Chapter 03: Cloning and characterisation of an inducible reprogramming factor: OctER
3.1 Introduction

3.1.1 Oct-3/4 and Klf4 regulate the initial stages of reprogramming

During reprogramming of MEFs to iPS, the cells transition through a number of loosely defined states that can be used to identify sub-populations during the reprogramming process. In the early stages of reprogramming, there is a down-regulation of fibroblast marker Thy1, which is followed by upregulation of pluripotency marker SSEA-1 (Brambrink et al., 2008; Stadtfeld et al., 2008). As reprogramming proceeds, there is a sequential induction of endogenous pluripotency genes and a reduction in dependence on the exogenous transcription factors, whose retroviral promoters are completely silenced during the final stages of reprogramming (Figure 3.1) (Brambrink et al., 2008; Stadtfeld et al., 2008). Using these landmarks, isolation of intermediate populations for detailed studies has been reported. By studying the differential gene patterns of the various intermediate populations found during the course of reprogramming, a road map on the route taken by MEFs to become pluripotent has been reported (Polo et al., 2012). However, it should be recognised that these are all heterogeneous populations, and the broadly defined stages may contain a series of sub-stages.

![Diagram](image)

Figure 3.1 Major landmarks that occur during the reprogramming process

The schematic gives an overview of the major events that occur during reprogramming MEFs to iPS. The banded black arrow represents the time in days from transduction of the Yamanaka factors to the final reprogramming stage. The black bar arrow indicates the dependence of exogenous for complete reprogramming, with the reduction in size indicating reduced dependence of exogenous factors. The weighted red bars indicate the start of expression timings of alkaline phosphatase and SSEA-1. The weighted green bars indicate the expression timing of Oct or Nanog reporter lines that are generally used to indicate successful reprogramming. Image adapted from (Brambrink et al., 2008)
The reprogramming roadmap can be broadly divided into three stages, initiation, stochastic and maturation (Figure 3.2). The initiation stage is defined by mesenchymal to epithelial transition, increased proliferation rates and metabolic changes (Polo et al., 2012). The maturation phase is defined by activation of endogenous pluripotency genes and remodelling of DNA methylation patterns at promoters of genes such as Nanog, Oct-3/4 and Zfp42 (Polo et al., 2012). During the intervening stochastic phase, there is a gradual increase in the levels of pluripotency associated genes such as Alpl & Fbx15 and transient activation of developmental genes such as Prx, Tbx21 & Pitx2 (Polo et al., 2012).

Of the Yamanaka quartet, c-Myc was the first factor to be found dispensable for reprogramming (Nakagawa et al., 2008; Wernig et al., 2008). Due to its oncogenic nature, the use of c-Myc in reprogramming made it a less attractive option for regenerative medicine. This was further compounded by the fact that in one study, up to 20% of the mice formed using iPS developed tumours that were attributable to c-Myc transgene reactivation (Okita et al., 2007). Reprogramming without c-Myc results in lower reprogramming efficiency and delayed formation of iPS colonies (Wernig et al., 2008). Yet, the quality of reprogramming increases, with a reduction in partially reprogrammed cells and a higher yield of completely reprogrammed cells (Nakagawa et al., 2008). Since reprogramming MEFs to iPS cells is possible without c-Myc, and the cell cycle switch still occurs during the initiation phase, the other three Yamanaka factors Oct-3/4, Klf4 and Sox2 are sufficient to switch the cell cycle from a slow cycling state to a fast cycling state, along with other pluripotency programs.

Sox-2 was found to be more important for the maturation phase of reprogramming than for the initial stages of reprogramming (Lin et al., 2011; Buganim et al., 2012). By using Oct-3/4, Klf4 & c-Myc it was possible to reprogram MEFs to a stable partially reprogrammed state which were alkaline phosphatase and SSEA-1 positive but negative for pluripotency markers such as Nanog (Lin et al., 2011). These stable intermediates could be similar to partially reprogrammed cells seen in the original study by Takahashi and Yamanaka and subsequent studies (Takahashi and Yamanaka, 2006; Mikkelsen et al., 2008; Sridharan et al., 2009). Upon expression of Sox2 in the stable intermediates, it was possible to convert them to a completely reprogrammed state that was positive for endogenous Oct-3/4 & Nanog (Lin et al., 2011). This would indicate that a key role of Sox2 in reprogramming, is the activation of the 'core' network of pluripotency factors, Oct-3/4
and Nanog. As Sox2 is not essential for maintaining the Oct-Sox enhancer network once activated, and overexpression of Oct-3/4 can maintain pluripotency in Sox2 null mES cells, Sox2 is also most likely not involved in switching the cell cycle from a slow cycling state to a fast cycling state during the initiation phase of reprogramming.

