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Cellular Reprogramming as a Tool for Harvesting Patient Specific Stem Cells

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Author's declaration

I hereby declare that this dissertation thesis is my own original work and that I indicated by references of all used information sources. I also agree with depositing my dissertation in the Medical Library of the Charles University, Faculty of Medicine in Hradec Kralove and with making use of it for study and educational purpose provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

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Abbreviation

hiPSC	human induced pluripotent stem cells
OCT4	octamer-binding transcription factor 4
SOX2	sex determining region Y box 2
KLF4	kruppel-like factor 4
SSEA-4	stage-specific embryonic antigen-4
TRA-1-60	podocalyxin
hESC	human embryonic stem cells
DPSC	dental pulp stem cells
nDPSC	natal dental pulp stem cells
miRNA	micro RNA
SOC	scaffold oligonucleotide connector
HF	human fibroblasts
WI38	human embryonic lung fibroblasts
DsRed2	*Discosoma sp* red fluorescent protein
CLA	cyclic ligation assembly

1. Introduction

1.1. Classification of different types of stem cells

Stem cells are undifferentiated group of cells that possess the capacity to self-renew and differentiate into various types of cells. Stem cells hierarchy is determined based on their differentiation potential. Embryonic stem cells capture the peak i.e. they have the maximum differentiation potential, further downhill are tissue specific stem cells with partial differentiation potential and, at the base are terminally differentiated cells with very restricted or no differentiation capacity. Apart from these two types, a special type of stem cells called as cancer stem cells exists which possess characteristics of normal tissue specific stem cells and which predominantly causes relapse and metastasis of tumours. Stem cells are classified as either totipotent, pluripotent, multipotent, oligopotent or unipotent depending on its potency.

Totipotent stem cells are the least differentiated cells and are only present until morula stage of development. They can give rise to an entire functional organism by differentiating into both embryonic and extraembryonic tissues (Rossant 2001).

Pluripotent stem cells are one step downstream of totipotent stem cells, having been lost the potential to differentiate into extraembryonic tissues. Hence, they are hierarchy placed below totipotent stem cells. Even after losing totipotency, these cells retain the capacity to differentiate into three germ layers i.e. ectoderm, endoderm and mesoderm, that form all the tissues and organs of the body (De Miguel et al. 2010). Pluripotent stem cells were first isolated from inner cell mass of the blastocyst (Evans et al. 1981). These cells are referred as embryonic stem cells (ESC). In the year 2006, Prof Yamanaka with his path breaking discovery, introduced a new method to obtain pluripotent stem cells (Takahashi et al. 2006). He coined the term “induced pluripotent stem cells (iPSC)” to these pluripotent stem cells. iPSC closely resemble their pluripotent counterparts i.e. ESC.

Multipotent stem cells reside in each tissue and are derivative of either of the three germ layers. Development, tissue regeneration, tissue homeostasis and defence are the key roles accomplished by multipotent stem cells. Dermis, synovial fluid, periosteum, bone marrow, adipose tissue, Wharton’s jelly, umbilical cord blood, and peripheral blood (Augello et al. 2010) are few examples

of tissue types from which multipotent stem cells are commonly isolated. Common examples of multipotent stem cells are as follow:

Germ layer of origin	Multipotent stem cells
Endoderm	Pulmonary epithelial stem cells, gastrointestinal tract stem cells, pancreatic stem cells, hepatic oval cells and prostatic gland stem cells
Mesoderm	Hematopoietic stem cells, mesenchymal stem cells, bone marrow stem cells, cardiac stem cells, satellite cells of muscle
Ectoderm	Neural stem cells, dental pulp stem cells, neural crest stem cells, hair follicle stem cells

Table 1. Examples of multipotent stem cells and the germ layer from which they arise.

Oligopotent stem cells are derivative of multipotent stem cells and can differentiate and form two to three lineages within a specific tissue. They inherit self-renewal property from multipotent stem cells. Oligopotent stem cells have been reported in ocular surface of pigs and are able to differentiate into corneal and conjunctival cells (Majo et al. 2008). Bronchoalveolar duct junction cells are termed oligopotent as they give rise to bronchiolar epithelium and alveolar epithelium (Kim et al. 2005).

1.1.1. Pluripotent stem cells

Human embryonic stem cells (ESC) isolation and expansion was first reported in the year 1998 by Dr. James Thomson (Thomson et al. 1998). They are derived from inner cell mass of the blastocyst (Evans et al. 1981) and can differentiate into tissues of the all three germ layers. They can be propagated in undifferentiated state for a prolonged period in culture (Yao et al. 2006). Oct4, Sox2 and Nanog transcription factors are indispensable for maintaining pluripotent state (Wang et al. 2012, Liang et al. 2008). Human embryonic stem cells can be maintained in feeder free and serum free defined culture medium for prolonged period without induction of differentiation (Ludwig et al. 2006).

