CHAPTER 5 : RETINOIC ACID RECEPTORS and the REGULATION OF OCT4 EXPRESSION

5.1 Introduction

5.1.1 Retinoic Acid signalling during Development

In **Chapter 4**, I described that BNC2 and RARβ can effectively replace ectopic requirement of Oct4 during reprogramming. Although the reprogramming efficiency of BNC2 was superior to RARβ, co-operation between RARβ, c-Myc, Klf4 and Sox2 in the reprogramming cocktail led to the generation of iPSC colonies expressing endogenous Rex1 in a homogenous manner. Together with evidence describing that RARs can associate with Oct4 regulatory elements and affect Oct4 expression (Ben-Shushan et al., 1995; Wang et al., 2011c), it is tempting to speculate that Retinoic Acid (RA) signalling may play a role in the reprogramming process. With this in mind, **Chapter 5** aims to unravel the mechanisms behind RARβ and its family members in their abilities to influence the acquisition of pluripotency.

Retinoic Acid (RA) signalling has been illustrated in developmental processes in a multi-dimensional manner. Identified as the first candidate diffusible morphogen in vertebrates, attempts to dissect the role of RA during development have been inundating (Thaller and Eichele, 1987). Initial exemplification of the obligatory need of Vitamin A, a precursor of RA, during embryonic development (Hale, 1933; Warkany and Schraffenberger, 1946; Wilson and Warkany, 1948; Wilson et al., 1953) led to the discovery that specific elimination of retinoic acid during development leads to malformation of the neural crest, ocular and nervous system (Dickman et al., 1997; White et al., 1998). Conversely, excessive amounts of retinoic acid have been demonstrated to initiate aberrations in embryonic development across species, from *Xenopus laevis* to zebrafish, mice and humans (Sive et al., 1990; Holder and Hill, 1991; Papalopulu et al., 1991; Morriss-Kay et al., 1991; Conlon and Rossant, 1992; Lammer et al., 1985).

From a canonical perspective, RA elicits a cascade of events through its association to Retinoic Acid Receptors (RARs). RARs are molecular components which recognise and bind to RA (Giguere et al., 1987; Petkovich et al., 1987; Benbrook et al., 1988; Brand et al., 1988; Krust et al., 1989; Zelent et al., 1989). There are three members in the RAR family, namely RAR-alpha (RARα), RAR-beta (RARβ) and RAR-gamma (RARγ). All three family members share features such as a ligand binding domain (LBD) that associates with RA and a DNA binding domain (DBD) which tethers to Retinoic Acid Response Elements (RAREs) delineated by the motif (A/G)G(G/T)TC(A/G) and its variants (reviewed by Chambon, 1996). Analagous to RA, RARs are essential during embryonic development. Due to close similarities between RARs, functional redundancy is apparent where mutations in single RARs do not instigate developmental defects. In contrast, simultaneous ablation of two RARs results in overt malformation and death *in utero* (Ghyselinck et al., 1997; Lohnes et al., 1994; Kastner et al., 1994; Mendelsohn et al., 1994).

With an affinity for RAREs, RARs reside in the nucleus and bind to cognate recognition motifs. In the absence of its ligand, RARs are postulated to associate with co-repressor complexes, thus inhibiting the initiation of transcription. Conversely, in the presence of its ligand, RA binds to the LBD of RARs and results in the dissociation of co-repressor complexes. This is ensued by the recruitment of co-activator modules and transcriptional machinery to prompt the transcriptional process (reviewed by Rochette-Egly and Germain, 2009).

Apart from the canonical signalling pathway, non-genomic responses of RARs have been delineated. Acting through kinase cascades, RARs have been demonstrated to activate p38 MAPK, MSK1 and PI3K (Alsayed et al., 2001; Gianni et al., 2002; Gianni et al., 2006; Bruck et al., 2009; Masia et al., 2007). These signalling events are independent of the transcriptional capabilities of RARs and have been attributed to two serine phosphorylation sites within the LBD and N-terminal domain (Bruck et al., 2009; Rochette-Egly et al., 1997; Bastien et al., 2000). In addition to non-genomic modes of

action, RARs can also achieve ligand independence. These incidences include co-operation with other receptors and epigenetic modifications. To this end, RARα and Estrogen Receptor (ER) have been demonstrated to co-occupy regulatory elements within the chromatin in an ER dependent manner (Ross-Innes et al., 2010). In addition, RARα has been described to influence transcriptionally permissive histone modifications at the promoter locus of Mest in the absence of RA (Laursen et al., 2012).

5.1.2 Retinoic Acid Receptors and Oct4 regulation

Amid the plethora of genomic interactions exhibited by RARs at its cognate binding sequences, RAREs have been elucidated within the cis-regulatory elements of Oct4 (Schoorlemmer et al., 1994; Pikarsky et al., 1994; Okazawa et al., 1991). The discovery of RAREs within the Oct4 regulatory elements was incited by the amalgamation of various pieces of information, namely (i) the importance of Oct4 during early development (Nichols et al., 1998) and (ii) the loss of pluripotency upon high doses of retinoic acid (Okamoto et al., 1990). With this in mind, our current understanding of the regulation of Oct4 expression, with primary focus on RA signalling, will be illustrated below.

5.1.2.1 Regulation of Oct4 expression levels

The fleeting and obligatory expression of Oct4 during early stages of embryonic development has motivated the elucidation of its functional network. With the advent of sophisticated analysis methods such as chromatin immunoprecipitation coupled to sequencing, the transcriptional landscape during early stages of embryonic development has been feasible. Using mouse and human ESCs as a surrogate to study early development, transcriptional complexes associated with pluripotency have been unearthed (Boyer et al., 2005; Loh et al., 2006; Kim et al., 2008; Wang et al., 2006; Marson et al., 2008b; Chen et al., 2008). These analyses revealed the presence of a core transcriptional apparatus consisting of Oct4, Sox2 and Nanog which actively participates in the maintenance of pluripotency. Examination of the genomic regions bound by pluripotency-associated transcription factors at high resolution revealed that the distal enhancer of Oct4

depicts the densest binding locus. Transcriptional regulators which tether to this region include Stat3, Klf4, Smad1, Esrrb, Nanog, Sox2, Tcf3 and c-Myc (Chen et al., 2008; Marson et al., 2008b). In addition, p300 binding and activating methylation marks at lysines 4 and 36 of histone 3 are heavily represented in the enhancer, promoter and transcribed regions of Oct4 within ESCs (Marson et al., Chen et al., 2008; Schnetz et al., 2010). This strengthens the notion that Oct4 regulatory elements are heavily occupied in pluripotent cells, hence unravelling of the regulatory mechanisms at these elements will illuminate the molecular switches involved in the acquisition of induced pluripotency and differentiation.

5.1.2.2 Retinoic Acid Receptors mediate Oct4 expression

To initiate the understanding of Oct4 transcriptional regulation, phenomenon described to accompany changes in Oct4 expression levels was employed as a learning platform. To this end, RA-induced differentiation which leads to a decrease in Oct4 expression levels pioneered initial studies and sought to determine the effect of RA signalling on Oct4 regulation (Schoorlemmer et al., 1994; Pikarsky et al., 1994; Okazawa et al., 1991).

Using a combination of DNase footprinting, chloroamphenicol acetyltransferase (CAT) assays and computational prediction to study the effects of RA-induced differentiation, three regions that encompass RAREs were disclosed. They were subsequently labelled as the distal enhancer, proximal enhancer and proximal promoter, positioned approximately 2kb, 1kb and 100bp upstream of the Oct4 transcriptional start site respectively. These elements were portrayed to be active in EC cells and murine embryos, and inactive in the presence of high doses of retinoic acid (Schoorlemmer et al., 1994; Pikarsky et al., 1994; Okazawa et al., 1991; Yeom et al., 1996).

The three regulatory elements control the expression of Oct4 at different stages of development. The distal enhancer drives Oct4 expression within the pre-implantation epiblast, whereas the proximal enhancer is responsible for inciting Oct4 expression in the

post-implantation epiblast (Yeom et al., 1996). EpiSCs reflect a sub-population from the late epiblast and rely on the proximal enhancer for expression of Oct4, whereas derivatives from the early epiblast depend on the distal enhancer for Oct4 expression (Tesar et al., 2007; Guo et al., 2010; Han et al., 2010a). Despite dissimilar regulatory circuits, the expression levels of Oct4 are similar between EpiSCs and ESCs (Tesar et al., 2007). Although the control of Oct4 expression has been delineated in various pluripotent states, regulatory elements which govern Oct4 expression during the acquisition of pluripotency have not been explored.

The RARE situated at the proximal promoter (RAREoct) has been described to be pivotal in the regulation of Oct4 expression through competitive binding between Coup-TFI, Coup-TFII and the RARs (Ben-Shushan et al., 1995). Coup-TFI and Coup-TFII have the capacity to tether to RAREoct and repress Oct4 expression, whereas increasing amounts of RARs counteract this phenomenon by displacing negative regulators and inciting Oct4 expression. Additional regulatory components have since been elucidated to adhere to RAREoct (Fuhrmann et al., 1999; Fuhrmann et al., 2001; Barnea and Bergman, 2000). For example, an SF1 (Nr5a1) recognition site was identified adjacent to RAREoct and the application of CAT assays reveals that a combination of SF1 and RARα or RARγ results in enhanced transcriptional activity at RAREoct (Barnea and Bergman 2000). As SF1 shares similar recognition sites to LRH1 (Nr5a2), it was further delineated that LRH1 competently occupies RAREoct (Gu et al., 2005) and co-operation between LRH1 and RARγ instigates rapid and efficient generation of iPSCs (Wang et al., 2011c). As exploration of the role of RARs in the acquisition of induced pluripotency is in its infancy, it will be intriguing to extend these findings to RARβ and determine its ability to function as a surrogate for ectopic Oct4 during reprogramming.

5.1.3 Chapter Aim

This chapter aims to delineate the mechanisms behind RARβ in its ability to replace ectopic Oct4 during the initiation of reprogramming events. As RARs have been

implicated in the regulation of Oct4 through their association with Oct4 cis-regulatory elements, these findings serve as a basis to understand the role of RARβ in negotiating the requirement for ectopic Oct4 during the acquisition of pluripotency and may offer a distinct perspective of the reprogramming process.

5.2 Results

5.2.1 Retinoic Acid enhances reprogramming efficiency

As an initial step to dissect the role of RARβ in the acquisition of pluripotency, its ligand, all trans-RA (ATRA), was assessed for its ability to imitate the effects of RARβ and elicit an effect on the reprogramming process. To this end, ATRA was introduced to the conventional reprogramming cocktail of Oct4, c-Myc, Klf4 and Sox2. High concentrations of RA have been well-established to incite differentiation into neural lineages (Okada et al., 2004; Schuldiner et al., 2001). As such, low concentrations of ATRA were employed. 0.1nM and 1nM of ATRA were introduced at the initial stages of reprogramming, and number of colonies generated acted as a surrogate to determine reprogramming efficiencies. To heighten the sensitivity of the screen, high passage MEFs were utilised and few colonies were observed when the conventional reprogramming cocktail was introduced on its own (**Figure 5.1**). This allows clear delineation of any effect on reprogramming efficiencies. Interestingly, addition of 1nM ATRA escalated the number of iPSC colonies generated, whereas 0.1nM of ATRA did not yield a similar effect. A similar phenomenon was observed when a separate form of RA, 9-cis RA, was used. 9-cis RA has been described to be reciprocal ligand recognised by RXRs (Heyman et al., 1992; Levin et al., 1992), suggesting that RXRs may also be involved in reprogramming events. The experiment was replicated three times and a similar effect was observed. **Figure 5.2** illustrates a quantitative view of the colony numbers generated by the addition of 1nM RA. These results ascertain the role of RA signalling in the reprogramming and provide supporting evidence for the ability of RARβ to competently act as a substitute for exogenous Oct4 during reprogramming.

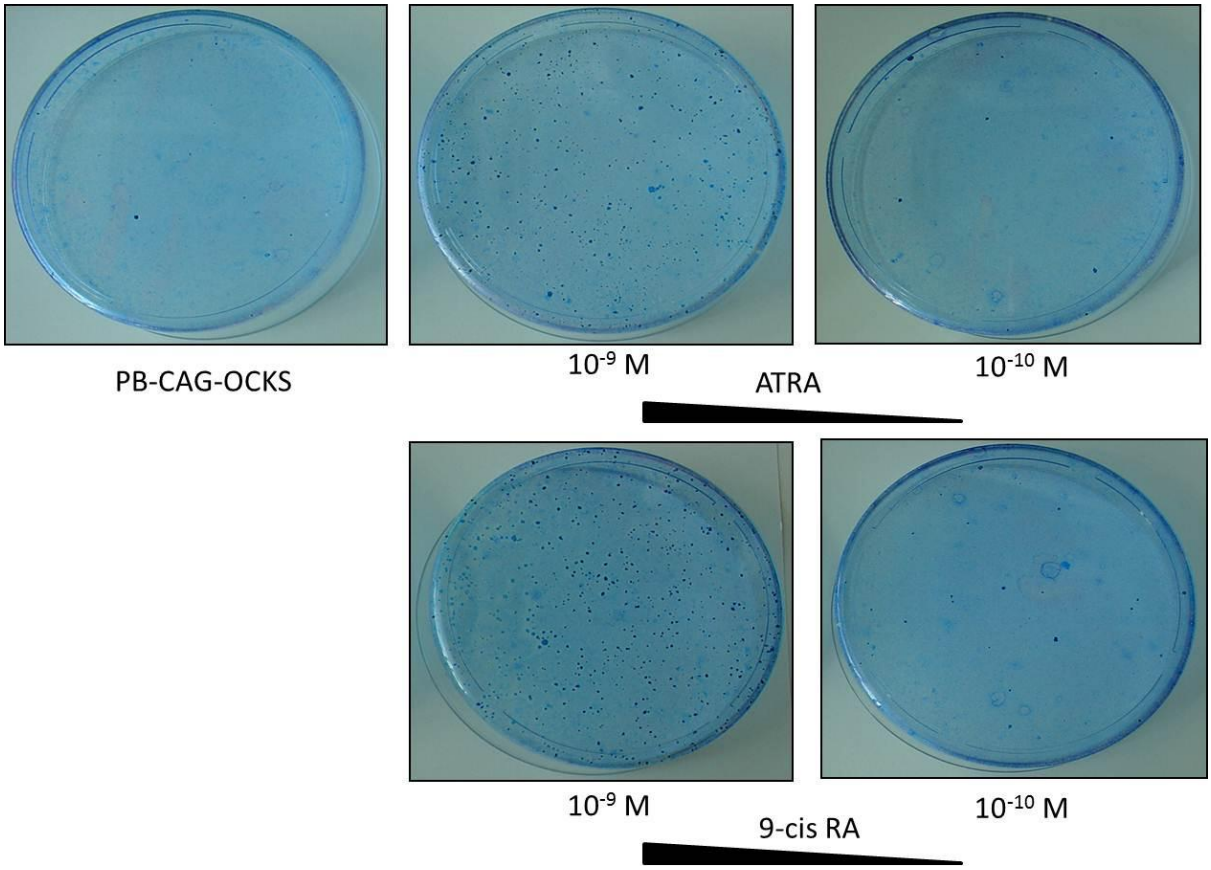

Figure 5.1 Addition of all-trans retinoic acid affects reprogramming efficiencies To assess the influence of all-trans retinoic acid (ATRA) on reprogramming, Oct4, c-Myc, Klf4 and Sox2 were transfected into MEFs and electroporated MEFs were seeded onto a mitotically inactive feeder layer in the presence of ATRA . Cells were exposed to the indicated ATRA concentrations for 5 days. 21 days after reprogramming, the cells were stained with bromophenol blue for visualisation.

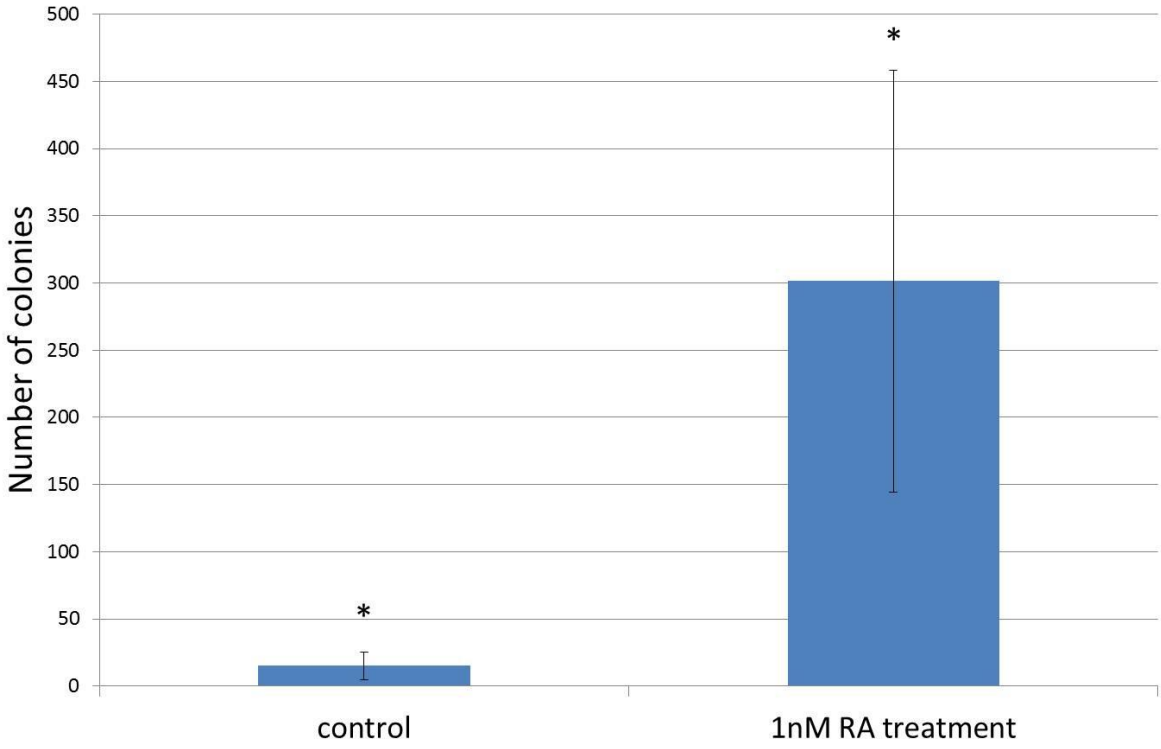

Figure 5.2 Addition of 1nM all-trans retinoic acid improves reprogramming efficiencies Tripicates were conducted to study the effect of 1nM ATRA on reprogramming efficiencies. 1nM ATRA was introduced for 5 days to MEFs which had been transfected with Oct4, c-Myc, Klf4 and Sox2. 21 days after transfection, cells were stained with bromophenol blue and counted. The numbers of observed colonies are reflected in the bar chart above where the error bars signify the standard deviation calculated from the values obtained from 3 separate experiments. Statistical analysis was performed using a student's T-test. *$p<0.05$

5.2.1 RARβ binds to the enhancer and promoter regions of Oct4

Having delineated that the natural ligand of RARβ can enhance reprogramming efficiencies, it was pertinent to understand the mechanisms behind the ability of RARβ in acting as a substitute for exogenous Oct4 in the acquisition of induced pluripotency. Recent findings have highlighted the association of RARα and RARγ to the distal enhancer element of Oct4 (Delacroix et al., 2010). As RARβ was omitted from the study, it is intriguing to determine if RARβ possesses similar qualities. To this end, chromatin immunoprecipitation (ChIP) was performed using an antibody specific to RARβ. As RARβ is not highly expressed in ESCs, MEFs were used as a starting material. Fragmented genomic regions which were tethered to RARβ were eluted and analysed. Using GenePalette to identify consensus motifs recognised by the RAR family, eleven potential RAREs were annotated 10kb upstream of the Oct4 transcriptional start site (TSS) (**Figure 5.3**). Primers against each of the eleven sites were designed and introduced to the ChIP eluate for amplification. **Figure 5.4** illustrates quantification of the amplified products corresponding to eleven putative RAREs preceding Oct4 TSS. RAREs between 100bp and 2.17kb upstream of Oct4 TSS displayed positive association to RARβ. This region comprises of the sites which have been previously demonstrated to show susceptibility to control by RARs, namely the distal enhancer, proximal enhancer and proximal promoter (RAREoct) (Yeom et al., 1996). To inject an additional level of validation, DNA immunoprecipitated using the abovementioned RARβ antibody was sequenced using primers corresponding to 2.17kb upstream of Oct4 TSS, exhibiting identity to the distal enhancer of Oct4. (**Figure 5.5**).

