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iPSC GENERATION, PRIME TO NAÏVE REVERSION & CHARACTERIZATION AND PRIMORDIAL GERM CELL DIFFERENTIATION OF NORTHERN WHITE HORN RHINO

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1. Abstract:

Germline specification underlies reproduction and evolution, but it has been proven difficult to study since it occurs shortly after blastocyst implantation and also because the molecular mechanisms underlying PGC specification are poorly understood due to inaccessibility of cell materials and lack of *in vitro* models for tracking the earliest stages of germ cell development. This process can be modeled with induced pluripotent stem cells (iPSCs) by differentiating them into primordial germ cell-like cells (PGCLCs) [1].

Since the first demonstration of iPSC formation in 2006 by professor Yamanaka and his team, induced pluripotency has become a prominent tool in biomedical research. And when they are differentiated into progenitors, it has been extensively shown how these are important as the first germ cell population established during development and are immediate precursors for both the oocytes and spermatogonia[2]. Although, most of the studies currently done are focused towards mouse and humans very little has been targeted towards other large mammalian species. The objective is thus to focus on generation of PGCs in another mammalian species that have a similar genetic makeup to that of humans. Rhinos, are a species that has a similar genetic makeup to that of humans, they express certain genetic markers that are also found in humans' explicitly and so, for this study, we have focused on attempts to generate PGCLCs from iPSCs of Northern White Horn Rhino (NWR, *Ceratotherium simum cottoni*). The NWR is almost extinct, rather, it is the most endangered mammal in the world with only two females' surviving[3] and that's why we would want to save it from extinction. The Drukker lab has been successful in collecting and storing samples from the last male rhino that died recently and so there is a fair chance to reprogram those cells into iPSCs. Possibility to differentiate these cells into PGCs by confirming their viability to be termed as actual PGC representative *in vivo* would be a leap forward towards saving these animals from extinction. It will thus also be highly motivating to do further research to perform further differentiation steps to finally produce proliferating germ cells having the ability to generate gametes.

2. Introduction:

Induced pluripotency refers to a condition where normally non-pluripotent somatic cells are forced to regress into a pluripotent state. The experiments for iPSC generation were first carried out by professor Yamanaka *et al.*, whereby it became popular to use this for extensive research[4]. Primordial germ cells, the precursors of sperm and eggs, originate during weeks 2–3 of early post-implantation development[5] which can generate a new organism that is capable of creating endless new generations through germ cells and are uniquely programmed for transmission of genetic and epigenetic information to subsequent generations[7]. This germ cell lineage differentiates into spermatozoa or oocytes and serves as both the origin of totipotency and the foundation for heredity and evolution. PGCs generated from induced pluripotent stem cells in vitro also hold promise, with broad applications for studies of germline cells. Specification of the germ cell fate during development marks the inception of such vital processes in biology, and it has been an intensive focus of studies using a number of organisms[2, 6].

The mechanism for germ cell development in mammals has most extensively been studied using the mouse as a model organism, providing essential information practically applicable to all mammals, including humans. On the other hand, among diverse mammalian species, there exist significant differences in the precise mechanisms for germ cell development, which necessitates careful species-by-species studies for a precise understanding of germ cell development in a given species. In this regard, there is a critical lack of information as to the mechanism for germ cell development in rhinos, mainly due to the difficulties/limitations in accessing relevant experimental materials[8]. It would therefore represent a promising breakthrough over these limitations if rhino germ cell development could be reconstituted in vitro from rhino pluripotent stem cells (rPSCs), including rhino induced pluripotent stem cells (riPSCs) [9]. Another hurdle in this regard is that the rhino genome has been constituted *de novo* and so exact genome analysis data is not present yet. Conventional approaches toward establishing a self-sustaining NWR population over the last two decades have been repeatedly unsuccessful. To date only one study related to ovum pick up and embryos production in rhinoceros has been reported[10]. There has been no report of the production of rhinoceros embryo from fertilization to the pre-implantation stage[11, 12]. For this reason it could be exciting to use this tool to generate PGC from iPSCs developed from somatic cells and used as a viable strategy to rescue genes from the iconic, almost extinct, Northern White rhinoceros and may also have broader impact if applied with similar success to other endangered large mammalian species.

3. Literature Review:

3.1. Induced Pluripotent Stem Cells:

3.1.1. Stem cell potency:

Stem cells are cells that are capable of self-renewal, proliferating extensively and differentiating towards several other cell types[12]. Depending on the differentiation capacity, stem cells are classified into different categories: totipotent, pluripotent, multipotent, and oligo-potent stem cells [13, 14]. Among them, zygotes are considered to be totipotent cells, giving rise to all cells, including pluripotent embryonic stem cells and extraembryonic tissues [14, 15] and on the other hand pluripotent stem cells are defined as cells capable of differentiating into any of the three germ lines and thus all adult cell types [8, 14]. Multipotent cells are derived from pluripotent cells and capable of differentiating into some cell types derived from a single germ layer, such as mesenchymal stem cells which form adipose tissue and bone, among others [13-15]. Oligo-potent cells are considered tissue-resident progenitor cells with the potency to form a limited number of terminally differentiated cells[13]. Examples of oligo-potent cells are lymphoid stem cells, forming B and T cells[17, 18]. It may be noted that oligo-potent cells are not always defined as stem cells if a stem cell is considered a cell able to proliferate indefinitely[19].

3.1.2. Induction:

Cells resembling pluripotent embryonic cells (ESC) have previously been generated from somatic cells with two notable methods, nuclear transfer to oocytes, and fusion with ES cells[20]. A breakthrough in reprogramming somatic cells came about when several studies displayed that fibroblasts from mouse and human are reprogrammable with defined factors like utilizing a viral vector[9, 21-25], episomal plasmids and RNA transfection. Subsequently, reprogramming has succeeded with multiple other cell types[26-28]. The induction of somatic cells into iPS cells is frequently carried out using four transcription factors, OCT3/4, SOX2, Klf4, and c-Myc, also referred to as the Yamanaka factors. Other combinations of factors have also been employed, including 1-4 factors in most cases[26]. All factors are not always required, but OCT3/4 and SOX2 are generally employed[26, 29].

c-Myc is a factor occasionally replaced or left unused, as it is a known proto-oncogene and thus problematic with possible clinical applications when transduced with an integrating[22, 26]. However, as it is shown that transgene integration into cell genome is not necessary for induced pluripotency, non-integrating methods have been devised to eliminate insertional[22]. One of these methods exploits the use of non-integrating Sendai RNA virus[30]. Other methods include the use of plasmids, episomes, reprogramming proteins, small molecules, and miRNAs[25, 31]. Sometimes incomplete silencing or re-activation of transgenes occurs with retroviral induced cells and may cause dependence on the transgene expression, tumorigenesis, and interference with development and differentiation[26, 33]. A schematic illustration of the miRNA procedure is presented in **fig 1**.

When expressed in cells, the factors affect transcription, activating an expression network of several pluripotency-associated factors and series of events proceeding towards pluripotency (Kim et al. 2008; Wei et al. 2009). OCT3/4 and SOX2 are considered as core factors working synergistically, while c-Myc and Klf4 are thought to modify the chromatin structure for OCT3/4 and SOX2 binding[9]. In addition, c-Myc is proposed to universally amplify active gene expression, thereby enhancing the induction process (Nie et al. 2012). Obstacles that somatic cells must overcome in the course of reprogramming include the inhibition of somatic regulators, senescence and apoptotic pathways, the induction of proliferation, the activation of pluripotency loci, and independence from exogenous factor expression[26].