Figure 3.2 Reprogramming MEFs to iPS can be divided into three phases, initiation, stochastic and maturation

By isolating populations based on cell surface markers such as Thy1+, SSEA-1 or using reporter genes such as Oct-eGFP, it is possible to build a profile of changes that take place during the reprogramming process. The x-axis is time from induction, the positive y-axis are cell that successfully reprogram, while the negative y-axis consists of cells that are refractory to reprogramming. The black arrows on the bottom mark the rough boundaries for the three phases, initiation, stochastic and maturation. The bumps represent areas where significant changes for mRNA, microRNA, histone modifications were found. Changes in DNA methylation mostly take place in the maturation phase and bivalent domains were generated gradually after an initial burst. Forced expression of OKSM in refractory cells leads to successful reprogramming. Image adapted from (Polo et al., 2012)
As the evidence suggests that c-Myc & Sox2 are not essential for the initial stages of reprogramming, Oct-3/4 & Klf4 were the most likely factors to be responsible for increasing the cell cycle kinetics and regulation in the initial stages of reprogramming. While overexpressing Oct-3/4 alone in somatic cells leads to slower proliferation, in mES cells, Oct-3/4 plays a major role in generating and maintaining the rapid cell cycle (Figure 1.9). The expression levels of Oct-3/4 are also tightly maintained in mES cells, as any deviation leads to differentiation (Niwa et al., 2000). Further, the role of Klf4 is complicated by the fact that it can act as both a repressor and an activator. Finally, redundancy amongst Klf family members makes it challenging to attribute functions to one factor. For all these reasons, Oct-3/4 emerged as the best candidate to employ in probing the changes that take place in the initial stages of reprogramming.

### 3.1.2 Need for an inducible system to probe early events in reprogramming

In order to study the dynamics of the cell cycle changes that take place in the initial stages of reprogramming, it would be important to use a synchronised reprogramming system. *In vitro* cultures are generally asynchronous populations which makes studying changes in the cell cycle challenging as they could be masked by out-of-phase cell populations. Synchronising cells to enrich for homogenous populations by using cell cycle inhibitory drugs could be used but with limited effect. Most drug-synchronised populations will become asynchronous in a few days since drugs have to be removed for release into the cell cycle. Further, since the changes that take place in the initiation phase of reprogramming require testing over long durations, this would not be a feasible option. Multiple rounds of synchronisation could lead to cell toxicity and would negatively impact the results of the study.

In order to overcome these limitations, an inducible reprogramming system was used. An inducible system would allow for the creation of a homogenous reprogramming population that can be used to study the changes in the cell cycle dynamics. An ideal inducible system would be one in which only on addition of the inducer, the functional protein of interest would be immediately available in the cell to act on its target genes. A tetracycline based inducible reprogramming vector had already been developed by two groups (Brambrink et al., 2008; Maherali et al., 2008; Stadtfeld et al., 2008). There are two types of tetracycline induced systems, TetON and TetOff (Gossen and Bujard, 1992;
Gossen et al., 1995). The reprogramming systems already developed were both TetON systems, where upon addition of the inducer tetracycline or doxycycline, synthesis of mRNA began, followed by protein translation and maximal protein activity was seen 72 hours post induction (Gossen and Bujard, 1992; Gossen et al., 1995; Brambrink et al., 2008; Maherali et al., 2008; Stadtfeld et al., 2008), by which time the system is already heterogeneous. Further, all cell culture media containing FBS is contaminated with low levels of tetracycline, a drug routinely used in veterinary care. The long duration for maximal protein activity and leakiness due to serum based tetracycline contamination meant that the tetracycline inducible reprogramming system would not be suitable to study the early phases of reprogramming.

The 4-Hydroxytamoxifen (OHT) based inducible system has advantages since it is less leaky and rapid compared to the tetracycline based system (Littlewood et al., 1995; Kringstein et al., 1998). The OHT induced system is based on an engineered estrogen receptor which is responsive only to OHT, and not endogenous estrogen (Littlewood et al., 1995). This prevents any leaky expression that may result from any serum-based contaminants. The gene of interest is fused to a modified estrogen receptor, while expression of the fusion gene is under the control of a constitutive promoter of our choice. The choice of promoter gives an added advantage as we can control the levels of fusion gene expression at this stage by using either a high expressing or low expressing constitutive promoter.