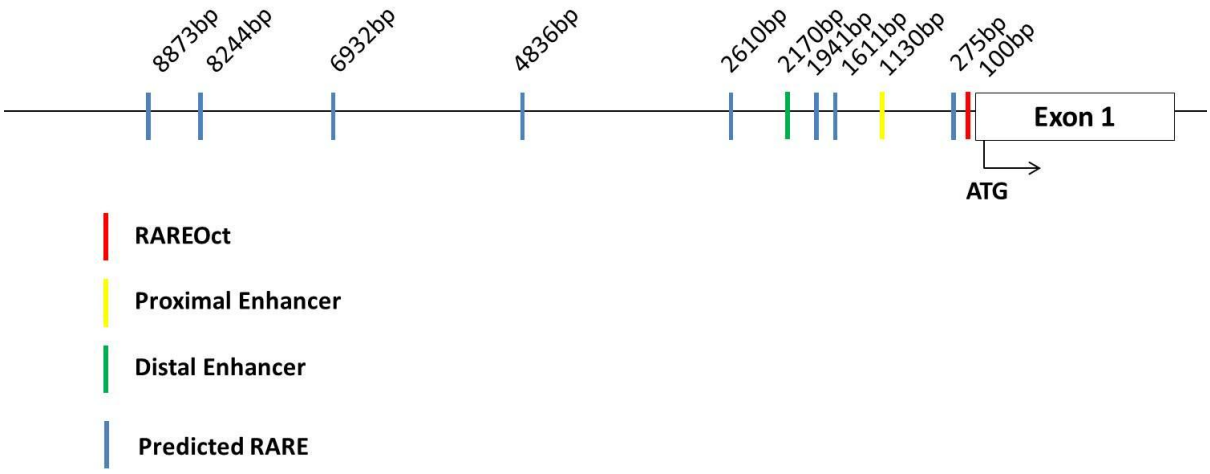

Figure 5.3 Predicted Retinoic Acid Response Elements (RAREs) upstream of Oct4 RAREs which fulfil the consensus motif (A/G)G(G/T)TC(A/G) were identified using GenePalette. Predicted RAREs are displayed in blue boxes and their distances from the transcriptional start site are indicated. The boxes in red, yellow and green depict RAREs representative of the proximal promoter (RAREoct), proximal enhancer and distal enhancer respectively.

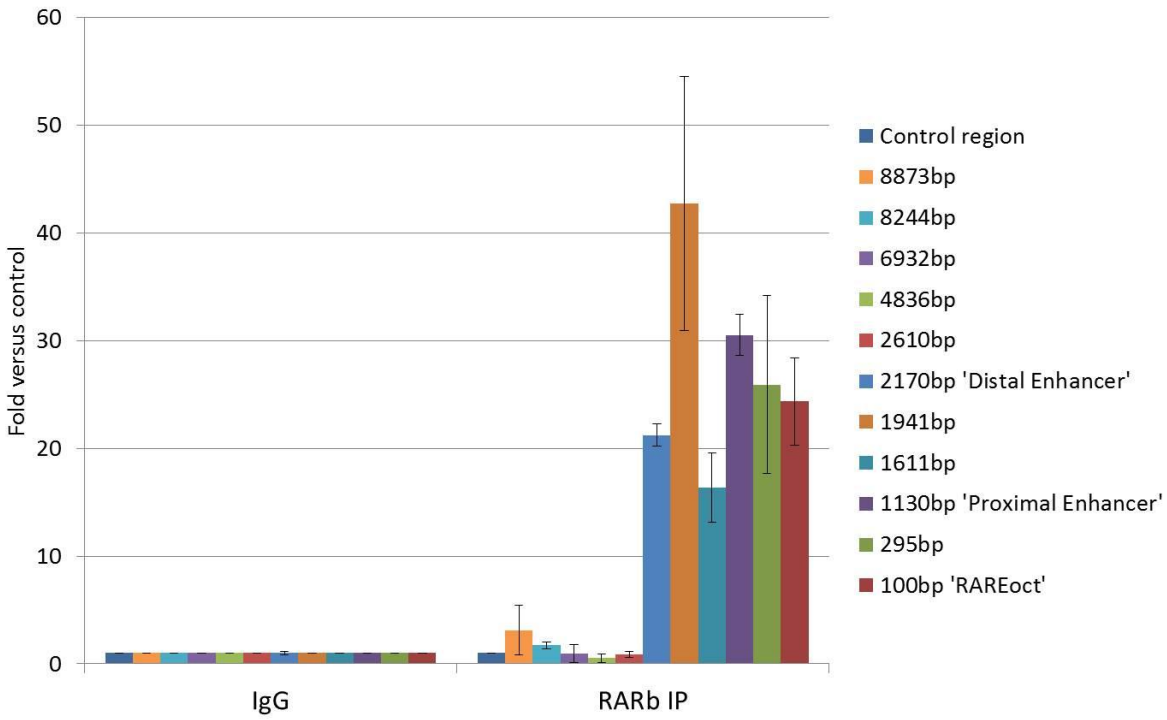

Figure 5.4 RARβ binds to RAREs up to 2kb upstream of Oct4 Chromatin Immunoprecipitation (ChIP) was performed using antibodies against RARβ in MEFs. Using eluate obtained from the pull-down, qPCR was conducted using primers designed against the 11 predicted RAREs. The values were normalised against a control region 24kb upstream of Oct4, and IgG.

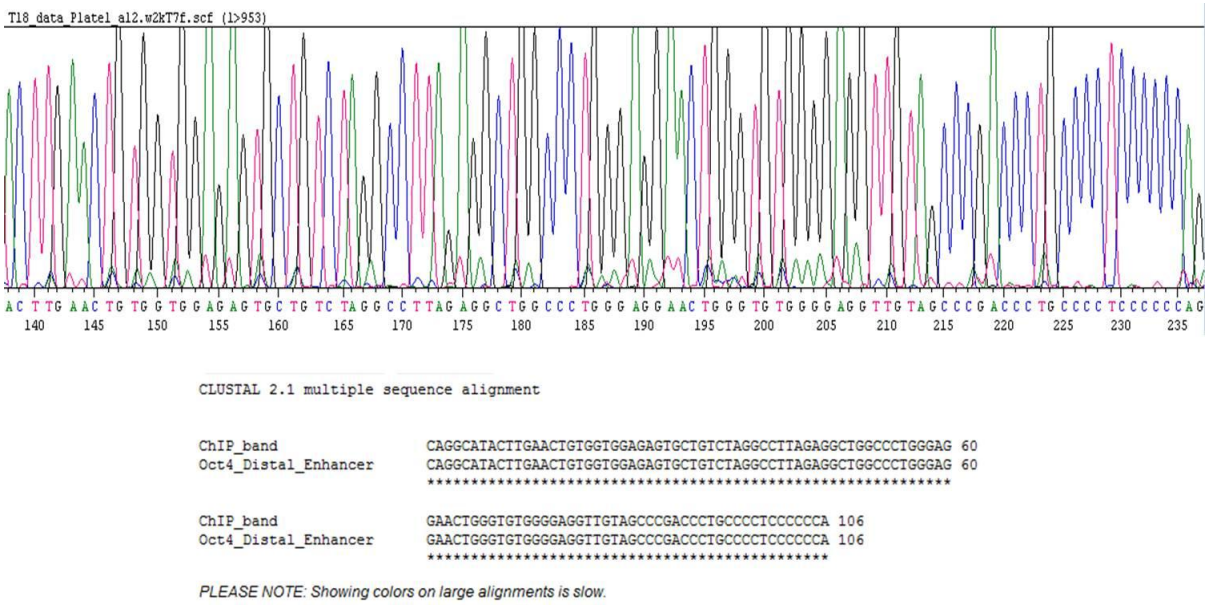

Figure 5.5 Sequence of amplified band using primers against Oct4 Distal Enhancer Chromatin Immunoprecipitation (ChIP) was performed using antibodies against RARβ in MEFs. Using eluate obtained from the pull-down, PCR was conducted using primers designed against the distal enhancer of Oct4. Amplified products were visualised on an agarose gel before extraction and purification. The purified amplicon was sequenced (Top) and aligned to the genomic sequence of Oct4 using ClustalW2 (Bottom).

5.2.2 RARβ elicits transcriptional activation through interaction with the distal enhancer of Oct4

To determine if binding of RARβ to the regulatory elements of Oct4 elicits transcriptional activation, various DNA fragment sizes of the Oct4 genomic locus were cloned into luciferase reporter constructs (**Figure 5.6**). Having delineated that binding of RARβ was restricted to 2.17kb upstream of the Oct4 TSS, I chose to study DNA spanning 2.4kb which encompassed the distal and proximal enhancers of Oct4 and RAREoct (DE-PE-RAREoct). In addition, a 1.3kb fragment comprising of both Oct4 proximal enhancer and RAREoct (PE-RAREoct), and a 466bp fragment solely consisting of RAREoct (RAREoct), were inserted into reporter constructs preceding cDNA encoding for firefly luciferase. In order to study the effect of RARβ on the enhancer elements alone, additional constructs were generated. First, a 450bp genomic fragment containing merely the distal enhancer was inserted adjacent to the luciferase reporter construct (DE-minP). A basic promoter consisting of a TATA box (minP) was included preceding the luciferase reporter gene to allow for efficient promotion of gene expression in the presence of enhancers. Extending this construct, genomic fragments of increasing length, namely a 2kb fragment consisting of both the distal and proximal enhancer (DE-PE-minP), and a 2.4kb fragment encompassing the distal and proximal enhancer and RAREoct (DE-PE-RAREoct-minP), were inserted in front of the minimal promoter and cDNA encoding for firefly luciferase.

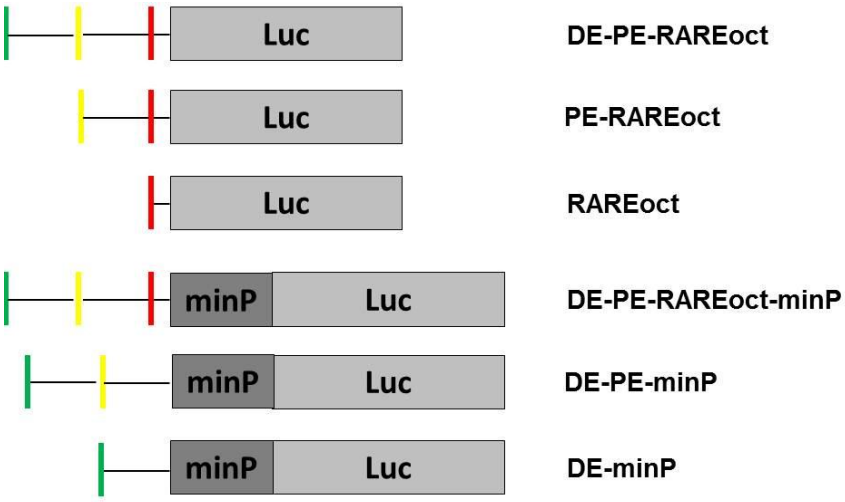

Figure 5.6 Schematic Diagram of luciferase constructs generated Luciferase constructs comprising of a combination of elements upstream of Oct4 were designed. The red, yellow and green boxes depict RAREs representative of the proximal promoter (RAREoct), proximal enhancer and distal enhancer of Oct4 respectively. The top three constructs involve the modification of pGL3-basic, which allows the insertion of a promoter of interest adjacent to a luciferase reporter gene. The bottom three constructs involve the modification of pGL4.23, which allows the insertion of desired enhancer regions adjacent to a minimal promoter (minP) preceding a luciferase reporter gene.

It was previously described that a genomic fragment of length 18kb which contained the regulatory elements of Oct4 was able to recapitulate the expression of Oct4 in a developing mouse embryo (Yeom et al., 1996). To verify the functionality of the luciferase reporter constructs, each luciferase vector was introduced into MEFs or ESCs, representing somatic or pluripotent cell types. **Figure 5.7** demonstrates that DE-PE-RAREoct is highly activated in ESCs, as reflected by high luciferase activity. Conversely, the same construct is repressed in MEFs. Similarly, PE-RAREoct is twice as active in ESCs than MEFs. On the contrary, RAREoct was observed to be repressed in ESCs but active in MEFs. This could be attributed to the presence of repressors such as Coup-TFI or Coup-TFII in ESCs which have been described to bind to RAREoct (Ben-Shushan et al., 1995). **Figure 5.8** fortifies these observations where DE-PE-RAREoct-minP is 8-fold more active in ESCs than MEFs. In parallel, DE-minP and DE-PE-minP exhibited 2-fold and 3-fold increased activity in ESCs compared to MEFs respectively, suggesting synergy between the distal enhancer and RAREoct for the manifestation of maximum Oct4 expression.

Having demonstrated the functional integrity of the luciferase constructs, these reporter vectors were introduced to MEFs in combination with reprogramming cocktails. **Figure 5.9** offers a graphical representation of the luciferase readings triggered by the presence of the reprogramming factors. Given that the distal enhancer encompasses several recognition sites for a spectrum of transcription factors, including c-Myc, Klf4 and Sox2 (CKS), it was expected that the presence of CKS activated DE-PE-RAREoct. The observed activation of DE-PE-RAREoct was augmented when RARβ was co-introduced with CKS. Intriguingly, the proximal enhancer and proximal promoter regions of Oct4 were not affected in the presence of RARβ. On the contrary, RARγ has recently been implicated in the regulation of Oct4 expression levels via interaction at the proximal promoter (Wang et al., 2011c). In a bid to explore if RARγ could function in a similar manner as RARβ, RARγ was introduced into the experimental study. Interestingly, when RARγ was introduced in place of RARβ, a similar phenomenon was observed where CKS and RARγ triggered activation of DE-PE-RAREoct, reinforcing the observation that RARs elicit a transcriptional effect at the Oct4 distal enhancer.

The co-operative role between LRH1 and RARγ in the acceleration and enhancement of reprogramming events has recently been established (Wang et al., 2011c). To determine if LRH1 has a similar additive effect in the presence of RARβ and CKS, five factors (CKS, RARβ and LRH1) were introduced into MEFs and observed for transcriptional activation as indicated by luciferase activity. Interestingly, the addition of LRH1 to the reprogramming cocktail containing CKS and RARβ enhanced luciferase activity. However, comparable to previous findings, the increased luciferase activity was only observed in the luciferase construct that encompasses the distal enhancer of Oct4 (DE-PE-RAREoct) (**Figure 5.9**). An analogous result was observed when RARγ was applied instead of RARβ, where the presence of LRH1, RARγ and CKS displayed elevated levels of luciferase activity.

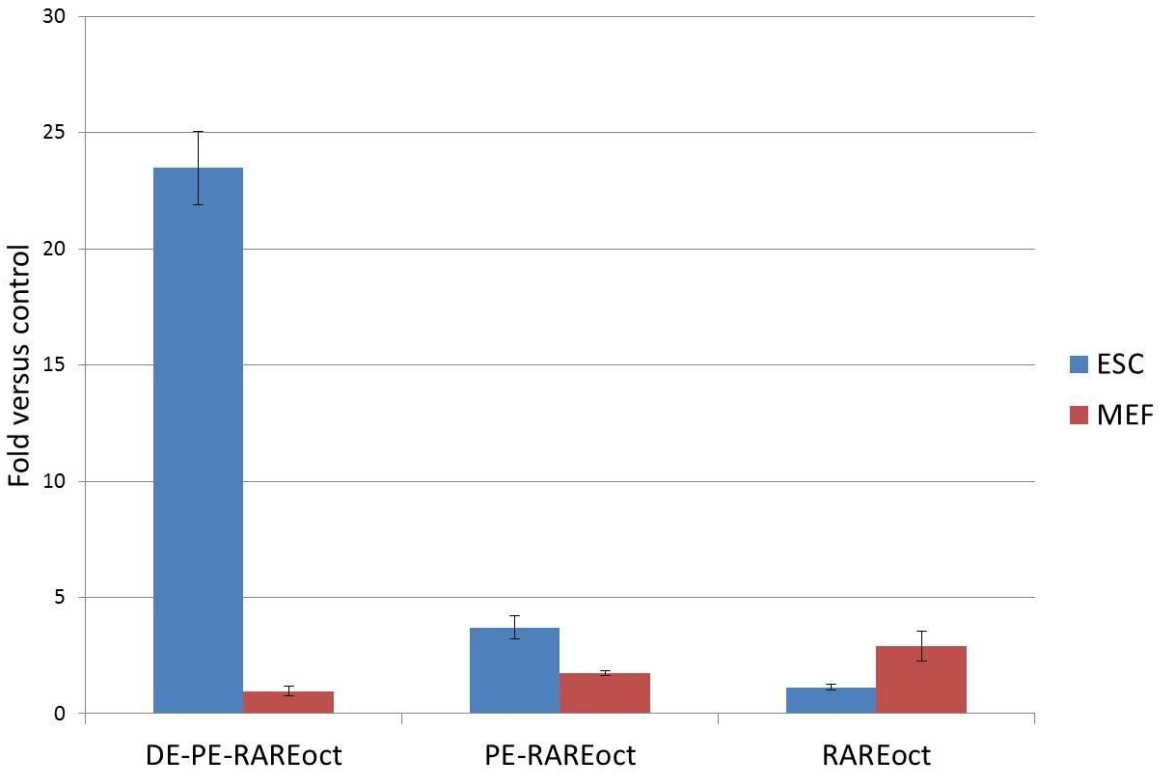

Figure 5.7 Activity of luciferase constructs in MEFs and ESCs To determine the functionality of each luciferase construct, the reporter vectors were separately transfected into MEFs or ESCs. After 48 hours, transfected cells were lysed and analysed for luciferase actvity through the addition of luciferin. Renilla was co-introduced with each luciferase reporter construct to account for transfection variability. The values plotted on the graph were normalised to an empty pGL3-basic vector and their corresponding Renilla control readings.

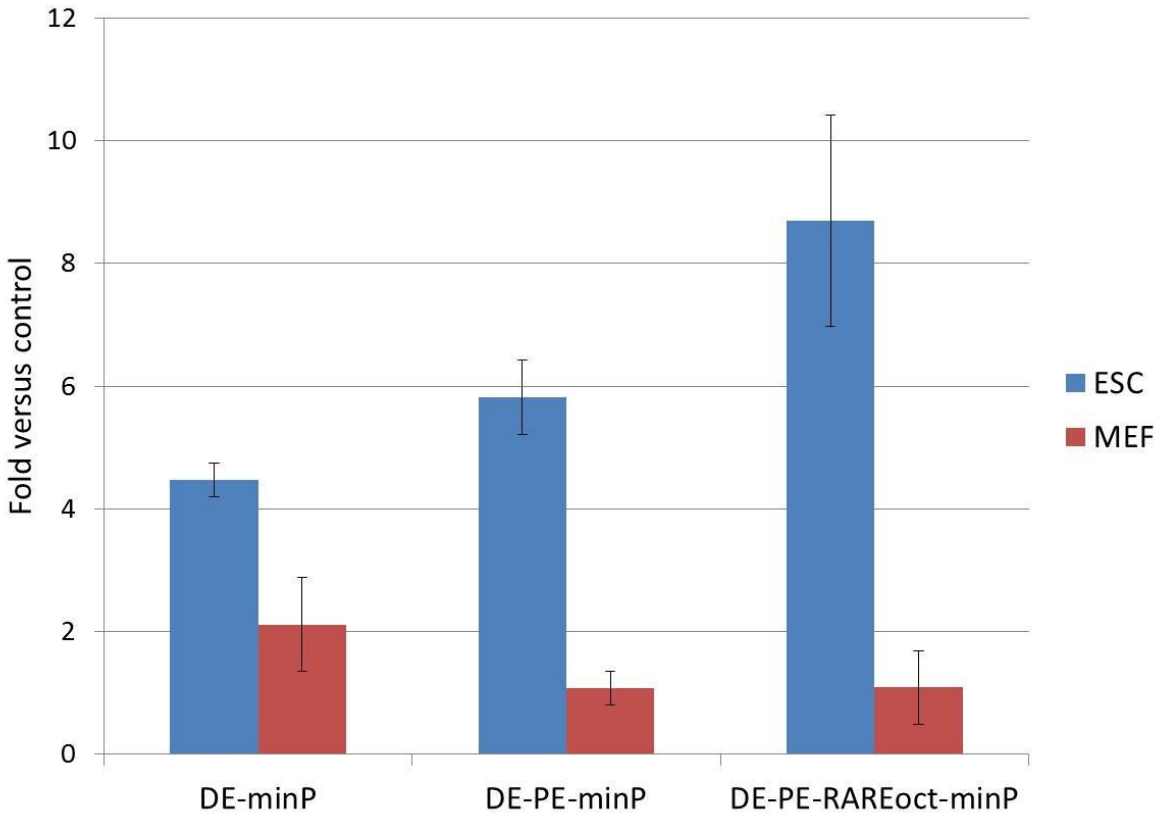

Figure 5.8 Activity of luciferase constructs in MEFs and ESCs To determine the functionality of each luciferase construct, the reporter vectors were separately transfected into MEFs or ESCs. After 48 hours, transfected cells were lysed and analysed for luciferase actvity through the addition of luciferin. Renilla was co-introduced with each luciferase reporter construct to account for transfection variability. The values plotted on the graph were normalised to pGL4.23 and their corresponding Renilla control readings.