1.1.2. Identification of minimal set of transcription factors essential for reprogramming

Induced pluripotent stem cells are class of pluripotent stem cells obtained by transcription factor mediated reprogramming of somatic cells. Prof Shinya Yamanaka had reported that ectopic

expression of octamer-binding transcription factor 3/4 (*OCT4),* SRY-related high-mobility group box protein- 2 (*SOX2),* Kruppel-like factor 4 (*KLF4)* and oncoprotein (*c-MYC)* transcription factors was able to dedifferentiate murine fibroblasts to pluripotent stem cells state (Takahashi et al. 2006).

24 potential reprogramming factors			
Ecat	**Gdf3**	**Sox2**	**Ecat8**
Dppa5	**Sox15**	**Rex1**	**cMyc**
Fbx15	**Dppa4**	**Utf1**	**Oct4**
Nanog	**Dppa2**	**Tcl1**	**Stat3**
Eras	**Fthl17**	**Dppa3**	**B-catenin**
Dnmt31	**Sall4**	**Klf4**	**Grb2**

Table 2. List of 24 set of genes that were considered as potential reprogramming factors.

Journey of identifying minimal set of factors capable of inducing pluripotency was daunting. Out of the large pool of transcription factors upregulated in ES cells, they narrowed down to 24 factors (Takahashi et al. 2016). To further streamLine the number of factors, they introduced individual gene as well as mixture of all 24 genes. It was found that none of the factors alone could reprogramme the murine embryonic fibroblasts (MEF) but combination of all 24 factors was able to form colonies resembling ES cells. In the following experiments, a single factor was removed from the set of 24. In total, they tested 24 combinations of 23 factors/genes each. This strategy narrowed down the factor list from 24 to 4 genes. It was found that, elimination of *OCT4*, *SOX2*, *KLF4* and *c-MYC* genes completely inhibited formation of ES resembling pluripotent colonies. It was finally revealed that the cocktail of four transcription factors *OCT4*, *SOX2*, *KLF4* and *c-MYC* were able to induce pluripotency upon ectopic expression in somatic cells. Prof. Shinya Yamanaka reported, reprogramming of murine embryonic fibroblasts in the year 2006 followed by human fibroblasts in the adjoining year. The list of 24 set of genes that were considered as potential reprogramming factors are mentioned in Table 2.

1.1.3. Induced pluripotent stem cells

Pluripotent stem cells can be obtained from embryos or by epigenetic reprogramming. Epigenetic reprogramming can be performed in three ways; somatic cell nuclear transfer, cell fusion and by

ectopic expression of defined set of transcription factors. Prof. Shinya Yamanaka introduced induced pluripotent stem cell (iPSC) technology, in which he showed that ectopic expression of four transcription factors could dedifferentiate tissue specific somatic cells to pluripotent stem cells (Takahashi et al. 2006). For his path-breaking discovery, he received Noble prize for physiology or medicine in the year 2012. iPSC technology bypasses all the ethical concerns raised by use of human embryonic stem cells. In addition, it makes it easy to derive patient-specific pluripotent stem cells, and transplantation of differentiated iPSC can be achieved without immune rejection. Personalized medicine is another avenue were iPSC technology finds its use.

iPSC are functionally identical to embryonic stem cells with unlimited self-renewal capacity and can contribute to lineages from all three germ layers. In 2007, Yamanaka reported that human fibroblasts could be reprogrammed to pluripotent stem cells by introduction of Oct4, Sox2, c-Myc and Klf4 transcription factors using retroviral vector. Combination and number of factors required for reprogramming varies with the type of starting cell line. Oct4, Sox2, Nanog and Lin28 is another combination of transcription factors used for reprogramming somatic cells (Yu et al. 2007). Murine fibroblast can be reprogrammed with Oct4, Sox2, Klf4 i.e. without the use of c-Myc (Wernig et al. 2008). Just two factors i.e. Oct4 and Klf4 can reprogram murine neural stem cells because they express higher endogenous levels of Sox2 and c-Myc (Kim et al. 2008). In the following year, Kim et al. reported that Oct4 alone could reprogramme adult neural stem cells (Kim et al. 2009b, Kim et al. 2009c). Kim et al. reported that direct delivery of reprogramming protein could reprogramming human fibroblasts, which was against the common notion of delivering the factors by viral vector (Kim et al. 2009a).