The fusion gene is constitutively expressed, producing a fusion protein which is kept inactive as it rapidly forms a complex with Hsp90 and is sequestered to the cytoplasm (Littlewood et al., 1995). On addition of OHT to the media, the fusion protein is released from the inactivation complex and can enter the nucleus to perform its function. By contrast, in the tetracycline system, on introduction of the inducer, the gene is first transcribed, the protein is then translated and can finally function, on the other hand, in the OHT induction system, on addition of the inducer, the protein is directly activated resulting in a faster response time. These characteristics make it suitable for use in the proposed study.
3.1.3 A flexible linker to preserve Oct-3/4 dynamic structure

Oct-3/4 belongs to the POU family of transcription factors that contains a bipartite DNA binding domain referred to as the POU domain. POU was named after the pituitary-specific Pit-1, the octamer-binding proteins Oct-1 and Oct-2, and the neural Unc-86 from *C. elegans* (Jerabek et al., 2014). The POU domain consists of two subdomains, the POU-specific domain (POUs) and POU-homeodomain (POUHD) which are connected by a 17 amino acid long linker (Remenyi et al., 2003; Esch et al., 2013). The POUs and POUHD domains bind to the sequences ATGC and (A/T)AAT respectively, which together form the octamer recognition sequence 'ATGCAAAT' (Jerabek et al., 2014). Oct-3/4 can bind to and activate its targets as a monomer or a homodimer and can also form a heterodimer via the DNA-binding domain with Sox2 (Figure 3.3) (Jerabek et al., 2014). These various combinations allow just two transcription factors to regulate a large network of pluripotency genes in mES cells (Figure 3.3).

In order to create an inducible fusion protein, it was important to incorporate a linker to connect the protein with the modified OHT binding domain. Direct fusion of the OHT domain with Oct-3/4 has high probability of misfolding, reduced activity or low protein production; therefore it was important to select the correct linker. There are three types of linkers that can be used to create the fusion protein: flexible, rigid or cleavable. A cleavable linker would not be preferred as it would negate the inducible nature of the OHT induction system. A rigid linker has the advantage that it would keep the OHT domain a fixed distance away from the POU domains, thereby preventing any hindrance in their functionality. However, due to the dynamic and highly flexible nature of Oct-3/4 protein, a rigid linker may cause functional deficits. Therefore a flexible linker was used. In theory, the flexible linker would allow for sufficient movement amongst the various domains, as well keep the OHT domain away from the POU domains, allowing them to function freely for DNA binding and transactivation.
Figure 3.3 Models of Oct-3/4/DNA complexes and Oct/Sox2/DNA complexes

The POU
domain is shown in orange, POU
HBD is in blue, Oct-3/4 linker is in purple and HMG is in green. (A) Oct-3/4 monomer bound to an octamer consensus site. (B)(C) Oct-3/4 dimer bound to an octamer consensus site. (D) Oct-3/4-Sox2 heterodimer bound to the UTF1 motif. (E) Oct-3/4-Sox2 bound to the FGF motif. (F) Oct-3/4-Sox2 bound to a compressed motif. The variance from the canonical octamer sequence in the UTF1 motif and the spacer sequence between the Oct-3/4 and Sox-2 binding sites are highlighted in red. These models show the various configurations that Oct-3/4 can bind to its motif. To create a fusion Oct-3/4, it would be essential that the linker allow such dynamic positions, for which a flexible linker would be ideal. Image adapted from (Jerabek et al., 2014).
3.2 Results

3.2.1 Vector cloning strategy

The OctER vector was designed based on the following

1) Primary MEFs would be used for all the studies, which are difficult to transfect. To be able to introduce DNA into MEFs, a viral vector based system would have to be used.

2) Successful reprogramming requires robust expression of the Yamanaka factors to reprogram, at the same time silencing of exogenous factors is important for successful reprogramming (Papapetrou et al., 2009; Carey et al., 2011; Tiemann et al., 2011; Nagamatsu et al., 2012). A CMV promoter was used for this purpose, as it expresses robustly and is also silenced in mES cells (Xia et al., 2007; Qin et al., 2010).

3) As multiple vector designs were being tested, it would be preferable to use a modular cloning strategy. The modularity would enable easy switching between different parts of the expression cassette as well between different vectors.

4) Oct-3/4 is a dynamic protein and would require a flexible linker. For this, a linker with Gly-Gly-Gly-Gly-Ser repeats was used. By increasing or decreasing the number of repeats, the linker length could be optimised to reduce steric hindrance.