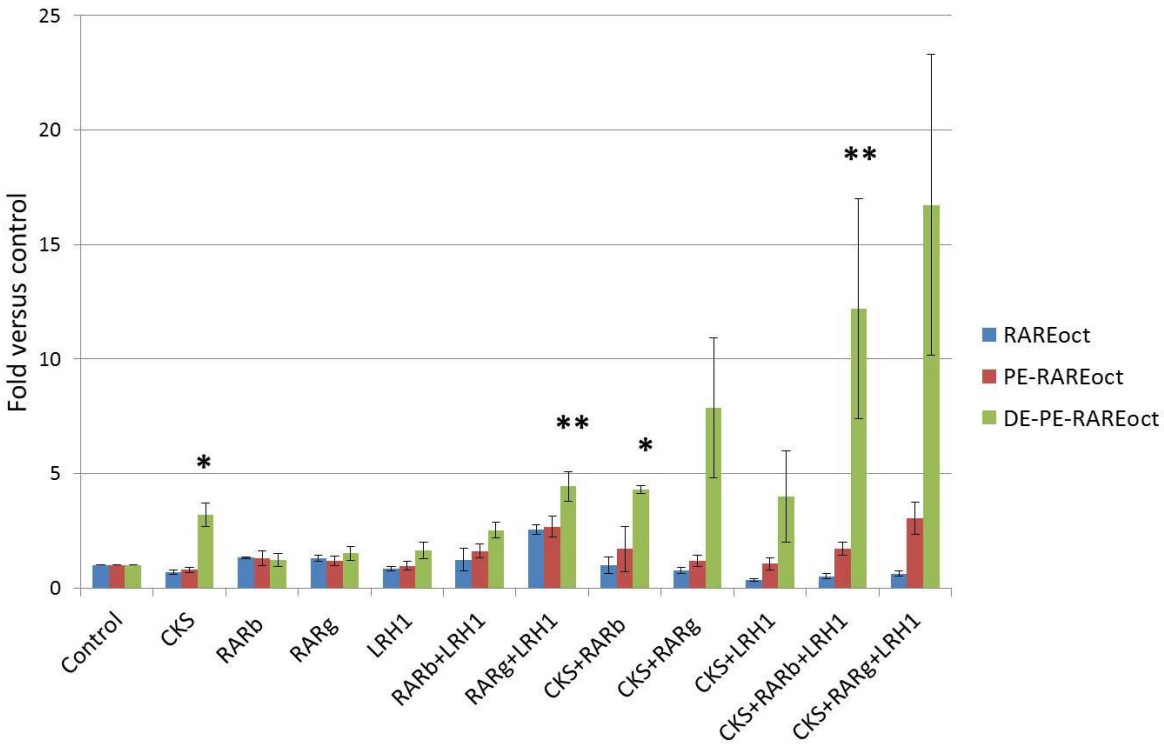

Figure 5.9 RARβ and RARγ act synergistcally with LRH1 to transcriptionally activate the distal enhancer of Oct4 Combinations reflective of the reprogramming cocktails were transfected into MEFs. The combinations of transgenes introduced are labelled along the horizontal axis. Each of the three luciferase constructs (RAREoct, PE-RAREoct, and DE-PE-RAREoct) was co-introduced with each transgenic combination. After 48 hours, the cells were lysed and assessed for luciferase activity. The values are displayed in the bar chart, where the three colours depict the different luciferase construct analysed. The readings are normalised against Renilla and pGL3-basic. Statistical analysis was performed using a student's T-test. *p=0.05; **p<0.05

5.2.3 RARs can function as replacements to exogenous Oct4 during the generation of iPSCs

Having described that both RARγ and RARβ positively regulate the distal enhancer of Oct4, I next investigated if RARγ and RARα could similarly trigger reprogramming events in the absence of ectopic Oct4 expression. Using a doxycycline inducible system, individual RAR family members were introduced into MEFs, in the absence or presence of LRH1. LRH1 was included on the premise it can act synergistically with RARγ and RARβ to activate Oct4 expression levels, as suggested by **Figure 5.9**. To distinguish reprogrammed cells, AP which acts as a general stem cell marker was employed. **Figure 5.10** illustrates the number of AP expressing colonies obtained from the various reprogramming mixes. The presence of RARs or CKS individually did not generate any iPSC colonies. In contrast, combination of CKS and each RAR family member incited the formation of iPSC colonies (RARγ-iPSCs and RARβ-iPSCs). The presence of LRH1 and CKS was observed to trigger reprogramming events (LRH1-iPSCs), reproducing observations which were previously established (Heng et al., 2010). Analagous to findings obtained from **Figure 5.9**, addition of LRH1 to reprogramming cocktails consisting of CKS and each RAR family member promoted the number of reprogrammed colonies (RARγ+LRH1-iPSCs and RARβ+LRH1-iPSCs), reiterating the co-operation between LRH1 and RARs in the activation of the Oct4 regulatory elements.

These observations were reaffirmed in triplicates using larger volumes of starting material. **Figure 5.11** provides a numerical representation on the various reprogramming efficiencies. Mirroring previous observations in **Figure 5.10**, RARβ and RARγ were competent at replacing exogenous Oct4 to generate iPSCs and this ability was augmented with the inclusion of LRH1. However, the conventional reprogramming cocktail (Oct4 and CKS) was the most efficient at producing iPSC colonies, surmising that the stoichiometric ratios of the replacement factors were sub-optimal.

To ensure that iPSCs generated in the presence of LRH1 and/or RARγ were sustainable and exhibited pluripotency, transcript levels of pluripotency markers in these cells were determined. **Figure 5.12** illustrates that iPSCs generated in the presence of LRH1 and RARγ, where exogenous Oct4 was absent, exhibited comparable levels of pluripotency markers to ESCs and Oct4-iPSCs, reinforcing that RARγ and LRH1 can derive pluripotent derivatives in the absence of ectopic Oct4.

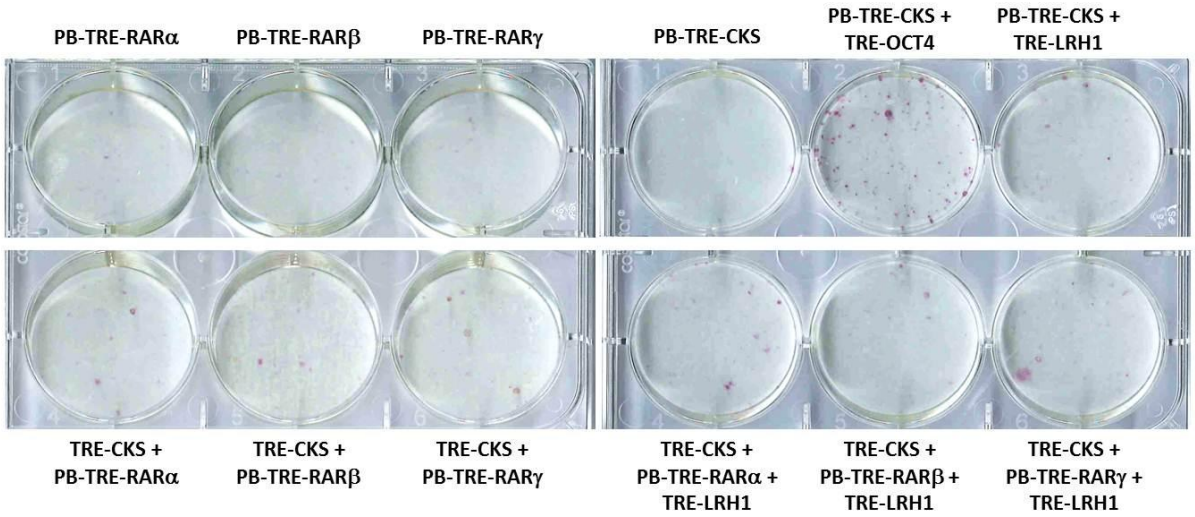

Figure 5.10 RAR family members act synergistcally with LRH1 to increase reprogramming efficiencies in the presence of PB-TRE-CKS To determine the reprogramming capabilities of RARβ, RARγ and RARα, each factor was introduced into MEFs, in co-operation with PB-TRE-CKS and PB-CAG-rtTA . Transfected cells were maintained over three weeks on a feeder layer to observe for colonies. iPSC colonies were stained for the expression of AP, as depicted in red.

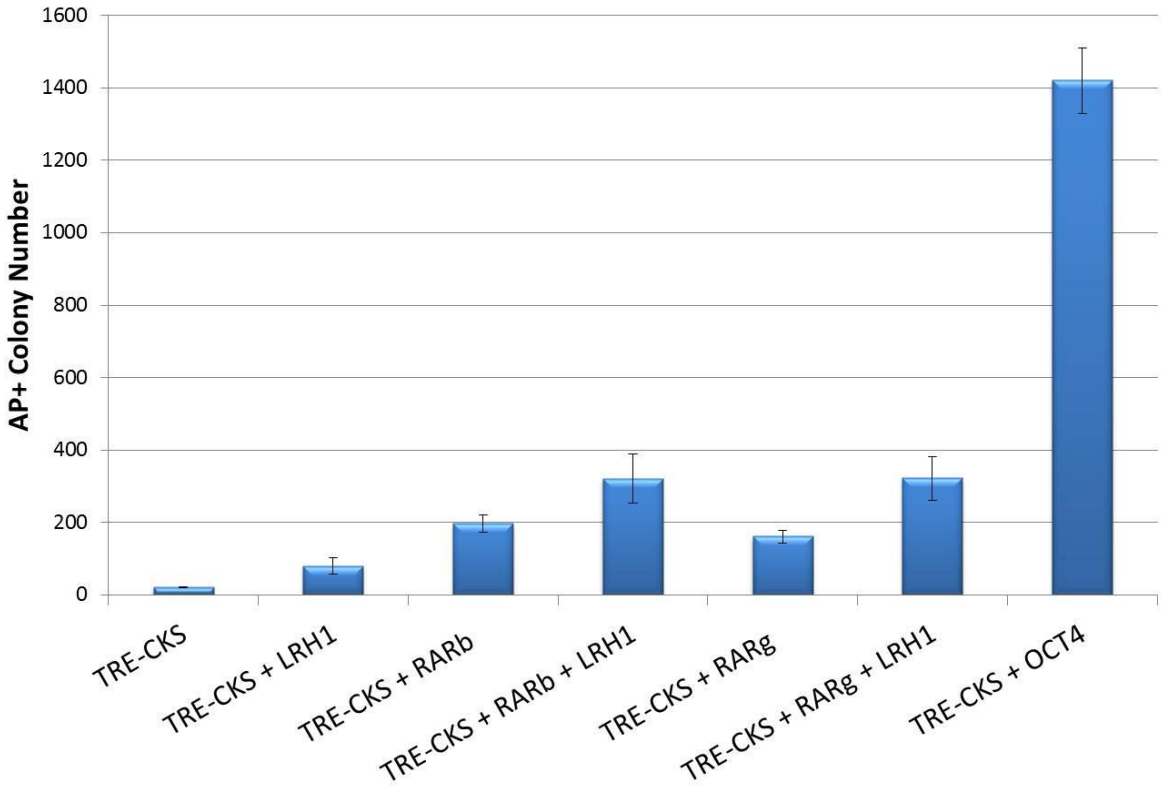

Figure 5.11 Comparison of reprogramming efficiencies triggered by RARβ and RARγ, in synergy with LRH1 To compare the reprogramming capabilities of RARβ and RARγ, both factors were introduced into MEFs, in the presence or absence of LRH1, and the addition of PB-TRE-CKS and PB-CAG-rtTA . Transfected cells were maintained over three weeks on a feeder layer to observe for colonies. Number of colonies generated in each transgenic combination were calculated and indicated in the bar chart. Triplicates for each reprogramming combination was performed and the error bars indicate the standard deviation derived from the three independent experiments.

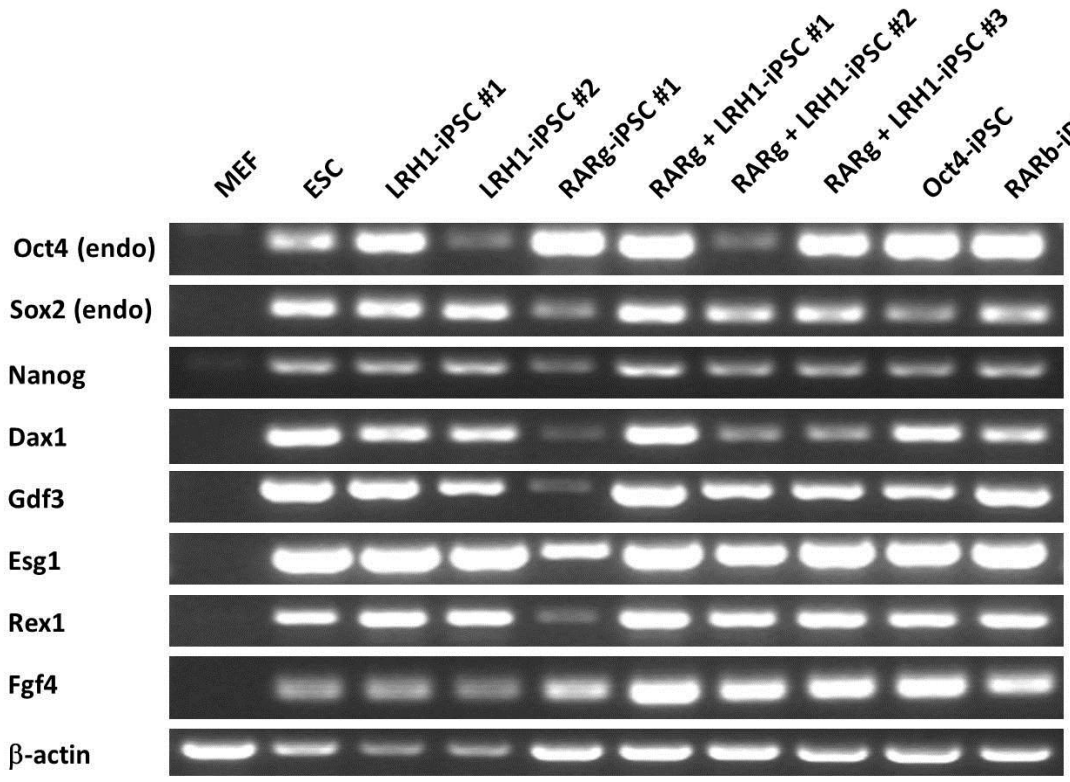

Figure 5.12 Expression levels of pluripotency markers in iPSCs generated by RARγ and/or LRH1 iPSCs were generated using combinations of RARβ, RARγ and LRH1, in the presence of PB-TRE-CKS. iPSC colonies were picked and expanded in doxycycline free-growth medium. cDNA was obtained from established iPSC clones and expression of pluripotency markers were assessed by PCR and visualised on an agarose gel. The intensities of the bands are indicative of the amount of transcript present.

5.2.4 Oct4, c-Myc, Klf4, Sox2, RARγ and LRH1 (6F) are the most potent activators of the Oct4 distal enhancer

This chapter has described that RARs and LRH1 play a positive transcriptional role by tethering to the Oct4 distal enhancer in the absence of ectopic Oct4. Recent work has described the remarkable ability of Oct4, c-Myc, Klf4, Sox2, RARγ and LRH1 (6F) in improving reprogramming speed and efficiencies (Wang et al., 2011c). As ectopic Oct4 has been omitted from the experiments thus far, it was interesting to determine if the reprogramming advantage instigated by 6F was also attributable to transcriptional activation at the distal enhancer. To this end, reprogramming cocktails inclusive of Oct4 were co-introduced with luciferase constructs described in **Figure 5.6** into MEFs. Luciferase assays were performed at 48 hours post transfection and **Figure 5.13** illustrates the results of the luciferase assays. Evident from the graphical representation of luciferase readings, the presence of 6F initiated luciferase activity of DE-PE-RAREoct up to 20-fold, when compared to activity incited by the presence of Oct4, c-Myc, Klf4 and Sox2 (4F). Interestingly, substitution of RARγ with RARβ also led to a 10-fold increment in luciferase activity compared to 4F. These results reinforce the notion that RARs can regulate Oct4 expression, especially when facilitated by the introduction of ectopic Oct4 and LRH1.

To align the luciferase results to a functional test, an identical spectrum of reprogramming combinations in **Figure 5.13** was introduced into MEFs and observed for the formation of iPSC colonies. **Figure 5.14** illustrates a numerical comparison of iPSC colonies obtained from the respective reprogramming conditions. Analagous to data retrieved from **Figure 5.13**, the addition of RARβ and RARγ to the conventional reprogramming mix (4F) improved reprogramming efficiencies. The efficiency was substantially further increased in co-operation with LRH1. These results complement previous findings and suggest that RAR family members elicit similar effects in assisting reprogramming events, but the 6F combination is most competent at inciting Oct4 expression and reprogramming efficiencies.

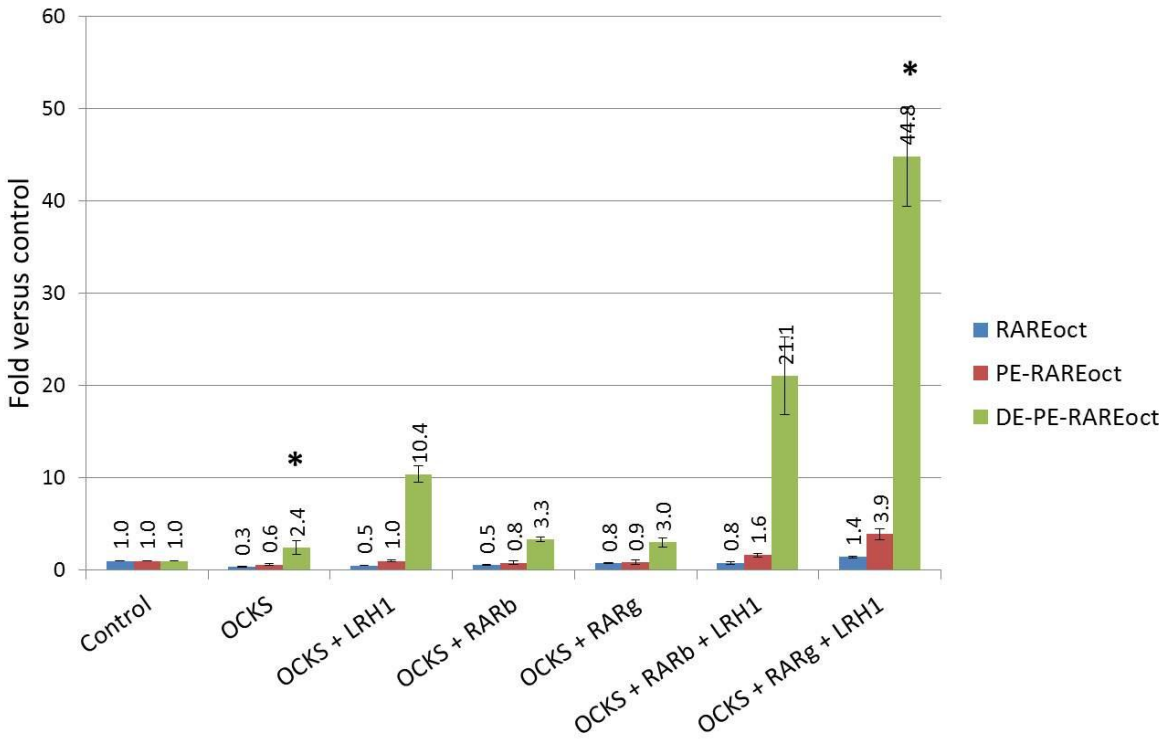

Figure 5.13 RARβ and RARγ act synergistcally with LRH1 to transcriptionally activate the distal enhancer of Oct4 in the presence of conventional four reprogramming factors
Combinations reflective of the reprogramming cocktails were transfected into MEFs. The combinations of transgenes introduced are labelled along the horizontal axis. Each of the three luciferase constructs (RAREoct, PE-RAREoct, and DE-PE-RAREoct) was co-introduced with each transgenic combination. After 48 hours, the cells were lysed and assessed for luciferase activity. The values are displayed in the bar chart, where the three colours depict the different luciferase construct analysed. The readings are normalised against Renilla and pGL3-basic. Statistical analysis was performed using student's T-test. *$p<0.05$

