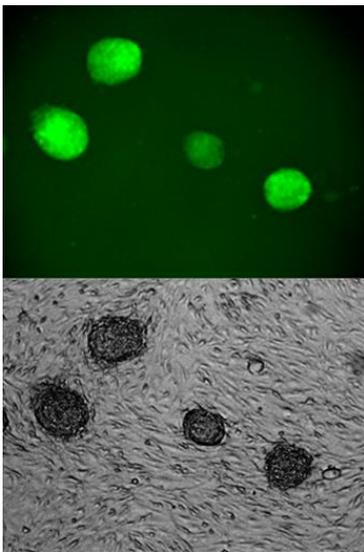


# Secondary iPSC reprogramming to study the role of Wt1 gene

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## Abstract

Wilms tumour suppressor gene (WT1) is essential for normal development of a kidney, it was also discovered as a major controller of mesenchymal-epithelial balance in general development of organs. In this project we use Wt1 and its role in the mesenchymal-epithelial balance to study the mechanism of iPSC reprogramming. Primary iPSCs were generated by the expression of four factors; cMyc, Klf4, Oct4 and Sox2 using a Piggy Bac transposition-based doxycycline-inducible reprogramming system in mouse embryonic fibroblast (MEFs) with Nanog<sup>KIP</sup> and Wt1<sup>GFP</sup> reporter alleles. Mouse models with Nanog<sup>KIP</sup> and Wt1<sup>GFP</sup> reporter alleles are used to follow the expression of Nanog and Wt1 gene throughout the reprogramming process. CRISPR-Cas9 has been applied to precisely knockout (KO) the Wt1 gene from one of the selected primary iPSC clone (parental). Chromosome count of primary iPSCs was performed together with a test for their pluripotency and differentiation potential before they were used to produce chimeras to derive secondary MEFs. A secondary iPSC reprogramming was performed on both parental and Wt1 null secondary MEFs. Wt1 was found to be indispensable for iPSC reprogramming, Wt1 null MEFs maintained their mesenchymal morphology throughout the reprogramming process, whereas a significant proportion of wild type MEFs successfully reprogrammed into iPSCs with elevated expressions of pluripotency markers under self-renewal culture conditions.

### **Abbreviations**

<i>2i</i>	<i>Two inhibitors(CHIR &amp; PD)</i>
<i>BFP</i>	<i>Blue fluorescent protein</i>
<i>Cdh1</i>	<i>E-Cadherin</i>
<i>DOX</i>	<i>Doxycycline</i>
<i>EBs</i>	<i>Embryoid bodies</i>
<i>EDTA</i>	<i>Ethelenediaminetetraacetic acid</i>
<i>EMT</i>	<i>Epithelial to mesenchymal transition</i>
<i>ESC</i>	<i>Embryonic stem cell</i>
<i>GFP</i>	<i>Green fluorescent protein</i>
<i>GMEM</i>	<i>Glasgow minimum essential media</i>
<i>iPSC</i>	<i>Induced pluripotent stem cell</i>
<i>KO</i>	<i>Knock-out</i>
<i>LIF</i>	<i>Leukemia Inhibitory factor</i>
<i>MEF</i>	<i>Mouse Embryonic Fibroblast</i>
<i>MET</i>	<i>Mesenchymal to epithelial transition</i>
<i>mRNA</i>	<i>Messenger ribonucleic acid</i>
<i>PB</i>	<i>Piggy Bac</i>
<i>PBS</i>	<i>Phosphate Buffered Saline</i>
<i>qPCR</i>	<i>quantitative Polymerase Chain reaction</i>
<i>RFP</i>	<i>Red fluorescent protein</i>
<i>sgRNA</i>	<i>Single guided ribonucleic acid</i>
<i>TET</i>	<i>Tetracycline</i>
<i>TVP</i>	<i>Trypsin Versene Phosphate</i>

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## Introduction

The recent discovery of reprogramming somatic cells into pluripotent stem cells has advanced the world of regenerative medicine. The iPS reprogramming can be achieved by introducing specific transcription factors; cMyc, Klf4, Oct4, Sox2 into embryonic and adult fibroblasts (Takahashi & Yamanaka, 2006). The development towards the generation of patient's specific stem cells is still in its infancy and several aspects about this technology have to become more clear. During reprogramming, fibroblasts transition morphologically from mesenchymal into epithelial-like cells, these transitions are called mesenchymal to epithelial transitions (MET). Numerous genes are involved in these transitions but in this study we focus on *Wt1* and its role in reprogramming.

For a long time, researchers have been studying the Wilms tumour 1 (*Wt1*) gene. *Wt1* plays many roles in the cellular developmental and cell survival processes such as RNA metabolism, splicing, translation and transcription. As the name suggests, it was identified as a tumour suppressor gene in Wilms tumour; a paediatric cancer that is caused by the absence or loss of *Wt1* gene or other genes that creates a loss in the developmental control of embryonic nephron progenitor cells (NPCs). Since *Wt1* is considered as the controller of mesenchymal-epithelial balance in development of organs, there are reasons to believe that *Wt1* plays a role in the formation of induced pluripotent stem cells (iPSCs) (Li et al., 2010).

In this study, further experiments are conducted to understand the role *Wt1* in reprogramming. MEFs from mouse models with *Nanog*<sup>KIP</sup> (Dolt et al., 2013) and *Wt1*<sup>GFP</sup> (Hosen et al., 2007) reporter alleles are used to follow the expression of *Wt1* by means of GFP (green fluorescent protein) during each stage of reprogramming. The expression of *Nanog* by means of mKate2 (a far-red fluorescent protein) expression indicates the state of pluripotency and overall tests the efficiency of reprogramming in the cells. PiggyBac (PB) transposition-based doxycycline-inducible reprogramming system (Woltjen et al., 2009) is applied to make primary iPSCs. CRISPR-Cas9 system is used to precisely knockout (KO) *Wt1* gene from one of the selected primary iPSC clones (parental). The parental and the *Wt1* KO primary iPSCs are later used to generate chimeras and secondary MEFs for both lines. Secondary reprogramming can be initiated by addition of doxycycline. The differential gene expression profile of marker genes from both lines was generated for comparison. Several techniques were used such as Gibson assembly to modify and build the constructs, fluorescent activated cell sorting (FACS) to help select cells of interest based on the expression of the fluorescent characteristics and Real time-qPCR (RT-qPCR) to test the expression profile of the generated iPSCs.

Research question: Study the role of *Wt1* gene in iPSC reprogramming by using mouse embryonic fibroblasts (MEFs) derived from embryos with combined *Nanog*<sup>KIP</sup> and *Wt1*<sup>GFP</sup> reporter alleles.

Sub-questions:

- *Is Wt1 required for iPSC reprogramming?*
- *What are the dynamics of Wt1 expression during reprogramming?*
- *Why some cells activate Wt1, whereas, others do not ?*
- *Will the cells that activate Wt1 be the only ones that reprogram successfully?*
- *What happens to the cells that do not activate Wt1?*

# Theoretical background

## iPSC reprogramming

Embryonic stem (ES) cells replicate indefinitely and can differentiate into all three germ layers; mesoderm endoderm and ectoderm (Pluripotency). ES cell derived tissues can improve the transplantation therapy for several diseases. And the ES cell derived in vitro organoid system can also be used to model several genetic diseases like cancers, diabetes and other genetic disorders. However, the use of ES cells has both biological and ethical limitations, for example graft rejection by the recipients.

The technology of iPSC reprogramming is a much more efficient way to generate patient specific pluripotent stem cells. This was discovered by Nobel prize winner scientist; Yamanaka. His lab used four transcription factors; cMyc, Klf4, Oct4, Sox2 (MKOS) to induce pluripotency in somatic cells. The reprogramming process takes about 12-24 days and there are three phases; initiation, maturation and stabilization. Initiation is marked by an early mesenchymal to epithelial transition (MET). MET is the loss of mesenchymal features and gain of epithelial characteristics due to changes in the cell-cell and cell-matrix interactions. The opposite mechanism of MET is called EMT (epithelial to mesenchymal transitions). E-cadherin (Cdh1) protein is responsible for maintaining the epithelial state while Snail protein represses the transcription of Cdh1 and other key epithelial regulators. Cdh1 expression is upregulated in MET and Snail expression is upregulated in EMT. During somatic/fibroblast reprogramming towards iPSCs, the main transition that happens is MET because fibroblasts like many other somatic cells are mesenchymal and iPSCs are epithelial. Among many other roles of the four transcription factors during iPSC reprogramming, they also regulate the expression of Snail and Cdh1. Sox2 and Oct4 downregulate the transcription of Snail gene and Klf4 induces the transcription of Cdh1 and other epithelial markers, while c-Myc enhances proliferation and transformation of the cells. During iPSC reprogramming, most of the mesenchymal cells directly go through MET, whereas some epithelial cells go through EMT to finally go through MET to become iPSCs (Liu, Song, Yu, & Zhao, 2014). See **figure-1**.

## WT1 gene and its role in iPSC reprogramming

WT1 gene is located on chromosome 11p13 and is commonly known as a tumour suppressor gene of Wilms tumour in kidney. Wilms tumour are paediatric kidney cancers that affect 1 in 10,000 children below the age of five. The suppression or malfunctioning of WT1 result in abnormalities during nephron development (nephrogenesis) which is the functional unit of a kidney (Davies et al., 2004).

WT1 is a complex gene with 36 multifunctional protein isoforms, hence its involvement in many different biological processes. One of these processes is transcription, WT1 was studied in different types of cells and it was identified as a transcriptional activator and repressor depending on the cellular context. For instance, WT1 acts as a transcriptional co-activator in sex determination and acts as a co-repressor in cholesterol biosynthesis in a developing kidney. This role linked WT1 to many more diseases like Alzheimer's diseases (Roberts, 2005).

The function of WT1 that will be focused on in this project is its important role in the balance of mesenchymal-epithelial transitions (Hohenstein & Hastie, 2006). As described in earlier paragraphs, iPSC reprogramming in somatic cells like fibroblasts consist of morphological transitions named MET and EMT. WT1 was discovered to play an important role in inducing those transitions by stimulating differentiation in some cells and by preserving their progenitor state in other. Cellular differentiation is process by which a progenitor cell develops into a mature, distinct and functional cell. In addition, WT1 is an activator of Snail and a repressor of Cdh1 in the epicardial and embryonic stem cells (Martinez-Estrada et al., 2010), which are the two crucial genes involved in MET and EMT. This aspect of WT1 enlightens many developmental processes like nephrogenesis. In summary, WT1 is a gene that functions both in development and in adulthood (Hohenstein & Hastie, 2006). The iPSC reprogramming technology is still in its earlier phase of development and still requires improved understanding of the process in order to enhance the overall efficiency. There are many reasons to believe that WT1 has a crucial role in iPSC reprogramming, this study will contribute to the knowledge of iPSC reprogramming process.

## PiggyBac transposition in reprogramming

Transposition is a way by which a DNA segment called transposon can change its relative position to another site to play an important role within the entire genome of a cell (Zhao et al., 2016). There are two types of transposons namely; retrotransposons that work in a "copy-and-paste" mechanism and DNA transposons that work in a "cut-and-paste" mechanism. The copy and paste mechanism consist of transcription of desired DNA then the RNA is reverse transcribed into cDNA and later it is integrated in a new site. During cut and paste mechanism, a cut of the desired DNA is made and then integrated in the new position(Zhao et al., 2016). There are many transposons used by scientists in molecular biology including Sleeping beauty (SB) and Frog prince that were isolated from inactive transposons of fish and frog genomes respectively. PiggyBac (PB) is a transposon extracted from cabbage looper moth *Trichoplusia ni*. PB has many advantages compared to other transposons. Firstly, it has a higher transposition efficiency in many organism including yeasts and mammals. Secondly, PB has no tendency of leaving footprints after transposition (Excision) like SB. PB has a size of approximately 2.4 kb with identical 13bp inverted terminal repeats that are positioned at every end of the transposon vector and additional 19bp internal repeats. PB is an example of DNA transposons type that uses a "cut-and-paste mechanism" for transposition and the transgene is inserted between Inverted terminal repeats. An enzyme called PB transposase recognizes the Inverted terminal repeats and allows the transposition(Zhao et al., 2016).