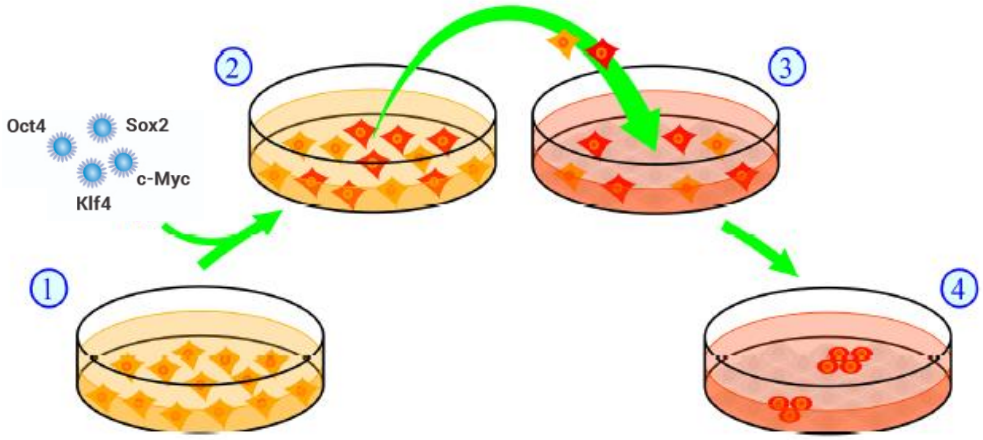


Fig 1: Induction protocol. Phases: (1) The isolation and culture of somatic cells. (2) Transferring of reprogramming factors. (3) Harvesting the cells and culturing in pluripotent cell conditions (on a feeder layer). (4) The formation of iPS cell colonies. (Image: Y. Tambe 2007)

3.1.3 Applications:

iPS cells have prominent applications in cell and tissue modeling, drug discovery, gene repairing, and disease pathogenesis studies in vitro. In contrast to embryonic stem cells, iPS cells have the feature of being patient and disease-specific enabling the study of inherited conditions with iPS-cell derived cells and tissues without ethical concerns and limited availability surrounding embryonic cells and oocytes. Also, regenerative therapeutical applications could be conducted without the issue of tissue rejection[9, 20, 26 and 34]. **Fig 2** shows a scheme describing the applications of iPSC. Human ES cells and iPS cells do not seem to exhibit significant differences in global gene expression patterns[35]. It is shown, however, that some iPS cells may retain a transient epigenetic memory especially in early passages[36, 37]. In other mammals for instance and for this case in NWR, iPSCs could prove to be a vital tool in preserving these species from extinction as this allows to generate progenitor cells which has the capability to be further modified and directed towards gamete-specific differentiation. That could be potentially a better way than conventional IVF as compared to the sperm cells in the bank proliferating fresh cells might have a better chance of fertilization. Such an approach will be the key to perform further experiments towards attempts of generating new individuals without necessarily interfering with the genetic makeup.

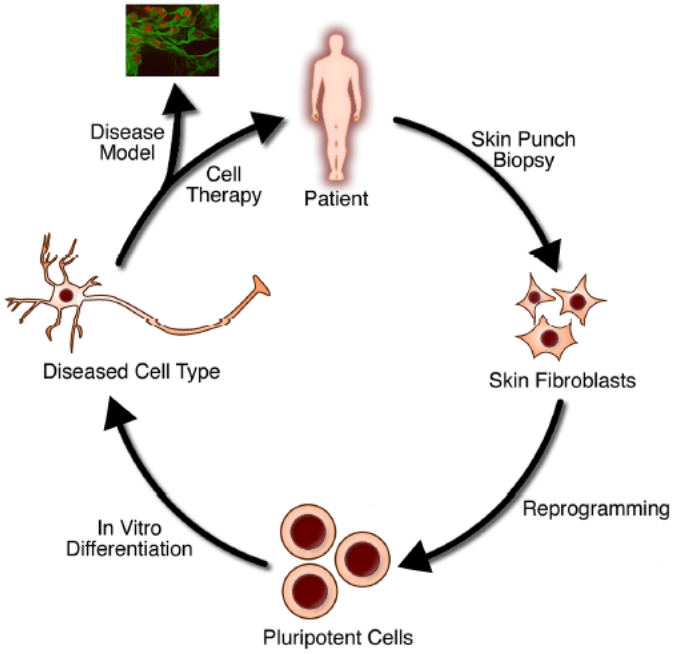


Fig 2: Applications for induced pluripotent stem cells. (Rodolfa 2008, modified)

3.2. Methods of characterization:

3.2.1 Profile of pluripotency:

The characterization of cells includes a set of defined tests to reach certainty on the cell type. iPS cells are frequently characterized with the criteria applied to ES cells[38]. As previously mentioned, there are some reported differences between iPS and ES cell characteristics considering expression patterns, but slight differences exist also between different iPSC and ESC lines. In the course of extended culture, however, iPS cells come to resemble ES cells probably due to the completion of reprogramming. (The International Stem Cell Initiative 2007) Features assessed by characterization include cell and colony morphology, growth rate, the expression of surface markers and other antigens, the expression of pluripotency marker genes, methylation statuses, enzyme levels, and differentiation into somatic cell types in vitro and in vivo (table 1).

Among pluripotency markers, Nanog and OCT3/4 are regarded as archetypal pluripotent stem cell markers (The International Stem Cell Initiative 2007). In addition, it is noted that OCT3/4 and SOX2 work together through feedback loops regulating both their own transcription and other pluripotency-associated genes[39].

Feature	Status/Marker/Function
Morphology	Cells: high nucleus/cytoplasm ratio Colonies: round, tight, smooth borders
Growth rate	Comparable to ESC, Td ≈ 40-50 h
Surface marker proteins	SSEA3, CD 38
Undifferentiated ES markers	Nanog, OCT3/4, REX1, SOX2, GDF3, FGF4, ESG1, DPPA2, hTERT
Unmethylated promoters of pluripotency-associated genes	Nanog, OCT3/4, REX1

TABLE 1: Pluripotency indicating conditions frequently studied in cell line characterization protocols[8, 38]

3.2.2. Factors Maintaining Pluripotency in Cell Culture:

Culturing conditions including culturing wells, media, incubator environment, and supportive matrices affect strongly the condition of the cell culture. Feeder cells are used frequently in cell culture. Feeders are cells secreting substances that support the cultured cells and provide a matrix to attach. They are rendered incapable of proliferation with chemical or physical means. These types of media have been crucial for stem cell experiments since the beginning of studies in iPSCs. Although, feeder media is suitable for supporting of stem cell culturing but it may cause transferring of animal pathogens and inducing immune response. So, feeder free culturing of iPSCs on matrigel in supplemented media (without using MEF conditioned medium) resolves these problems and could help in easy applications of iPSCs in further researches[40].

For our project mTeSR™1 cGMP, feeder-free maintenance medium for human iPS cells were used. mTeSR™1 is a highly specialized, serum-free and complete cell culture medium. With pre-screened raw materials that ensure batch-to-batch consistency and robust feeder-free protocols for ES and iPS cell culture, mTeSR™1 provides more consistent cultures with homogeneous, undifferentiated phenotypes. mTeSR™1 is manufactured under a cGMP quality management system compliant to 21 CFR 820, ensuring the highest quality and consistency for reproducible results. It is the most widely published feeder-free cell culture medium for human embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells), with established protocols for applications ranging from derivation to differentiation.