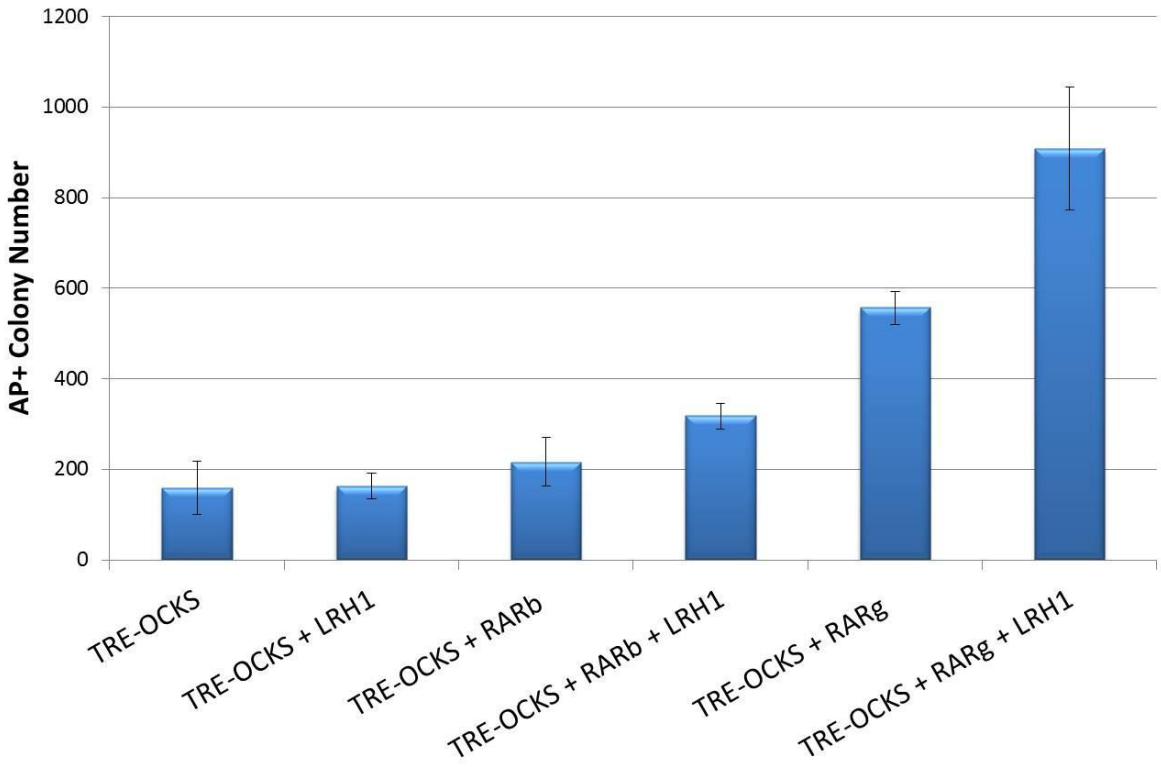

Figure 5.14 Comparison of reprogramming efficiencies triggered by RARβ and RARγ, in synergy with LRH1 and the four conventional reprogramming factors To compare the reprogramming capabilities of RARβ and RARγ, both factors were introduced into MEFs, in the presence or absence of LRH1, and the addition of PB-TRE-OCKS and PB-CAG-rtTA. Transfected cells were maintained over three weeks on a feeder layer to observe for colonies. Number of colonies generated in each transgenic combination were calculated and indicated in the bar chart. Triplicates for each reprogramming combination was performed and the error bars indicate the standard deviation derived from the three independent experiments.

5.2.5 LRH1 binds to the regulatory elements of Oct4

The synergy between RARγ and LRH1 in activating Oct4 expression at RAREoct has been attributed to co-operative binding (Ben-Shushan et al., 1995; Wang et al., 2011c). With the discovery that RARβ and RARγ work in unison with LRH1 to promote Oct4 expression at the distal enhancer, it is imperative to determine if LRH1 is able to tether to similar upstream regions of Oct4. To pursue this question, ChIP was performed in ESCs using an antibody against LRH1. Using the same set of primers described earlier (**Figure 5.3**), 11 putative RAREs within 10kb upstream of the Oct4 TSS were amplified. **Figure 5.15** illustrates quantification of the amplified products at the 11 potential sites. Remarkably, there exists a close resemblance between the patterns observed for LRH1 and RARβ. Motifs situated beyond 2.17kb of the Oct4 TSS were poorly represented, whereas regions closer to the Oct4 TSS were heavily represented. This supports the notion that LRH1 competently binds to the distal and proximal enhancers of Oct4 and RAREoct, explicating its ability to co-regulate the Oct4 distal enhancer with RAR family members.

Results from the luciferase assay suggest that RARγ interacts more effectively with LRH1 than RARβ to activate transcription at the Oct4 distal enhancer (**Figure 5.9** and **Figure 5.13**). This could be attributable to mutual exclusivity in their inherent expression profiles. Examination of transcript levels of the RAR family members and LRH1 in ESCs and MEFs illustrate that RARβ and LRH1 are unlikely to co-express in a natural setting (**Figure 5.16**). In contrast, LRH1, RARα and RARγ are expressed in ESCs, surmising the innate ability of RARγ and LRH1 to co-operate and trigger transcription at the Oct4 distal enhancer (**Figure 5.9** and **Figure 5.13**). These discrepancies in expression levels reflect the effective pairing between RARγ and LRH1, but not RARβ and LRH1. Subsequent co-immunoprecipitation experiments using endogenous proteins could allow better understanding of the natural synergy between RARs and LRH1. As RARα and RARγ are expressed in similar patterns, it will be interesting to determine if RARα and LRH1 display synergistic abilities to promote the transcriptional activation of Oct4.

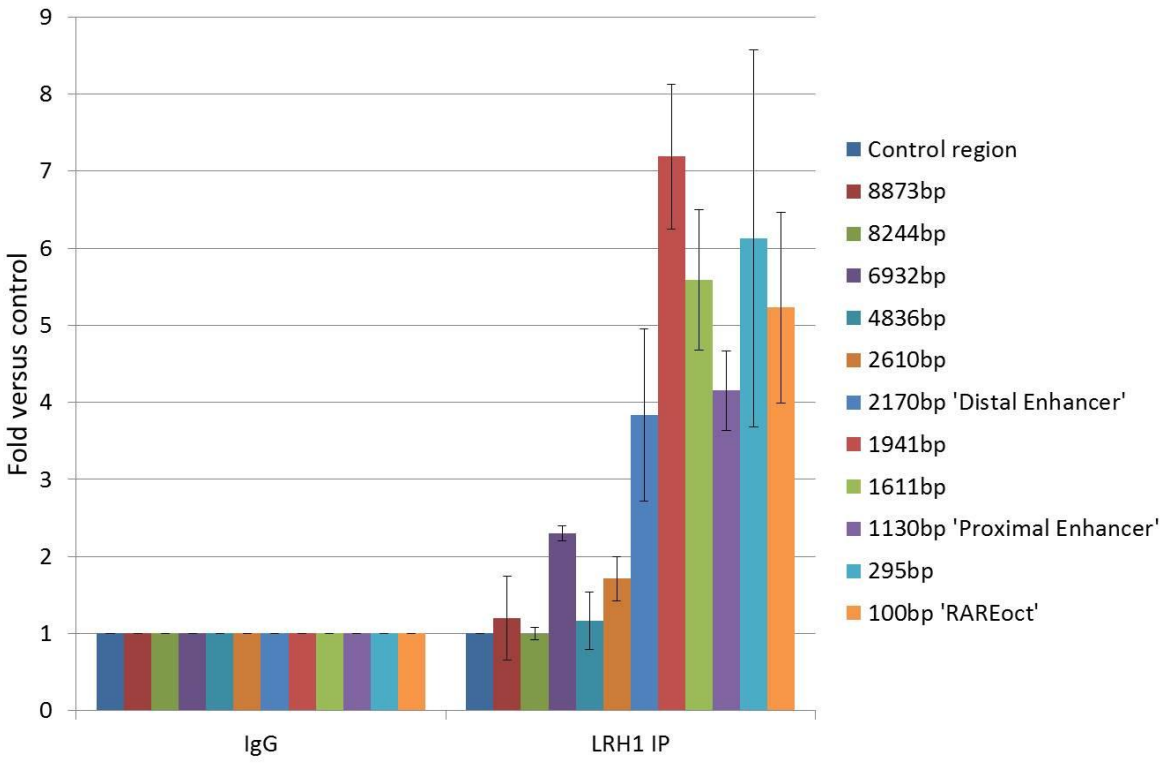

Figure 5.15 LRH1 binds to RAREs up to 2kb upstream of Oct4 Chromatin Immunoprecipitation (ChIP) was performed using antibodies against LRH1 in ESCs. Using eluate obtained from the pull-down, qPCR was conducted using primers designed against the 11 predicted RAREs. The values were normalised against a control region 24kb upstream of Oct4, and IgG.

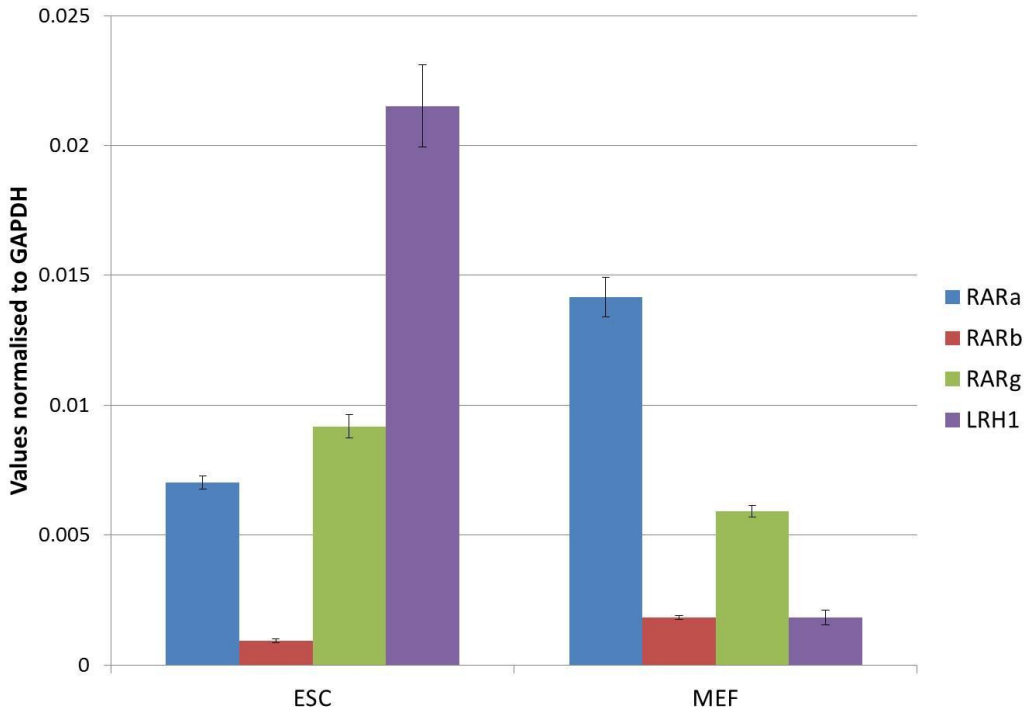

Figure 5.16 Expression profile of RAR family members and LRH1 in MEFs and ESCs Primers were designed against exon junctions of RARα, RARβ, RARγ and LRH1. Using cDNA from ESCs and MEFs, the transcript amounts of RARα, RARβ, RARγ and LRH1 were quantified through qPCR using corresponding primers and SYBR-Green and normalised against GAPDH expression levels.

5.2.6 Oct4 expression is obligatory during reprogramming

Although RAR family members have been portrayed to induce nuclear reprogramming of mouse fibroblasts to a primitive pluripotent state and eradicate the requirement for exogenous Oct4 expression, it is not clear if endogenous Oct4 expression is obligatory during the reprogramming process. To address this concern, temporal control of Oct4 in ZHBTc4 ESCs was employed (Niwa et al., 2000). **Figure 5.17** delineates the genetic modifications received by ZHBTc4 ESCs. In essence, both endogenous copies of Oct4 had been replaced by antibiotic resistance through homologous recombination. To sustain pluripotency, exogenous Oct4 cDNA controlled by a tetracycline repressible system was introduced into ZHBTc4 ESCs. These genetic manipulations allow self-renewal and the maintenance of pluripotency. In addition, Oct4 levels can be altered according to the addition of doxycycline, a potent derivative of tetracycline, where escalating amounts of doxycycline represses Oct4 expression to an increasing extent.

To determine if endogenous Oct4 is required during reprogramming, ZHBTc4 ESCs were differentiated for 6 days in the presence of 0.1μM ATRA and 1μg/mL doxycycline before reprogramming factors were introduced to determine competence in the acquisition of pluripotency (**Figure 5.18**). A combination of high doses of retinoic acid and doxycycline instigated differentiation of compact ESC colonies into flat extended cells. It has been previously established that exposure to doxycycline triggers trophoblast differentiation (Niwa et al., 2000), whereas high amounts of ATRA have been described to instigate differentiation cues (Strickland and Mahdavi, 1978; Schuldiner et al., 2001; Okada et al., 2004). A combination of two differentiation stimuli results in a population of differentiated cells that are distinct from ESCs and exhibit a complete loss of Oct4 (**Figure 5.19**). With the ablation of Oct4 expression, reprogramming cocktails were introduced to assess the reprogramming potential of the differentiated ESCs. Upon the introduction of reprogramming factors, the transfected cells were maintained over two weeks in the absence or presence of doxycycline and scored for the number of iPSC colonies generated.

As a control experiment, I made an initial attempt to determine the pliability of Oct4 expression after the withdrawal of doxycycline. ZHBTc4 ESCs which had been differentiated using ATRA and doxycycline over 6 days were seeded in the absence of doxycycline. Cells were collected at 1, 2, 3 and 7 days after doxycycline withdrawal and assessed for Oct4 expression (**Figure 5.19**). Upon 24 hours of doxycycline treatment at 0.1μg/mL, Oct4 expression was markedly reduced to levels resembling MEFs. Interestingly, after 7 days of doxycycline withdrawal, Oct4 transcript amounts were only regained to 20% of the initial intensity, and reversion to ESC-like morphology was not observed. This could be attributed to epigenetic modifications of the Oct4 transgene in ZHBTc4 cells during the differentiation process.

To assess the acquisition of pluripotency in the absence of Oct4, differentiated ZHBTc4 cells were transfected with various combinations of reprogramming factors (**Figure 5.20**). Continuous exposure to doxycycline and repression of Oct4 ablated the reprogramming potential of RARs and LRH1, implying the obligatory requirement for Oct4 during the initiation of reprogramming events. The absence of reprogramming events was rectified through the introduction of ectopic Oct4, reinforcing the notion that Oct4 is essential during reprogramming. However, the observation that ectopic expression of Oct4 was sufficient to initiate reprogramming events suggests that endogenous expression of Oct4 is unnecessary. Apart from ectopic introduction of Oct4, doxycycline which repressed Oct4 expression was withdrawn to stimulate Oct4 expression and generate iPSC colonies in the presence of CKS and RARs (**Figure 5.20**). This observation was compounded with the co-introduction of LRH1. Surprisingly, withdrawal of doxycycline in cells which only encompassed ectopic CKS could not initiate the formation of iPSCs. This could be attributable to an incomplete re-expression of Oct4 following doxycycline withdrawal as described in **Figure 5.19**. To offer a graphical representation of colony numbers, **Figure 5.21** depicts a cumulative bar chart reflecting the number of iPSC colonies obtained from four separate reprogramming experiments.

Having determined that Oct4 expression is essential to trigger the generation of iPSCs, it was next explored if increasing Oct4 expression levels could affect reprogramming efficiency. To this end, compounded amounts of Oct4 facilitated by introduction of ectopic Oct4 and re-expression of Oct4 transgene in the absence of doxycycline, were demonstrated to escalate reprogramming efficiencies (**Figure 5.20**). This proposes that amount of Oct4 is a limiting factor for the initiation of reprogramming. Additionally, inclusion of RARs and LRH1 into the reprogramming mix further enhanced reprogramming efficiencies (**Figure 5.20**), once again emphasising the capacity of RARs and LRH1 to promote the generation of iPSCs As Oct4 expression in ZHBTc4 cells is not controlled by its genomic regulatory elements, it suggests that RARs have alternative roles in the reprogramming process. To this end, RARs have been described to function through non-canonical pathways and may account for the discrepancies observed (Alsayed et al., 2001; Gianni et al., 2002; Gianni et al., 2006; Bruck et al., 2009; Masia et al., 2007; Ross-Innes et al., 2010; Laursen et al., 2012).

Overall, these results surmise the unequivocal need for the presence of Oct4 during reprogramming. Ectopic expression of Oct4 through the delivery of a foreign vector is sufficient to trigger reprogramming events but heightened amounts of Oct4 can enhance the generation of iPSCs. Furthermore, inclusion of RARs into the reprogramming cocktail can augment reprogramming efficiencies, substantiating previous experimental observations and proposing a new dimension to the role of RARs in the reprogramming process.

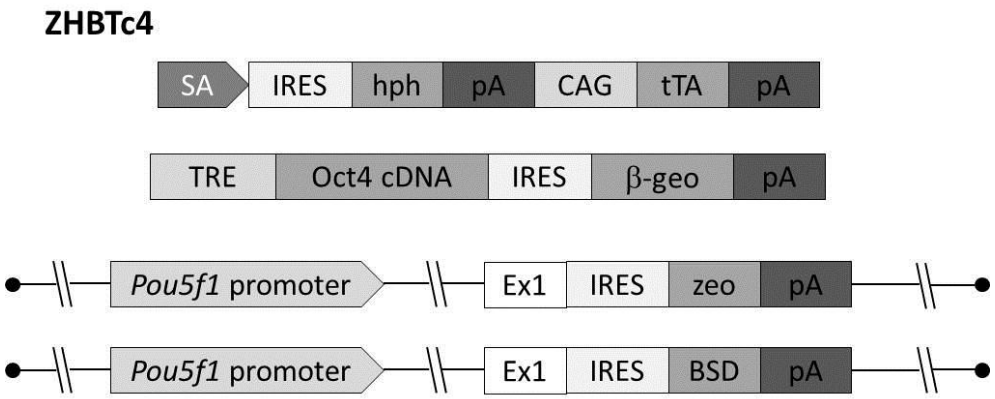

Figure 5.17 Genomic modifications of ZHBTc4 ESCs ZHBTc4 ESCs comprise of transgenes encoding (i) tetracycline transactivator and (ii) Oct4 cDNA driven by a tetracycline response element. The endogenous Oct4 loci are disrupted through homologous recombination and exon 1 is spliced with zeocin and blasticidin resistance genes.

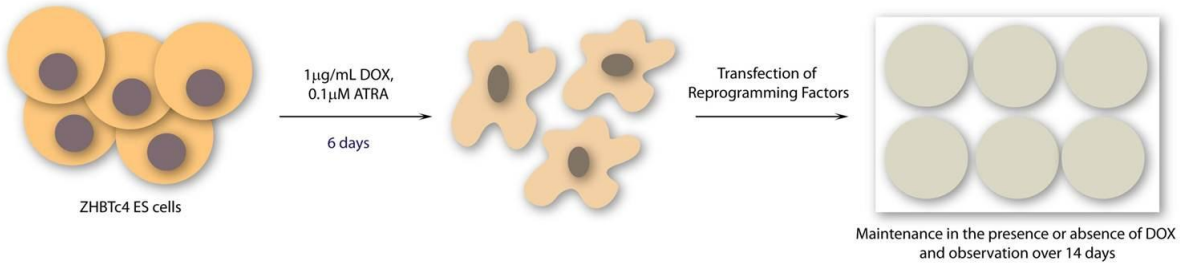

Figure 5.18 Experimental Strategy to determine involvement of Oct4 in reprogramming To determine the role of Oct4 in reprogramming, Oct4 expression levels were manipulated using ZHBTc4 ESCs. 1μg/mL DOXYCYCLINE and 0.1μM ATRA were introduced to ZHBTc4 ESCs for 6 days to trigger differentiation. Combinations of reprogramming factors were transfected into the differentiated cells and maintained in ESC growth media for 14 days. Colonies obtained were stained for the presence of AP and quantified.