Cellular reprogramming is a complex process and the cell undergoing it must pass through necessary functional events that are as follows: (i) active proliferation without apoptosis and cell senescence (Banito et al. 2010), (ii) mesenchymal-to-epithelial transition (MET) (Li et al. 2010, Samavarchi-Tehrani et al. 2010), and (iii) a metabolic shift from oxidative phosphorylation to glycolysis to facilitate quicker ATP production (Pfeiffer et al. 2001, Zhang et al. 2012). Completion of these events finally leads to progressive appearance of stem cell markers [e.g., alkaline phosphatase and surface antigens SSEA-3 and TRA-1-60], and finally the complete activation of the core pluripotency network (Brambrink et al. 2008, Cacchiarelli et al. 2015, Stadtfeld et al. 2008b).

1.2. iPSC are unique subtype of pluripotent stem cells

Superficially, iPSC appear to be indistinguishable from their pluripotent counterpart, embryonic stem cells. Irrespective of their origin or method of generation, all iPSC express unique repeated gene expression profile (Chin et al. 2009). Gene expression similarity between hESC and hiPSC varies according to different passages of hiPSC. Gene expression profile of late passage hips is more closely related to hESC than early passage hiPSC (Chin et al. 2009). Extended culturing of hiPSC is essential to attain gene expression profile similar to hESC. Even then, they retain a gene expression signature unique from hESC. Difference in expression profile for specific set of genes observed between iPSC and hESC is due to differential promoter binding by the reprogramming factors (Chin et al. 2009). Together, this discussion signifies that iPSC have a slightly unique gene expression profile and that is why it should be considered a unique subtype of pluripotent cell.

Chin et al. had performed gene expression and methylation analysis on H1 hESC cell line and its isogenic iPSC lines. The results revealed no significant difference between hESC and hiPSC at the gene expression and methylation level (Chin et al. 2009).

1.3. Different somatic cell types that can be used for reprogramming

To produce human iPSC from somatic tissue, different starting cell types are available. For example, fibroblasts, white blood cells, renal epithelial cells, keratinocytes etc. The above-mentioned cell types can be easily isolated without major invasive procedures.

1.3.1. Fibroblasts

In general, any actively dividing somatic cell type can be used for reprogramming (Haase et al. 2009). Fibroblasts is widely chosen cell line for reprogramming because they are inexpensive to procure and easy to handle. Moreover, nature and characteristics of fibroblasts are deeply studied, and they are well established in several fields of research.

Fibroblasts are connective tissue cells of mesenchymal origin, which synthesize extracellular matrix and precursor molecules. They provide structural framework and play an important role in wound healing. Human fibroblasts can be isolated from connective tissues of various organs and

tissues of the body, for example, dermal (Lowry et al. 2008), cardiac (ventricle or atrium) (Saxena et al. 2008), lung (Salazar et al. 2009), or periodontal ligament (Kumada et al. 2010).

Fibroblasts can be easily cultivated, and they have minimum requirement for serum and media. They outgrow from the tissue piece within one to two weeks (Streckfuss-Bomeke et al. 2013). The low methylation status of the promoter regions of OCT4 and NANOG in fibroblasts as well as reprogramming favourable transcriptional and epigenetic states might be involved in enhancing reprogramming ability (Streckfuss-Bomeke et al. 2013). Apart from these few advantages, fibroblasts suffer from major disadvantages as listed below.

Most commonly, normal human dermal fibroblasts are obtained from either adult skin biopsies or neonatal foreskin biopsies from circumcisions. Moreover, biopsies are painful and must be performed under supervision of medical professionals.

Reprogramming efficiency of fibroblasts is very low about 0.01–0.5% and complete reprogramming process needs approximately three to five weeks (Takahashi et al. 2007). In addition, with higher passages, the reprogramming efficiency is reduced, and the risk of accumulated genomic alterations is increased. Even though, acquisition of fibroblasts is invasive and has low reprogramming efficiency, still it is widely used for reprogramming because the biopsies from which fibroblasts are abundant and readily available.

1.3.2. White blood cells

White blood cells can also act as starting cell source for reprogramming. Two types of cell population can be harvested from peripheral blood for reprogramming; $CD34^+$ hematopoietic stem cells and peripheral blood mononuclear cells. Proportion of hematopoietic stem cells in peripheral blood is very low. Hence, to increase proportion of $CD34^+$ hematopoietic stem cells in peripheral blood, injection of cytokine like granulocyte colony-stimulating factor (G-CSF) is administered to the patient. This stimulate the production and mobilization of stem cells into peripheral blood. Reprogramming of $CD34^+$ hematopoietic stem cells to iPSC was reported by Loh et al (2009). Generation of patient specific iPSC is the main advantage of this procedure. Serious disadvantages limiting widespread use of $CD34^+$ hematopoietic stem cells are mainly because of the isolation procedure. This includes, adverse effects arising due to administration of G-CSF

(Cashen et al. 2007). Moreover, the immobilization and isolation procedure are painful, highly time consuming and expensive.