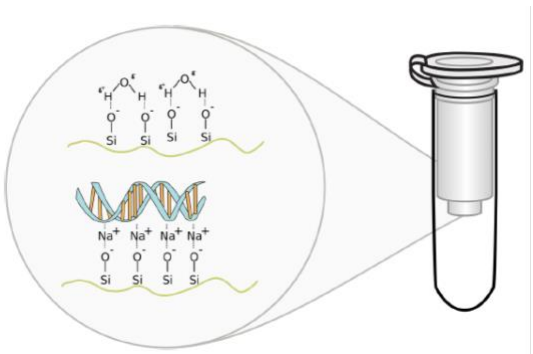
3.3. Characterization Strategies:

3.3.1. Marker Amplification using qPCR by RNA extraction and generation of cDNA:

Intact, high-quality, and high-yield RNA is required for many purposes in molecular biology[41, 42]. Compared to large molecule DNA extraction, shorter RNA molecules tolerate more mechanical stress and can be isolated from cells with disruptive methods. However, RNA is susceptible to degradation by omnipresent ribonucleases and factors such as heat, elevated pH, divalent cations, and extended storage periods[41, 42]. Today, the most widely used techniques are the phenol-chloroform extraction and the silica-gel column-based method. The RNeasy Mini Kit provides fast purification of high-quality RNA from cells, tissues, and yeast using silica-membrane RNeasy spin columns with a binding capacity of 100 µg RNA.

To produce starting material for a polymerase chain reaction, isolated RNA has to be transcribed into complementary DNA (cDNA) [43]. The transcription is carried out with reverse transcriptase (RT) enzyme in a solution containing random primers and deoxynucleotides[2]. Reverse transcriptase produces single-stranded DNA molecules on an RNA template[3].

PCR is an extremely sensitive method, detecting minuscule amounts of target DNA[43]. qPCR is a modification of the conventional PCR where the amplification of the product can be monitored real time. This gives further insight in how much amplification is occurring when and provides additional information on the activity of certain genetic markers to check if thev are activated or not. The data then received can be analyzed to ascertain the results.



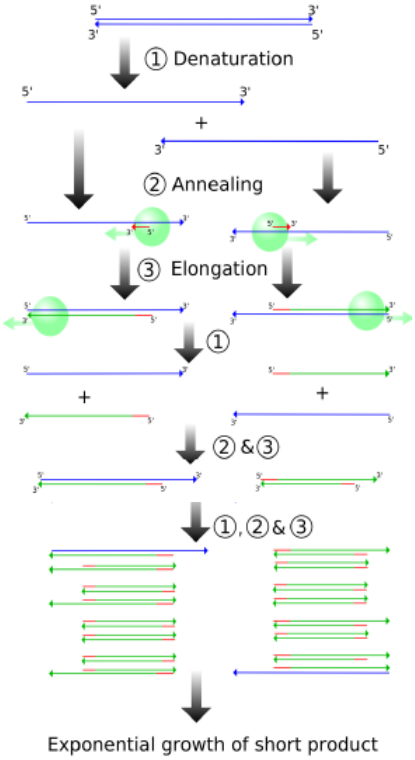


Fig 3: **A** Silica-based spin column. (Image: Wikimedia Commons 2008)
B PCR phases. (Image: Ball 2007)

3.3.2. Immunofluorescence staining:

Antibodies, also known as immunoglobulins, are proteins produced by plasma cells that bind specifically to particular substances referred to as antigens[4, 5]. Antibodies share the same fundamental structure[3]. Antibodies are utilized in biosciences to identify specific molecules in biological matrices. Immunofluorescence staining refers to immunochemical methods based on the use of fluorescent dyes attached to antibodies to visualize the protein of interest. The location of the antigens in a sample is ascertained when the sample is illuminated with a dye-specific excitatory wavelength. Fluorescent label may be directly attached to the antibody or indirectly to a secondary antibody which, in turn, binds to the primary antibody[5]. An indirect method is elucidated in **fig 4.**

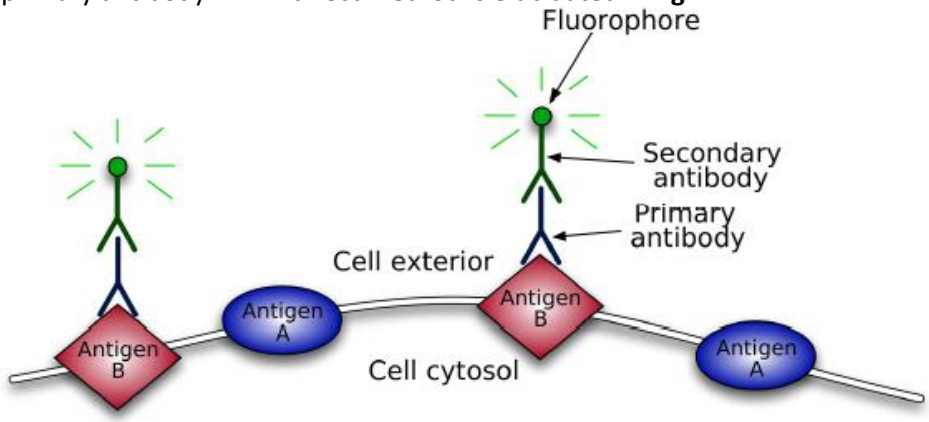


Fig 4: Indirect immunofluorescence staining. (Image: Munch 2007)

3.3.3. Flow Cytometry:

The basic principle of flow cytometry is the passage of cells in single file in front of a laser so they can be detected, counted and sorted. Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths Surface antigens as well as the internal proteins can be used as a reporter of gene action and analyzed such as to derive the characteristic of a particular type of cell type.

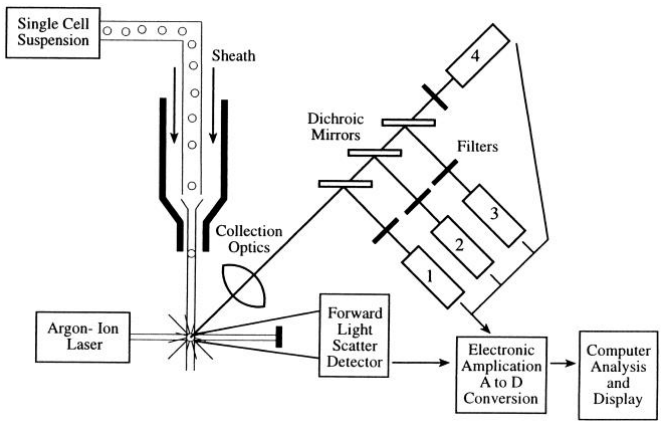


Fig 4: Outline of working principle of a flow cytometer

PSCs grow and are routinely passaged as clumps of cells, a characteristic that confers survival and maintenance of pluripotency[44]. Manipulation of PSCs via FACS, which requires cells to be completely dissociated, is therefore challenging. In this project we use the FACS machine to identify and prove naïve conversion of the NWR cells from the primed state and also the progenitor differentiation after their induction[45]. Clonal and sub-clonal selection at single cell per well into a 96-well plate directly from FACS would result in a panel of potential iPSC lines that can then be further characterized. Day 4 or day 5 embryoids are collected into 15 ml conical centrifuge tubes using wide-bore pipette tips. For dissociating the embryoids, Accutase is used. The dissociated cells are diluted in feeder cell medium and centrifuged at 400x g, 5–10 min. Then the dissociated cells are resuspended in 50 µl of FACS solution consisting of 1% (v/v) FBS in PBS. The cells are then fixed for 30mins using the Inside Perm solution. After the incubation, the stained cells are washed and centrifuged in FACS solution at 400x g for 8–12 min. They are then coated with antigens and analyzed in the FACS machine (FACS Aria III).