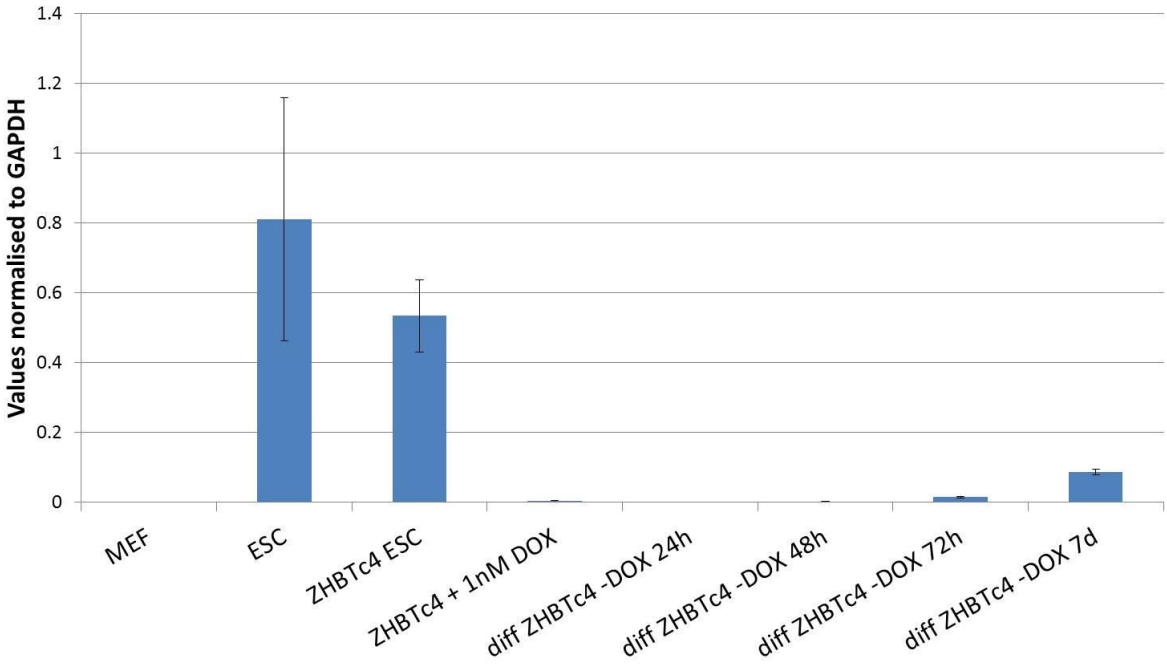

Figure 5.19 Manipulation of Oct4 expression levels in ZHBTc4 ESCs To verify the changes in Oct4 expression levels in ZHBTc4 ESCs, Oct4 transcript amounts were quantified using qPCR. Lane 1 shows negligible Oct4 levels in MEFs. Unmodified ZHBTc4 ESCs (Lane 3) exhibit Oct4 expression comparable to ESCs that carry both endogenous copies of Oct4 (Lane 2). Introduction of 1nM DOXYCYCLINE to ZHBTc4 ESCs over a period of 24 hours (Lane 4) drastically reduces Oct4 expression levels. Lanes 5-8 correspond to the re-expression of Oct4 in differentiated ZHBTc4 cells upon the withdrawal of DOXYCYCLINE over a 7 day period.

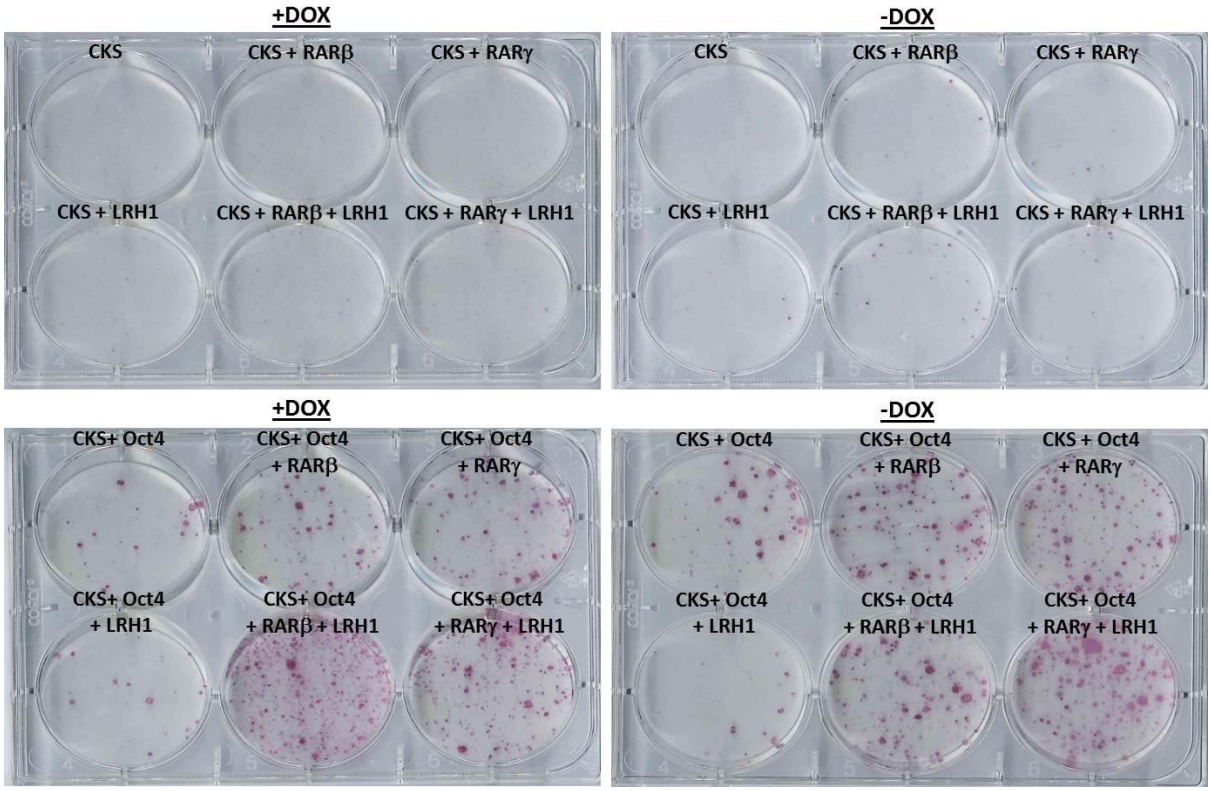

Figure 5.20 Reprogramming of differentiated ZHBTc4 ESCs in the presence or absence of Oct4 ZHBTc4 ESCs were differentiated in 0.1μM ATRA and 1μg/mL DOXYCYCLINE for 6 days before combinations of CAG-promoter driven reprogramming factors were introduced by electroporation. Transfected cells were maintained in ESC growth media for 14 days, before reprogrammed colonies were stained for the presence of AP. AP activity, as indicated by red colour is a marker for pluripotency.

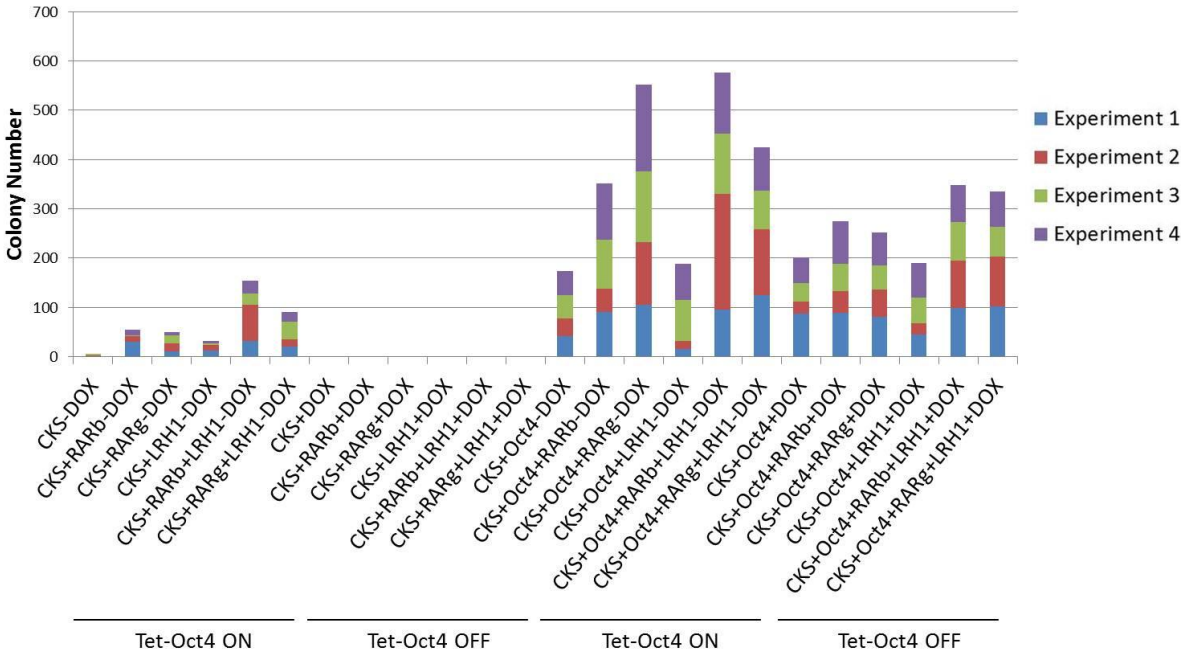

Figure 5.21 Bar chart reflecting reprogrammed colonies from differentiated ZHBTc4 ESCs ZHBTc4 ESCs were differentiated in 0.1μM ATRA and 1μg/mL DOXYCYCLINE for 6 days before combinations of reprogramming factors were introduced by electroporation. Transfected cells were maintained in ESC growth media in the absence (Tet-Oct4 ON) or presence (Tet-Oct4 OFF) of doxycycline for 14 days, before reprogrammed colonies were stained for the presence of AP. Number of colonies expressing AP is reflected in the bar chart. Each colour represents an independent experiment. Cumulative values for four separate experiments are illustrated.

5.2.7 Understanding the kinetics of activating Oct4 regulatory elements during reprogramming

RARs have been described as potent triggers of the reprogramming process, with RARγ and LRH1 exhibiting impactful capacities to positively regulate the Oct4 distal enhancer and RAREoct. With this in mind, it is pertinent to dissect the molecular kinetics of Oct4 activation assisted by RARγ and LRH1 during reprogramming. In order to assess the kinetics behind reprogramming, recordings of a homogenous population of cells undergoing reprogramming would be ideal. To this end, iPSCs were generated through the introduction of 4F or 6F in a doxycycline inducible approach. Both populations of iPSCs were differentiated through the addition of 0.1μM ATRA over 6 days (**Figure 5.22**). This creates a consistent platform to examine snapshots of the initial stages of reprogramming. Differentiated iPSCs were then exposed to doxycycline to elicit the re-expression of reprogramming factors. Comparison of 4F and 6F reprogramming kinetics highlights differences attributed to the presence of RARγ and LRH1. Cells were collected at various time points after doxycycline treatment to inspect epigenetic alterations at the Oct4 regulatory elements. Extending this, luciferase assays were performed in these cells to determine the activity of reporter constructs described in **Figure 5.6** at 24, 48 and 72 hours after doxycycline treatment. This method permits the dissection of changes akin to epigenetic modifications and activating stimuli at the regulatory components of the Oct4 locus during the first 72 hours of reprogramming and illuminates the participation of RARγ and LRH1 during the acquisition of pluripotency.

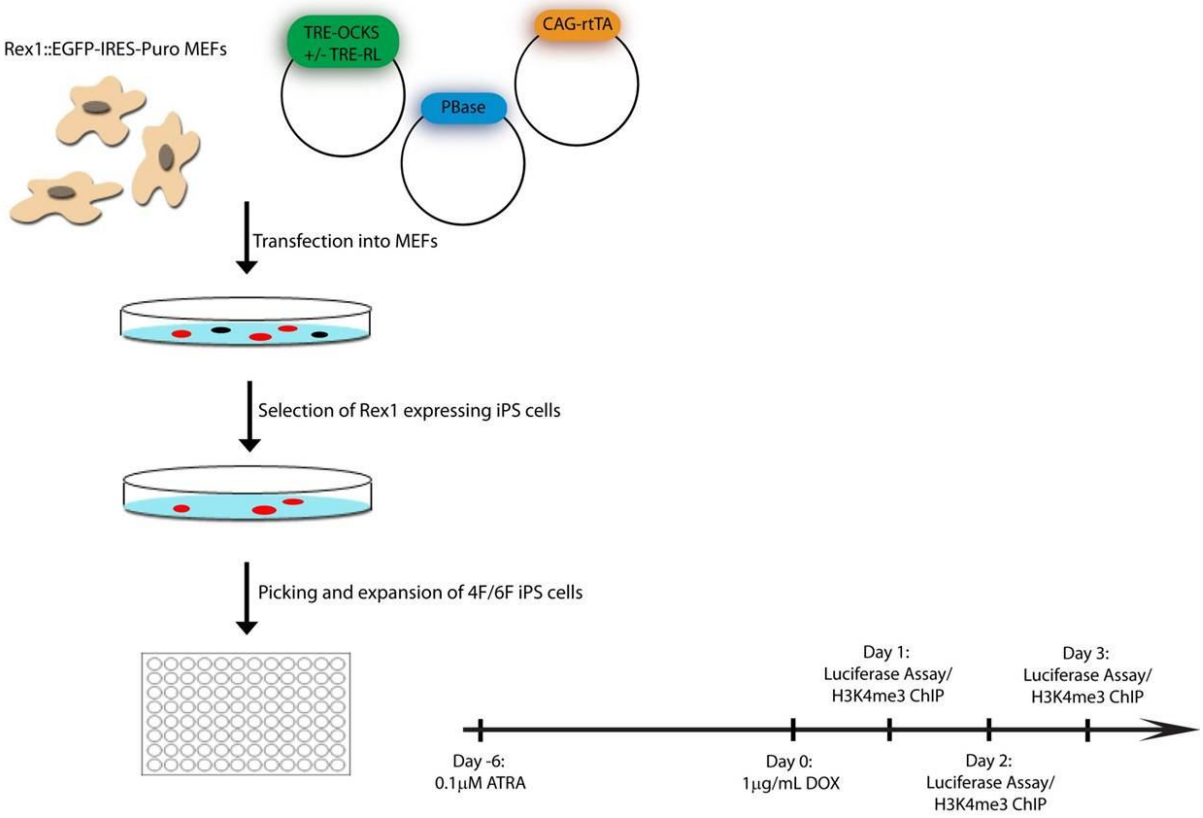

Figure 5.22 Experimental Strategy to study the kinetics of Oct4 activation during reprogramming Using a doxycycline (DOXYCYCLINE) inducible platform, 4F (Oct4, c-Myc, Klf4 and Sox2) or 6F (Oct4, c-Myc, Klf4, Sox2, RARγ and LRH1) were transfected into Rex1::EGFP-IRES-Puro MEFs. iPSCs expressing Rex1 were selected for using Puromycin and surviving colonies were picked and expanded. Upon expansion, DOXYCYCLINE was withdrawn to establish iPSC lines independent of the reprogramming transgenes. The cells were differentiated using 0.1μM ATRA for 6 days, before DOXYCYCLINE was re-introduced to trigger the re-expression of transgenes. At 24, 48, and 72 hours after transgene expression, cells were analysed for the activation of Oct4 enhancer/promoter regions using luciferase assays and chromatin immunoprecipitation of H3K4me3.

To determine if six days of ATRA treatment was sufficient to elicit differentiation of iPSCs and silence Oct4 transcription to levels that resemble MEFs, luciferase constructs described in **Figure 5.6** were introduced into differentiated 4F and 6F iPSCs. In unmodified 4F and 6F iPSCs, transcriptional activities of luciferase constructs were similar to ESCs (**Figure 5.23**). On the other hand, differentiation of iPSCs led to silencing of luciferase constructs to levels similar to MEFs (**Figure 5.24**). These results reaffirm the dependability of differentiated iPSCs as an assessment of regulatory elements upstream of Oct4 using luciferase assays.

Having determined that six days of ATRA treatment was sufficient to reduce luciferase activity in differentiated iPSCs to levels similar in MEFs, the experiment was repeated and doxycycline was introduced for various lengths of time, immediately after six days of ATRA treatment. The addition of doxycycline acted as a stimulus for the re-expression of reprogramming factors and initiated the acquisition of pluripotency in differentiated 4F and 6F iPSCs. **Figure 5.25** and **Figure 5.26** offer a graphical representation of luciferase readings measured using luciferase reporter constructs described in **Figure 5.6**, at specified durations after the addition of doxycycline in differentiated 4F and 6F iPSCs.

At 24 hours, luciferase constructs that comprised of either the distal enhancer (DE-minP) or proximal promoter (RAREoct) alone triggered transcriptional activation in differentiated 6F iPSCs, suggesting that expression of 6F could activate both the distal enhancer and RAREoct independent of each other. However, after 48 hours, these levels plateau. In contrast, a marked increase in luciferase activities was observed in luciferase constructs driven by the presence of both the distal enhancer and RAREoct (DE-PE-RAREoct and DE-PE-RAREoct-minP), 48 hours after re-expression of reprogramming transgenes in differentiated 6F iPSCs. Luciferase activity continued to escalate at 72 hours, proposing synergistic co-operation between the distal enhancer of Oct4 and RAREoct. The presence of the proximal enhancer did not significantly affect transcriptional activity, reinforcing previous observations that RARs and LRH1 did not significantly regulate Oct4 expression through the proximal enhancer (**Figure 5.9** and **Figure 5.13**). Interestingly,

differentiated 4F iPSCs did not result in the activation of luciferase reporter constructs between 24 and 72 hours, suggesting that observed differences in the luciferase activities in differentiated 6F iPSCs were attributable to the presence of the extra two factors, RARγ and LRH1.

From the luciferase assay, it is evident that the Oct4 distal enhancer and RAREoct are rapidly activated upon reprogramming, attributable to the expression of RARγ and LRH1. To offer a distinct perspective to the kinetics of reprogramming elicited by the presence of RARγ and LRH1, transcriptionally permissive histone modifications at the distal enhancer of Oct4 and RAREoct were examined in differentiated 4F and 6F iPSCs at 24, 48 and 72 hours after transgene re-activation. To this end, immunoprecipitation of trimethylated lysine 4 of histone 3 (H3K4me3) would allow the co-precipitation of actively transcribed genomic regions (**Figure 5.22**). Detection of Oct4 distal enhancer elements and RAREoct would suggest that these regions have undergone epigenetic modifications to permit transcription.

To allow a fair comparison to findings from the luciferase assay (**Figure 5.25** and **Figure 5.26**), 4F and 6F iPSCs were similarly differentiated in 0.1μM ATRA before doxycycline was introduced to trigger the re-expression of reprogramming factors. Analogous to the results obtained from the luciferase assay, both the Oct4 distal enhancer and RAREoct were associated with H3K4me3 in differentiated 6F iPSCs after 24 hours of doxycycline treatment (**Figure 5.27**). However, between 24 and 48 hours, pervasive epigenomic modifications ensued and both the Oct4 distal enhancer and RAREoct in 6F cells were heavily tethered to H3K4me3 activating marks. This association exhibited up to an eight-fold increment when compared to unmodified 6F iPSCs. However, between 48 and 72 hours, H3K4me3 levels were reduced to resemble unmodified 6F iPSCs. Reminiscent of the luciferase assay, the Oct4 regulatory elements in differentiated 4F iPSCs were not associated to H3K4me3 up to 72 hours of transgene re-activation, again demonstrating the notion that rapid activation of the distal enhancer and proximal promoter of Oct4 was triggered by the presence of RARγ and LRH1.

Overall, two distinct techniques were employed to study the kinetics behind the initiation of reprogramming events and findings from both experiments concur, implicating RARγ and LRH1 in the prompt activation of Oct4 within 24 hours of reprogramming. To associate the rapid activation of Oct4 with a functional outcome, differentiated 4F or 6F iPSCs were seeded onto a feeder layer in the presence of doxycycline and assessed for the formation of iPSCs. At the end of 7 or 14 days, AP expressing colonies were counted and compared to determine differences in reprogramming efficiencies (**Figure 5.28**). In the absence of doxycycline, both differentiated 4F iPSCs and 6F iPSCs gave rise to iPSC colonies, suggesting aberrant silencing of transgenes. Overall, differentiated 6F iPSCs generated 12.5 and 8 fold more iPSC colonies than differentiated 4F iPSCs at 7 days and 14 days respectively, reiterating the capacity of RARγ and LRH1 in the prompt activation of Oct4 regulatory elements and initiation of reprogramming events.