![Figure 3.4 Schematic of OctER showing the position of the main domains](image)

The POU$_S$ domain is shown in orange, POU$_{HD}$ is in blue, Oct-3/4 linker is in purple, the flexible linker is in red, and the OHT binding domain is in green. The linker was fused to the C-terminal of Oct-3/4. The domains are not drawn to scale.
3.2.2 Cloning of OctER

To clone the OctER construct (Figure 3.4), the following fragment were assembled: Oct-3/4 from pCX-OKS-2A (Addgene plasmid #19771) (Figure 3.5), the CMV promoter from pLVX-Puro (Clontech) (Figure 3.6) and the OHT binding domain from pBABE-cMycER (kind gift from Dr. Evan) (Figure 3.7) They were amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, cat# 11304011) (Littlewood et al., 1995). Primers were designed to amplify each of the fragments with overhangs that contained the corresponding restriction sites (Figure 3.8). For the primers used to amplify the OHT binding domain, along with restriction sites, the overhang also contained the flexible linker repeat sequences for the N-terminal of the OHT binding domain. All primers used for amplifying the various fragments are in the appendix. Each fragment was ligated into pGEM-T shuttle vector (Promega, cat# A3600) using rapid ligase (Bangalore Genei) and transformed into DH5α. 8 colonies per fragment were tested by colony PCR to screen for positive clones. A miniprep was performed for all the positive clones and their sequences were verified by Sanger sequencing (Bioserve). Clones that contained no mutations were selected, and a restriction digest was performed to remove the fragments with the required overhangs from the shuttle vector. The digested products were run on a 1% agarose gel, fragments were excised based on their size and were purified using Wizard SV Gel and PCR Clean-Up System (Promega, cat# A9282).

4 OctER cassettes were cloned (Figure 3.9)

1) OctER with a flexible linker containing 2 repeats (10 amino acids)
2) OctER with a flexible linker containing 3 repeats (15 amino acids)
3) OctER with a flexible linker containing 4 repeats (20 amino acids)
4) OctER with a flexible linker containing 6 repeats (30 amino acids)

For the viral vector backbone, FUGW (Addgene plasmid #1488) was used (Figure 3.10). The UbC promoter and eGFP were digested out to create an empty backbone into which each of the OctER cassettes were ligated (Figure 3.11). Each of the plasmids were sequence verified to ensure correct orientation and the absence of mutations.
Figure 3.5 Vector map of pCX-OKS-2A
Oct-3/4, Klf4 and Sox2 are shown in dark green, the ORF is shown in orange. Size of the plasmid and the scale bar around the plasmid are in bp. All features are drawn to scale. Image created using Geneious v5.6

Figure 3.6 Vector map of pLVX-Puro
The CMV promoter is shown in light green. Size of the plasmid and the scale bar around the plasmid are in bp. All features are drawn to scale. Image created using Geneious v5.6
Figure 3.7 Vector map of pBABE-cMyc-ER
OHT binding domain is shown in dark blue. Size of the plasmid and the scale bar around the plasmid are in bp. All features are drawn to scale. Image created using Geneious v5.6

Figure 3.8 Schematic of the lentiviral OctER cassette highlighting its modular structure
The 5’ and 3’ LTRs (long terminal repeats) of the lentiviral vector are shown in orange, CMV promoter is shown in yellow, Oct-3/4 is in green, the flexible linker is in blue, the OHT binding domain is in purple. The restriction sites used to design the modular structure are shown. By using a range of unique restriction sites, it was possible to easily switch parts within the cassette and transfer the cassette between different vectors.
Figure 3.9 Schematic of the various OctER proteins displaying the increasing length of the flexible linker

Oct-3/4 is shown in dark green, the flexible linker is shown in purple, and the OHT binding domain is shown in dark blue. The scale bar on the top is the number of amino acids. All structures are drawn to scale. Image created using Geneious v5.6

Figure 3.10 Vector map of FUGW used as the backbone for cloning in OctER cassettes

The restriction site PaeI and EcoRI are marked. The CMV-OctER cassette was cloned in to this site. Size of the plasmid and the scale bar around the plasmid are in bp. All features are drawn to scale. Image created using Geneious v5.6
30 bp to 90 bp
(10 aa to 30 aa)

Figure 3.11 Vector map of OctER with the various flexible linkers
Oct-3/4 is shown in dark green, flexible linker is shown in purple, OHT binding domain is shown in dark blue, the OctER ORF is shown in orange. Size of the plasmid and the scale bar around the plasmid are in bp. All features are drawn to scale. Image created using Geneious v5.6. aa – amino acids.