Lymphocytes and monocytes are the two main types of peripheral blood mononuclear cells; commonly referred as PBMC. These cells are separated from the whole blood by density gradient centrifugation. Mature lymphocytes undergo genomic rearrangement to produce infinite combination in their receptors which helps in identifying presence of foreign entities in our body. Tan et al showed that a single drop of peripheral blood contains sufficient number of cells for performing cellular reprogramming (Tan et al. 2014). This procedure was shown to have higher reprogramming efficiency amongst various other approaches of reprogramming from peripheral blood. Very low reprogramming efficiency and risk of T-cell lymphomas development makes it less likely choice for use in reprogramming (Serwold et al. 2007, Staerk et al. 2010).

Umbilical cord blood is another source of somatic cells; it contains very young population of cells with minimal nuclear and mitochondrial mutation (Haase et al. 2009). High cost of harvesting and conservation limits the use of umbilical cord blood for reprogramming.

1.3.3. Renal epithelial cells

Renal epithelial cells are exfoliated via normal process and are passed out of the body through urine. Non-invasive method of collection and high reprogramming efficiency are few of the advantages of this cell type. Number of renal epithelial cells found in the urine is very low and in vitro expansion of these cells is very expensive. In addition, there is high risk of contamination occurring during collection. As well as the reprogramming efficiency reduces after five passages; all the above facts limits the use of renal epithelial cells for reprogramming (Xue et al. 2013, Zhou et al. 2011).

1.3.4. Keratinocytes

Keratinocyte are the major cell population constituting the epidermis of the human skin. They mainly occur in epidermis and hair follicles. An intact outer root sheath contains enough cells to successfully expand them to a number sufficient for reprogramming. Special low calcium medium formulations are required for culturing keratinocytes to prevent them from undergoing senescence.

Keratinocytes proved to be a better cell source for reprogramming than fibroblasts because they reprogram faster and have higher reprogramming efficiency due to high basal expression of c-Myc and Klf4 genes (Gandarillas et al. 1997, Segre et al. 1999). Promising features like easy and non-invasive isolation and high reprogramming efficiency makes keratinocytes an interesting cell type for iPSC generation. A certain degree of expertise is required for culturing keratinocytes from plucked hair. The hair should be plucked with adequate root and an intact outer root sheath. Currently this is the only obstacle which is preventing wide spread use of keratinocytes as starting cell line for reprogramming.

1.3.5. Other cell types used for reprogramming

Few unique cells lines like stomach cells, pancreatic β cell and hepatocytes were also successfully reprogrammed into iPS cells, albeit at very low efficiencies (Aoi et al. 2008, Stadtfeld et al. 2008a). Moreover, these cell lines are not easily accessible and are not available in large quantities, which limits their use as starting cell source for reprogramming.

Various differentiated cell types have been reprogrammed to pluripotent stem cell like fibroblasts, pancreatic β-cells, B-lymphocytes, melanocytes, keratinocytes etc.; thus, demonstrating their ability to transform from differentiated state to induced pluripotent state. However, the efficiency of dedifferentiation to pluripotent state and number of iPSC factors required totally depends upon the cell type selected. Thus, clinical application of iPSC technology can be made realistic by combining right cell type and transgene-free methods.

Depending on type of study intended one must choose the appropriate cell line for reprogramming. For instance, one can study the effect of somatic cell characteristics on efficiency of reprogramming. A cell line having a genetic disease background can be used for reprogramming to get an insight of disease development and progression.

1.4. Use of stem cells as a cell source for reprogramming

Tissue specific stem cell have an edge over somatic cells in terms of being efficient starting cell source for reprogramming (Ebrahimi 2015b). This is because they have relatively fast cell cycle kinetics, plasticity and endogenously express few pluripotency factors, albeit at lower levels compared to embryonic stem cells. It has been revealed that tissue specific stem cells only need to

overcome few barriers in the path towards attaining pluripotency as they endogenously express few of the factors essential for reprogramming (Ebrahimi 2015b). Furthermore, only key reprogramming factors i.e. OCT4 and SOX2 are required to accomplish reprogramming as opposed to somatic cells, which require cocktail of all the four factors.