4. Materials and Method:

4.1. Cell Culture method:

4.1.1. Origin of the Cells:

Cells from the fibroblasts of Northern White Horn Rhino have been used for reprogramming and iPSC is generated using episomal transfection and for further downstream PGC differentiation in this project. Clones were picked after reprogramming and the ones used for this project are named as: 4(12) and 5(15). Another batch of fibroblast cells were also thawed later for reprogramming using mRNA transfection method (**fig. 6**).

4.1.2. Culture conditions:

The cell cultures were maintained in a cell culture laboratory under aseptic conditions. The culturing plates were kept in an incubator set to 37 °C temperature. The temperature was monitored on a daily basis. The carbon dioxide levels in the incubator were set to 5.0%[46]. The incubator was humidified by keeping sterile water on a tray inside the incubator.

All cells were cultured on regular plastic 6-well plates coated with matrigel and in fibroblast media before iPSC induction and mTeSR basal media after iPSC generation. The morphology of the cell colonies was assessed visually and colonies were photographed with an inverted phase contrast microscope system.

4.1.3. Passaging:

The cell lines were enzymatically passaged when confluent to prevent the colonies from overgrowing. Passage numbers were used to follow the approximate age of the cultures, one unit standing for approximately one week. First, the new 6 well plates were coated with matrigel. The coating was prepared by applying 1 ml of matrigel onto the wells and letting it bind for an hour in the incubator. The cell culture supernatant from the old wells were aspirated followed by a wash with 1 mL of DPBS without Ca2+ and Mg2+ per well. 1ml of Stem MACS enzymatic solution was then added onto each well and gently rocked to distribute the solution evenly. The plates are then incubated for 5mins in room temperature and the detachment process monitored under the microscope to keep in mind that the colonies do not detach completely but until the colony edges lift off. When lifting off starts Stem MACS solution is aspirated out and fresh media (1 ml) put onto the plate and the colonies were gently rinsed with a 1ml tip. The cell suspension was then transferred into a 15 mL conical tube and carefully pipetted 2–3 times to break up the colonies into smaller cell clusters. A small centrifugation is then performed at 200 rpm for 3 mins to help the cells settle down. The supernatant is removed and fresh 1ml media added for resuspension. Finally, in 1:10 dilution concentration the cells are plated onto the new 6 well plates having 2ml fresh media. The media is replaced each day for maintenance.

4.2. Workflow of cells:

4.2.1. Generation of iPSC from fibroblasts:

Fibroblast cells are thawed in normal fibroblast growth medium (FGM) which contains DMEM, 10% FBS, 1% L-glutamine and 1% 10mM non-essential amino acid. The cells are then allowed to grow in a 10 cm plate coated in matrigel and get confluent inside an incubator in optimal conditions with everyday media change. When the cells are 80-90% confluent they are detached from the plate using Trypsin-EDTA and counted in order to plate desired number of cells onto new 6-wellplates for reprogramming. Two different dilutions are plated in duplicates ($3x10^4$ and $4x10^4$ cells) in new plates and allowed to grow in the fibroblast media.

Fig 5: NM-RNA reprogramming cocktail setup

For reprogramming the cells, the Stemgent StemRNA-3rd gen reprogramming kit is used. A well of fibroblast the culture plate is selected based on the number of cells and the confluence. The media in the well is replaced to reprogramming media (Nutristem medium) from FGM and put into the incubator for 5mins. One NM-RNA Reprogramming cocktail single use aliquot is thawed at room temperature and immediately placed on ice and labelled as tube A. To tube A, 234.6 µl of Opti-MEM is added and gently mixed by tapping. In other tube labelled as tube B, 6 µl of RNAiMAX transfection

reagent is added to 244 µl of Opti-MEM (**fig. 5**). Finally, after mixing, the entire contents of tube B is transferred to tube A drop-wise at meniscus level. The mixing is now done by tapping the end and the mixture is incubated at room temperature for 15mins. 500 µl of the NM-RNA Reprogramming transfection complex is added to the well having reprogramming media by tilting the plate and pipetting drop-wise into the medium and mixed in the end by rocking in X-Y direction. The plate is then returned into the incubator overnight. In the next day the media is aspirated and fresh reprogramming media is transferred and the same steps of transfection are performed approximately in the same of the day as before. This is done for 4 days for reprogramming to complete. From day 5 and till day 10 - 14 only media change is performed daily using reprogramming media to allow complete and distinct colony formation. When distinct colonies appear, they are picked up and put into new wells for them to proliferate.

4.2.2. Reversion of primed iPSCs into Naïve state:

Naïve pluripotent stem cells (N-PSC) have improved functionality compared to the primed state as they are in the ground state of pluripotency without any bias towards differentiation towards a particular lineage. To induce naïve PSCs from primed PSCs, the medium needs to be replaced with the naïve medium, at approximately 24 h after passaging primed PSCs with basal PSC dissociation medium. The naïve media should be replaced every day. **NOTE:** After 24hrs of seeding onto the new plate a half medium change is necessary to allow the cells to get used to the changing of medium. When colonies become confluent, the cells are passaged onto new plates for continuing conversion and this time the passaging is done in the naïve medium itself. For visual effect the naïve induced cells can be seen to have a pinkish colour during pellet formation compared to the white pellet in primed state. Finally, these competent PSCs can be used thereafter for PGCLC induction. The figure shows a visual interpretation of changing into naïve states. For validating the naïve and primed state the cells are analyzed using qPCR and FACS. Two different naïve induction methods are used in this project to compare the efficiency of each of them. One is the RSeT media supplied by Stem Cell Technology company and the other is the mTeSR media with addition of supplements to make it +5i to support the naïve conversion. The figures in section describe how cells look in each of the media and why one is chosen over the other.

4.2.3. Differentiation of Naïve NWR iPSCs into PGCs:

For the differentiation of naïve iPSCs into PGCs the Irie *et al.* protocol has been adopted and used. [47]Confluent naïve cells are selected for PGC induction. The cells are first collected with Accutase and spinned down to form a pellet. The pellet is then resuspended in 3ml of medium and the cells are counted by manual cell counting. In a 96 well ultra-low attachment plate around 2000-4000 cells per well are then plated per well and centrifuged at 200g for 2mins to ensure that the cells are settled on the plate to promote the formation of embryoids. The embryoids can be allowed to develop without the necessity for changing the medium for up to 5 days.