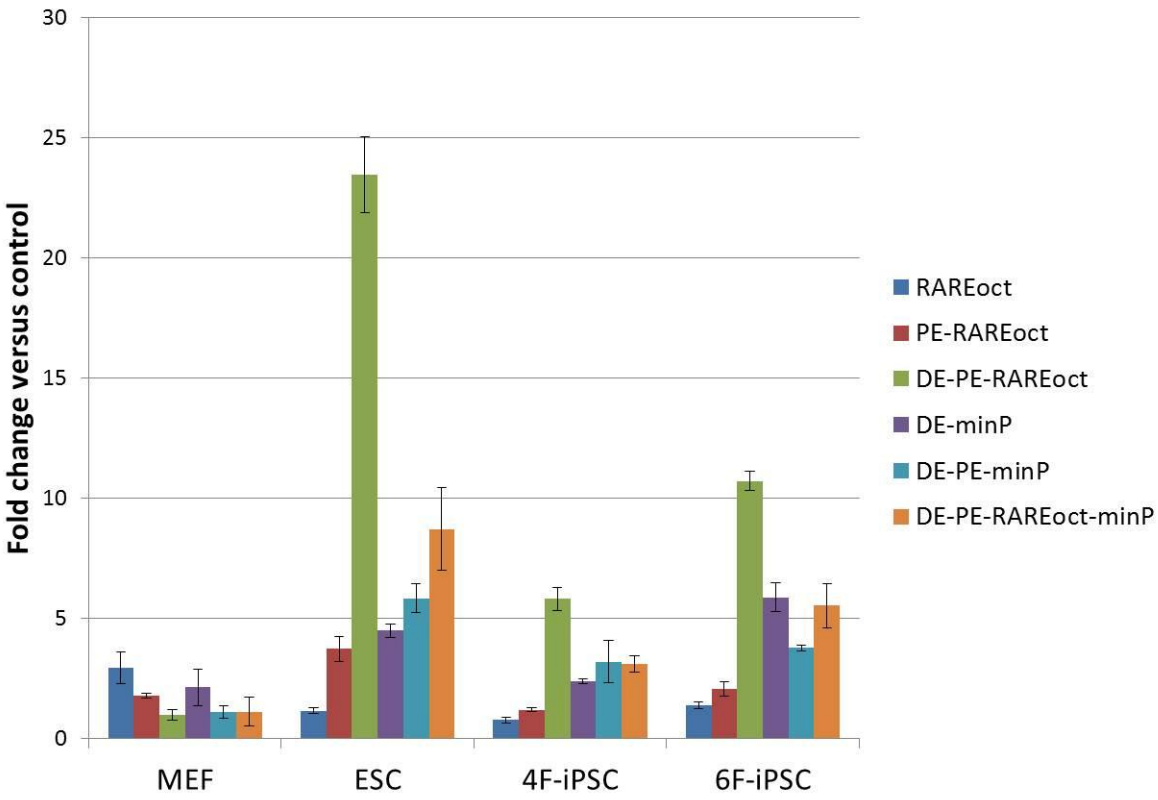

Figure 5.23 Activity of luciferase constructs in unmodified 4F and 6F iPSCs To determine the activity of luciferase constructs depicted in **Figure 5.6**, 10μg of each construct was co-electroporated with 1μg of Renilla luciferase driven by a constitutive TK promoter. Each coloured bar denotes a luciferase construct. The constructs were introduced into MEFs, ESCs and 4F and 6F iPSCs, which were obtained as described in **Figure 5.19**. The firefly luciferase values were normalised to their corresponding Renilla readings and respective empty vector. Normalised values are indicated on the bar chart.

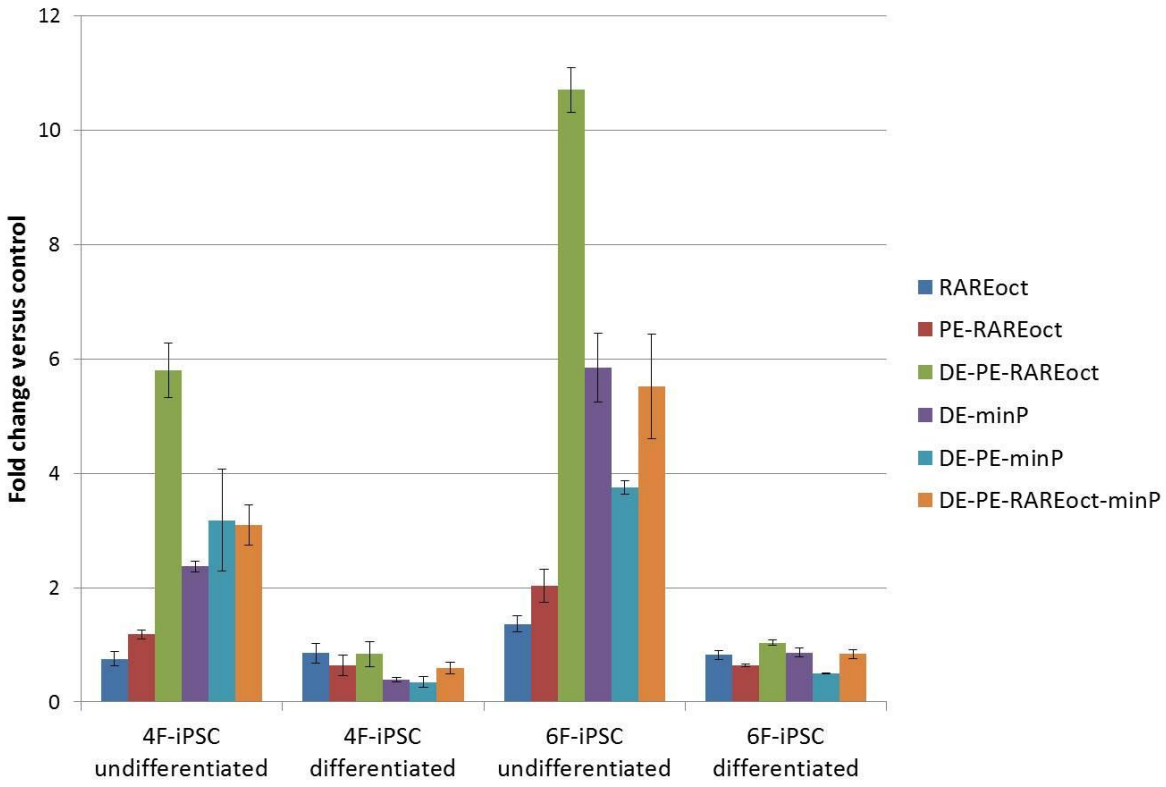

Figure 5.24 Differentiation of 4F and 6F iPSCs reduces luciferase activity To ensure that 6 days of 0.1μM ATRA treatment was sufficient to induce differentiation, luciferase constructs listed in **Figure 5.6** were introduced into both undifferentiated and differentiated iPSCs. 10μg of each luciferase construct was co-electroporated with 1μg of Renilla contstruct into undifferentiated and differentiated derivatives of 4F and 6F iPSCs. Firefly luciferase readings were normalised to Renilla luciferase values and the respective empty vector. Normalised values are shown on the bar chart.

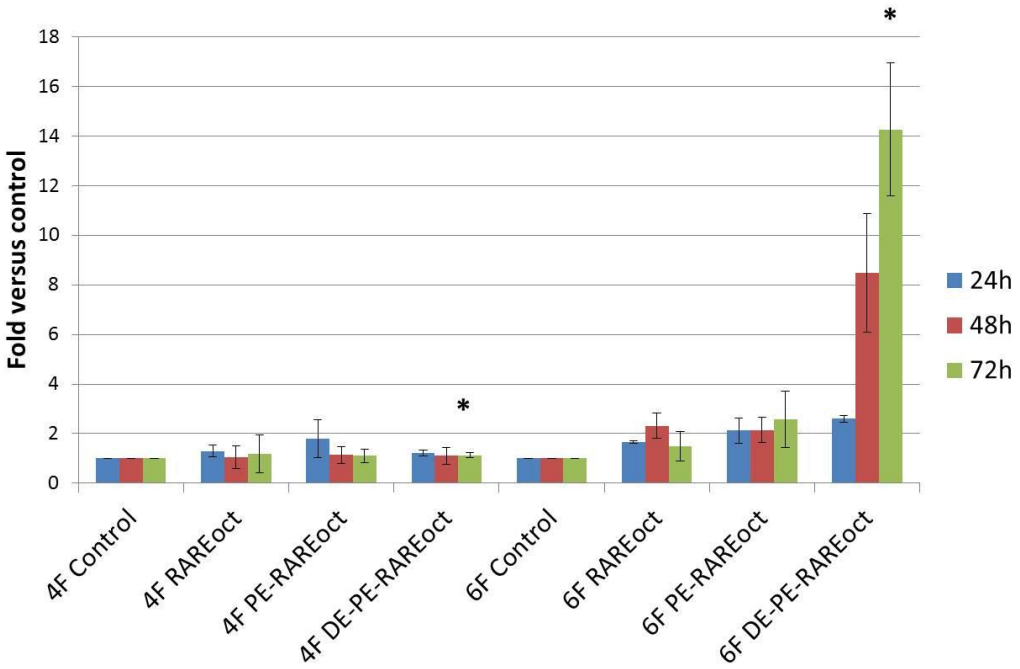

Figure 5.25 Oct4 Distal Enhancer and Proximal Promoter are activated within 24h of ectopic transgene expression To determine the onset of activation at the distal enhancer, proximal enhancer and proximal promoter of Oct4, luciferase constructs as labelled on the horizontal axis were introduced into differentiated 4F and 6F iPSCs. Transfected cells were exposed to DOXYCYCLINE for 24, 48 and 72hours until luciferase activity was assayed. Renilla luciferase constructs were co-introduced with the luciferase reporter vectors for normalisation. Values observed in the bar chart reflect normalisation to Renilla luciferase readings, empty pGL3 vector and activity in the absence of DOXYCYCLINE. Statistical analysis was performed using a student's T-test. *$p<0.05$

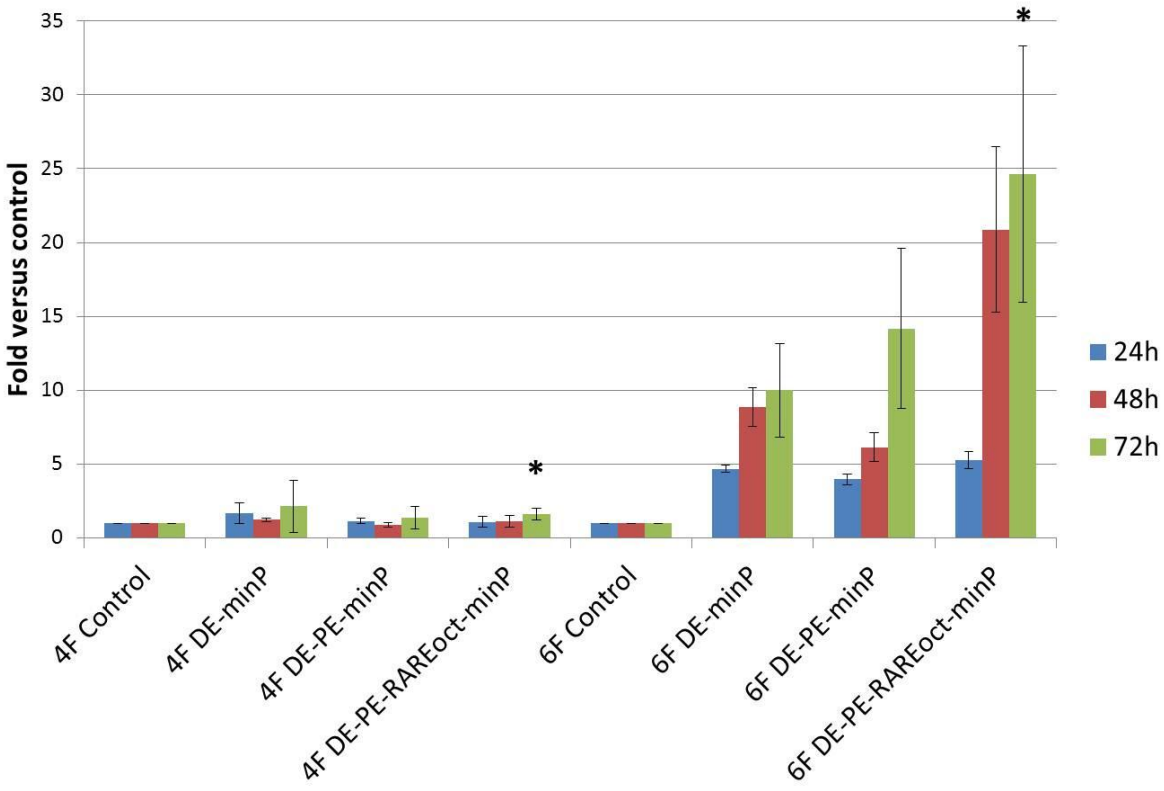

Figure 5.26 Oct4 Distal Enhancer and Proximal Promoter are activated within 24h of ectopic transgene expression To determine the onset of activation at the distal enhancer, proximal enhancer and proximal promoter of Oct4, luciferase constructs as labelled on the horizontal axis were introduced into differentiated 4F and 6F iPSCs. Transfected cells were exposed to doxycycline for 24, 28 and 72hours until luciferase activity was assayed. Renilla luciferase constructs were co-introduced with the luciferase reporter vectors for normalisation. Values observed in the bar chart reflect normalisation to Renilla luciferase readings, empty pGL4.23 vectors and activity in the absence of doxycycline. Statistical analysis was performed using a student's T-test. *$p<0.05$

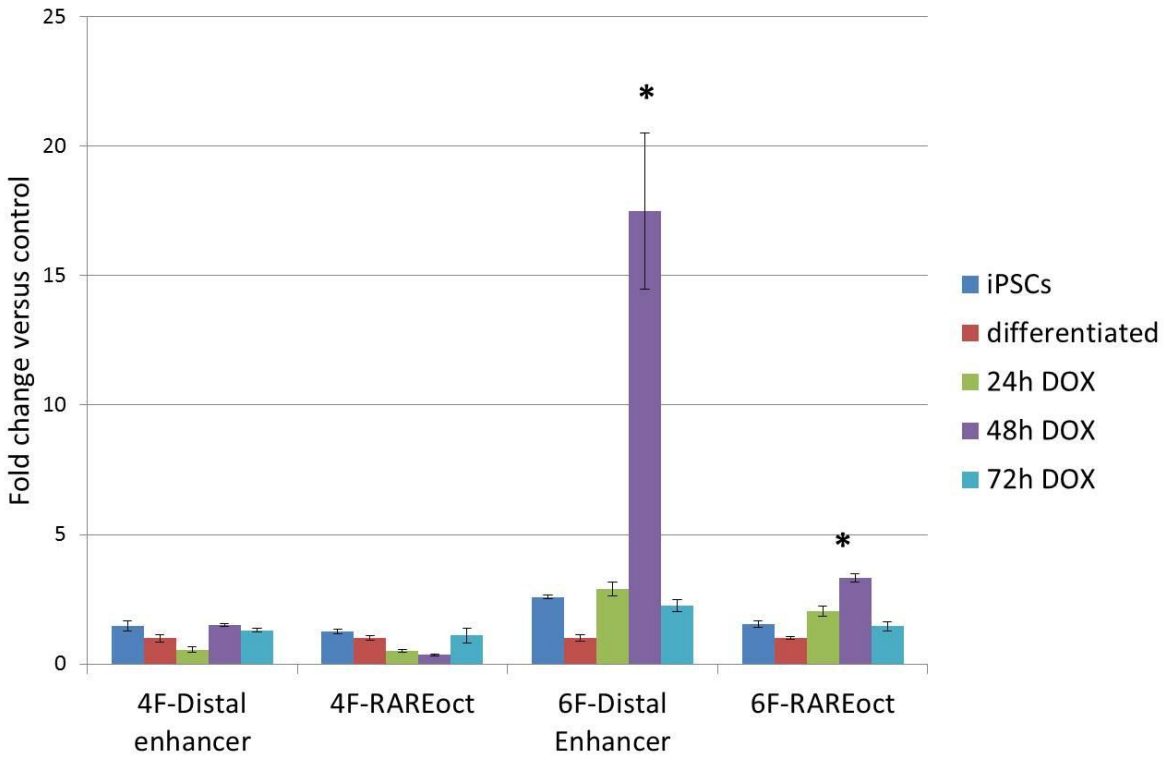

Figure 5.27 Oct4 Distal Enhancer and Proximal Promoter are associated to activating histone marks within 24h of ectopic transgene expression To determine the epigenomic landscape of the Oct4 distal enhancer and proximal promoter during reprogramming, differentiated 4F and 6F iPSCs were exposed to doxycycline for 24, 48 and 72 hours. After incubation for the respective lengths of time, chromatin immunoprecipitation of H3K4me3 activating marks was performed. Using the eluate, qPCR was performed using primers against the distal enhancer or RAREoct, as indicated on the bar chart. Readings were normalised against IgG and plotted on the bar chart. Statistical analysis were performed using a student's T-test. *$p<0.05$

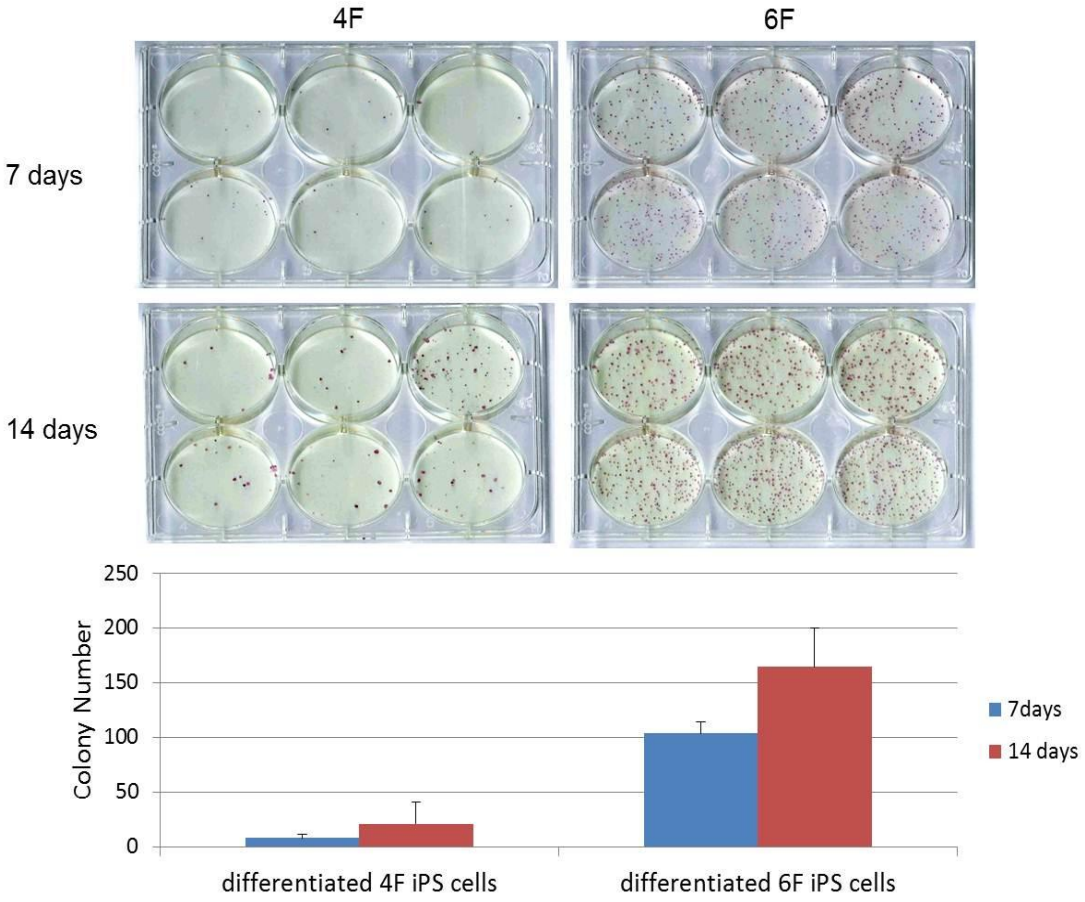

Figure 5.28 Comparison of reprogramming efficiencies in differentiated 4F and 6F iPSCs iPSCs generated through the introduction of 4F and 6F in a doxycycline dependent manner were differentiated for 6 days in ATRA. Once differentiated, 50 x 10^3 cells were seeded into each well of a 6 well plate. The cells were exposed to 1μg/ml doxycycline to re-express 4F or 6F reprogramming factors. (Top) After 7 or 14 days, the reprogrammed cells were stained for the expression of AP. The expression of AP is indicated by the colour red. (Bottom) Quantification of colony numbers.