PB based plasmids can be used for regulation and control of transgenic expression of desired proteins. One of such system is Tet (tetracycline) system, it consists of two complementary alternatives; the tTA (tetracycline trans activator)-dependent circuit (Tet-Off system) and the rtTA(reverse tetracycline trans activator) dependent circuit (Tet-On system). The Tet gene expression system functions when a recombinant tetracycline-controlled transcription factor either tTA (for Tet-Off) or rtTA (for Tet-On) binds to the Tet-op promoter, subsequently repression or induction of the target gene respectively (**figure-2**). Gene expression is regulated by the presence or absence of tetracycline or one of its derivatives such as doxycycline (Dox). In this project, PB-TET(PiggyBac-tetracycline/doxycycline inducible promoter)-MKOS plasmid is used to reprogram fibroblast to primary iPSCs through dox-inducible expression of four transcription factors c-Myc, Klf4, Oct4 and Sox2 with intercalating 2A peptides . 2A peptides are self-cleaving peptides used for controlled co-expression of the transcription factors from the same transcript in the cells during reprogramming ("TET System: Controlled Gene Expression,"). Another PB plasmid constitutively expressing rtTA protein was used alongside but it only initiates the transcription from the TET promoter upon addition of doxycycline (Woltjen et al., 2009). Further, 2A peptide and blue fluorescent gene (BFP) was cloned in same reading frame at 3' end of rtTA in the PB rtTA plasmid. Therefore, BFP expression can help detect and sort secondary MEFs that are derivatives of the primary iPSCs from the chimeras.

## Analysis of pluripotency and differentiation potential of primary iPSCs by RT-qPCR

The two major similarities between ES cells and iPSCs in vitro are self-renewal, and a capacity to differentiate into cells consisting of all three somatic germ layers. To assess the differentiation potential, iPSCs were differentiated into Embryoid bodies (EBs). EBs are three-dimensional cell aggregates of pluripotent cells that differentiate into three embryonic germ layers; Endoderm lineage (inner layer), Mesoderm lineage (middle layer) and Ectoderm lineage (outside layer) recapitulating the embryo development. During embryonic development tissues like intestines and liver are derived from endoderm germ layer, kidneys and heart tissue are derived from mesoderm, whereas the ectoderm lineage gives rise to neuronal, skin and other related cell types. EBs were formed by preventing iPSCs from adhering to the surfaces of the multi-wells cell culture plates and centrifuged to allow aggregation of the cells. Furthermore, cell to cell interaction is essential for EBs formation, the technique has a rapid aggregate formation initiated by a centrifugation step in the protocol of Multi-well and EB microfabrication (Rungarunlert, Techakumphu, Purity, & Dinnyes, 2009). These aggregates of iPSCs when further grown in the absence of LIF would result in the maturation of EBs. In this project the ultra-low attachment U bottom 96-well plates have been used to form EBs with high homogeneity.

RT-qPCR is an advanced method to detect and measure a target DNA during each cycle of PCR. The PCR reaction consists of an oligonucleotide probe with a reporter fluorophore (fluorescent dye) at the 5' end and a quencher dye at the 3' terminus mix together with primers and a DNA polymerase such as Taq DNA polymerase. A close proximity of the reporter fluorophore to the quencher of the probe

decreases the fluorescent signal of the reporter, this is based on a principle called fluorescence resonance energy transfer (FRET) (Arya et al., 2005). The PCR reaction starts with denaturation at 95°C then followed by annealing at 60°C when both the primers and the probe anneal to the target DNA. In the next step, as the Taq DNA polymerase extends the target DNA downstream (3' end) of the primer, it cleaves the probe into fragments separating the reporter fluorophore from the quencher. This increases the fluorescent signal of the fluorescent reporter dye simultaneously allowing the amplification of the target DNA. During each cycle fluorescent reporter dye is released hence the fluorescence intensity increases exponentially, proportionate to the amount of target DNA amplified (Arya et al., 2005).

Pluripotency and Differentiation potential of iPSCs was analyzed by RT-qPCR for genes specific for pluripotency and each of the three germ layers during differentiation respectively. Abundance of mRNA for beta-Actin, a house keeping gene, was used as reference as they have a constitutive level of expression in different types of tissues at most stages of development. The relative abundance of Nanog and Oct4 mRNA was assessed as markers for pluripotency. Nanog gene is highly expressed in the ground state level of pluripotency in the ES cells and Oct4 works in collaboration with Nanog and has a major function in self-renewal of ES cells (Addis et al., 2010) (Xu et al., 2016). Zinc finger protein 521 (Zfp521) is a gene that is expressed in the early neural development. It acts as a stage development marker in the transition of epiblast-like cells to neuroectoderm (BurrIDGE et al., 2007). Brachyury (T) is a gene that is essential in the development of posterior mesoderm in mouse embryos. T is essential for embryo maturation, no expression of T in embryos will lead to death on the 10<sup>th</sup> day of gestation. Mixl1 gene has an important role during gastrulation in the formation of the primitive streak and marks the cells that will form mesoderm and endoderm. Primitive streak is a thickened epiblast layer that marks the start of gastrulation (Hart et al., 2002). During in vitro differentiation, T and Mixl1 are expressed from day 2 then drop on day 5 (Vidricaire, Jardine, & McBurney, 1994). Sox1 is one of the members of SoxB1 (Sox1, Sox2 and Sox3). Sox1 promotes neuronal differentiation i.e. Sox1 is expressed in ectoderm cells that will turn into neural cells (Kan et al., 2007; Pevny, Sockanathan, Placzek, & Lovell-Badge, 1998). GATA-4 is a transcription factor that regulates the development, proliferation, differentiation and survival of cardiac myogenesis (Yao et al., 2013). Eomesodermin (Eomes) is a transcription factor member of the T-box family regulating the function and development of cytotoxic lymphocytes such as NK and CD8<sup>+</sup> T cells (Lino, Barros-Martins, Oberdorfer, Walzer, & Prinz, 2017). Eomes has a crucial role in the development of definitive endoderm (Teo et al., 2011).

## Genome editing using CRISPR-Cas9

CRISPR-Cas9 system is a new genomic engineering technology, it provides a precise programmable editing of the genome of an organism. Cas9 is a DNA nuclease guided by RNAs, it promotes Double stranded breaks (DSBs) at a specific genetic locus (**figure-3**).

Single guide RNA (sgRNA) consists of CRISPR RNA array (crRNA) and Trans-activating crRNA (tracrRNA). crRNA is made of 20 base pairs of repetitive elements (direct repeats) that are spaced by short sequences named protospacers. Within the DNA target, each protospacer is always associated with protospacer adjacent motif (PAM). The role of tracrRNA is to facilitate the maturation of crRNA which make up the sgRNA (Ran et al., 2013). sgRNA bind to the target complementary DNA template and guides the endonuclease Cas 9 for a DSB. The DNA damage repair follows the DSB either by Non-homologous end joining (NHEJ) or Homology directed repair (HDR). NHEJ occurs in the absence of repair template, the DSBs re-ligate each other to result into a deletion or insertion mutations that can also lead to an early stop codon and result into a truncation(deletion) of a protein. The other pathway is the high-fidelity pathway, HDR that occurs in the presence of a repair template, the template can be either a double stranded DNA with complementary arms to facilitate insertion or single stranded DNA oligonucleotides. HDR is more reliable than NHEJ and has a high efficiency in dividing cells (Ran et al., 2013). CRISPR-Cas9 system in the project has been used to knockout Wt1 gene by inducing two DSBs at the coding region of exon1 and at the start of intron1 in selected primary iPSC clones, NHEJ will be used as the DNA repair pathway.

## Generation of Chimeras for both parental and Wt1 KO iPSCs lines

Chimera can be defined as a single organism or tissue composed of cells that hold two or more different genotypes. To study the function of many genes in a living organism, chimeras are generated from ES cells with a desired targeted mutation. Generation of mouse germline chimeras occurs in three steps

**(figure-4)** ("Blastocyst Injection," 2013). First ES cells with a desired mutation are cloned and injected into a recipient pre-implantation mouse embryo called blastocyst. Later, these blastocysts are surgically transferred into a recipient pseudo-pregnant foster mother to allow maturation of these embryos (Capecchi, 2005). In this project, parental and Wt1 KO primary iPSCs lines were used instead of ES cells to generate chimeras separately. From these chimeras MEFs were generated and the MEFs with iPSC contribution were selected by treating the cells with G-418; a neomycin antibiotic as Neomycin resistant-gene is present in the PB-TET-MKOS construct integrated in the primary iPSCs. These secondary parental and Wt1 KO MEFs were further used to study the role of Wt1 during secondary iPSCs reprogramming.

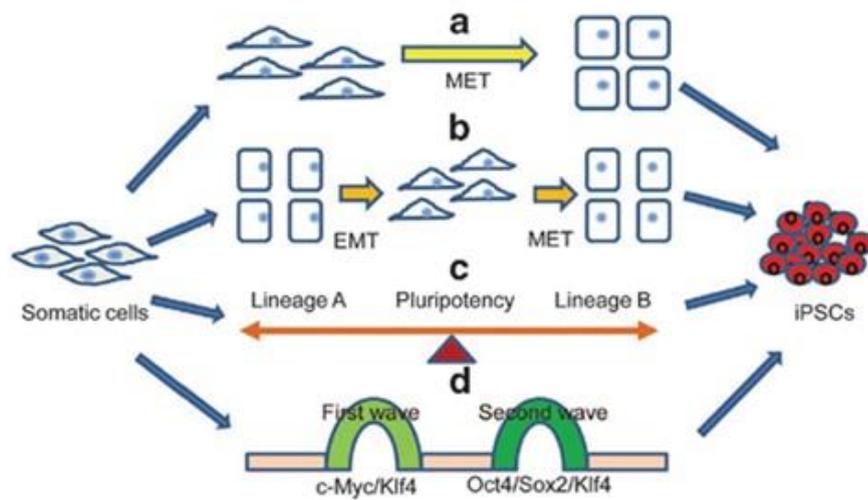
## Secondary reprogramming mechanism

Primary iPSC reprogramming is a technology that is affected by several factors. Some of them include the efficiency of transfection and the cell type heterogeneity in MEFs. To overcome the aspect of transfection efficiency, a second generation of iPSC reprogramming has been applied in this project. Secondary reprogramming consists of secondary fibroblasts derived from the chimeras generated using primary iPSC lines and all the MEFs after selection carry the Dox inducible system to initiate the reprogramming, this makes the procedure more homogeneous. Secondary reprogramming also allows to answer questions related to cell type heterogeneity in MEFs like; what types of cells will reprogram? what happens to cells that do not reprogram? do they all go through MET? what are the crucial genes activated during reprogramming? And, finally will help answer the question clearly whether if Wt1 has a role in reprogramming.

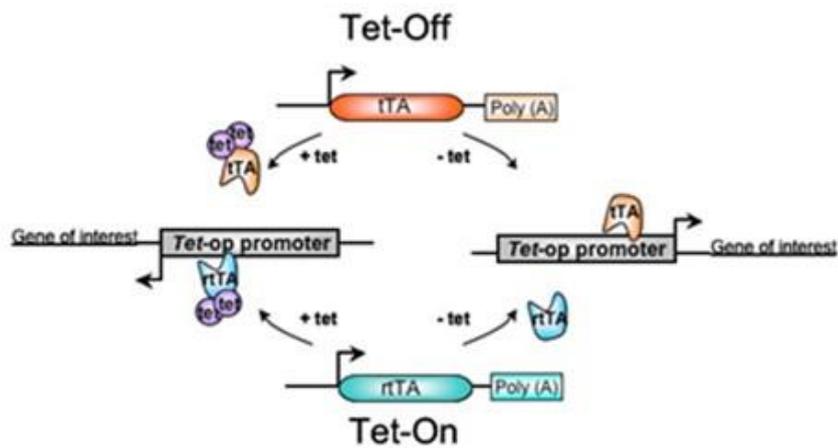
Secondary reprogramming takes about 18 days to produce the first iPSC clones to mature. For first set of 8 days Dox is added to the media followed by another 10 days without Dox for maturation of iPSCs. During secondary reprogramming iPSCs are cultured on ES media that consists of serum, LIF (leukemia Inhibitor factor) and other additional supplements specified in the material and methods chapter. In addition, a specified cell culture media is used called 2 inhibitors (2i) medium that consists two inhibitors; CHIR (99021) and PD (0325901). These two inhibitors bring and maintain ES cells in their ground state pluripotency (Sim et al., 2017). According to a recent single-cell gene expression study on MEFs going through secondary reprogramming demonstrated the developmental trajectories; a small set of cells initiate MET and most of the cells that go through MET end up reprogramming into trophoblast or Neuronal lineages when cultured in serum containing ES medium at later stages. Same set when cultured at later stages in 2i medium reprogrammed into matured iPSCs. Remaining cells that do not go through MET branch out into a different lineage of cells called stromal (Schiebinger et al., 2019). Stromal cells are a mixture of cells that maintain their mesenchymal morphology during the period of 18 days. RT-qPCR analysis was performed for expression profile of the genes specific to the above mentioned lineages during secondary reprogramming and how the loss of WT1 affects the lineage switching (**Figure-5**).