4.3. Characterization of Cells:

4.3.1. RNA extraction & cDNA for qPCR analysis:

RNA extraction with spin column method

RNA extraction from the cells was carried out using RNeasy Mini Kit from Qiagen. The kit is a spin column system based on a silica matrix and extraction. Cell samples were obtained from a single 6-well plate culturing well. Prior to taking the sample, the cells were washed once with PBS. PBS was then aspirated and 350 µl of RLT buffer was added. Applying the buffer lysed the cells one volume of ethanol was then added to the lysate to adjust RNA binding conditions. The mix was then loaded to Nucleo-Spin column and centrifuged for 15s at >8000g.700 µl of RW1 buffer was then added onto the column and again spinned at >8000g for 15s. The column was then washed with 500 µl RPE buffer for 2mins and centrifuged at 8000g. After all the washes the flow-through was discarded before adding the later solution. After that the collection tube was replaced. Finally, RNA was eluted into a sterile 1.5 ml collection tube with 30-50 µl RNase-free water. The procedure was carried out for each sample. The protocol is summarized in table 2.

Phase	Reagent	Centrifugation at >8000g
Lyse cells	350 µl RLT buffer	
Adjust RNA binding conditions	1 vol. 70% ethanol	
Bind RNA	RNeasy mini spin column	15 s
Desalting and equilibration	700 µl RW1 buffer	15 s
Wash buffer	500 µl RPE buffer	2 mins
Dry the membrane	New collection tube	1min
Elution of RNA	RNA-free water	1 min

Table 2: Total RNA isolation protocol for RNeasy mini kit from Qiagen

Nano Drop spectrophotometer was used to measure the concentration and purity of the RNA extracts. The results of the measurements are listed in table 3. Prior to the usage, RNA samples were stored at -80 °C.

Cell Line	Passage	RNA concentration ng/µl	Volume used for transcription
4(12) primed	20	15.9	5 µl
4(12) Naïve (mTeSR)	22	93.7	1 µl
4(12) Naïve (RSeT)	19	18.8	5 µl

Table 3: RNA concentrations and volume for cDNA transcription

c-DNA transcription:

cDNA transcription was carried out using Thermo Scientific Verso cDNA Synthesis Kit. The master mix was prepared for the reverse transcription reactions according to table 4. 15 µl of the master mix was added to PCR tubes for each reaction. The amount of RNA for each reaction was determined to be the maximum from each cell line ≤ 1 µg, since all samples did not yield to 100 ng/µl concentration required for 1 µg amount. So, the volume of each extract was calculated accordingly (table 3). The total reaction volume was 20 µl. The temperature program for the thermocycler is in table 5.

Reagent	Volume
5x cDNA synthesis buffer	4 µl
dNTP Mix	2 µl
RNA primer	0.5 µl
RT enhancer	0.5 µl
Verso Enzyme mix	0.5 µl
RNAse free water (more water for Naïve in mTeSR)	7.5 µl
Total volume of master mix	15 µl

Table 4: Master Mix of components for reverse transcription in one reaction

Step	Temperature	Time
1	42 °C	30 min
2	95 °C	2 min
3	4 °C	Hold

TABLE 5: Temperature program for cDNA transcription

Analysis of Gene Expression

To study the expression status of genes, previously transcribed cDNA was used or the PCR protocol. Master mixes were aseptically prepared for the reactions into eppendorf tubes according to table 6. The DNA polymerase was from Thermo Scientific. Primers used were designed to attach to the transgene OCT4, DNMT1, NaNOg, STELLA, TET1, TET2 and exon spanning primers for OCT4, NaNOg and STELLA.

Reagent	Volume
Syber dye	5 µl
Water	3 µl
Forward primer	1 µl
Reverse primer	1 µl

Table 6: Master mix for qPCR analysis

A housekeeping gene, GAPDH, was used as a control. GAPDH, producing glyceraldehyde-3-phosphate dehydrogenase, is a maintenance gene and known to be perpetually expressed in cells to a degree (Warrington et al. 2000; Barber et al. 2005)[48, 49].

A total volume of 10 µl of the master mix was used for each reaction. The master mixes were pipetted into 384 well plate for qPCR and 1 µl of the sample cDNA solutions were added. Reaction tubes were mixed by tapping and spun in a micro-centrifuge. The PCR program is described in table 7.

Steps	Temperature	Duration	
1	50 °C	2 min	
2	95 °C	10 min	
3	95 °C	15 s	40 Cycles
4	60 °C	1 min	
5	95 °C	15 s	
6	60 °C	1 min	
7	4 °C	Hold	

Table 7: qPCR program for data analysis

The generated results were analyzed using Microsoft Excel and are described in section 5.2.

4.3.2. FACS Analysis of Antibodies:

Fixation of cells

For the FACS analysis we had the rhino primed cells, rhino primed +activin induced cells and naïve rhino cells. There were 4 antibodies that were used for the characterization of the respective states; OCT4, TFAP2C, SOX 17 and BLIMP1.

The chosen wells for FACS analysis were first washed with PBS and then acutased to generate single cells, necessary for analysing. The disrupted cells were collected in a 15 ml falcon tube and centrifuged at 200g for 2mins. The supernatant was aspirated and the cells were resuspended in 0.5 ml FACS buffer (PBS+ 1%FBS) and 500 µl of inside fix solution to fix the cells. The cells were incubated for 20mins for the fixation and after that they were centrifuged again and washed with the FACS buffer. The cells were then centrifuged to form a pellet and now resuspended using 800 µl of Perm solution. 100 µl of each sample was pipetted onto a well in a 96 well plate for the antibody binding.

Primary Antibody Induction

Primary antibodies were first prepared according to table 8 and then distributed equally onto each well for binding. Antibody was not added in two wells from each sample to have an unstained population for gating purpose in the FACS analysis and to later add only secondary antibody for isotype gating. The plate was then incubated for 1hr to allow the primary antibody to bind to the cells. Based on the host of the primary antibody, the secondary antibody was chosen accordingly for the later steps.

Primary Antibody/ host	Volume prepared
OCT4/Rabbit	300 μl Perm solution + 6 μl OCT4 antibody
SOX17/ Mouse	300 μl Perm solution + 6 μl SOX17 antibody
BLIMP1/ Rat	240 μl Perm solution + 6 μl BLIMP1 antibody
TFAP2C/ Mouse	300 μl Perm solution + 6 μl TFAP2C antibody

Table 8: Primary antibody mix for cells in total distributed in ll the samples

Secondary Antibody Induction

After an hour of incubation with the primary the plate was centrifuged at 200g for 3 mins and the excess solution was removed. They were again washed in 200 μl of FACS buffer by resuspending and again centrifuged to remove the supernatant. Secondary antibodies were then prepared according to table 9 and distributed onto the wells along with the isotype well. It was then incubated for 30mins and then centrifuged and washed with FACS buffer for the final analysis in FACS AriaIII. The results of the FACS analysis is described in section 5.3.