Moving upstream from differentiation to pluripotency possess lot of barriers and bottlenecks which makes it an inefficient process (Hochedlinger et al. 2009). Every differentiated cell has an inherent mechanism which protects it from aberrantly activating pluripotency network (Cahan et al. 2014, Morris et al. 2014, Tomaru et al. 2014). Speaking of barriers affecting reprogramming, differentiation state of starting cell source acts as an important barrier. Other barriers that directly affect efficiency of reprogramming are p53, p21, p57, $p16^{Ink4a}$/$p19^{Arf}$, Mbd3, etc. (Ebrahimi 2015a). Specialized cells acquire programs suitable to the function they perform. These programs in scientific terms referred to as somatic gene regulatory networks, and the epigenetic state severely curtails reprogramming (Eminli et al. 2009). As opposed to somatic cells, tissue specific stem cells are relatively undifferentiated with pronounced plasticity (Ebrahimi et al. 2011, Korbling et al. 2003). Various studies have pointed out that tissue specific stem cells are more amenable and efficient in reprogramming than somatic or mature cells (Eminli et al. 2009, Giorgetti et al. 2009, Kim et al. 2009c, Niibe et al. 2011, Wang et al. 2013, Yulin et al. 2012). Interesting fact to note about tissue specific stem cells is that they express pluripotency regulatory like embryonic stem cells. Although, these factors are not expressed to levels compatible to embryonic stem cells, still they render tissue specific stem cells more amenable to reprogramming over mature cells (Ellis et al. 2004, Galan-Caridad et al. 2007). Furthermore, they show high plasticity and do not express lineage specific genes which adds amenability to undergo reprogramming (Al-Habib et al. 2013, Ebrahimi et al. 2011, Greco et al. 2007, Riekstina et al. 2009). In sharp contrast to differentiated cells, tissue specific stem cells have more reprogramming enhancers than barriers. Expression of stemness related genes, permissible chromatin state, a decreased level of barriers (e.g. TGF-β and MAP kinase pathways) and increased levels of genetic and epigenetic facilitators (e.g. KDM2B) (Vidal et al. 2014) are some of the intrinsic features that act as reprogramming enhancer. In summary, expression of pluripotency factors, lack of lineage specific gene expression and permissible chromatin state makes tissue specific stem cells more efficient to reprogramming over differentiated cells.

Fewer reprogramming factors can be used to reprogramme tissue specific stem cells after assessing the expression level of endogenous reprogramming factor (Kim et al. 2009b, Kim et al. 2009c). Mesenchymal stem cells isolated from human bone marrow (Greco et al. 2007, Riekstina et al. 2009), adipose tissue, heart, dermis (Riekstina et al. 2009) and dental pulp (Al-Habib et al. 2013, Ebrahimi et al. 2011) have been confirmed to express key pluripotency genes such as Oct4, Sox2 and Nanog. Examples of stem cells that have been reprogrammed are as follows. $CD133^{+}$ stem cells can be efficiently reprogrammed to pluripotent stem cells state using only two factors i.e. Oct4 and Sox2 (Giorgetti et al. 2009). While, neural stem cells can be reprogrammed using Oct4 alone as they endogenously express high levels of Sox2, c-Myc, Klf4 and SSEA1 (Kim et al. 2009b, Kim et al. 2009c). Hematopoietic stem cells and endometrial stem cells were reprogrammed to iPSC and were shown to be more amenable to reprogramming as compared to fibroblasts (Eminli et al. 2009, Park et al. 2011).

1.4.1. Mesenchymal stem cells

From the above introductory para, we can clearly see that mesenchymal stem cells are prominent stem cells choice probably because of their easy availability and relatively inexpensive culturing technique. Bone marrow, umbilical cord, adipose tissue and teeth are few sources from which mesenchymal stem cells can be isolated (Prockop 1997, Rodriguez et al. 2005).

1.4.2. Hematopoietic stem cells

Reprogramming efficiency of various hematopoietic cell populations (derived from bone marrow) such as long-term hematopoietic stem cells, HSC and progenitor cells, myeloid progenitor cells, granulocyte/macrophage progenitors, megakaryocyte/erythrocyte progenitors, common myeloid progenitors , common lymphoid progenitors, pro-B lymphocytes, pro-T lymphocytes and differentiated B lymphocytes, T lymphocytes, macrophages, granulocytes was studied by Eminli et al. (Eminli et al. 2009). In this comprehensive study, it was found that hematopoietic stem and progenitor cells are 300 times more efficient in forming iPSC colonies than B and T lymphocytes (Eminli et al. 2009). Moreover, the efficiency reported was towering 28 percent (Eminli et al. 2009). The study also provided a direct correlation between differentiation state and reprogramming efficiency. Previous notion relied on proliferation rate, as a key factor affecting reprogramming efficiency.