3.2.3 Determination of optimal linker length for a functional OctER
Oct-3/4 and Sox2 form a heterodimeric complex that activates a large number of genes important for maintaining the pluripotent state such Nanog, Lefty1 & Utf1 (Kuroda et al., 2005; Rodda et al., 2005; Nakatake et al., 2006). By testing if OctER can form a heterodimer with Sox2 and activate a pluripotency gene promoter, the appropriate OctER construct for subsequent experiments could be determined.
To test the functionality of the OctER constructs, a luciferase reporter system based on the mouse Nanog promoter (kind gift from Dr. Tada) (Kuroda et al., 2005) was used. The Nanog promoter consists of an Oct-3/4 - Sox2 binding site located -180 bp to 166 bp upstream of the transcription start site (Figure 3.12). By testing each of the constructs using the luciferase reporter, the optimal linker length for maximal activity of OctER could be determined.

![Diagram](image_url)

**Figure 3.12 Schematic of the luciferase reporter based on the mouse Nanog promoter**

Genomic region from -332bp upstream to 50bp downstream of the Nanog TSS was ligated to the luciferase gene (Kuroda et al., 2005). The Oct-3/4 - Sox2 binding site is located -180bp to 166bp upstream of the TSS. The Oct-3/4 binding site is indicated in green, Sox2 binding site is indicated in orange, the black forward arrow denotes the TSS, the genomic region from TSS to 50bp is denoted in blue, and the luciferase gene is denoted as a black outlined box. The domains are not drawn to scale.

Cos-7 cells were used for the assay, which are an African green monkey kidney fibroblast-like cell line that does not natively express Oct-3/4, Sox-2 or Nanog (Kuroda et al., 2005). The various OctER constructs along with equimolar quantities of Sox2 (Addgene plasmid# 15953), and a fixed amount of the Nanog promoter luciferase reporter and renilla plasmid (Promega, cat# E2231) were transfected into Cos-7. For controls, it was tested if OHT itself would induce luciferase activity, and Nanog promoter with Sox2. 24 hours post transfection, the media was changed and 100 nM OHT was added to the induced cells. For uninduced/Sox2 alone, ethanol served as a vehicle control. 24 hours post induction, 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).

All the luminescence readings were normalised to the basal Nanog reporter activity without OHT (Figure 3.13). It was observed that OHT did not induce the Nanog luciferase reporter. Sox2 by itself could activate the Nanog reporter but its activity was relatively low compared to induced conditions. All the uninduced conditions also exhibited this residual activity which is most likely due to the effect of Sox2. The induced conditions showed an increase in luciferase activity directly proportional to the length of the linker, with increasing linker length producing increasing luciferase activity. While there was a significant difference in activity between the OctER with 9 aa flexible linker and 15 aa flexible linker, the difference between 15aa flexible linker to 20 aa flexible linker was negligible. This indicated that the 5 extra amino acids in the 20 aa flexible linker were not sufficient to provide further spatial distance between the OHT binding domain and Oct-3/4. As the OctER with 30 aa flexible linker produced maximal activity compared to other OctER constructs, this construct was chosen for all the experiments. In all subsequent experiments, OctER implies OctER with a 30 amino acid flexible linker.
Figure 3.13 Linker length influences OctER activity
The data shows the % relative luciferase activity normalised to the Nanog luciferase reporter without OHT. OHT by itself does not induce any luciferase activity, while Sox2 induces a low level of activity which is also seen in all the uninduced conditions. With increasing length of the flexible linker, there was a corresponding increase in luciferase activity. OctER with a 30 aa flexible linker exhibited the maximal activity and was used for all subsequent experiments. The length for each linker are in brackets, Sox – Sox2, (aa) - amino acids, indicating linker length), Unind – uninduced, Ind – induced. Values represent mean ± SEM, N=3, * p < 0.05

3.2.4 Analysis of OctER at the protein level
Western blot analysis was used to compare the expression levels of OctER with a wild type Oct-3/4. Cos7 cells were transfected with OctER and a viral construct expressing Oct-3/4 under the CMV promoter. 24 hours later, the OctER transfected cells were split for induced/uninduced and pulsed with 100 nM OHT/ethanol respectively. 24 hours post induction, all the cells were lysed using 2x Laemmli buffer, equal amounts of protein lysate were loaded on a 10% SDS-PAGE and the samples were processed for western blotting. The levels of OctER and Oct-3/4 were tested using an antibody that can identify both proteins (Figure 3.14).