Secondary Antibody	Volume Prepared
Secondary anti-Rat	2x 600 μl Perm + 0.6 μl Secondary
Secondary anti-Rabbit	2x 600 μl Perm + 0.6 μl Secondary
Secondary anti-Mouse	3x 600 μl Perm + 0.6 μl Secondary

Table 9: Secondary antibody mix for cells in total distributed in all the samples

5. RESULTS:

5.1. Morphology:

5.1.1. iPSC colony formation using mRNA transfection:

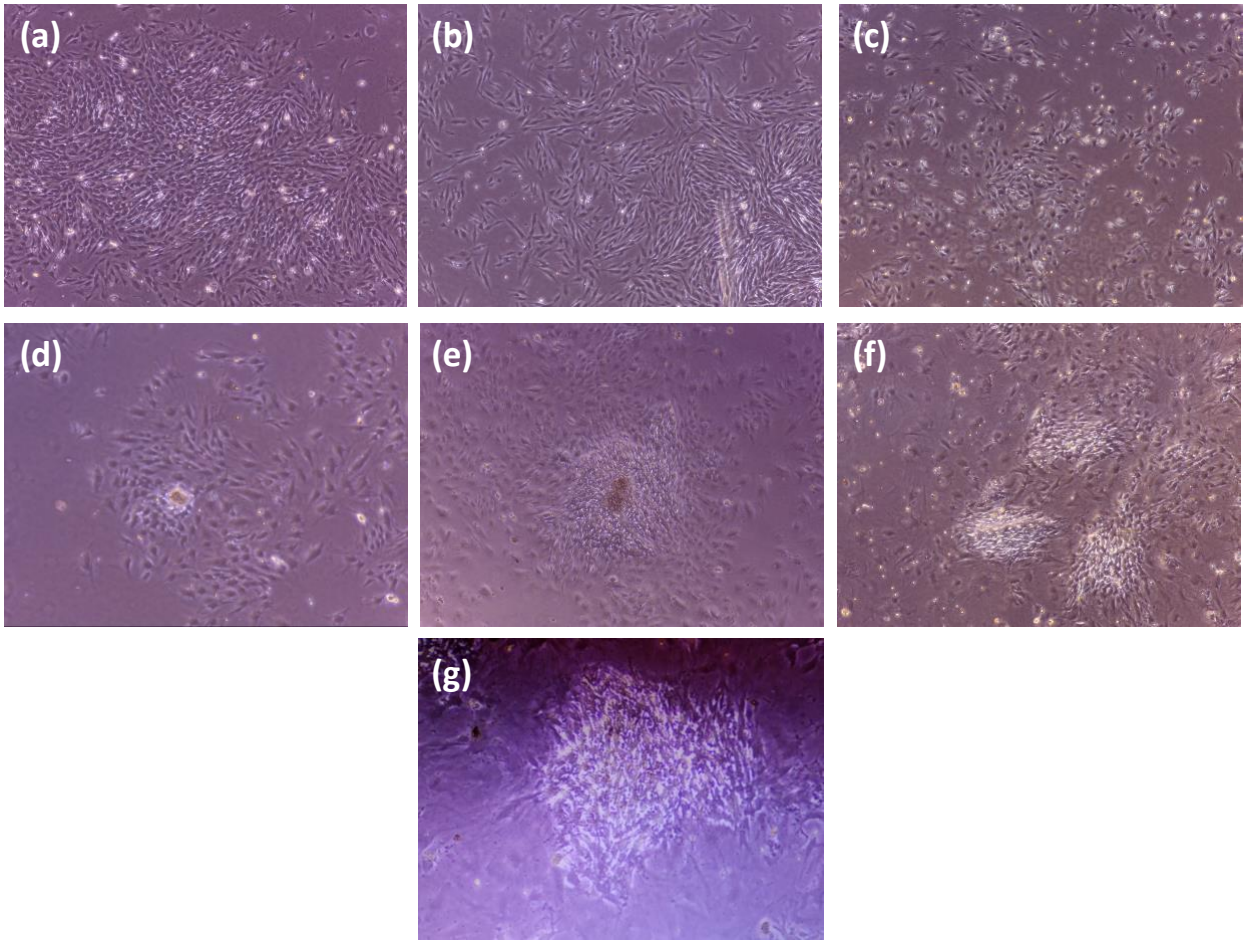


Fig 6: Pictures showing the development of iPSC colonies using mRNA transfection method. Figures (a) to (f) depict cells reprogram stage from day 1 to day 4 transfection while (e) and (f) show beginning of colony formation in day 6 and 8 respectively. Figure (g) shows the state of cells in day 11.

5.1.2. Naïve Reversion:

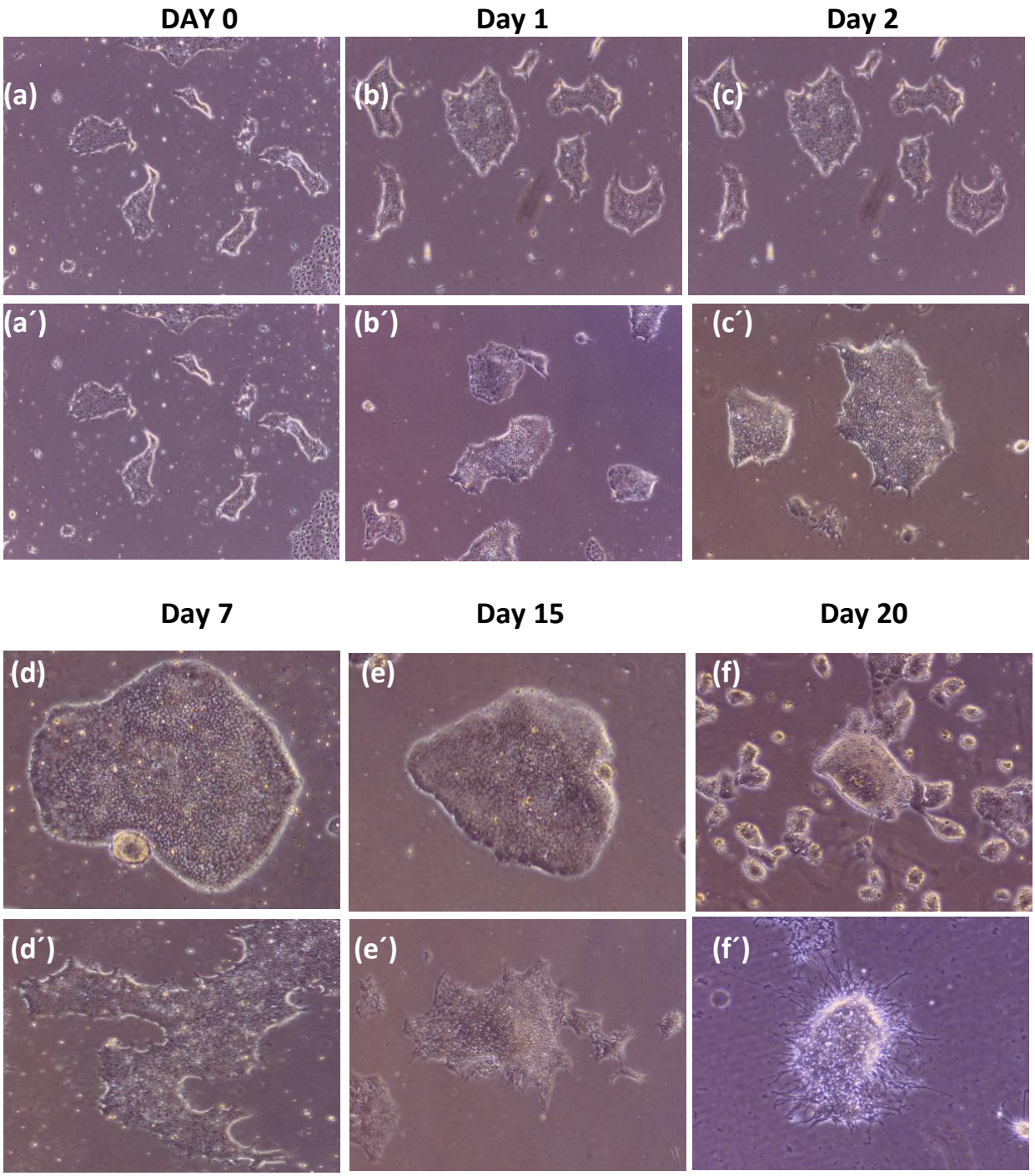


Fig 7: Pictures (a) to (f) show Rhino iPS cells growing in mTeSR naïve medium whereas (a´) to (f´) shows another set of rhino iPS cells split under similar conditions but growing in RSeT medium. All pictures have been captured in the specific day stated above with splitting done when wells are confluent.