## Flow cytometry and FACS

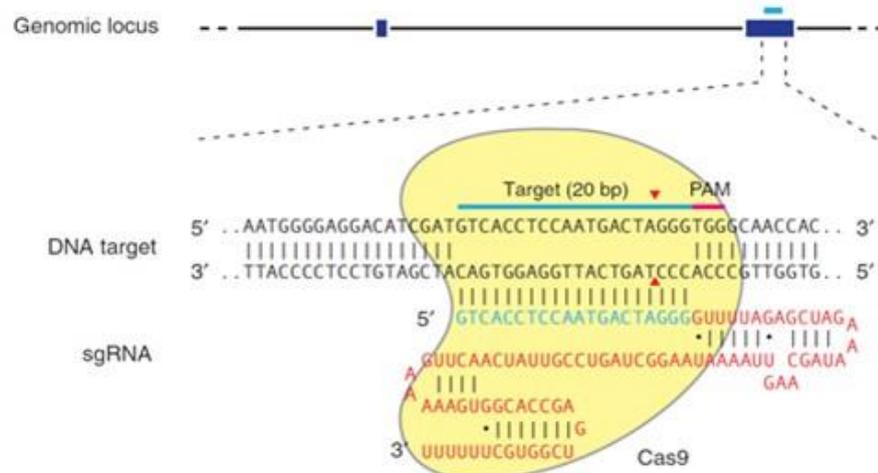
Flow cytometry is a technique that uses laser light beam to analyze the physical and chemical characteristics of cells in a mixed fluid. The fluorescent marker in the cell will give an intensity that represents the amount and shape of components in a specific cell ("Flow Cytometry Fundamental Principle, How FACS works,"). The flow cytometer consists of 3 parts: the flow cell, optics and electronics (data system). The flow cell is where the sample is injected with a specific fluid that will carry and align the cells to make them pass through a narrow channel to be analysed by the laser light. Light sources and filters are part of the optics. When a specific sample is injected, every particle is passed through the laser beams and emit lights according to their compatible wavelengths. The electronics part of the flow cytometer consist of different detectors that record data on the specificity of the particles, some detectors measure forward scatter lights and others measure side scatter lights to record the size, shape and compositions of the particles ("Flow Cytometry Fundamental Principle, How FACS works,"). Fluorescence-activated cell sorting (FACS) is a type of flow cytometry that sorts a mixture of biological cells into one or more containers according to specific fluorescent markers in each cell ("Flow Cytometry (FCM)/FACS Fluorescence-activated cell sorting (FACS),"). FACS is specifically used to detect the amount of protein expressed in cells and the number of cells that express that protein, the cells will be then separated in different chambers. This technique has been used to identify and sort cells expressing the reporter genes. BFP from PB-rtTA-2A-BFP present in primary iPSCs and GFP reporting the expression of Wt1 during reprogramming.



**Figure-1** MET and EMT transitions in iPSC reprogramming.



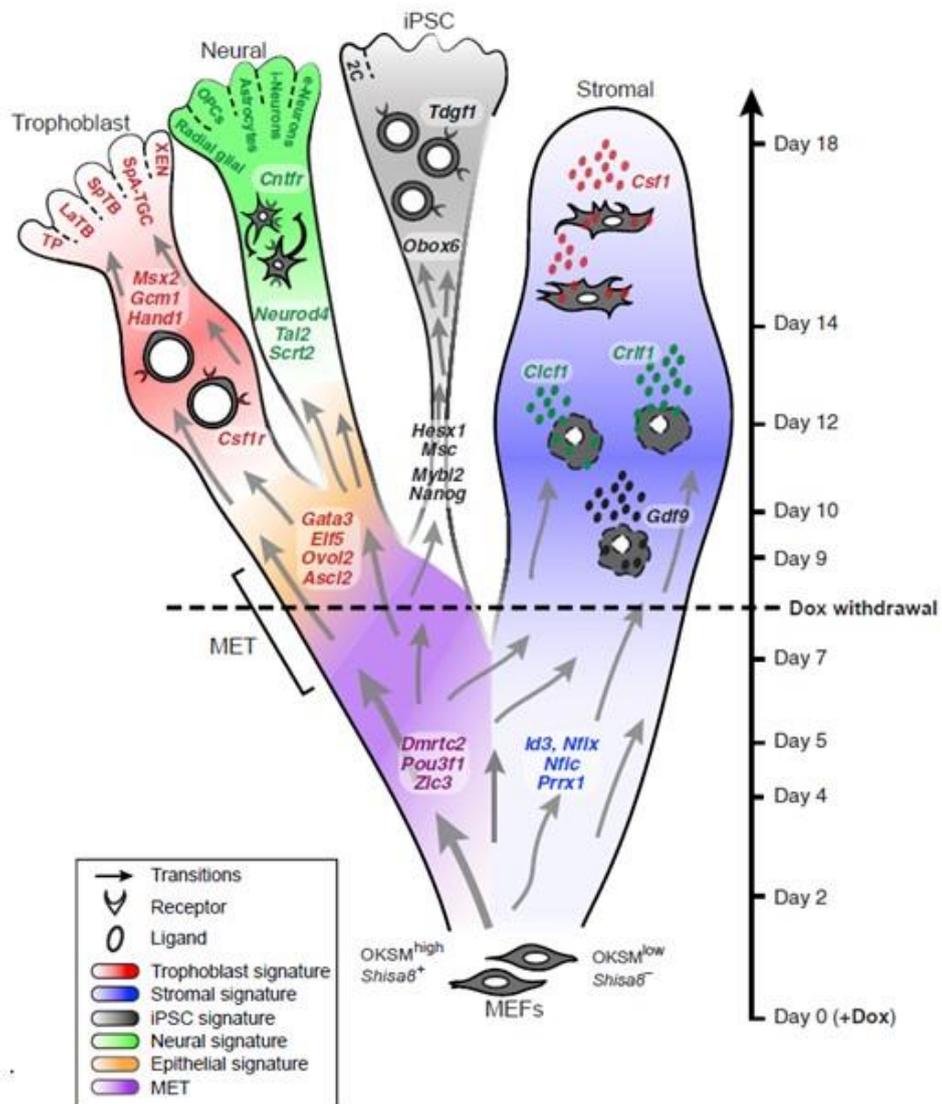
**Figure-2** schematic view of Tet-Off and Tet-on system.



**Figure-3** Illustration of CRISPR-Cas9 mechanism



**Figure-4** Overview of the generation of mouse germline chimeras from ES cells that contain a specific mutation.



**Figure 5.** Trajectory of fibroblasts during secondary reprogramming

# Material and methods

## Primary iPSCs

Primary iPSC lines were generated from MEFs with Nanog<sup>KIP</sup> and Wt1<sup>GFP</sup> reporter alleles as reported in the earlier Internship Research Report submitted in January 2019.

## Making EBs

- I. Lift the iPSCs (Trypsin 5mins)
- II. Re-plate on gelatin coated cell culture flasks for 20minutes (to deliberately remove the irradiated MEFs from ES cells)
- III. Collect the media by flushing 2-3 times in case some iPSCs attached.
- IV. Count the cells and calculate for plating 3000cells per well of 96 well ultra-low attachment U bottom plates with 100 microliter differentiation media.
- V. Spin the plate down at 200 g for 5minutes to aggregate the cells
- VI. Top up with 50 microliter media every other day.

On day 4

- VII. Collect the pellets in an Eppendorf by flushing  $\pm 2$  times with a pipette.
- VIII. Centrifuge at 200g for 5 minutes then snap freeze on dry ice and store the pellets at  $-80^{\circ}\text{C}$ .

On day 8

- IX. Collect the pellets in an Eppendorf by flushing  $\pm 2$  times with a pipette.
- X. Centrifuge at 200g for 5 minutes.
- XI. Snap freeze on dry ice and store the pellets at  $-80^{\circ}\text{C}$ .

## RNA isolation

- I. Wash the required cells with PBS then add Trypsin for 5mins in the incubator to detach the cells. After centrifuge at 300g for 5mins, aspirate the supernatant and snap freeze on dry ice and store the pellets at  $-80^{\circ}\text{C}$ .
- II. Disrupt the pellet of cells by adding 350  $\mu\text{l}$  of Buffer RLT(lysis buffer)
- III. Homogenize the lysate by pipetting up and down until the pellet is nearly lysed.
- IV. Add 350  $\mu\text{l}$  of 70% ethanol to the lysate and mix well then directly transfer the solution onto the RNeasy MinElute spin column (provided in the QIAGEN kit) with a 2ml collection tube.
- V. Immediately centrifuge for 30seconds at 8000g then Discard the flow through
- VI. Add 350  $\mu\text{l}$  of Buffer RW1(Washing Buffer) to the RNeasy MinElute spin column then centrifuge for 30seconds at 8000g then Discard the flow through.
- VII. Add 10  $\mu\text{l}$  DNase I stock solution to 70  $\mu\text{l}$  Buffer RDD separately. Mix by inverting the tube. Do not vortex.
- VIII. Add this DNase I incubation mix (80  $\mu\text{l}$ ) directly to the RNeasy MinElute spin column and incubate for 15minutes at room temperature  $20-30^{\circ}\text{C}$ .
- IX. Add 350  $\mu\text{l}$  of Buffer RW1 then centrifuge for 30seconds at 8000g. Discard the flow through from the collection tube.
- X. Add 500  $\mu\text{l}$  of Buffer RPE (with  $\sim 75\%$  ethanol) then centrifuge for 30seconds at 8000g. Discard the flow through and the collection tube.
- XI. Place a new collection tube. Add 500  $\mu\text{l}$  of 80% ethanol then centrifuge for 2minutes at 8000g. Discard the flow through from the collection tube.
- XII. Place a new collection tube then centrifuge at full speed for 5minutes (optional: open lid). Discard the flow through and collection tube.
- XIII. Place the RNeasy MinElute spin column in a new 1,5ml collection tube(Eppendorf) then add 15  $\mu\text{l}$  of RNase-free water, allow 5 minutes at room temperature. After centrifuge for 1minute at full speed. Repeat this step twice. Final volume 30  $\mu\text{l}$  of RNA

\*This protocol was retrieved from QIAGEN kit used in this experiment

## cDNA Synthesis

- I. After the isolation of RNA, quantify the RNA by using Nanodrop then add the component in **Table-1**
- II. Denature for 5minutes at 65°C. Spin then put on ice then add all the component in **Table-2**.
- III. Incubate the total 20 µL at 25°C for 5minutes because random primers are used then incubate at 42°C for one hour.
- IV. For inactivation of enzymes incubate at 65°C for 20minutes.
- V. Store the cDNA at -20°C

## RT-qPCR analysis

RT-qPCR was performed as described in **Table 3 and 4**. For each reaction, 10µl of the reaction mix and 2µl of cDNA template was used. See **Appendix** for the lists of specific genes for pluripotency and differentiation.

## Chromosome spreads and karyotyping

At 70-80% confluence cells were incubated with 0.1µg/ml colcemid for 1.5hour to retain the cells in their metaphase stage of mitosis. And then incubated for 10 mins in 0.075 M of KCL (used as a hypotonic solution to swell the cells) before spreading chromosomes followed by fixative (solution with a ratio of 3:1 of 100% methanol: 99%acetic acid respectively). The cells were squashed on to a glass slide and placed in 70% acetic acid to remove the cytoplasm for 1minute. The slides were left to dry and stained with DAPI to visualize the chromosomes.

## Genomic editing of primary Nanog<sup>KiP</sup> Wt1<sup>+GFP</sup> iPSC line by CRISPR-Cas9 to knock out Wt1

CRISPR-Cas9 system was used to edit parental primary iPSC line after they have been successfully reprogrammed. The aim of this technique is to knockout the Wt1 gene by deleting a region of exon1 similar to WT1<sup>GFP/+</sup> knock-in mice. And the parental and the Wt1 KO primary iPSCs will be used to generate chimeras. Major steps for CRISPR Cas9 system include;

- The designing of both mRNA encoding for Cas9, sgRNA, appropriate genotyping primers.
- Cloning of the sgRNA to a plasmid for the formation of a plasmid with both sgRNA and Cas9 (pX459).
- Transfection of primary iPSCs with pX459 cloned sgRNA.
- Detection of genomic microdeletion by PCR (Ran et al., 2013)

### Annealing sgRNA Oligo's for cloning into pX459

- I. Ordered oligo's (**Appendix**) were resuspended in 1 n mol/µl with TE (Tris EDTA buffer).
- II. Take 1 µl of each oligo plus 48 µl Oligo Anneal Buffer as in **Table-5**
- III. HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered with 1M KOH
- IV. PCR Oligo Anneal Program see **Appendix**
- V. The ligation reaction mix annealed oligo and pX459 was prepared as described in **Table-6** and was left overnight at room temperature.

## Transformation

- I. Defrost the chemical competent cells on ice
- II. Add 1µl ligation mix to the cells, mix very carefully not pipetting
- III. Place on ice for 30 min

- IV. Then Heat shock for 30 sec in a 42°C water bath
- V. Place immediately on ice water for 2 minutes
- VI. Add 1 ml preferably SOC (Super Optimal broth with Catabolite repression) incubate for 1 hr in 37°C shaker
- VII. Immediately plate out on 4 LB ampicillin plates of 10cm
- VIII. Incubate in 37°C incubator

### Colony PCR and Sequencing analysis

- I. First label some colony and pick by touching a single clone on the plate. Use fresh tips! Prepare PCR tubes with about 30-50µl of LB (Remember to shake)
- II. Prepare your PCR reaction mix with primers, dNTPs and MilliQ(Table-7). After add 1µl of the prepared colony sample.
- III. For reaction mix of 10 µl, add 1µl of 10µM primer, ~2 µl of template (higher than 50 ng/µl) and ~7 µl of deionized H<sub>2</sub>O. See **Table-7 & 8**.