Cord blood is another rich and readily accessible source of hematopoietic stem cells. It has been reported that CD133+ cord blood stem cell can be reprogrammed to iPSC using only two factors i.e. Oct4 and Sox2 (Giorgetti et al. 2009). $CD133^{+}$ cord blood stem cell has been reported to express pluripotency associated genes such as *OCT4*, *NANOG*, *SOX2*, *REX1*, *CRIPTO*, *SALL2*, *DPPA4*, *ZNF589* and *DNMT3A/B* (Kucia et al. 2007, Nikolova et al. 2007, Zhao et al. 2006), however the expression levels are much lower as compared to embryonic stem cells. Furthermore, the promoter regions of *OCT4* and *NANOG* are more accessible to ectopic transcription factors as they contain lower repressive marks compared to fibroblasts. These results indicate that cord blood derived stem cells are efficient in undergoing reprogramming owing to their endogenous expression of pluripotency factors and permissive chromatin organization.

Collectively, HSC expresses characteristics which make them more amenable to reprogramming as compared to somatic or terminally differentiated cells. However, it is also important to note that derivation of HSC from bone marrow is an invasive process and is not routinely performed. While, cord blood can act as an alternative source, but the process of isolation and cryopreserving cord blood cells is expensive.

1.4.3. Adipose stem cells

Adipose tissues are abundant in human body and these tissues are very rich source of multipotent stem cells, which are referred as adipose stem cells (Guilak et al. 2006, Zuk et al. 2002). Moreover, these stem cells are able to differentiate into adipogenic, osteogenic, chondrogenic, and myogenic cell lineages (Bunnell et al. 2008, Guilak et al. 2006). Large number of adipose stem cells can be derived from single lipoaspiration. This reduces the time required for obtaining sufficient cell number essential for reprogramming. Adipose stem cells are quicker and efficient at reprogramming, as compared to fibroblasts (Sun et al. 2009).

Source of adipose stem cells is limited because the lipoaspiration procedure is considered as elective surgery and is usually not covered by insurance. In addition, the cells that are extracted are highly heterogeneous which require sorting to enrich the stem cells population thereby making it an expensive cell source.

1.4.4. Dental pulp stem cells

Dental pulp stem cells are ecto-mesenchymal in origin. They are commonly derived from exfoliated primary teeth and extracted third molars. Like hematopoietic stem cells, DPSC also express some pluripotent stem cells markers like Oct4, Sox2 and Nanog (Kerkis et al. 2006b). DPSC are more closely related to embryonic stem cells than to MSC and terminally differentiated fibroblasts (Beltrao-Braga et al. 2011). Surprisingly, human DPSC were able to functionally contribute to several tissues in human/mouse chimeras when transplanted into mouse blastocysts (Siqueira da Fonseca et al. 2009). Furthermore, DPSC are more efficient and rapidly undergo reprogramming into pluripotent stem cells as compared to other fibroblasts (Yan et al. 2010). These characteristics make DPSC more amenable to reprogramming into iPSC. DPSC will be explained in more detail in the following section (See section 1.5).

1.4.5. Neural stem cells

Neural stem cells are distinct cell types that endogenously express SOX2, c-MYC, and KLF4 as well as AP and SSEA-1 (Kim et al. 2009c). Moreover, the expression levels of SOX2 and c-MYC are more as compared to murine embryonic stem cells (Kim et al. 2009c). Interesting, Neural stem cells can be reprogrammed to iPSC using only *OCT4* transcription factor (Kim et al. 2009c). All the above characteristics makes neural stem cells an ideal cell source for reprogramming. Unfortunately, NSC isolation procedure is extremely invasive and such surgeries are very rarely performed.

Our place of work was the first in the Central Europe, which started to culture and characterise NSC in vitro since 1995 (Mokry et al. 1995; 1996; 1999; 2008), transplant them into murine brain (Mokry et al. 2005) and study behaviour of endogenous NSC in situ (Mokry et al. 2003). For propagation and ex-vivo expansion of NSC we adapted the neurosphere assay from Reynolds and Weiss (1992a; 1992b).

1.5. Dental pulp stem cells

1.5.1. General characteristics of DPSC

Dental pulp stem cells (DPSC) are multipotent stem cells that can differentiate into chondrocytes osteoblasts and adipocytes.

Isolation of dental pulp stem cells was first reported in 2000 by Gronthos et al. and were shown to be highly proliferative and displayed a hierarchy for cellular differentiation and multipotentiality (Gronthos et al. 2000). Dental pulp is derived from mesectoderm or ectomesenchyme, hence characteristics possessed by DPSC bears close resemblance to neural crest (Kerkis et al. 2012, Sasaki et al. 2008).

Dental pulp stem cells can be isolated from deciduous teeth and teeth extracted during routine dental procedure. Method of isolation is easy, non-invasive and free of any ethical concerns. These isolation characteristics gives an advantage to DPSC over other mesenchymal stem cells and other cell types.