5.1.3. PGC differentiation:

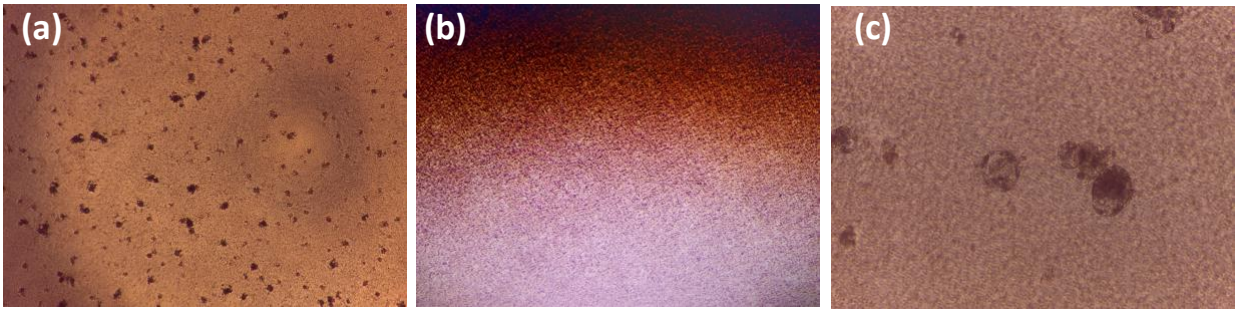


Fig 8: Pictures (a) to (c) shows PGC induction under different concentration of BMP4 and with different harvesting enzymes. (a) PGC induction with 500ng/ml BMP4 harvested with Acutase, (b) PGC induction with 100ng/ml BMP4 harvested with Acutase and (c) PGC induction with 50ng/ml BMP4 harvested with StemMACS.

5.2. qPCR Analysis:

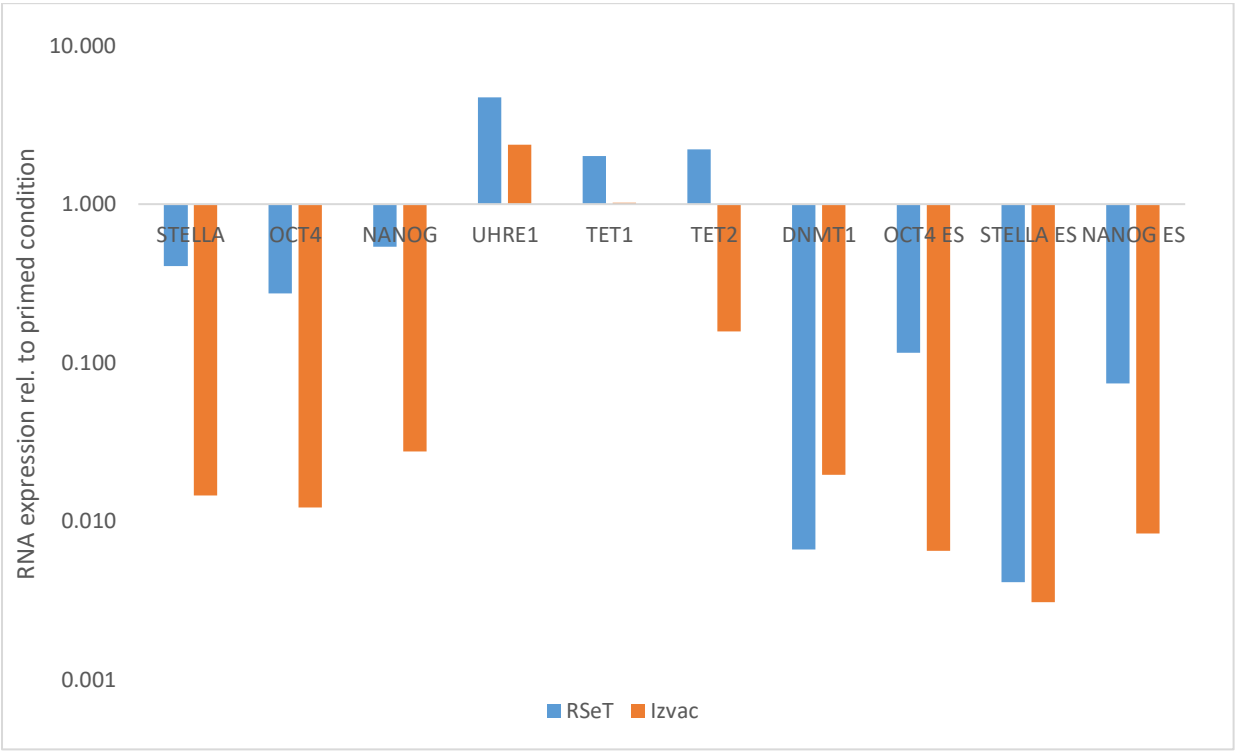
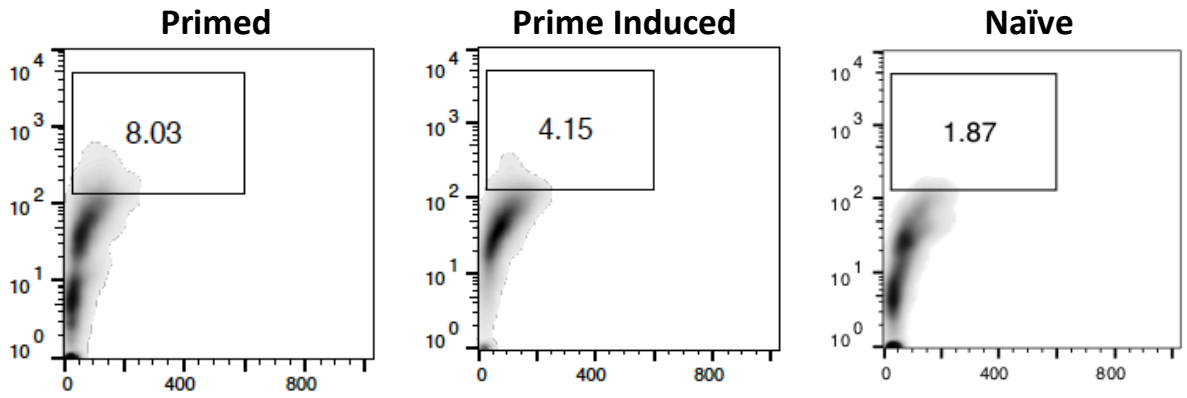


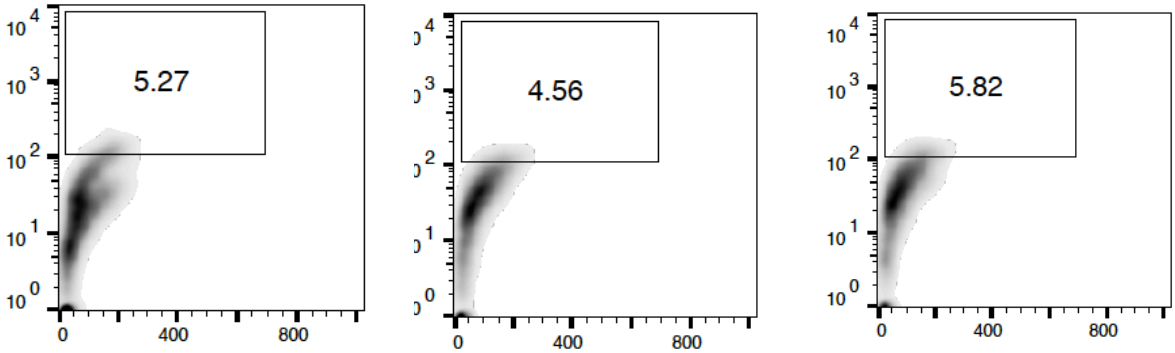
Fig 9: qPCR data showing RNA expression of different naïve cells relative to the primed state.