### Transfection by lipofectamine

Required:

- Lipofectamine 2000, 1 mg / ml
- GMEM without additives (or any Base media of your cells)
- ES cell medium
- PBS
- TVP

### Method for 6-well plate applied

Place primary iPSCs in culture on a surface so that on the day of transfection a healthy growing semi confluent culture can be harvested.  $1.5-2 \times 10^6$  cells are required for transfection.

Make the solutions in round tubes(15ml):

A: 125 µl of GMEM + max 6 µg of DNA mix well and allow incubation for 5 min RT

B: 125 µl GMEM + max 14,4 µl lipofectamine (DNA: lipofectamine should be 1: 2.4) resuspend the lipofectamine before pipetting well and resuspend solution B also well with the pipette tip. Allow 5 min RT to incubate.

Add solutions A and B together again by resuspending well and incubate 20 min RT

After 20minutes of incubation (A+B), Change the media of iPSCs then add 125µl of A+B solution, swirl then leave it overnight in the incubator. Wash the media the next day.

### Clonal segregation and Colony Picking

To avoid mosaicism, these cells were passaged at least 2 times before picking

- I. Prepare a flat bottomed 96 well plate with irradiated MEFs 2 day before the picking day
- II. Take a U-bottomed 96 well plate and fill 25µl PBS per well, place in a 37°C incubator for 10-15minutes
- III. Replace ES medium with PBS on the picking plate
- IV. Set the P20 pipette to 5µl and start picking then transfer to the U-bottomed 96well plate with PBS
- V. After picking 8-12 colonies, trypsinize with 25µl 10X TVP per well with a colony and incubate for 5minutes at 37°C.
- VI. Add 50 µl of ES media and resuspend until single cell solution then transfer to the flat bottomed 96 well plate with irradiated MEFs with 150 µl ES media
- VII. Culture the cells overnight and refresh with media the next day

### Genomic DNA isolation for genotyping

- I. When the cells are 50-60% confluent remove the media for DNA isolation
- II. Add 75 µl of lysis buffer (25mM NaOH and 0,2mM EDTA) per well

- III. Incubate at 95°C for 30mins and at 25°C to cool the sample for 10 mins using a PCR program machine to heat the lid as well.
- IV. Spin down the samples
- V. Add 75 µl of neutralization buffer (40mM Trizma)
- VI. Pipette up and down to mix
- VII. Proceed with a screening PCR

### **Genomic DNA Isolation for detection of microdeletion**

#### Materials:

- 1 x Tail buffer: 50 mM Tris-HCl, pH=8 (5 ml 1 M/100 ml) 100 mM EDTA (20 ml 0.5 M/100 ml)
- 100 mM NaCl (2ml 5M/100ml) 1% SDS (5 ml 20%/100 ml)

#### Procedure:

- I. Add 400 µl tailbuffer with prot K to tailpiece and incubate overnight at 55 °C.
- II. Mix well, until no clumps are visible anymore.
- III. Add 190 µl saturated NaCl.
- IV. Mix well and centrifuge 10 minutes at 13000 rpm.
- V. Take 500 µl of the supernatant and put into a new tube.
- VI. Add 500 µl isopropanol, mix by inverting the tube until the DNA precipitates and spin 10 minutes at 13000 rpm.
- VII. Discard supernatant and wash the pellet with 300 µl ethanol 70%.
- VIII. Remove the ethanol; let the pellet dry (not completely).
- IX. Dissolve pellet in approximately 100-150 µl TE.

### **Screening by PCR for detection of micro-deletion**

The isolated genomic DNA was screened by PCR. See **Table-9 and 10** for PCR reagents and cycling protocol. Then the products were put on and agarose gel to confirm the size (bp) of the expected band. See **Appendix** for the primers used.

## **Generation of embryonic chimeras**

### **Preparation of iPS cells for injection**

- I. Seed irradiated MEFs on a full 4-well plate, 4 days before the injection day
- II. Thaw 2cm<sup>2</sup> of the selected clone on all 4 wells with an increasing amount per well (40%,30%,20%,10%) 2days before injection.
- III. The following day, refresh media
- IV. On injection day, Lift the cells with Trypsin and neutralize with ES media
- V. Spin for 5 minutes at 300g
- VI. To remove MEFs from the cell suspension, re-plate the cells on a 4-well for 15minutes
- VII. Collect the cells in a 15ml flask

Injections were performed by Margot Linssen in the Hohenstein lab.

### **MEFs isolation and culture**

Karamjit Singh Dolt and Margot Linssen helped with the isolation of MEFs and I proceeded further with the plating and antibiotic selection.

On Day 13.5,

- I. Collect the head for genotyping on a 24-well plate
- II. Remove the gut, liver and blood vessels
- III. Take the rest, chop into pieces then add 4ml of Trypsin EDTA
- IV. Incubate for 15minutes at 37°C
- V. Put 0,1% gelatin on a P75 flask for 20 minutes
- VI. After 20 minutes, remove gelatin and add 10ml of MEF media
- VII. After 15 minutes incubation, add 4ml of MEFs media to neutralize then spin for 5minutes at 300g
- VIII. Resuspend the cells in 5ml of MEF media then plate on the P75 with media then place it in

- the 37°C incubator, refresh the media next day.
- IX. Isolated MEFs were chimeric (consists of different genotypes), cells that were derived from the primary iPSCs can be selected by treatment of 175µg/ml G-418 for 4 days. G-418 is a selective antibiotic for the neo gene (Neomycin) present in the PB-TET-MKOS plasmid transfected for primary iPSCs generation.
  - X. Genomic DNA was isolated from the tails of the embryos for contribution detection PCR. The materials and PCR conditions are described in **Table-9 and 10**. Then the products will be analyzed using agarose gel electrophoresis. See **Appendix** for a picture of the genotyping gel

### Freezing MEFs and iPSCs

- I. Make the freezing mix
- II. That contains = 60% Fibroblast medium(-LIF), 20%FBS and 20%DMSO
- III. Ratio=8:1:1 (GMEM:FBS:DMSO)
- IV. Aspirate the ES medium wash with PBS and add the correct amount of Trypsin then incubate at 37°C for 5 minutes
- V. Neutralize the reaction by adding the same amount of ES Medium as trypsin. Flush the well to resuspend the cells (North-South-East-West moving across the well)
- VI. Transfer the solution into a collection tube and spin at 1200rpm for 5 minutes (150-200g)
- VII. Aspirate the supernatant then resuspend in 400 µl of the freezing mix (or if there is more freezing samples add up the volume)
- VIII. Mix and place the vial in the freezing container in -80°C freezer overnight and place it in the liquid nitrogen to keep it for longer.

### Secondary iPSC reprogramming

MEFs (passage 1 from isolation) were first sorted against GFP then seeded on a 6-well plate with a cell density of 20,000cells/well. Secondary iPSC reprogramming was initiated with the addition of Doxycycline at Day0 to induce the expression of the four factors (Sox1,Oct-4,cMyc and klf-4). Doxycycline hydrochloride hemi-hydrate hemi-ethanoate (Doxycycline Hyclate D-9891) was purchased from Sigma Aldric and diluted in water to an end concentration of 2.0 µg/ml and freshly added to the ES media. Media was refreshed every other day with DOX until day 8, then the cells were cultured on normal ES media until day 18 (**figure-6**). From Day 18 the reprogramming cells are cultured in normal ES media and 2i medium separately, see **table-11 and 12** for the supplements in the two media respectively. 2 inhibitors namely; CHIR (99021) and PD (0325901) purchased from Tocris Bioscience. B27(17507-004) and N2(17502-048) were supplied by Gibco.

## **Flow cytometry/FACS to follow the intensity of GFP<sup>+</sup> in reprogramming cells**

Sample collection for flow cytometry and sorting

- I. Make a resuspension solution;  
That contains = PBS with 0.5% Bovine Albumin Serum (BSA)
- II. Aspirate the ES medium wash with PBS and add the correct amount of Trypsin then incubate at 37°C for 5 minutes
- III. Neutralize the reaction by adding the same amount of ES Medium as trypsin. Flush the well to resuspend the cells (North-South-East-West moving across the well)
- IV. Transfer the solution into a collection tube and spin at 300g for 5 minutes
- V. Aspirate the supernatant then resuspend in 500 µl of the resuspension solution. Filter the cell suspension then place it on ice till measurement.
- VI. Analysis was performed using FlowJo software.

## **Fluorescence Imaging**

Reprogramming process was observed by Invitrogen EVOS cell imaging system microscope and inverted fluorescent microscope Nikon TI Eclipse with 4 channels; Bright field, moxBFP, eGFP and mKate2 under 4x, 10x and 20x magnification. The images were processed and analyzed by Fiji and NIS viewer.

## **Analysis of the trajectories taken by secondary MEFs during reprogramming**

During reprogramming, cells were pelleted on several days to isolate RNA and make cDNA for RT-qPCR. RT-qPCR was performed as described in **Table 3 and 4**. For each reaction, 10µl of the reaction mix was mixed with 2µl of cDNA. See **Appendix** for the lists of specific genes for Stromal and MET.

**Table-1** Component for denaturation

Component	Volume
Total RNA	~500ng
Random Primer mix (60µM)	2µl
10nM dNTP	1µl
Nuclease-free water	To a total of 10µl

**Table-2** Component for reverse transcription

Component	Volume
10X M-MuLV buffer	2µl
M-MuLV RT(200U/µL)	1µl
RNase Inhibitor (40U/µL)	0.2µl
Nuclease-free water	6.8µl

**Table-3** Reaction mix for qPCR

Component	Volume
2X primetime Gene expression master mix	6µl
10X Target Gene Specific Prime time qPCR assay	1.2µl
10X beta-Actin Prime Time qPCR assay	1.2µl
MilliQ water	1.6µl

**Table-4** Cycling protocol

Step	Cycles	Temperature	Standard Cycling
Polymerase activation	1	95°C	3 mins
Denaturation	35-45	95°C	15 secs
Annealing and extension		60°C	1 mins
Hold, if needed	1	4°C	Up to 24 hrs

**Table-5** Oligo Anneal Buffer

Reagent	Volume	Final concentration
1M Potassium Acetate	2ml	100mM
20mM MgAcetate	2ml	2mM
300mM HEPES PH 7.39	2ml	30mM
H2O	14ml	-

**Table-6** Ligation Reaction mix

Component	Volume
Annealed oligo's	1µl
Bpil digested pX459 (~10ng/µl)	1µl
10X T4 Ligase buffer	1µl
T4 Ligase	1µl
MilliQ water	6µl

**Table-7** Reaction mix for colony PCR

Component	Volume
Template DNA from the colony in LB	1µl
10x Buffer Pim mix (dNTPs)	2µl
Forward primer 10µM	1µl
Reverse primer 10µM	1µl
Polymerase (Dream Taq)	0.1µl
MilliQ water	14.9µl

**Table-8** PCR cycling protocol

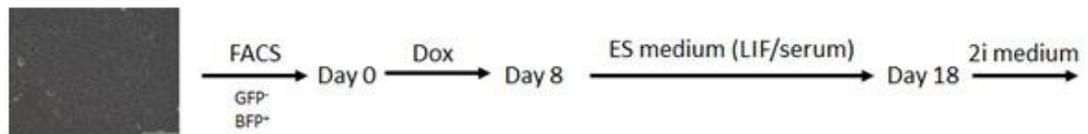
Step	Cycles	Temperature	Standard Cycling
Polymerase activation	1	98°C	3 mins
Denaturation	35	95°C	30 secs
Annealing		55°C	20 secs
Extension		72°C	1 min
Final extension		72°C	2 mins
Hold, if needed	1	4°C	5 mins

**Table-9** Reaction mix for Q5 qPCR

Component	Volume
Genomic DNA	1µl
10mM dNTPs	0.4µl
Forward primer 10µM	1µl
Reverse primer 10µM	1µl
5X Q5 reaction Buffer	4µl
Q5 Hot Start High fidelity polymerase	0.2µl
MilliQ water	12.4µl

**Table-10** Q5 PCR cycling protocol

Step	Cycles	Temperature	Standard Cycling
Polymerase activation	1	98°C	30 secs
Denaturation	35	98°C	10 secs
Annealing		55°C	20 secs
Extension		67°C	30 secs
Final extension		72°C	2 mins
Hold, if needed	1	4°C	5 mins



**Figure-6** Secondary reprogramming experimental procedures.

**Table-11** Reagents for the preparation of medium

Reagents	For a total of 100ml Medium
GMEM	88ml
Fetal Bovine Serum Gold (FBS)	10ml
Sodium Pyruvate 100mM	1ml
Non-essential amino acids 100x	1ml
Pen/Strep	1ml
Beta-Mecarptoethanol 50mM	0.2ml
*LIF (only for reprogramming)	1ml
Doxycycline 2.0µg/ml (750x)	133µl

**Table-12** Reagents for the preparation of 2i medium

Reagents	For a total of 100ml 2i Medium
DMEM/F12	50ml
Neurobasal plus	50ml
Glutamine(200mM)	1ml
N2 (100X)	1ml
B27 without Vitamin A (50x)	2ml
Beta-Mecarptoethanol 50mM	0.2ml
LIF (only For ES cells)	1ml
CHIR 3µM	30µl
PD 1µM	10µl
Pen/Strep	1ml

## Results

### In-vitro differentiation potential of parental primary iPSCs

After the generation of primary iPSCs from primary MEFs by transfecting three plasmids namely; PB-CA-rtTA-2A-BFP, PB-TET-MKOS and PB transposase, the iPS clones were picked and propagated. During these tests, two iPS clones were selected for testing the in-vitro differentiation potential. Clones B5 and C5 that were generated from transfection of plasmids PB-CA-rtTA and PB-rtTA-2A-BFP respectively in addition to PB-TET-MKOS and PB transposase. EBs were made from B5 and C5 iPS clones and cell pellets were collected at Day4 and at Day8, and together with respective primary iPSCs with and without Dox, RNA was isolated to synthesize single strand cDNA. cDNA was used to measure the relative expression of various pluripotency and differentiation markers by RT-qPCR.