Our group has been very active in dental pulp stem cell research from 2003. Significant part of this research was done by Dr. Tomas Soukup. He established and standardised the protocol for DPSC isolation. In addition, he also devised platform for characterising DPSC using flow cytometry (Karbanova et al. 2011, Suchanek et al. 2009).

1.5.2. Phenotypic characteristics

DPSC display fibroblastic characteristics. They maintain stem cell characteristics such as plasticity, normal karyotype and rate of expansion for at least 25 passages (Kerkis et al. 2006a). Using flow cytometry, cell surface markers analysis of DPSC shows that they are positive for CD73, CD90, CD105 (endoglin), CD13, CD29 (integrin b1), CD44, HLA-A, B, and C. They are negative for CD45, CD34, CD14, CD54, CD133 (Chen et al. 2012, Oktar et al. 2011). Besides expressing mesenchymal stem cell marker profile, it has been shown that DPSC express pluripotency markers including Oct4, Nanog, c-Myc, Sox2, stage specific embryonic antigens (SSEA-3, SSEA-4), and tumour recognition antigens (TRA1–60 and TRA-1–81) (Govindasamy et al. 2010, Kerkis et al. 2006a, Liu et al. 2006, Nakamura et al. 2009). However, it is important to note that these expression levels are lower than the ones observed in embryonic stem cells (Lengner et al. 2007). Even though DPSC express cell surface marker profile like MSC, there are differences in gene expression levels for cell signalling, cell communication, and cell metabolism (Yamada et al. 2010).

1.5.3. Multidifferentiation capacity

DPSC are multipotent in nature because they can differentiate into cells of various lineages. Differentiation can be triggered by exposing the cells to appropriate environmental conditions.

DPSC can differentiate into odontoblasts (Cordeiro et al. 2008, Couble et al. 2000, d'Aquino et al. 2011, Gronthos et al. 2000, Laino et al. 2005, Min et al. 2011), functionally active neurons (Arthur et al. 2008, Kadar et al. 2009, Kiraly et al. 2011, Nourbakhsh et al. 2011, Osathanon et al. 2011), mature melanocytes (Paino et al. 2010, Stevens et al. 2008), and smooth muscle cells (d'Aquino et al. 2007, Gandia et al. 2008, Kerkis et al. 2006a).

1.5.4. Reprogramming dental pulp stem cells to iPSC

Dental pulp stem cells can be isolated from extracted teeth using completely non-invasive procedure and without raising any ethical concerns. DPSC inherently express reprogramming factors (Liu et al. 2011), this might have a positive effect on reprogramming efficiency (Beltrao-Braga et al. 2011, Yan et al. 2010,). In addition, they also express Nanog, a member of core pluripotency family and alkaline phosphatase. DPSC have better chances of overcoming roadblocks toward pluripotency, because they continuously express reprogramming factor at lower levels and are positive for intermediate stage marker alkaline phosphatase (Brambrink et al. 2008, Papp et al. 2011).

1.6. Effect of mycoplasma contamination on somatic cells and pluripotent stem cells

Mycoplasma are prokaryotes that are classified under the class Mollicutes. They lack rigid cell wall and cannot be easily detected in cell culture, as they do not produce turbidity in the medium. The most frequent mycoplasmas that contaminate cell cultures are the human *M. orale*, *M. fermentans*, *M. hominis*, the bovine *M. arginini*, and the porcine species *M. hyorhinis*. This indicates that the sources for mycoplasma contaminations in cell culture are mainly laboratory personnel, contaminated serum or reagents and other contaminated cell cultures (Drexler et al. 2002, Uphoff et al. 2002).

Without inducing noticeable morphological changes, mycoplasmas affect several cell parameters including growth, morphology, metabolism, antigenicity and the genome of the cultured cells (Drexler et al. 2002, Razin et al. 1998, Rottem 2003).

Results presented by Markoullis et al. had shown that mycoplasma infected mESC showed chromosomal aberrations and these aberrations increased with the duration of infection (Markoullis et al. 2009). mESC showed significant decrease in cells expressing OCT4 and SSEA-1 while differentiation potential was severely affected at higher passages (Markoullis et al. 2009). Mycoplasma infected mESC caused reduction in number of germLine chimeras that were formed, and the pups born from infected mESC had reduced body weight, nasal discharge, osteoarthropathia, and cachexia (Markoullis et al. 2009). In conclusion, this data shows that mycoplasma contamination of mESC affects various cell parameters, germLine transmission, and postnatal development of the resulting chimeras (Markoullis et al. 2009).

From the above discussion, it is clear that mycoplasma hinders normal cell functioning as well as other characteristics of the cell. Hence, it is essential to screen the cells for mycoplasma before using them for any experiments.