5.3. FACS Analysis:

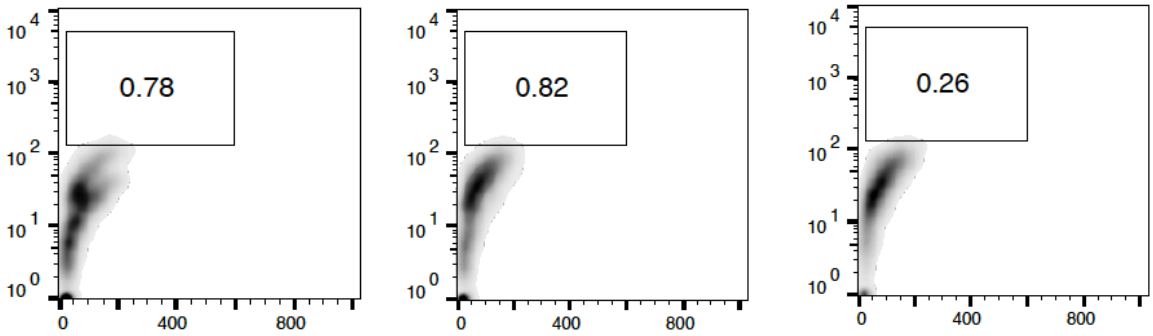
OCT4:



BLIMP1:



SOX17:



TFAP2C:

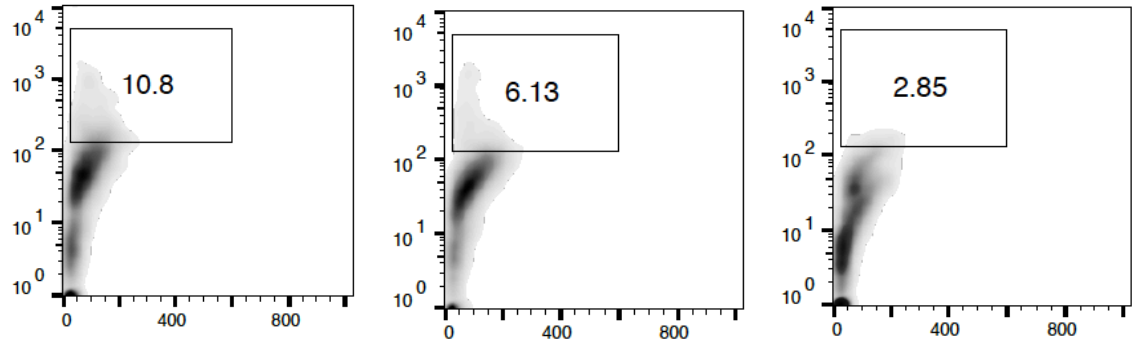


Fig 10: FACS analysis data for antibodies OCT4, BLIMP1, SOX17 and TFAP2C respectively measured for three different cell states; Prime, Prime + activin and Naïve.

6. Discussion:

The morphology of iPS cell colonies was as expected, exhibiting characteristics of a pluripotent stem cell, such as high nucleus-to-cytoplasm ratio, dense, flat colonies and smooth colonies and smooth colony borders. The characteristics differ notably from other cells, such as parental human dermal and mouse embryonic fibroblasts. Similar morphology has been described with other iPS cells in several studies[9, 22, 25].

RNA purity ratios ranged from 1.91 to 2.26 in iPS cell samples. RNA is considered to be pure around ratio 2,0 and contaminated with protein or DNA below 1,8 (Vomelová et al. 2009; Wilson & Walker 2010, 167). The measured purities can be considered satisfactory. It was advised from to include pre-heating the elution water to 37-50 °C and incubating the elution water 5 minutes prior to the centrifugation, for better yields. These notes should be taken into practice in the future while also pursuing to use samples with high cell count.

As the concentration of RNA isolated from the cells were not high it was imperative to use the maximum possible amount of RNA that can be added using the Verso kit. And for that reason, the samples were the concentration was below 20 ng/μl, a maximum of 5 μl of the RNA was utilized. To normalize the RNA amount with the other sample which had a concentration over 90ng/μl only 1 μl of RNA was used for cDNA generation and the volume was adjusted using RNA-free water.

The cDNA generation mix had a final volume of 20 μl, but the qPCR reaction required 22 μl of cDNA for each sample. For that reason, after before pipetting the cDNA onto the plate for qPCR analysis, 2 μl of RNA-free water was added in the cDNA tube, vortexed well and then spinned down. The qPCR was performed for all the primers in technical duplicates in order to avoid mistakes concerning pipetting error and to find mean Ct values for each reaction. In the results obtained, some Ct mean values were unusual compared to most of the other and in those cases the values that were comparable were taken for further analysis.

FACS data generated using the Flow-Jo software had some initial problems in determining. For example, the gating for each of the sample took a lot of trial and error to ascertain as the correct voltage values were not set in the very beginning. But on applying the correct gating strategies the results were obtained. Data suggests that firstly SOX17 cannot be used as a marker in Rhino cells. Possible reasons are that rhio genome has not been fully discovered as yet and even if the genome of a close relative of the NWR is recently published very little is known about the actual marker pattern. So which marker is the best fit to identify a particular state is not known. The OCT4 data, although is low in percentage but still shows a result that matches the hypothesis as in naïve state it is believed that OCT4 signal goes down at least in mouse iPS cells. The TFAP2C is believed to be essential in the naïve state as it is responsible in modulation of methylation in the DNA in the naïve cells to maintain pluripotency. BLIMP1 marker didn't work very well for this experiment and possibly requires another round of experimentation for further depth in understanding. But it does give a positive stain although in very low count.

PGC differentiation was not as expected, in the first two attempts because there was a possible contamination in the plate which led to the death of most of the cells, in the first attempt. A possible reason for contamination was thought to be that the cytokine aliquots used had been previously contaminated. So, all the cytokines were later checked for potential contamination in the GMEM medium but none had positive contamination result. It was also hypothesized that 500ng/ml of BMP4 was lethal towards the survival of the cells and their potential in making embroid bodies (EB). For that

reason, In the second attempt the BMP4 concentration was lowered to 100ng/ml but this time also there was no EB formation. It was then thought that as the cells were harvested using acutase, an enzyme believed to be naturally harsh to the cells, it might be better to use a less harsh enzyme and even lower concentration of BMP4. So in the third attempt, 50ng/ml BMP4 concentration was used to make the PGCLC medium and Stem-MACS was used to harvest the cells. Fig 9(c) suggest that this attempt had better standout morphological reference compared to the previous ones and so it was sent for analysis using FACS.

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