OctER produced a single band near the 71 kD marker which was close to the predicted molecular weight of 78 kD. Unusually, the levels of OctER uninduced were lower compared to induced. As these were whole cell lysates, it was expected that the OctER protein levels would be equal in both uninduced and induced. It is possible that along with sequestering OctER to the cytoplasm, the OHT binding domain may increase degradation of the fusion protein in the uninduced state (Fan et al., 2010). However, this is an advantage as it generates even lower background.
Figure 3.14 Analysis of OctER at the protein level

Cos7 cells were transfected with either OctER or Oct-3/4. The OctER cells were equally divided and pulsed with 100 nM OHT/ethanol for induced/uninduced respectively. Protein lysates were made 24 hours after induction and the protein lysate was examined by western blotting. OctER and Oct-3/4 were probed for using an antibody that detects both forms (top panel). The protein levels were normalised to GAPDH (bottom panel). Induced OctER expressed at similar levels to Oct-3/4. Unusually, OctER uninduced showed low expression levels of OctER. It is possible that along with sequestering OctER to the cytoplasm, the OHT binding domain may increase degradation of the fusion protein in the uninduced state. Predicted MW of OctER is 78 kD, Oct-3/4 is 43 kD and GAPDH is 40 kD. The protein ladder is represented by black bars and the measures are in kD.

3.2.5 OctER localises to the nucleus in an OHT dose dependent manner

Oct-3/4 contains a nuclear localisation signal (NLS) at the N-terminus of POUHD which facilitates its transport to the nucleus via importin α where it functions as a transcription factor (Oka et al., 2013). It was essential to determine if the NLS interfered with the sequestration of OctER to the cytoplasm during the uninduced state and was functional during the induced state. To determine nuclear localisation of OctER, MEFs were transduced with OctER and split into 9 plates. Each plate was pulsed with either a different amount of OHT (2.5 nM to 500 nM) or with ethanol in case of uninduced. 24 hours after induction, the cells were processed according to the Rapid, Efficient And Practical (REAP) method of subcellular fractionation (Suzuki et al., 2010). Nuclear,
cytoplasmic, and whole cell protein fraction were obtained. These fractions were loaded onto an 8% SDS-PAGE and processed for western blotting. The purity of the fractions was tested using Lamin B (nuclear marker) and GAPDH (cytoplasmic marker).

Each fraction was cleanly separated as there was no GAPDH contamination in the nuclear fraction and no Lamin B contamination in the cytoplasmic fraction, while whole cell fraction showed the presence of both GAPDH and Lamin B (Figure 3.15). The amount of OctER in the nuclear fraction was quantified, normalised to Lamin B levels and plotted with respect to uninduced (Figure 3.16). The amount of OctER in the cytoplasmic fraction was quantified, normalised to GAPDH levels and plotted with respect to uninduced (Figure 3.17). In the nuclear fraction, there was no OctER in the uninduced sample. With increasing amounts of OHT, there was a subsequent increase in the amount of OctER in the nuclear fraction (Figure 3.16). Thus it could be verified that OctER localisation to the nucleus was inducible in nature.

In the nuclear fraction, at 10 nM OHT, there was a 50% increase in the amount of OctER, almost 100% increase in at 25 nM, and 200% increase at 25 nM (Figure 3.16). The amount of OctER begins to plateau in the nucleus at 250 nM and 500 nM OHT which could most likely be due to saturation of OHT induction at these levels (Figure 3.16). The levels of OctER remain relatively constant in the cytoplasmic fraction but are still relatively lower than the nuclear fraction (Figure 3.16, Figure 3.17). These results verify that the levels of OctER present in the nucleus are OHT dose dependant.
Figure 3.15 OctER localises to the nucleus in an inducible manner

Representative image of subcellular fractions of MEFs expressing OctER. MEFs were transduced with OctER and equally divided into 9 parts which were pulsed with either varying amounts of OHT or ethanol for uninduced. The nuclear, cytoplasmic and whole cell fractions were isolated using REAP method for subcellular fractionation (Suzuki et al., 2010). Samples were loaded onto an 8% SDS-PAGE and processed for western blotting. The samples were probed for OctER, Lamin B (nuclear marker) and GAPDH (cytoplasmic marker). The amount of OHT used for induction is indicated on the top part of the image. The various fractions are labelled at the left of the image indicating the protein probed for.