From **Figure-7** it is evident that Oct4 and Nanog are highly expressed during the process of reprogramming (+Dox) and after they have fully reprogrammed (-Dox) B5 and C5 primary iPSC. The expression being higher in fully reprogrammed (-Dox) for both the lines. There was a significant drop in expression observed during differentiation by day 4 and continuously decreases further by day8. Oct4 gene is an important pluripotency marker with wider expression pattern from ground state of pluripotency up to the stage when ES cells are primed to differentiate while Nanog gene is highly expressed in ES cells at their ground state level. This explains the higher expression of Oct4 than Nanog in their undifferentiated state and the expression of Oct4 during in-vitro differentiation.

**Figure-8** demonstrates the expression of ectoderm markers Sox1 and Zfp-521. For Sox1 and Zfp-521 expression C5 showed increase as compared to B5 by Day 4 that continues to increase by Day 8.

**Figure-9** demonstrates the expression of Mesoderm lineage markers T and Mixl1. For T and Mixl1 expression C5 showed increase as compared to B5 by Day 4 that starts to drop by Day 8. **Figure-10** demonstrates the expression of Endoderm lineage markers Gata4 and Eomes. For Gata4 expression B5 showed drastic and abnormal increase as compared to C5 by Day 4 that continues to increase by Day 8. For Eomes expression C5 showed increase as compared to B5 by Day 4 that drops back by Day 8. Mixl1, T and Gata-4 are markers that are highly expressed in cells between day 2 and day 5 of in-vitro differentiation (Vidricaire et al., 1994; Yao et al., 2013) therefore the trend of variation in expression suggests that C5 has higher potential for mes-endoderm contribution. Both B5 and C5 illustrate the potential to differentiate into all three germ layers. In their undifferentiated state they do not express any differentiation markers.

In summary, Both C5 and B5 are pluripotent and have the ability to differentiate. But expression profile for most of the differentiation markers for C5 clone shows trends similar to literature and comparable to the positive control, so for the rest of the project this clone was used. The ability of iPSCs to differentiate confirmed that they should be able to generate a chimeric mouse like normal ES cell by blastocyst injection.

### Genomic editing (knock out) of Wt1 from primary Nanog<sup>KiP</sup> Wt1<sup>+GFP</sup> iPSC line by CRISPR-Cas9

The role of Wt1 in iPSC reprogramming is studied in this project, the first part of this project aimed at making a stable reprogramming technology by generating primary iPSCs. Primary iPSCs carry all the reprogramming cassettes.

To study the role of Wt1 in reprogramming the first question that needs to be addressed is how the loss of Wt1 affects reprogramming. CRISPR-Cas9 system in the project has been used to knockout Wt1 gene by inducing two DSBs at the coding region in exon1 and at the start of intron1 in selected primary iPSC clone. SgRNAs were designed by CRISPOR tool to precisely target the coding region in exon1 and the start of intron1. The oligos were ligated to a plasmid that carry Cas9 nuclease; PX459. By lipofectamine transfection, the recombined plasmids were transfected into the primary iPSCs. A screening PCR with primers that would amplify both regions of deletion was performed then put on agarose gel electrophoresis. **Figure-11** shows two gel pictures with clones that had a deletion; E2 (203bp) and other that did not; A1, A2 and A3 (1554bp). A sanger sequencing with primers on both ends of the expected deletion confirmed a 1351 nucleotides deletion at exactly 3 nucleotides upstream of the PAM sites (**Figure-12**).

## Chromosome count of parental and Wt1 KO primary iPSCs

iPSC reprogramming consists of many variables and components that could alter the genomic signature of cells. A chromosome count was performed to check whether the iPSCs maintained their chromosome number to 40 per cell nucleus. A percentage count of 60% and above is acceptable due to practical errors during spreading and the visibility of chromosomes. The percentage count (40 Chromosomes per nucleus) for C5 (parental) and C5-E2 (Wt1 knock out) was 88% and 83% falling into better than acceptable category (**Figure-13**).

## Generation of chimeras for both parental and Wt1 KO primary iPSCs

After primary iPSCs of both parental and Wt1KO were confirmed for state of pluripotency, differentiation potential and a high percentage count of exactly 40 chromosomes, they were used to generate chimeras. At day E13.5 and E12.5, the embryos were harvested from the parental and Wt1KO primary iPSCs micro-injections. As Chimeras are a mix of genotypes, to detect the contribution of iPSCs in the chimeras, a screening PCR was performed, and together with the total number of embryos harvested the percentage contribution of iPSCs was calculated. C5; parental primary iPSCs and C5-E2; Wt1KO iPSCs had a good contribution in the chimeras (**Table-13**) hence were used for secondary reprogramming. See **Appendix** for pictures of the embryos harvested with a blue fluorescence color contributed by the plasmid; PB-rtTA-2A-BFP used in the generation of primary iPSCs, and an agarose screening gel to detect the contribution of iPSCs in chimeras is also shown.

Embryonic fibroblasts were isolated and cultured with antibiotic G418 for 4 days to successfully exclude cells with no primary iPSCs contribution before the start of secondary reprogramming.

## Secondary iPSC reprogramming of parental and Wt1 KO secondary MEFs

The secondary MEFs for primary iPSC line C5 and C5-E2 were sorted against GFP, to exclude cells that already express Wt1 before the start of reprogramming. GFP negative cells were plated 20,000 cells per 6-well for each lines. The reprogramming was initiated by addition of Dox to the media. As the reprogramming begins, cells from both lines (C5 more than C5-E2) start to rapidly divide due to the activation of the transcription factor c-Myc present in the plasmid; PB-TET-MKOS. But as the reprogramming progresses, an evident difference between C5 and C5-E2 is observed. **Figure-14** clearly represents the progress of secondary reprogramming for both C5 and C5-E2. The expression of Wt1 was tracked by GFP expression in the cells.

Over the course of reprogramming the differences between C5 and C5-E2 became apparent. C5-E2 struggled to divide and grow while C5 was dividing rapidly. MET was observed in C5 from day 2 by the change of their morphology and a tendency to become epithelial. By day 4, a green fluorescence is observed in C5 cells that were transitioning, but in comparison to C5-E2, this was not observed. C5-E2 cells were rather only increasing size and forming astrocytes-like morphology with no epithelial signature detected. Upon removal of Dox at day 8, C5 cells continued their progress towards reprogramming to iPSCs while C5-E2 showed no sign of reprogramming. The first epithelial colony was observed at day 10 in C5, the colony was still a mix of epithelial and mesenchymal cells present in the middle and at the edges respectively. Out of this colony at day 14, we could observe highly green fluorescent epithelial-like colony supported by mesenchymal cells. On day 18, C5 had iPSC-like colonies while only mesenchymal cells were observed in C5-E2 cells with no GFP expression as a confirmation that Wt1 promoter was inactive.

The dynamics of Wt1 expression represented by GFP reporter was analyzed by flow cytometer throughout reprogramming. Pattern of percentage of GFP positive cells for C5 shows a peaks at the very start of reprogramming, between day1 and 2 then a drop continues until day 7 and starts to peak again from day 12 onwards. The pattern of C5-E2 suggests the similar pattern but cells do not proliferate further so flow cytometry data was not available for day14 onwards (**Figure-15**). The cells in C5-E2 are pushed to reprogram but due to the KO of Wt1 they do not and further experiments are required to study the mechanism behind this phenomena.

At day 18, secondary iPSCs only for C5 were split, one part was kept on ES medium and the other part was cultured in 2i medium for another 10 days to observe the expression of mkate2, the red fluorescent reporter for Nanog to assess the ground state of pluripotency. A dynamic expression of Wt1 and Nanog

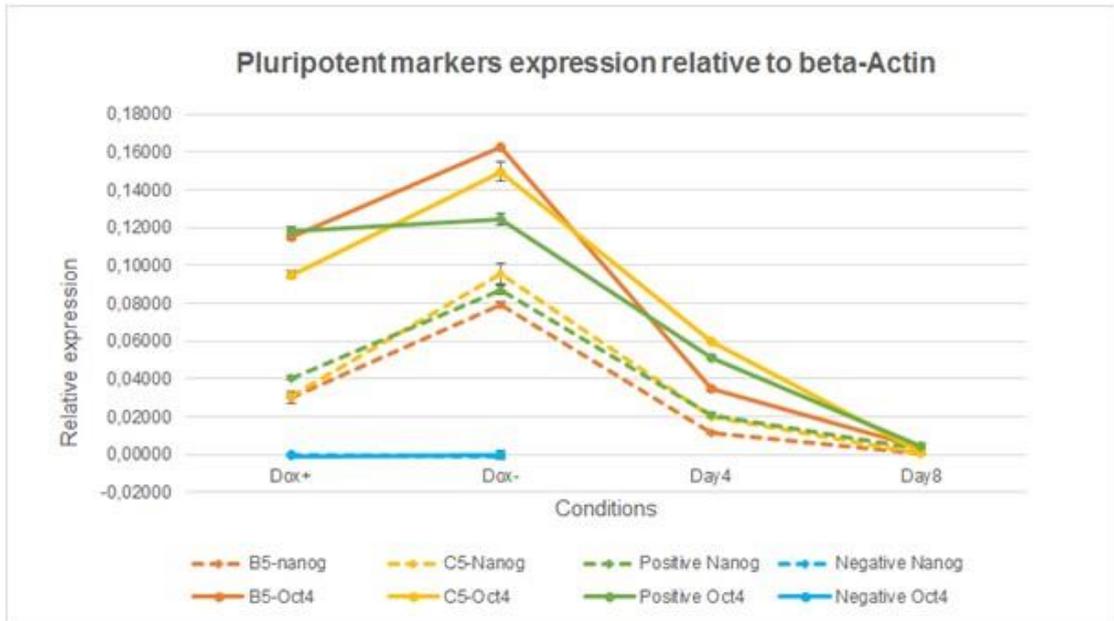
genes was observed in iPSCs on ES medium Vs 2i medium. iPSCs cultured on ES medium divided and formed colonies with a 70% GFP positive cells according to flow cytometry data but did not show a distinct expression of *mkate2*. These cells continually expressed *Wt1* until day 28. iPSCs cultured on 2i medium, gradually lost the expression of *Wt1* and start expressing *Nanog* (**Figure-16&17**). This could suggest that iPSCs only gain their ground state pluripotency when cultured in 2i medium and while in this state of pluripotency, *Wt1* drops in its expression.