5.2.8 6F triggers rapid expression of GFP controlled by endogenous Oct4 regulatory elements

Previous findings in this chapter delineated the role of RARγ and LRH1 in the activation of the Oct4 distal enhancer and RAREoct within 24 hours of reprogramming. To this end, a significant proportion of supporting evidence originated from the employment of luciferase assays. However, analysis of transcriptional activity using luciferase constructs which are ectopically introduced poorly reflects the dynamics that pertain to chromatin architecture at the endogenous locus. As such, it is imperative to draw parallels to transcriptional activity at the endogenous locus. To address this, MEFs that encompass a transgene consisting of Oct4 regulatory elements spanning 18kb controlling EGFP expression (Oct4-EGFP MEFs) were employed to generate a new population of 4F and 6F iPSCs (Ying et al., 2002). As such, EGFP expression acts as a surrogate to assess transcriptional activation of endogenous Oct4. 4F and 6F were introduced into Oct4-EGFP MEFs in a doxycycline inducible manner and reprogrammed colonies were expanded in the absence of doxycycline. Resultant iPSCs were designated as 4F-O-iPSCs and 6F-O-iPSCs respectively (**Figure 5.29**).

To determine if expression of RARγ and LRH1 leads to rapid activation of the endogenous Oct4 locus, a similar strategy to **Chapter 5.2.7** was employed to enable fair comparisons. 4F-O-iPSCs and 6F-O-iPSCs were differentiated using ATRA over 6 days before doxycycline was employed to trigger re-expression of the reprogramming factors. As EGFP expression acts as an indicator for Oct4 activity, cells which were exposed to doxycycline were observed under the microscope for EGFP expression.

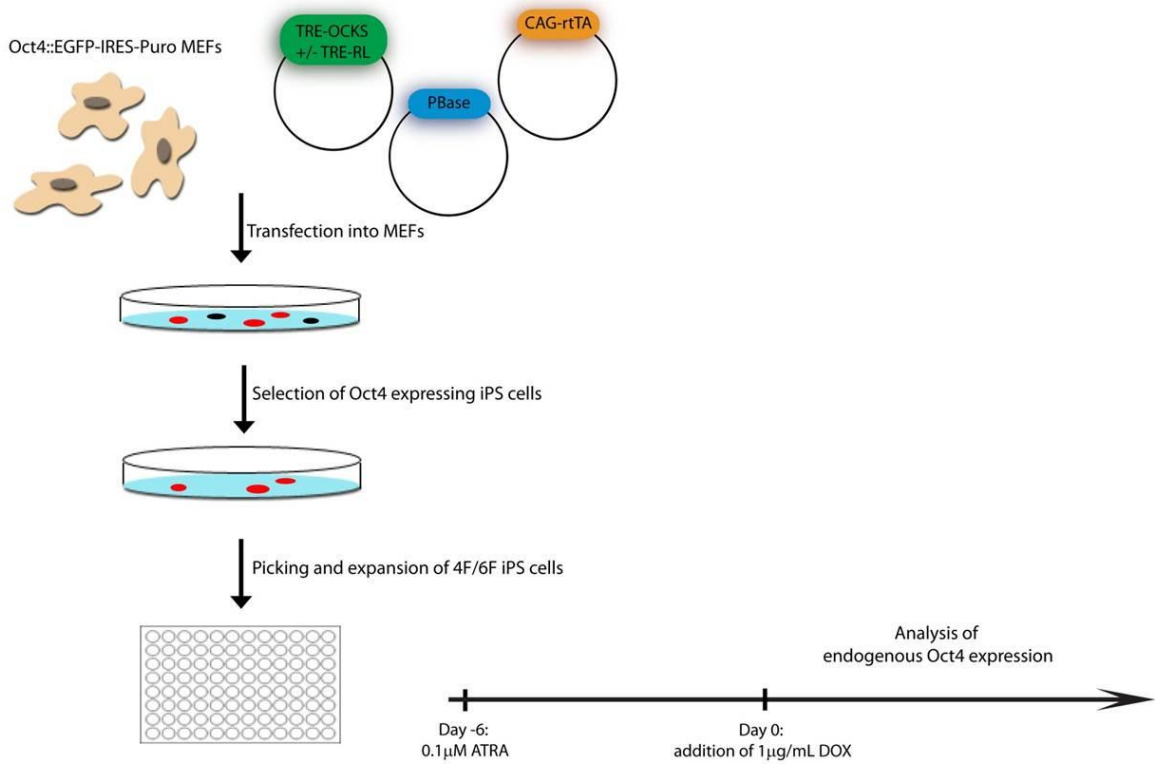

Figure 5.29 Experimental Strategy to observe expression of endogenous Oct4 during reprogramming MEFs which carry genes encoding EGFP expression and Puromycin resistance under the endogenous Oct4 promoter were innoculated with either 4F or 6F in a doxycycline inducible manner. Resultant iPSCs (4F-O-iPSCs and 6F-O-iPSCs) which were transcriptionally active at their endogenous Oct4 loci were selected for using puromycin. Surviving cells were picked and expanded. Differentiation was induced using 0.1μM ATRA for 6 days before doxycycline was introduced. Re-expression of Oct4 was tracked under the microscope over the course of doxycycline treatment.

As a control, it was first determined if 4F-O-iPSCs and 6F-O-iPSCs exhibited faithful recapitulation of endogenous Oct4 expression as denoted by EGFP expression. **Figure 5.30** illustrates homogenous iPSC colonies that express GFP, reiterating high levels of Oct4 expression. In addition, 4F-O-iPSCs and 6F-O-iPSCs express pluripotency markers in a similar pattern to ESCs (**Figure 5.31**), indicating the attainment of pluripotency. To omit the possibility of contamination between 4F-O-iPSCs and 6F-O-iPSCs, genomic material from both populations was inspected for the presence of incongruent transgenes. **Figure 5.32** illustrates that 4F-O-iPSCs and 6F-O-iPSCs consist of the right combination of transgenes, reaffirming the integrity of the iPSCs.

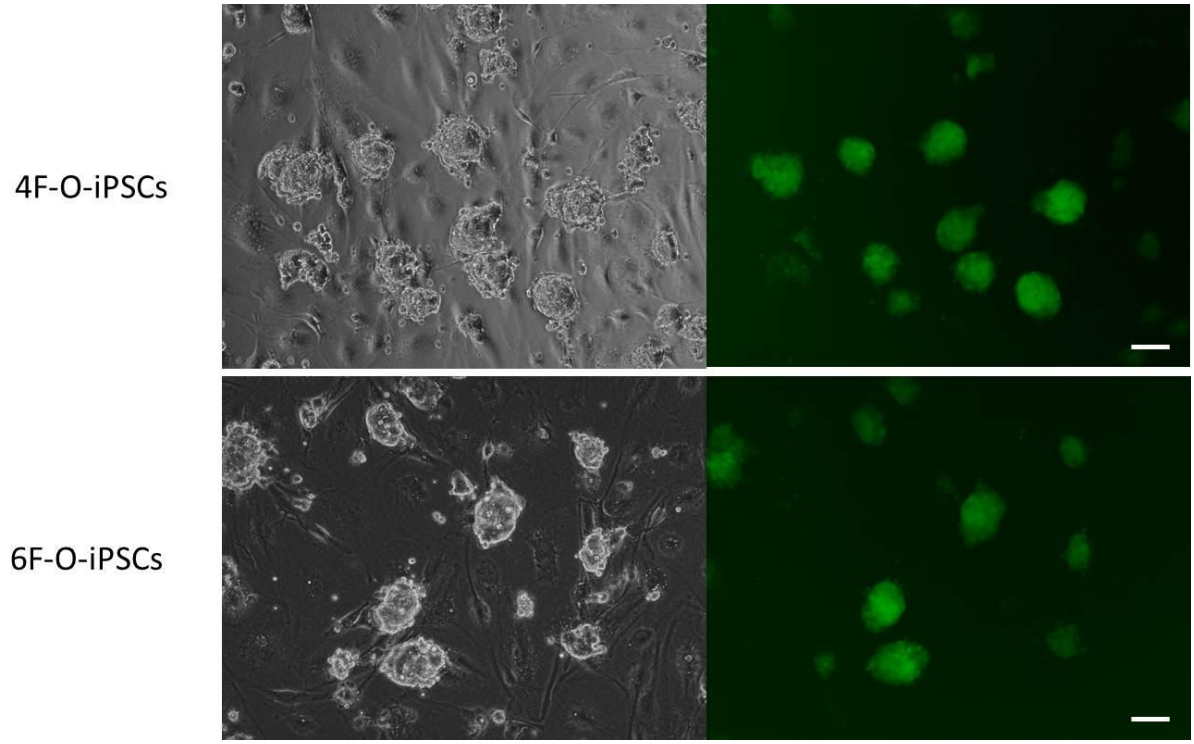

Figure 5.30 Microscope images of 4F-O-iPSCs and 6F-O-iPSCs iPSCs were obtained as described in **Figure 5.25.** iPSCs generated from the introduction of 4F and 6F into Oct4::EGFP-IRES-Puro MEFs were designated as 4F-O-iPSCs and 6F-O-iPSCs respectively. The cells were maintained in 2i-LIF growth media, in the absence of doxycycline. The images on the left reflect their morphology in brightfield, whereas the images on the right demonstrate EGFP expression from the endogenous Oct4 locus. Scale Bar: 50μm.

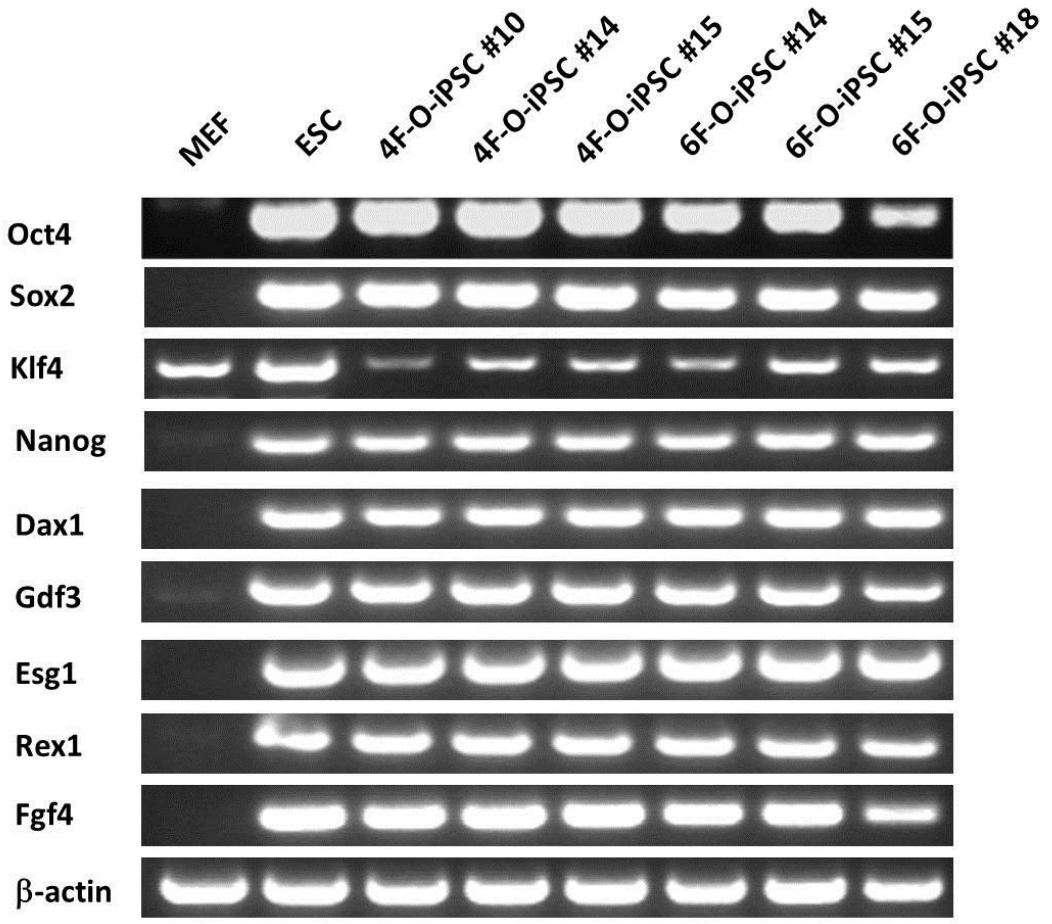

Figure 5.31 Expression levels of Pluripotency Markers in 4F-O-iPSCs and 6F-O-iPSCs RT-PCR of the various pluripotency markers was performed in MEF (lane1), ESCs (lane2), 4F-O-iPSCs (lanes 3-5), and 6F-O-iPSCs (lanes 6-8). Expression levels are described qualitatively by the intensity of the amplified products viewed using an agarose gel. The array of pluripotency markers tested is labelled to the left of the image.

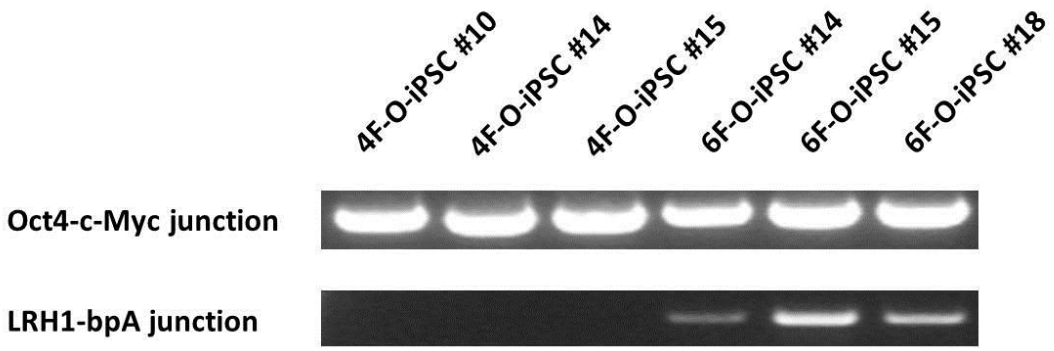

Figure 5.32 Presence of transgenes in 4F-O-iPSCs and 6F-O-iPSCs To ensure that the right combination of transgenes was present in 4F-O-iPSCs and 6F-O-iPSCs, genomic DNA was retrieved from 3 clones of 4F-O-iPSCs and 3 clones of 6F-O-iPSCs. Primers specific to the transgenes were designed. To detect insertions containing the vector encompassing 4F, primers against the junction of Oct4 and c-Myc were desgined. To detect insertions containing the vector containing RARγ and LRH1, primers were designed against the junction corresponding to LRH1 and bpA. PCR was performed using the primers indicated to the left and the amplified products were visualised using an agarose gel.

Having ascertained that 4F-O-iPSCs and 6F-O-iPSCs exhibited pluripotency, it was essential to ensure that differentiation of both iPSC populations would result in ablation of EGFP expression. As depicted by **Figure 5.34** and **Figure 5.35**, Oct4 expression levels were drastically reduced in the presence of ATRA, exemplifying the dependability of the system to study the effect of RARγ and LRH1 on the re-emergence of Oct4 expression.

As part of the experimental design, differentiated 4F-O-iPSCs and 6F-O-iPSCs were exposed to doxycycline to trigger re-expression of the reprogramming factors. However, as observed in **Figure 5.19**, differentiation of pluripotent cells may induce modifications which compromise transgene re-expression. To address this possibility, transgene re-expression in differentiated 4F-O-iPSCs and 6F-O-iPSCs was assessed. **Figure 5.33** demonstrates efficient re-expression of the reprogramming transgenes within 24 hours of doxycycline treatment, strengthening the reliability of the strategy chosen for the study of reprogramming kinetics in the presence of RARγ and LRH1.

Having obtained 4F-O-iPSCs and 6F-O-iPSCs which differentiate readily and exhibit the capacity for transgene re-activation, both iPSC lines were compared to determine if RARγ and LRH1 exhibited an effect on the expression of endogenous Oct4 during the initiation of reprogramming. To this end, 4F-O-iPSCs and 6F-O-iPSCs were differentiated in the presence of 0.1μM ATRA over 6 days before being exposed to doxycycline to re-establish transgene expression. Cells incubated in doxycycline were observed under a microscope at 24 hour intervals over a span of three days to capture transcriptional activation of the Oct4 locus, as indicated by green fluorescence. **Figure 5.34** and **Figure 5.35** depict representative images of the reprogramming events that occurred. In both populations of cells that harbour 4F and 6F, cells were amenable to morphological transformations within 72 hours, where an epithelial-like conformation was adopted. In contrast, EGFP expression was observed in reprogramming intermediates of differentiated 6F-O-iPSCs at 48 hours but not in equivalent 4F counterparts, suggesting that endogenous Oct4 transcription was activated at an earlier time due to the presence of RARγ and LRH1, thus conforming to previous findings.

As microscope images reflect the re-activation of Oct4 transcription at single cell resolution, it is pivotal to determine if a similar phenomenon is observed in a larger population of cells. To address this concern, differentiated 4F-O-iPSCs and 6F-O-iPSCs that had been exposed to doxycycline over a period of 1, 3 and 6 days were collected and analysed. **Figure 5.36** depicts a qualitative perspective of endogenous Oct4 transcript levels during the reprogramming of differentiated 4F-O-iPSCs and 6F-O-iPSCs. Concurring with previous observations, Oct4 transcripts were detected at 24 hours after doxycycline treatment of differentiated 6F-O-iPSCs, whereas the presence of Oct4 transcripts was detected at 72 hours after doxycycline induction in differentiated 4F-O-iPSCs. Overall, these findings reaffirm that co-operation between RARγ and LRH1 results in rapid activation of the distal enhancer of Oct4 and RAREoct, prompting transcriptional activity.

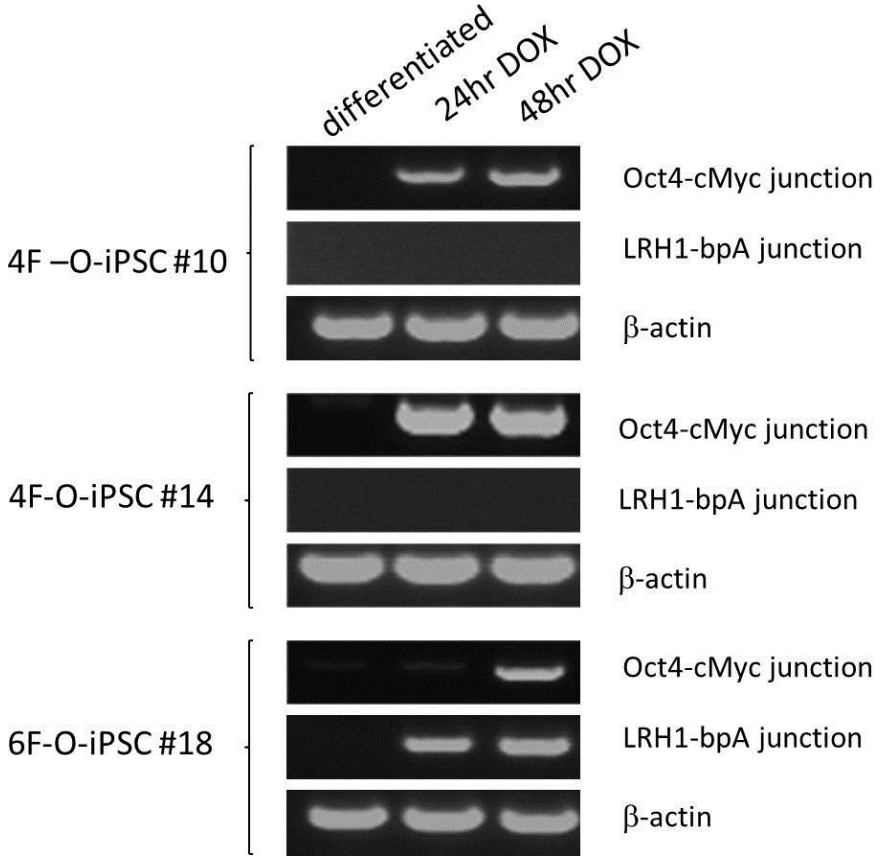

Figure 5.33 Re-activation of transgenes in differentiated 4F-O-iPSCs and 6F-O-iPSCs 4F-O-iPSCs and 6F-O-iPSCs were differentiated in the presence of 0.1μM ATRA over 6 days. To ensure that transgenes are readily re-expressed upon the addition of doxycycline, differentiated cells were seeded onto 6 well plates and 1μg/mL doxycycline was introduced for 24 and 48 hours. Cells were collected and RNA was extracted and converted to cDNA. PCR was performed using cDNA as a template to determine transcript amounts as recognised by primers specific to the junctions between cDNAs present only in the transgene.