Figure 3.16 OHT dose-dependent nuclear localisation of OctER (nuclear fraction)

The levels of OctER in the nuclear fraction were quantified, normalised to Lamin B and plotted with respect to uninduced. With increasing amounts of OHT, there is a subsequent increase in the levels of OctER in the nucleus. All protein levels were quantified from western blots using densitometric analysis and ImageJ. Values represent mean ± SD, N=3, *p < 0.05
Figure 3.17 OHT dose-dependent nuclear localisation of OctER (cytoplasmic fraction)

The levels of OctER in the cytoplasmic fraction were quantified, normalised to GAPDH and plotted with respect to uninduced. The levels of OctER remain relatively constant in the cytoplasmic fraction. All protein levels were quantified from western blots using densitometric analysis and ImageJ. Values represent mean ± SD, N=3, *p < 0.05
3.2.6 OctER is functionally inducible

To create a synchronised reprogramming system, the OctER would have to activate its target genes in an inducible manner. To test the functionality of the OctER constructs, a luciferase reporter system based on the mouse Nanog promoter (kind gift from Dr. Tada) (Kuroda et al., 2005) was used. The Nanog promoter consists of an Oct-3/4 – Sox2 binding site located -180 bp to 166 bp upstream of the transcription start site (Figure 3.12). Cos7 cells were transfected with equimolar quantities of OctER and Sox2, along with a fixed amount of the Nanog luciferase reporter & renilla plasmid. A transfection master mix was prepared to reduce any variability between conditions. Controls included Nanog promoter alone with OHT, and Nanog promoter with Sox2. 24 hours post transfection, the media was changed and a range of OHT from 2.5 nM to 1000 nM was added, while uninduced and Sox2 alone were pulsed with ethanol. 24 hours post induction, 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).

All the luminescence readings were normalised to the basal Nanog reporter activity without OHT (Figure 3.18). There was a dose dependent increase in luciferase levels with increasing amounts of OHT, demonstrating that OctER can activate the Nanog promoter in an inducible manner (Figure 3.18). 100 mM was found to be the optimal dose of OHT (Figure 3.18). At concentrations above 250 nM, the OctER activity plateaus or even reduces, suggesting that there was an upper limit for induction, and after a certain amount of OHT, the system is saturated (Figure 3.18). These results demonstrated the OctER functions in an inducible manner to activate the Nanog promoters.
Figure 3.18 OctER is functionally inducible

The data shows the % relative luciferase activity normalised to the Nanog luciferase reporter without OHT. OHT by itself does not induce any luciferase activity, while Sox2 induces a low level of activity which is also seen in uninduced. With increasing amounts of OHT, there is a corresponding increase in OctER activity as represented by % relative luminescence. At higher concentration of 250 nM, the activity plateaus suggesting saturation of signal. Sox – Sox2, Unind – uninduced. Values represent mean ± SEM, N=4, * p < 0.05

3.2.7 Expression of OctER in MEFs

As all the studies were to be performed in MEFs, OctER expression was further tested in MEFs. MEFs were transduced with OctER viruses and 48 hours later the cells were split into uninduced and induced. The next day, the MEFs were pulsed with either 100 nM OHT or ethanol for induced and uninduced respectively. 24 hours post induction, RNA was isolated from the cells, cDNA was synthesized and qRT-PCR was performed to test for expression levels of OctER. The primers used for quantification can amplify both endogenous as well as exogenous Oct-3/4, in this case OctER.

All the expression values were normalised to untransduced MEFs and the levels of OctER expression in uninduced/induced were compared to the endogenous levels of Oct-3/4 in mES (Figure 3.19). OctER RNA expressed robustly and the levels were almost equivalent to Oct-3/4 in mES (Figure 3.19). As the inducible nature of the OHT based system is at the
protein level and not at the RNA level, the RNA expression levels for both uninduced and induced were expected to be similar as was seen (Figure 3.19).

![Figure 3.19 OctER expresses at levels similar to mES cells in MEFs](image)

**Figure 3.19 OctER expresses at levels similar to mES cells in MEFs**

MEFs were transduced with OctER, equally divided and pulsed with either 100 nM OHT or ethanol for induced or uninduced respectively. The RNA was isolated, cDNA synthesised and gene expression quantified using qRT-PCR. Gene expression was normalised to untransduced MEFs. Both uninduced and induced express OctER at similar levels to endogenous Oct-3/4 in mES. unind – uninduced, ind – induced. Values represent mean ± SEM, N=3, * p < 0.05.

