mES and is crucial for maintaining the rapid proliferation seen in mES (Stead et al., 2002; Kalaszczyńska et al., 2009). Using the CCRE regulatory element present on Cyclin A promoter as a readout for Cyclin A expression, c-Myc showed the highest CCRE activity while Oct-3/4 strongly suppressed CCRE activity. When OctER and Klf4 were expressed, there was a synergistic effect which enhanced expression of Cyclin A. All of these results confirm the effects of these factors on MEF proliferation as seen in the WST-1 assay. Further, the bioinformatics analysis done in this
study suggested that Oct-3/4 targets the CCRE. These results confirm that Oct-3/4 and Klf4 most likely regulate the expression of Cyclin A via the CCRE. This is probably the first study to show the effect of Oct-3/4 and Klf4 on Cyclin A expression and also the first study to show the effects of the Yamanaka factors on Cyclin A and how this affects MEF proliferation.

5.3.4 OctER and Klf4 initiate the cell cycle switch during early reprogramming

From the gene expression analysis, it was seen that OctER and Klf4 alone had inhibitory effects on cell cycle genes with a general increase in negative cell cycle regulators and a decrease in positive cell cycle regulators. When Klf4 & OctER were combined there was an OHT dependent increase in positive cell cycle regulators such as Cyclin A and an OHT dependent decrease in negative cell cycle regulators such as p21. The gene expression data validates the cell proliferation data with OctER & Klf4 both showing a decreased proliferation rate and KOE showing an increase in proliferation in an OHT dependent

This suggests that Oct-3/4 and Klf4 have an individual antagonistic function in MEFs which is reversed when both are expressed together and they function in a synergistic manner. Oct-3/4 and Klf4 synergise to remove the cell cycle inhibitors and increase the levels of cell cycle enhancers thereby speeding up the cell cycle.

5.3.5 OctER and Klf4 initiate MET transition during early reprogramming

When KOE was active in MEFs, there was an upregulation of E-cadherin and a downregulation of N-cadherin, which is a hallmark of Mesenchymal to Epithelial (MET) (Maeda et al., 2005; Gravdal et al., 2007). This would suggest that along with initiating the cell cycle switch, Oct-3/4 & Klf4 also initiates MET. Klf4 is known to regulate the transcription of E-cadherin and our gene expression data in MEFs also confirmed this (Yori et al., 2010). OctER on the other hand showed a downregulation of N-cadherin but showed no expression of E-cadherin even though this was accompanied by a downregulation of Snail, a known suppressor of E-cadherin (Batle et al., 2000; Cano et al., 2000). This suggests, that in order initiate the MET, both factors are required due to some synergistic action that needs to be further explored.
When these results are taken together, it suggests that Oct-3/4 & Klf4 have an individual antagonistic function in MEFs which is reversed when both are expressed together and they function in a synergistic manner. Oct-3/4 & Klf4 synergise to remove the cell cycle inhibitors and increase the levels of cell cycle enhancers thereby speeding up the cell cycle. They synergise to initiate MET, which results in the loss of somatic cell characteristics though they do not facilitate induction of endogenous pluripotency factors. What this would suggest is that synergistic function Oct-3/4 & Klf4 during the initial stages of reprogramming is probably important to make the somatic cell more conducive to gaining pluripotency by facilitating a pluripotent like state with a faster cell cycle and repressed somatic characteristics. More work needs to be done to get a clearer picture of the synergistic role of Oct-3/4 & Klf4.

To summarise, this study shows for the first time that Oct-3/4 regulates the expression of Cyclin A via the CCRE region. This study is also for the first time shows that Oct-3/4 along with Klf4 most likely facilitates reprogramming by erasing some of the somatic cell characteristics, thereby providing a blank canvas for the combined Yamanaka factors to create a pluripotent state.