### Trajectories taken by secondary MEFs during iPSC reprogramming

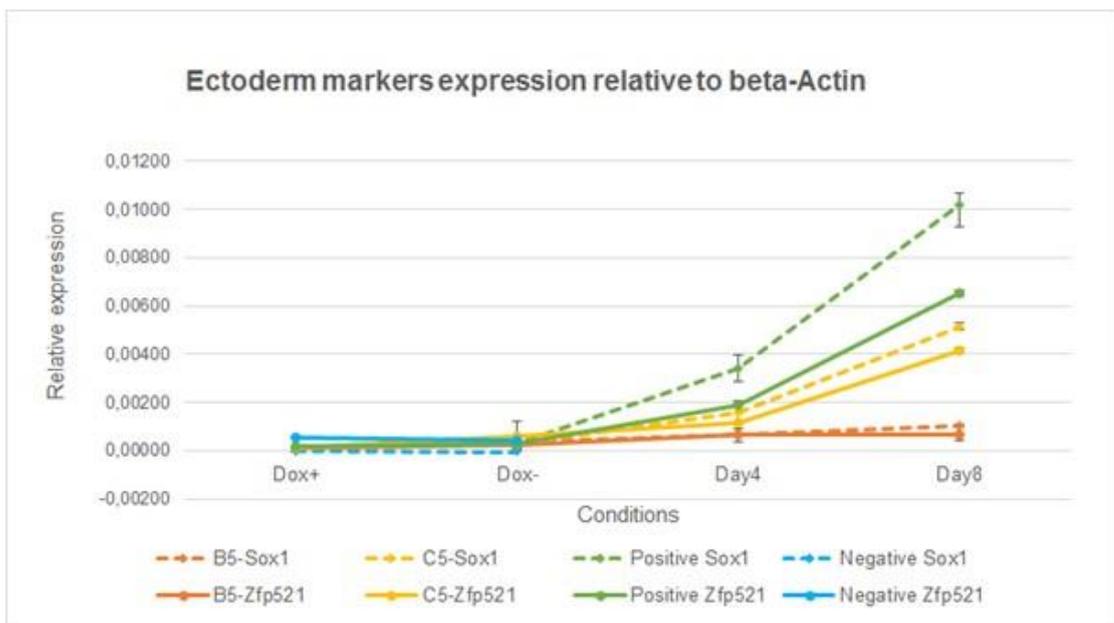
Our hypothesis before we started secondary reprogramming was that all cells will be prompt to reprogram since they all carry the reprogramming cassettes. From the first round of secondary reprogramming, we could speculate that only 10% of the total population of cells activate GFP but not all of them go through MET to finally reprogram (**Figure 5**). We investigated this phenomenon by sorting GFP negative and positive cells on day 4 and we analyzed them by RT-qPCR using specific markers for MET and stromal lineage. *Zic3* is a gene required for conservation of pluripotency, *Nfic* and *Prrx1* are connected in mesenchymal programs (Schiebinger et al., 2019). *Cdh1* and *Zic3* were used to follow cells that are going through MET while *Nfic* and *Prrx1* were used to follow the expression of cells that are taking a trajectory towards stromal.

The mRNA relative expression of *Zic3* and *Cdh1* displayed in **figure-18 and 19** clearly indicates *Wt1* positive cells going through MET which is in congruence with earlier discovery that *Wt1* has a crucial role in the mesenchymal-epithelial balance (Hohenstein & Hastie, 2006). The expressions are low for these genes but the differences in the expression are apparent. The relative mRNA expression displayed in **figure 20 and 21** suggests that *Wt1* expressing cells in addition to the non-*Wt1* expressing cells also have some contribution towards the stromal lineage.

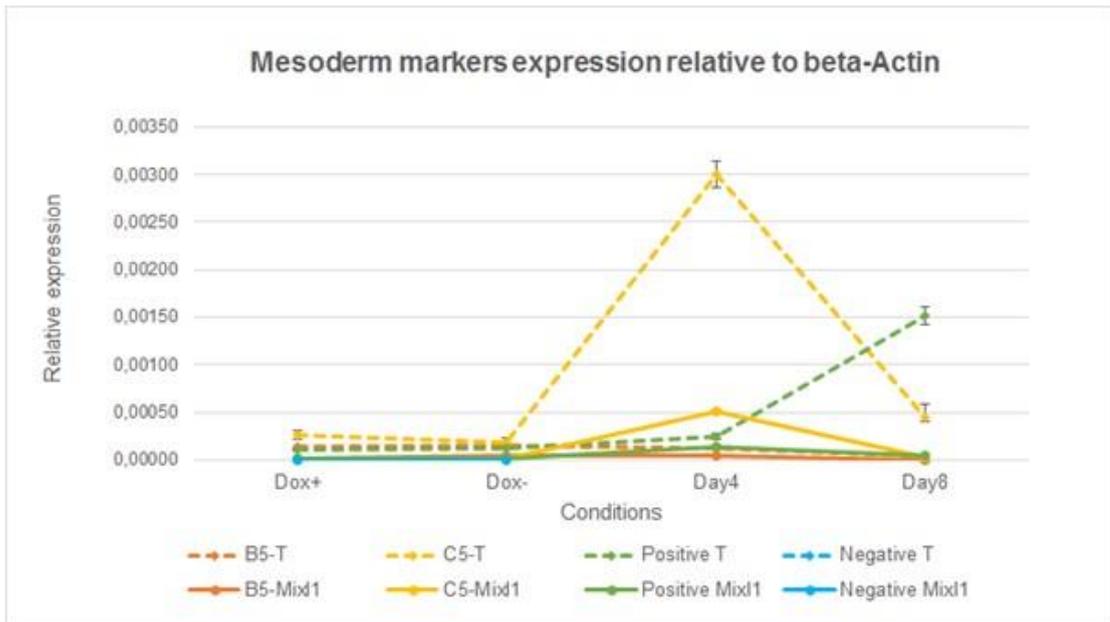
However, this could be an interesting observation that only 10% of all the cells are going towards MET although they are all induced to reprogram by Dox addition. The 90% cells left could be a mix of cells that have a low detectable range of *Wt1* expression and take on another trajectory. Overall, C5 shows a higher expression than C5-E2, this implies that the loss of *Wt1* in C5-E2 could have affected the signature of these cells. Furthermore, cells that are not activated by Dox to start reprogramming do not go through this trajectory and they have approximately the same expression as of day 0.



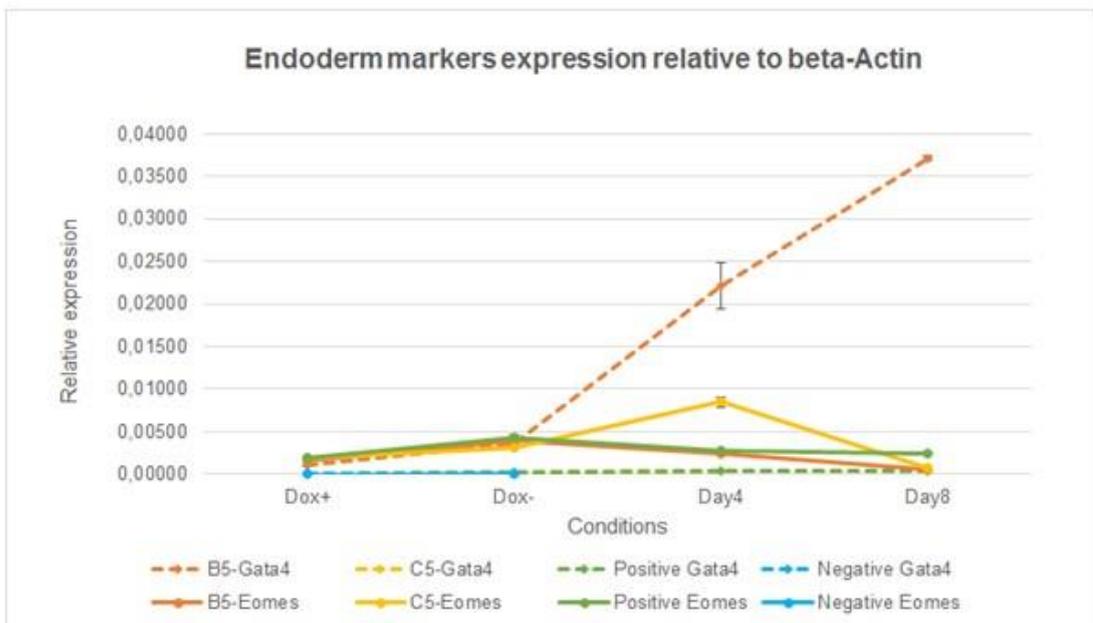
**Figure-7** Nanog and Oct-4 mRNA expression relative to beta-Actin of B5 and C5 iPS clones. ES cell line JM8+ used as positive control and fibroblasts as negative control.



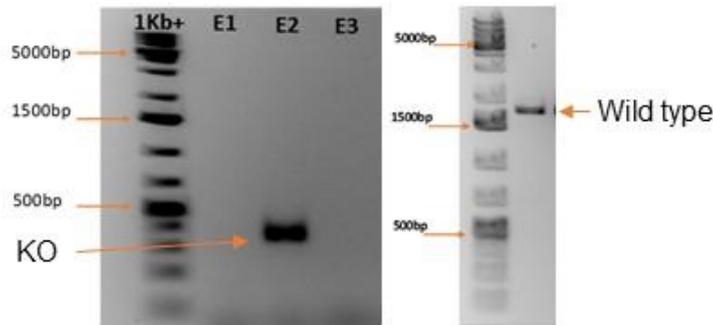
**Figure-8** Sox-1 and Zfp521 mRNA relative to beta-Actin, JM8+ used as positive control and Mouse embryonic fibroblasts as negative control.



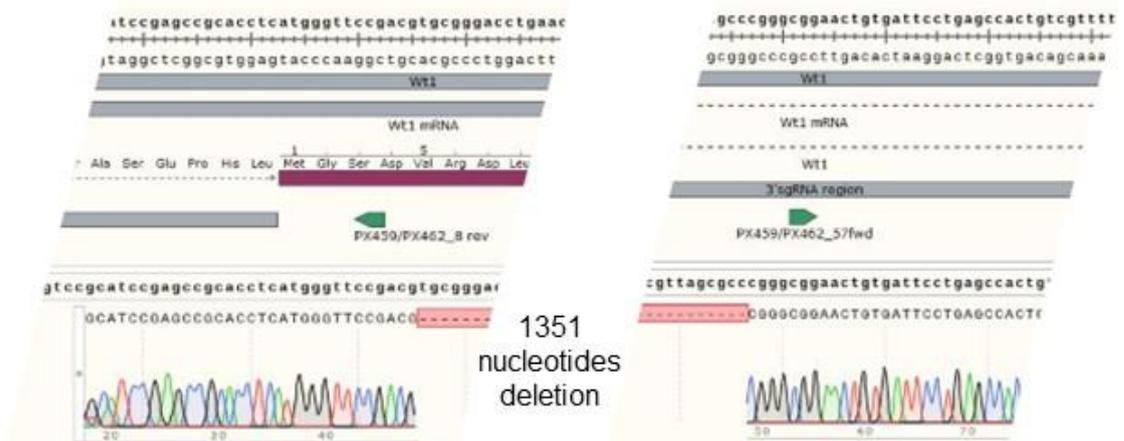
**Figure-9** T and Mixl1 mRNA expression relative to beta-Actin of B5 and C5 iPS clones. ES cell line JM8+ used as positive control and fibroblasts as negative control.



**Figure-10.** Gata4 and Eomes mRNA expression relative to beta-Actin of B5 and C5 iPS clones. ES cell line JM8+ used as positive control and fibroblasts as negative control.

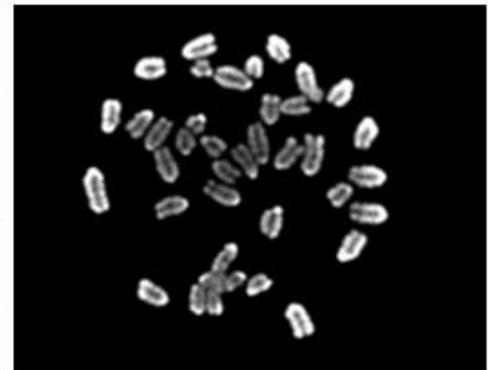


**Figure-11** Agarose Screening gel for WT1 KO



**Figure-12** Deletion map of Wt1 at the ATG start of Exon1 and at the start of Intron 1

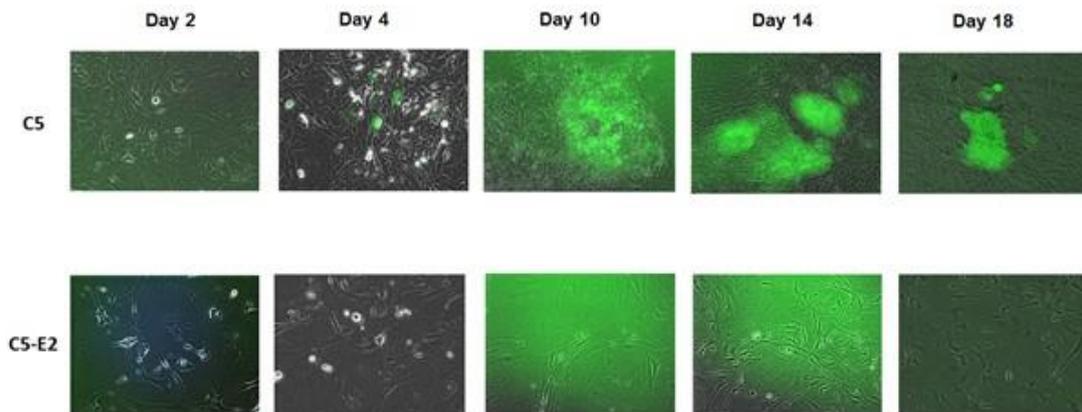
Clone	%40 chromosomes count
1FrtTA_B5	69
1FrtTA_B7	77
1FrtTABFP_C5	88
1FrtTABFP_C9	91
1FrtTABFP_C5-E2 (Wt1KO)	83