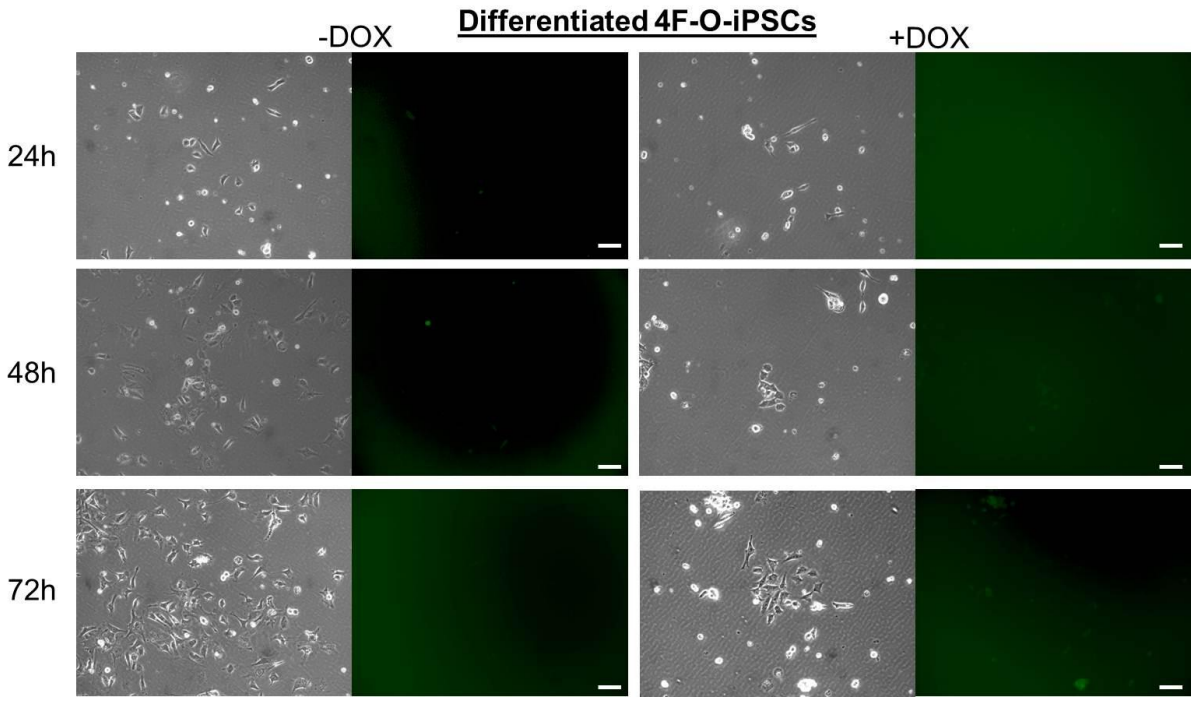

Figure 5.34 Re-establishment of Oct4 expression through the addition of doxycycline to differentiated 4F-O-iPSCs 4F-O-iPSCs were differentiated using 0.1μM ATRA for 6 days. 1μg/ml doxycycline (DOX) was added to the differentiated cells over a period of 72 hours. Changes in morphology and the re-expression of endogenous Oct4, as depicted by green fluorescence (200x), were traced every 24 hours. As a control, cells were grown in ESC media, in the absence of doxycycline. Scale bar: 50μm.

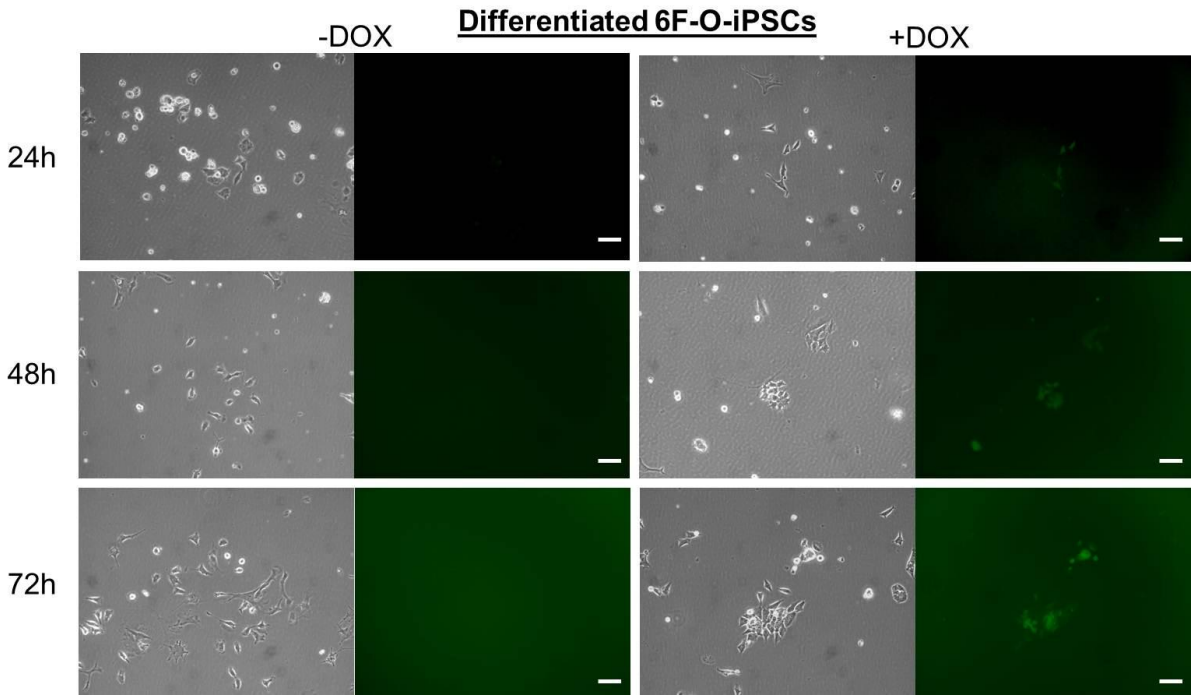

Figure 5.35 Re-establishment of Oct4 expression through the addition of doxycycline to differentiated 6F-O-iPSCs 6F-O-iPSCs were differentiated using 0.1μM ATRA for 6 days. 1μg/ml doxycycline (DOX) was added to the differentiated cells over a period of 72 hours. Changes in morphology and the re-expression of endogenous Oct4, as depicted by green fluorescence (200x), were traced every 24 hours. As a control, cells were grown in ESC media, in the absence of doxycycline. Scale bar: 50μm.

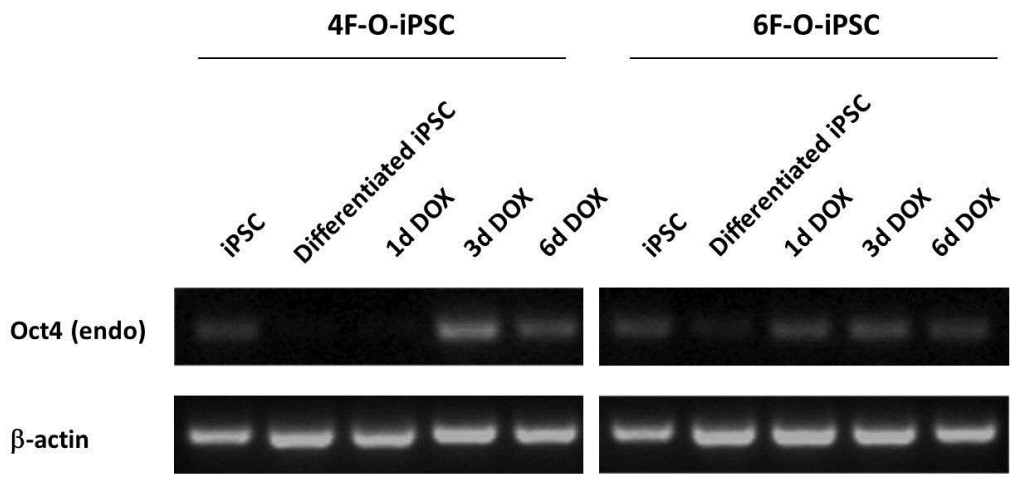

Figure 5.36 Analysis of Oct4 transcript levels after the addition of doxycycline to differentiated 4F-O-iPSCs and 6F-O-iPSCs 4F-O-iPSCs and 6F-O-iPSCs were differentiated using 0.1μM ATRA for 6 days. 1μg/ml doxycycline (DOX) was added to the differentiated cells over a period of 6 days. RNA was collected after 1, 3 and 6 days after doxycycline induction and the presence of Oct4 transcripts was assessed through PCR amplification using primers specific to endogenous Oct4. β-actin levels were also assessed as an internal control.

5.3 Discussion

5.3.1 Epigenetic changes in Oct4 regulatory elements

Chromatin remodelling has been portrayed to play a significant role in the initiation of the reprogramming process, as delineated by the profound effect elicited by chemicals which modify the epigenomic landscape (Mikkelsen et al., 2008; Huangfu et al., 2008a). Furthermore, components that participate in Brahma-related gene 1-Associated Factors (BAF) chromatin remodelling processes and Activation-Induced cytidine Deaminase (AID) dependent demethylation have been described to enhance reprogramming efficiencies (Singhal et al., 2010; Bhutani et al., 2010). In addition, impairment of transcriptionally repressive histone modifications by ablation of Utx1 has been described to affect pluripotency, cell fate decisions and reprogramming (Welstead et al., 2012; Mansour et al., 2012). Several studies have also depicted discrepancies in epigenetic profiles exhibited by iPSCs and ESCs, surmising a relationship between epigenetic disparities and variations in the pluripotent nature of reprogrammed cells (Lister et al., 2011; Stadtfeld et al., 2010).

Although it is evident that genome wide epigenomic alterations are pivotal during the acquisition of pluripotency, precise epigenetic changes have not been dissected. This chapter proposes that an initial amendment to the epigenomic landscape occurs at the distal enhancer of Oct4 and RAREoct. Activation of both regulatory elements was demonstrated to be facilitated by RARγ and LRH1, leading to the enhancement of reprogramming speed and efficiencies. Remarkably, the influence of RARγ and LRH1 ensues within 24 hours of reprogramming. This was examined from distinct perspectives, employing chromatin immunoprecipitation experiments which inspect transcriptionally permissive histone modifications at endogenous gene loci, to luciferase assays which assess the functional aspects of the regulatory elements. Ultimately, endogenous Oct4 transcriptional activity was assessed through the measurement of endogenous Oct4 transcripts and visualisation of EGFP as a marker. Results from these experiments concur and suggest that the presence of RARγ and LRH1 leads to rapid activation of the distal enhancer of Oct4 and RAREoct, resulting in efficient transcription of Oct4.

The distal enhancer of Oct4 and RAREoct were originally discovered due to their sensitivity to RA treatment (Schoorlemmer et al., 1994; Pikarsky et al., 1994; Okazawa et al., 1991; Sylvester and Schöler, 1994; Barnea and Bergman, 2000; Minucci et al., 1996). Exploration of the regulatory elements revealed that the distal enhancer influences Oct4 transcription in naïve pluripotent cells such as ESCs, but not EC cells or EpiSCs (Yeom et al., 1996; Tesar et al., 2007; Bao et al., 2009; Han et al., 2010a). Conversely, the proximal promoter plays a role in driving Oct4 expression in primed pluripotent cells such as EpiSCs (Tesar et al., 2007; Guo et al., 2010; Han et al., 2010a). As the generation of iPSCs entails the conversion of somatic cells to ESC-like derivatives, this chapter proposes that activation of the distal enhancer of Oct4 is critical in the initial stages of reprogramming. The addition of RARγ and LRH1 to the reprogramming mix was demonstrated to elicit a selective effect on the distal enhancer but not proximal enhancer of Oct4, thereby improving reprogramming conditions and reinforcing the significance of distal enhancer during the acquisition of naïve pluripotency.

On a separate note, studies on RAREoct have delineated positive and negative regulatory components which exert effects on Oct4 expression levels (Ben-Shushan et al., 1995; Fuhrmann et al., 1999; Fuhrmann et al., 2001). The RAR family has been portrayed to positively and negatively regulate the proximal promoter of Oct4 (Ben-Shushan et al., 1995; Barnea and Bergman, 2000; Sylvester and Schöler, 1994). As the locus is heavily occupied by regulators, the multifunctional role of RARs could be narrowed down to co-operating partners. This notion is reinforced by observations that the inclusion of LRH1 into various reprogramming mixes containing either RARγ or RARβ enhanced Oct4 transcriptional activity and reprogramming efficiencies. In addition, as ligand independent effects of RARs have been described (Ross-Innes et al., 2010; Laursen et al., 2012), it is plausible that the reprogramming capacities of RARs may be partly attributable to ligand independence. As RARα has been described to co-occupy regulatory regions with ER (Ross-Innes et al., 2010), it will be interesting to investigate the plausible role of ER signalling during somatic cell reprogramming. It has been established that Estrogen

Receptor Related Receptor-beta (Esrrb) can mediate reprogramming events in the mere presence of Oct4 and Sox2 (Feng et al., 2009). Although Esrrb remains to be an orphan receptor, these findings implicate the possibility of an estrogen related mechanism during the acquisition of pluripotency.

This chapter identified epigenetic modifications at Oct4 regulatory elements which accompany the initiation of reprogramming events. This was determined through the employment of chromatin immunoprecipitation of H3K4me3, followed by site specific quantification. Histone modifications allow an epigenetic snapshot of a genomic locus and have been demonstrated to embody dynamic changes during retinoic acid-mediated differentiation (You et al., 2011a). Trimethylated forms of histone H3 at lysine 4 (H3K4me3) have been identified at transcriptional start site of active promoters (Kim et al., 2005; Heintzman et al., 2007; Roh et al., 2005). By examining genomic regions which associate with H3K4me3, genes which are transcriptionally active can be isolated. To exploit this phenomenon, H3K4me3 was employed to examine molecular changes at the regulatory elements of Oct4. However, mono- and di-methylation at lysine 4 of histone 3 (H3K4me1 and H3K4me2) have been described to possess predictive power for the activation of enhancer regions (Kim et al., 2005; Heintzman et al., 2007). Enhancers in Oct4 regulatory regions are no exception and have been described to associate with H3K4me1 in ESCs (Whyte et al., 2012). As a result, examination of genomic regions encompassing H3K4me1/2 marks during the reprogramming process triggered by 4F and 6F may provide better insight to the activation status of the Oct4 enhancers. Nevertheless, the versatility of chromatin often entails looping between enhancer and promoter regions (Kagey et al., 2010) and analysis of the genomic locus corresponding to Oct4 in ESCs describes common components observed in promoter and enhancer regions, illustrating a native looped conformation in ESCs. With this in mind, H3K4me3 activating histone modifications are indicative of transcriptionally active gene loci, regardless of enhancer or promoter elements, and were employed in this study.

5.3.2 Identification of RAR-associated genomic regions

RARs were originally identified through homology to glucocorticoid and thyroid hormone receptors (Dejean et al., 1986; Giguere et al., 1987). Steroid hormone receptors possess DNA binding domains which recognise the canonical sequence (A/G)G(G/T)TC(A/G) and its variants. Traditional methods to identify RAR-regulated genes include analysis of transcriptional profiles stimulated by retinoic acid treatment, deletion mapping to identify the binding region, and predictions using computational techniques. With the advent of chromatin immunoprecipitation techniques coupled to sequencing (ChIP-seq), genome wide exploration of transcription factor binding events can be performed (Nielsen et al., 2008). To this end, genomic regions bound to RARα and RARγ have been elucidated using ChIP-seq (Delacroix et al., 2010; Ross-Innes et al., 2010). In contrast, there has been no attempt at delineating genome-wide RARβ binding regions. This study demonstrates an execution of ChIP using antibodies that recognise RARβ. The cis-regulatory elements of Oct4 were identified and verified using 3730 Sanger sequencing of amplified products corresponding to the regions-of-interest. With broader interest in genome-wide targets of RARβ, it will be intriguing to perform deep sequencing of eluates obtained from immunoprecipitation of RARβ. These findings may illuminate additional roles of RARβ in its ability to replace the requirement of exogenous Oct4 during the initiation of reprogramming events. Moreover, RARβ but not RARγ was identified from the genome wide screen performed in **Chapter 3**. Although this observation could be attributable to technical limitations, it is plausible that RARβ may participate in alternative pathways, or possess distinct interacting partners that facilitated its identification during the screen. To reinforce this notion, it has been established that RARβ and RARγ display specific expression patterns during development (reviewed by Dolle, 2009), suggesting that both RARs exhibit distinct cellular functions.

5.3.3 Implications of delineating transcriptional regulation of Oct4

Oct4 exists in a fleeting manner during development, where zygotic expression begins before 3.5dpc and abrogates between 4.5dpc and 10.5 dpc in somatic cells or in the germline respectively. Repression of Oct4 expression marks the first cell fate decision

where differentiation occurs to adopt a trophectoderm fate (Palmieri et al., 1994; Nichols et al., 1998). Deregulation of Oct4 expression levels leads to corrupted developmental cues, resulting in a spectrum of phenotypes, ranging from embryonic lethality to inappropriate differentiation accompanied by the loss of pluripotency (Nichols et al., 1998; Niwa et al., 2000). During the acquisition of pluripotency, the significance of ectopic expression of Oct4 is unequivocal (Nakagawa et al., 2008; Kim et al., 2009b; Kim et al., 2009c). The importance of Oct4 has been suggested to be attributable to its ability to elicit transcriptional activation (Hammachi et al., 2012). Findings from this chapter reiterate the importance of Oct4 two-fold. First, c-Myc, Klf4 and Sox2 alone were unable to elicit reprogramming events and second, silencing of Oct4 in ZHBTc4 cells abrogates the ability to acquire pluripotency. In addition to the generation of iPSCs, Oct4 has also been described to play roles in lineage switching (Efe et al., 2011; Szabo et al., 2010). As nuclear reprogramming entails immense amounts of chromatin remodelling, it will be interesting to inspect molecular changes at the regulatory elements of endogenous Oct4 during alternative methods of nuclear reprogramming and draw parallels to findings from this study.

5.3.4 Alternative platforms to study Oct4 activation during reprogramming events

The experimental strategy employed in this chapter focuses on secondary reprogramming events originating from differentiated iPSCs. A drawback of this method is exemplified by remnants of pluripotent cells which are recalcitrant to retinoic acid driven differentiation. The presence of these cells may mask the evaluation of obtained results. To circumvent this, various platforms can be utilised.

In order to obtain a homogenous platform to study the kinetics of reprogramming, secondary MEFs can be derived from chimeras derived from the injection of iPSCs in mouse blastocysts. Cells generated through this method would have been exposed to various developmental cues and no residual pluripotent cells should persist. To this end, several studies have employed the use of secondary MEFs to study the reprogramming process in detail (Hanna et al., 2009; Samavarchi-Tehrani et al., 2010). On the other hand,

secondary MEFs have participated in embryonic development and possess inherent barriers to reprogramming cues. This is demonstrated by the forced re-expression of reprogramming factors in secondary fibroblasts, where 20% of cells displayed early pluripotency markers and a mere 1.2% of these cells re-activated their endogenous Nanog locus after 16 days (Mikkelsen et al., 2008). As this chapter describes rapid epigenetic changes at the regulatory elements of Oct4, analysis of transcriptionally permissive histone modifications, H3K4me3, may not be reliably recapitulated using a population of secondary MEFs which exhibits resistance to reprogramming.

From another perspective, it will be interesting to explore if rapid activation of the Oct4 distal enhancer is observed during reprogramming of primary fibroblasts. However, heterogeneity in starting material may impede the ability to assess epigenetic changes. It has been suggested that reprogramming occurs in a stochastic fashion (Hanna et al., 2009) and various sources of starting material possess inherently distinct reprogramming efficiencies (reviewed by Hochedlinger and Plath, 2009). These confounding factors will pose difficulties in the examination of Oct4 epigenetic changes in a sub-population of cells undergoing nuclear reprogramming.

With these difficulties in mind, experiments conducted in this chapter serve as a building block to decipher epigenetic changes that accompany the acquisition of pluripotency. Differentiation of iPSCs using ATRA allows a consistent treatment to a population of cells, producing a powerful platform to study initial events during the course of reprogramming.

5.4 Conclusion

This chapter attempts to decipher the molecular mechanisms that explicate the ability of RARβ to replace exogenous Oct4 during the acquisition of pluripotency. 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 RARs, ChIP and luciferase assays were performed to demonstrate that RARβ binds to the RARE within the distal enhancer of Oct4 and activates Oct4 expression. Similar attributes were mirrored in RARγ, another RAR family member, which has been described to promote reprogramming speed and efficiencies in synergy with LRH1. 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 way 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, both the distal enhancer and RAREoct were examined during the initial three days of reprogramming to determine rapid epigenetic changes. Using ChIP and luciferase assays, it was demonstrated that both regions were 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 three days when only four reprogramming factors (Oct4, c-Myc, Klf4, Sox2) were used. These findings 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.