### 3.2.8 OctER localises to MEF nuclei in an OHT dependent manner

To determine if OctER localises to the nucleus in MEFs, MEFs were transduced with OctER viruses and 48 hours later were split into 10 plates. The next day, MEFs were pulsed with a range of OHT (10 nM to 1000 nM) for induced and ethanol for uninduced. 24 hours post induction, the cells were fixed and processed for immunostaining. Staining for OctER was done using an antibody against Oct-3/4, and at least 1000 cells per condition were imaged (Figure 3.20). The median intensity of nuclear signal for all the cells was calculated using CellProfiler (Figure 3.21). The median intensity was normalised to uninduced and a quadratic regression was performed to best fit the sigmoidal data.
**Figure 3.20 OctER localises to the nuclei in MEFs**

The top panel is uninduced and the bottom panel is induced. Staining shown for nuclei using DAPI and OctER using an antibody against Oct-3/4. Scale bar is 20 μm.
Extract information from filename and sort images into different categories

(DDPI into DNA, Alexa 488 into OctER)

Identify nuclei from images in 'DNA' category

Draw a boundary around the nuclei

Extend 7 pixels beyond the nuclei periphery and draw a second boundary

Mark area surrounded by first boundary as nucleus

Mark area in between first and second boundary as cytoplasm

Number all nuclei present in the image

Overlay nuclear and cytoplasm outlines onto corresponding images of 'OctER'

Measure intensity of all pixels in 'nuclear' and 'cytoplasm' in OctER

Calculate median pixel intensity for each

Export data to an Excel file

Figure 3.21 CellProfiler pipeline used for quantification of OctER nuclear localisation
From 10 nM to 250 nM, there is a linear correlation between the amount of OHT used for induction and the levels of OctER within MEFs nuclei (Figure 3.22). The linear correlation extends up to 500 nM OHT, after which the signal plateaus (Figure 3.23). The uninduced displays a residual amount of OctER levels as the Y-intercept is non-zero (Figure 3.22). This could be due to the NLS or low levels of leaky expression which were also seen in MEFs. The results validate that nuclear localisation of OctER is OHT dependent in MEFs, and can be used in creating a synchronised reprogramming system.

![Graph showing linear correlation between dose of OHT and OctER levels](image)

**Figure 3.22 OctER is OHT dependent for nuclear localisation in MEFs**
The graph displays the linear correlation between nuclear localisation of OctER to OHT amounts. The x-axis is dose of OHT used for induction in nM and the y-axis is % median intensity relative to uninduced.
Figure 3.23 OctER is OHT dependent for nuclear localisation in MEFs
The graph displays the correlation between nuclear localisation of OctER to OHT amounts. At lower concentrations of OHT, there is a linear correlation between OctER nuclear localisation and OHT which plateaus at higher amounts of OHT. The x-axis is amount of OHT used for induction in nM and the y-axis is % median intensity relative to uninduced.

3.3 Discussion
In order to study the changes that take place in the cell cycle during reprogramming MEFs to iPS, it would be ideal to use a synchronised reprogramming system. Using cell synchronisation drugs would not be feasible due to the long duration of the experiments and its toxic effects. Instead a synchronised reprogramming system can be created by converting any one of the Yamanaka factors into an inducible factor. By keeping all the other factors constant and modifying the levels of one factor, it should be possible to create a synchronised reprogramming system.

Oct-3/4 was chosen for the inducible switch as its levels are tightly regulated in mES where any in deviation in its levels caused causes differentiation (Niwa et al., 2000). Further, it is absolutely essential for reprogramming, as it doesn’t display any functional redundancy in reprogramming unlike the other Yamanaka factors. (Nakagawa et al., 2008).
3.3.1 Creating an OHT dependent inducible Oct-3/4

The 4-Hydroxytamoxifen (OHT) based inducible system was used to create the inducible Oct-3/4 as it displays a rapid response to its inducer as its inducibility is at the protein level unlike the Tetracycline based inducible system which is at the transcript level (Littlewood et al., 1995). As most of the changes in the cell cycle occur in the initiation stage, there was a strong possibility that these changes would not be captured using the slower tetracycline inducible system (Polo et al., 2012).

The Oct-3/4 was fused to a modified OHT binding domain via a 30 aa flexible linker to allow for the dynamic nature of Oct-3/4 binding on DNA which was then cloned into a viral backbone to ensure high success of gene delivery in MEFs (Littlewood et al., 1995; Jerabek et al., 2014).

3.3.2 OctER is functional in an inducible manner

The fusion Oct-3/4 (OctER) localised to the nucleus in both Cos7 cells and MEFs. The functionality of OctER was confirmed using a Nanog promoter based luciferase reporter (Kuroda et al., 2005). OctER could activate the Nanog promoter in an OHT dependant manner, suggesting that it could function along with Sox2 to regulate expression of an important pluripotency factor, Nanog.

An inducible OctER viral construct was successfully cloned and it was demonstrated via various assays that OctER can localise to the nucleus of MEFs in an OHT dependent manner. The OctER can form a heterodimer with Sox2 to activate Nanog promoter. Thus OctER is a functional transcription factor. With this, the ability of OctER to reprogram MEFs could be tested.