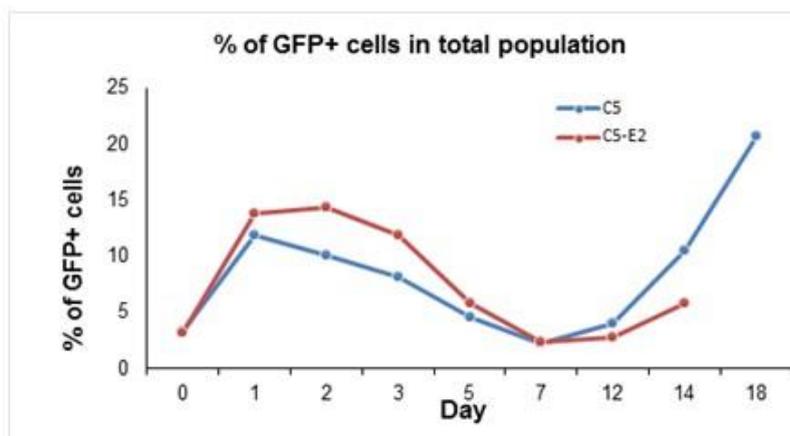
**Figure-13.** Percentage count and Chromosome spreads of iPSCs

**Table-13.** Percentage contribution in Chimeras for each primary iPSC line

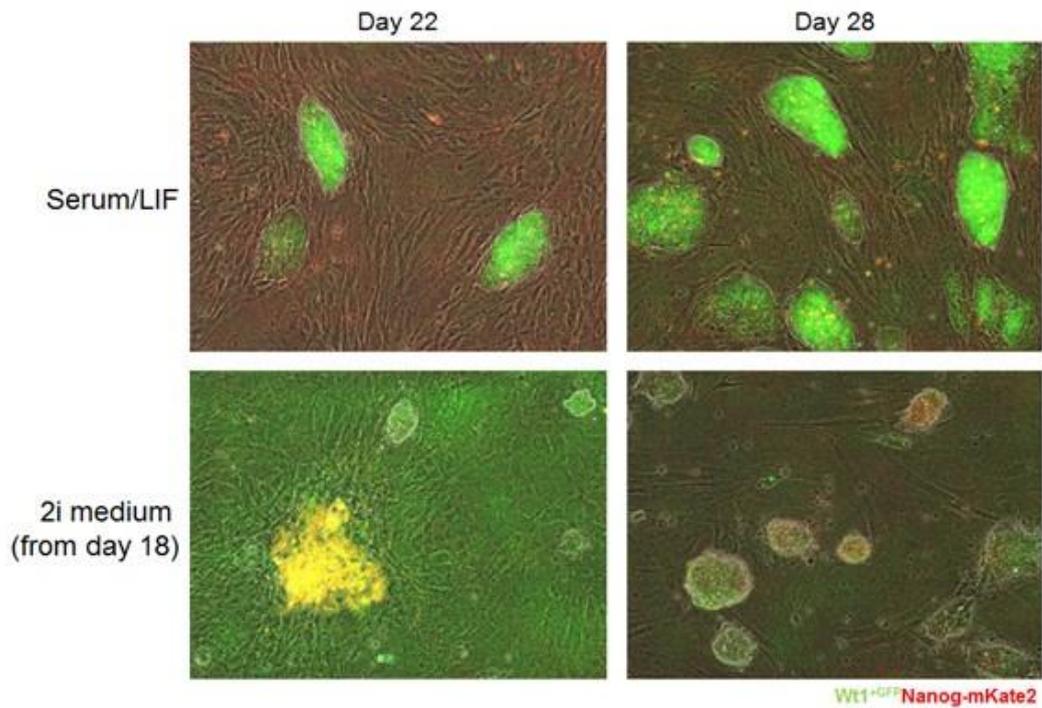
Clone	#Blastocysts injected	#Embryos Recovered at E13.5/12.5	# Embryos with any contribution	% Embryo with any contribution
C5	22	29	16	72,7
B5	26		7	26,9
C5-E2	32	18	18	100,0



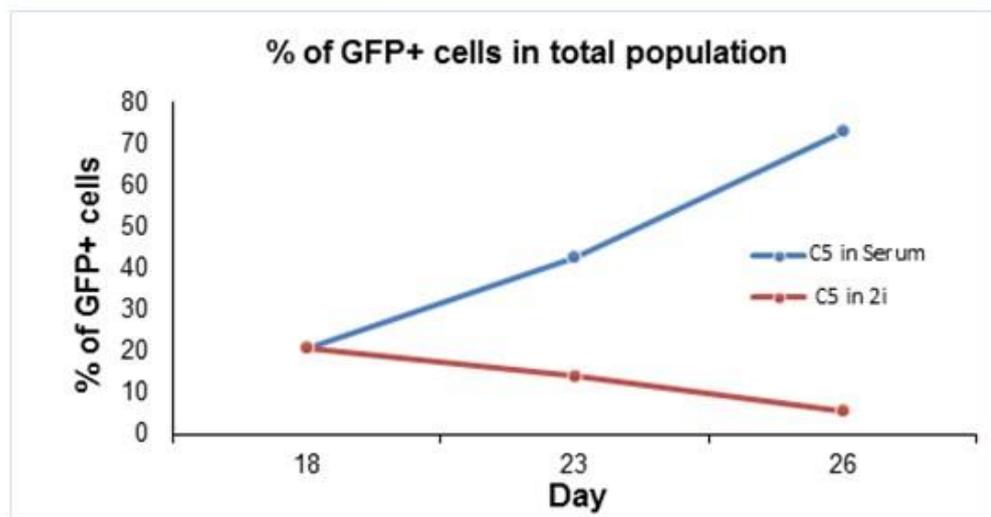
**Figure-14** Merged pictures of GFP expression of C5 and C5E2 over the course of reprogramming.



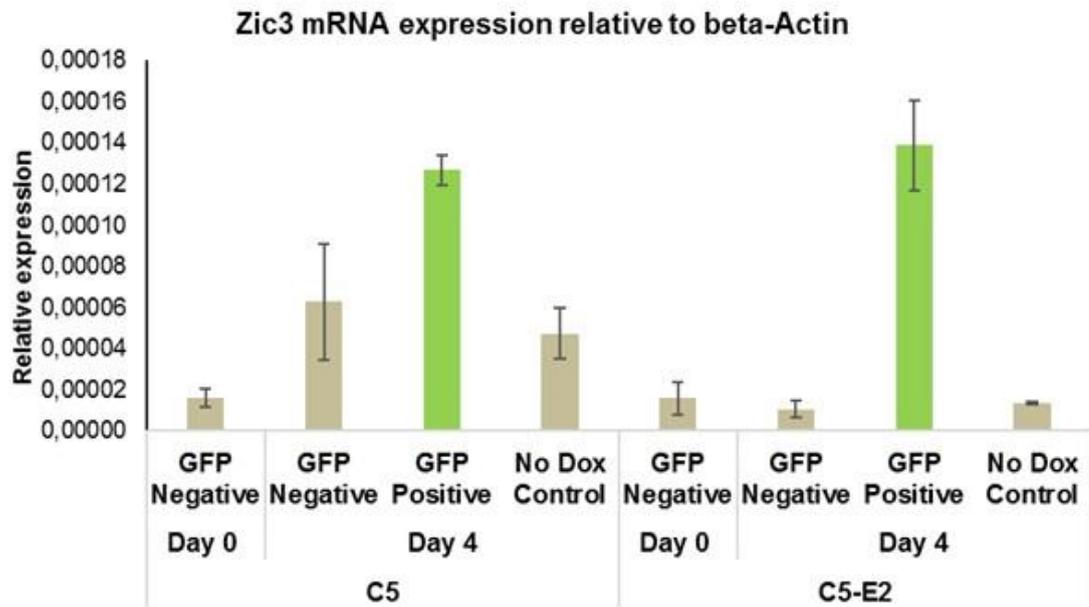
**Figure-15** Flowmetry expression of Wt1 by GFP during reprogramming of both C5 and C5-E2.



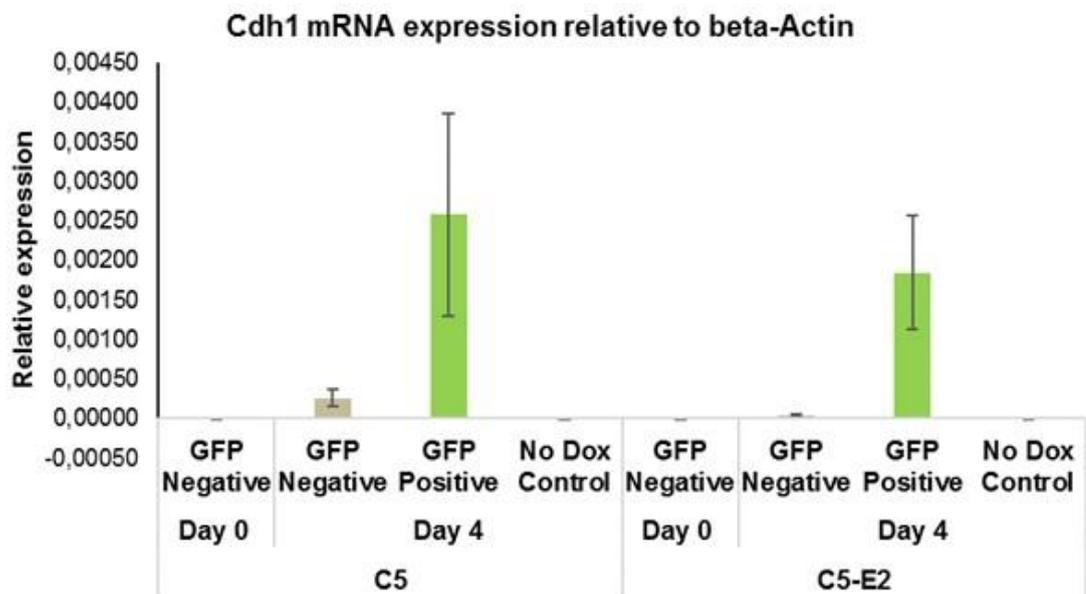
**Figure-16** Merged pictures of C5 in serum and on 2i medium showing the expression of Wt1 in green and Nanog in red.



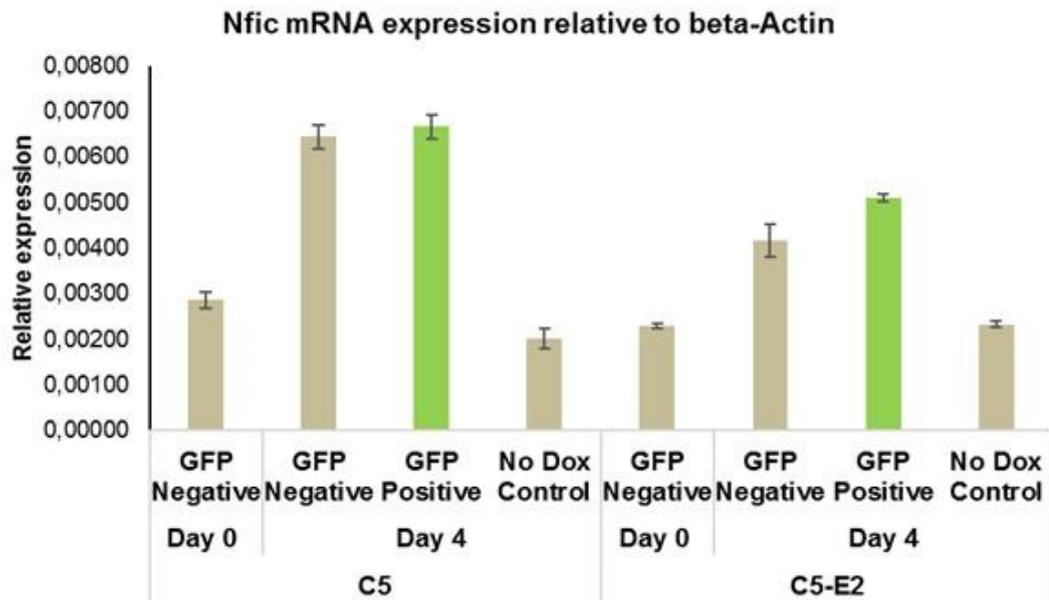
**Figure-17** Flowmetry expression of Wt1 and Nanog from day 18 till day26 of both C5 in serum and in 2i.



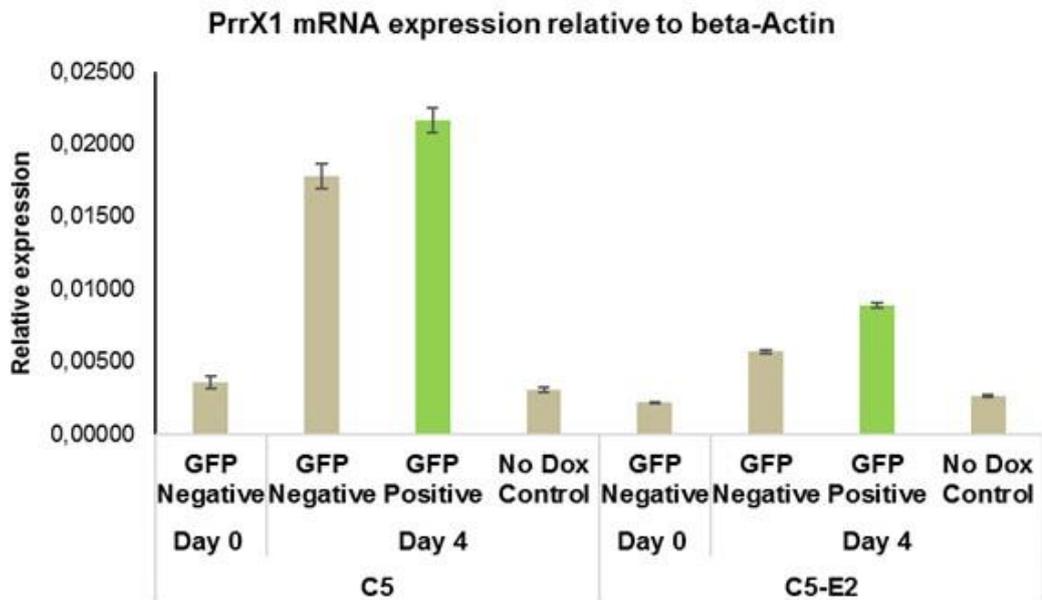
**Figure-18** Zic3 mRNA expression relative to beta-Actin of C5 and C5-E2 at day 0, day 4 sorted GFP+ and GFP- and without Dox.



**Figure-19** Cdh1 mRNA expression relative to beta-Actin of C5 and C5-E2 at day 0, day 4 sorted GFP+ and GFP- and without Dox.



**Figure-20** Nfic mRNA expression relative to beta-Actin of C5 and C5-E2 at day 0, day 4 sorted GFP+ and GFP- and without Dox.



**Figure-21** PrrX1 mRNA expression relative to beta-Actin of C5 and C5-E2 at day 0, day 4 sorted GFP+ and GFP- and without Dox.

## Discussion

The low efficiency of secondary iPSC reprogramming was unexpected. In the first part of this project, primary iPSC reprogramming was entitled as an irreproducible and inefficient mechanism because of the stress given onto the cells during transfection and all the other factors that reprogramming embodies. By performing secondary reprogramming our expectation was that all the cells will reprogram but that was not observed, instead an approximate percentage of 10% of the total population of cells activate GFP but not all of them go through MET to finally reprogram to iPSCs. We believe the other 90% of GFP negative cells act as a supportive layer or are involved in cell to cell interactions that contribute as a whole to the reprogramming process. During our first run, on day4 we sorted GFP positive from negative cells and both population did not reprogram. We speculate that the lifting of cells might have altered the reprogramming process and disrupted an interaction between both populations. Not all cells that go through MET will successfully reprogram, this was also stated in recent papers. There is still an unknown aspect of the type of cells that will reprogram. The efficiency of reprogramming can only be increased once this aspect is studied. Cells that go through MET gives not only rise to iPSCs but also neural and trophoblast cells (Schiebinger et al., 2019).

We believe that Wt1 plays a role before reprogramming starts as well because KOWt1(C5-E2) cells struggled to match up the numbers with the wild type line (C5). The second run of secondary reprogramming a ratio of 1:3 vials of  $1 \times 10^6$  cells of C5 to C5-E2 was used and still the total number of C5-E2 cells was less. To strictly confirm that Wt1 is indeed a gatekeeper for iPSC reprogramming, a conditional knockout of Wt1 will have to be used in the same secondary reprogramming process. In this case, fibroblasts will stay vigorous until they start to reprogram and Wt1 will be conditionally knocked out for reprogramming exquisitely. The embryonic fibroblasts isolated from C5-E2 could have struggled because of a day of difference in their isolations compared to C5. The isolation of embryos from C5-E2 was at day 12.5 of fertilization while C5 was at day 13.5, this was performed because from day 12.5 KO cell lines are predicted to show a phenotype and we only wanted to see a difference in their genotype that is whether they lost the expression of Wt1 gene. The embryos were indeed smaller in size compared to the wildtype.

During sorting of GFP positive and negative cells before the start of reprogramming, different populations were displayed. FACS was performed with two channels; BFP reporter of PB-rtTA-2A-BFP and GFP reporter for Wt1. There were roughly 4 populations; GFP negative, positive and intermediate that were also BFP positive and a separate population of BFP negative. Before reprogramming the cells were sorted for GFP negative to start with cells that do not express Wt1. A separate run of secondary reprogramming with GFP positive cells was performed but these cells did not reprogram to iPSCs. On day4, we collected only BFP positive population because these were derived from the primary iPSCs and together we collected GFP negative and highly positive. The separation of the two population was complicated because of the intermediate population. A better approach would have been to separate the population in three, take highly positive and negative GFP cells and collect the intermediate as a separate sample. This approach could help explain the phenomenon of similar expressions of stromal markers of GFP negative and positive population during RT-qPCR.

Although, RT-qPCR analyzes and shows a good representation of relative mRNA expression of a pool of cells, the genomic differences between the wild type and KOWt1 can be further studied. Further comparison will be carried out by performing a total RNA sequencing on not only wild type and KOWt1 but also on GFP positive and negative cells of each line. This approach will give more insight in the differences between the two cell lines.

The expression of mkate2; a red fluorescent reporter for Nanog gene in secondary iPSCs validates the success of the process. It indicates the ground state pluripotency of secondary iPSCs. The phenomenon of the drop in Wt1 expression when Nanog is expressed in 2i medium still need further studies. Could it be that Wt1 and Nanog genes are expressed alternatively and that one is silenced or hindered by the other?

Wt1 has been shown to play a major role in iPSC reprogramming, cells that activate Wt1 will be the ones that successfully reprogram (**Figure-14**). Parallel secondary reprogramming of wild type and KOWt1 primary iPSCs confirms the importance of Wt1 in MET, KOWt1 cells failed to go through MET

and hence lost their trajectory towards iPSC reprogramming. Wt1 might be a gateway for iPSC reprogramming because of its early expression in the flowmetry measurement. It can be concluded that Wt1 is both a tumour suppressor gene for Wilms tumour and a crucial gatekeeper gene in iPSC reprogramming. This work is still ongoing, but our research cannot be interpreted otherwise. Although this finding is a step closer to the full understanding of iPSC reprogramming, there is still a lot to discover.

The bigger purpose of this research is to give insights on the mechanism of iPSC reprogramming and contribute to the regenerative medicine aspect of research. The next step would be to perform the same mechanism in human fibroblasts. Several steps have been made to acquire secondary human iPSCs. If the same is observed during human iPSCs reprogramming, we are one step closer to medical trials and application.

## Acknowledgements

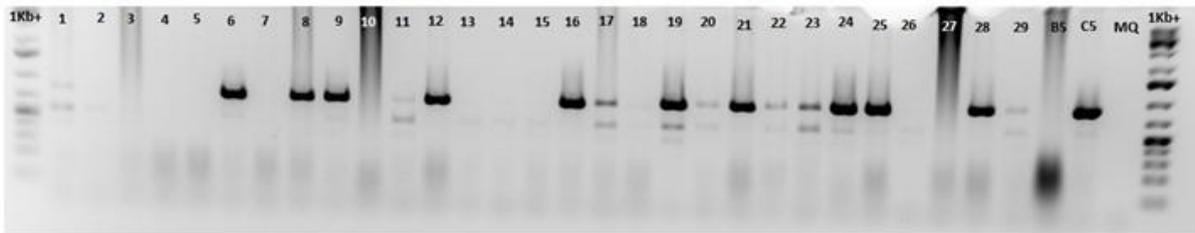
I would like to express my gratitude to my day to day supervisor Karamjit Singh Dolt for all the professional trainings together with a lot of insights on the aspect of working in a research environment, and a big thanks to our supervisor Peter Hohenstein for this internship opportunity and for all the milestones encouragement. I wish to thank lab technicians Jill Claassens and Conny Brouwers for all trainings and tips and a special thanks to Margot Linssen for all the mouse and injection work, the whole department of Human genetics at LUMC. I would like to appreciate the support from my school; HZ University of applied sciences and especially my supervisor José de Winter. Finally, my parents and everyone who contributed to my wellbeing and focus for this period.

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## Appendix



**Figure-22** A screening gel picture of chimeras to determine the contribution of primary iPSCs in secondary fibroblasts



**Figure-23** A picture of a head of a chimera derived from primary iPSCs

**Table-14** PCR Oligo Anneal Program

Temperature	Time
95	10mins
95-85	
85	1min
85-75	
75	1min
75-65	
65	1min
65-55	
55	1min
55-45	
45	1min
45-35	
35	1min
35-25	
25	1min

**Table-15** List of primers used in this project

<b>Single guided primers (Cas9)</b>	
8rev-Forward	5'-AAACACGTGCGGGACCTGAACGCG -3'
8rev-Reverse	5'-CACCGCGCGTT CAGGT CCCGCACG -3'
<b>Single guided primers (Cas9)</b>	
57fwd-Forward	5'-CACCGCTCTTTCGTTAGCGCCCGGG -3'
57fwd-Reverse	5'-AAACCCCGGGCGCTAACGAAAGAGC -3'
<b>Screening KO PCR primers</b>	
WT1ATG-Forward	5'-GTCCAGCGCCGAGAAT C-3'
WT1Intron-Reverse	5'- GCGTCTGTGTCAGTAAAGAATTCC-3'
<b>Secondary Mefs Screening PCR primers</b>	
GFP-Forward	5'-GACGTAACGGCCACAAGTT-3'
GFP-Reverse	5'-GAACTTCAGGGTCAGCTTGC-3'
<b>qPCR Assay probes and primers</b>	
<b>Nanog</b>	
Probe	5'-/56-FAM/CCACCGCTT/ZEN/GCACTTCATCCTTTG/3IABkFQ/-3'
Primer1	5'-TGCTGAGCCCTTCTGAATC-3'
Primer2	5'-TGCTGAGCCCTTCTGAATC 3'
<b>Oct4</b>	
Probe	5'-/56-FAM/CTGTCTATC/ZEN/GCCGCTACTTCCGC/3IABkFQ/-3'
Primer1	5'-CACCATTGCTCATCCAGAA- 3'
Primer2	5'-CTGCCTACCTGCTCTTTCTC 3'
<b>Sox1</b>	
Probe	5'-/56-FAM/ACAAAAGAC/ZEN/AAAAACCCAGCGAGGC/3IABkFQ/-3'
Primer1	5'-GTACAGTATTTATCGTCCGCAGA-3'
Primer2	5'-GGCAGTCATACAAAAGTTGGC-3'
<b>Zfp521</b>	
Probe	5'-/56-FAM/TGTCACCCA/ZEN/TGCCCAGAATCAGTC/3IABkFQ/-3'
Primer1	5'-CAGCACACAGTTGAAAACGC-3'
Primer2	5'-GACACTTGATACACTGGTAGGTC-3'
<b>Brachyury/T</b>	
Probe	5'-/56-FAM/TCATAACGC/ZEN/CAGCCCACCTATG/3IABkFQ/-3'
Primer1	5'-GATAACTGGTCTAGCCTCGGA-3'
Primer2	5'-GTACCATTGCTCACAGACCA-3'
<b>Mixl1</b>	
Probe	5'-/56-FAM/CACGCTGCT/ZEN/ACCCGAGTCCAG/3IABkFQ/-3'
Primer1	5'-CTTCCGACAGACCATGTACC-3'
Primer2	5'-GCTGAAATGACTTCCCACTCT-3'
<b>Gata4</b>	
Probe	5'-/56-FAM/CAACCCTGG/ZEN/AAGACACCCCAATCT/3IABkFQ/-3'
Primer1	5'-TTCGACAGCCAGTCCT -3'
Primer2	5'-ATTGACACACTCTCTGCCTTC -3'
<b>Eomes</b>	
Probe	5'-/56-FAM/TGCGGCAAA/ZEN/GCGGACAATAACATG/3IABkFQ/-3'
Primer1	5'- TTTCTGGAAGTGGTTCTGG -3'
Primer2	5'- GGAGCCAGTGTTAGGAGATTC -3'
Probe	5'-/56-FAM/TGCGGCAAA/ZEN/GCGGACAATAACATG/3IABkFQ/-3'
Primer1	5'- TTTCTGGAAGTGGTTCTGG -3'
Primer2	5'- GGAGCCAGTGTTAGGAGATTC -3'