1.7. Various methods used for reprogramming

Shinya Yamanaka originally discovered that forced expression of Oct4, Sox2, c-Myc and Klf4 could revert somatic cells to pluripotent state. Apart from this classical method of reprogramming, other methods like miRNA and small molecules can generate iPSC (Anokye-Danso et al. 2011).

The vectors used for delivering transcription factors and miRNA can be categorized as integrative and non-integrative, depending upon whether they integrate with the host genome or not. Retro viral and lenti-viral vectors are integrative vectors whereas episomal vectors, Sendai virus, adenovirus, minicircles, piggyBac, direct miRNA transfection, and mRNA and protein overexpression are non-integrative vectors. Comparative analysis between various methods used for reprogramming is shown in table 3.

Method	Integrating	Time (days)	Efficiency (%)	Multiple cell types reprogrammed
Retroviral	Yes	25–35	0.02–0.08	Yes
Lentiviral	Yes	20–30	0.02–1	Yes
Lentiviral (miRNA)	No	18–26	10.4–11.6	No
miRNA (direct transfection)	No	20	0.002	Yes
Adenoviral	No	25–30	0.0002	No
Sendai virus	No	25	0.5–1.4	Yes
mRNA	No	20	0.6–4.4	No
Protein	No	56	0.001	No
Episomal	No	30	0.0006–0.02	Yes
PiggyBac	Yes	14–28	0.02–0.05	No
Minicircles	No	14–16	0.005	No

Table 3. Comparison of published methods for the reprogramming human somatic cells to induced pluripotent stem cells. Adapted from Rao et al. 2012.

1.7.1. MicroRNA mediated reprogramming

MicroRNAs (miRNAs) are small non-coding RNAs with an average length of 22 nucleotides. They usually act by binding to the 3′ untranslated (3′UTR) region within mRNA molecules. This interaction leads to either degradation of the mRNA molecules or inhibits the translation of the transcript. Anokye et al. had reported that, 302/367 miRNA cluster standalone can reprogramme murine and human fibroblasts to pluripotent stem cells with efficiency far better than that of Yamanaka transcription factors (Anokye-Danso et al. 2011).

1.8. Construction of a vector for efficient expression of miRNA

302/367 miRNA cluster used for reprogramming is an intronic miRNA cluster. Commonly used expression vector does not provide optimum environment for their expression. Hence, we decided to construct a vector in which an intron was placed within a reporter gene. This construct would not only provide optimum environment for miRNA expression but also reveals if the miRNA has been processed appropriately. The later can be confirmed by detecting florescence of reporter gene.

The construction of expression vector was done using cyclic ligation assembly. The principle behind the assembly is as follows. This method employs a thermostable ligase and short scaffold oligonucleotides connectors (SOCs) that are homologous to the ends and beginnings of two adjacent DNA sequences (Roth et al. 2014).

A short description of the process reads as follows. First, denaturation of the two fragments is carried out in the presence of suitable SOC, which allows the SOC access to the complementary strands of the two sequences when the reaction is returned to an annealing temperature (Roth et al. 2014). Binding of the SOC to the complementary strands creates a temporary dsDNA structure with an absence of a single phosphodiester bond between the two complementary strands (Roth et al. 2014). The thermostable Taq Ligase (Takahashi et al. 1984) specifically ligates the complementary strands together to produce a continuous ssDNA structure without any loss or addition of nucleotides at the joint. SOCs serve only as initial scaffolds for fragment assembly, with successfully assembled products becoming additional templates as they assemble (Roth et al. 2014). The two ligated strands anneal to their complementary strands, again creating a single nick in a dsDNA structure. The amount of correctly assembled product increases exponentially as the reaction cycles between denaturing and annealing/ligation temperatures (Roth et al. 2014).

Precise site of intron cloning was found after analysing the sequence using splice prediction software. Thorough analysis of the DsRed2 sequence (reporter gene) revealed that the site around EcoO109I restriction site encompasses prominent intron splicing enhancer region. Software predicted highest probability for successful intron splicing, if the sequence was to be inserted exactly at EcoO109I restriction site.

1.9. Differentiation of hiPSC into three germ layers to confirm attainment of pluripotency

Pluripotency of iPSC is confirmed by checking their differentiation potential into cells constituting all three germ layers. Differentiation potential can be verified by either teratoma formation, embryoid body formation or targeted differentiation towards cell types representing the three germ layers. Teratoma assay is gold standard for testing pluripotency but its routine use in characterizing iPSC clones is limited because of extensive expenses associated with maintaining immune-deficient mice as well as the need for special approval for handling animals.

An alternative to teratoma assay is embryoid body formation (EB). In EB formation, iPSC or pluripotent stem cells are allowed to aggregate and are cultured as floating bodies. This triggers spontaneous differentiation into cells of all three germ layers over the period of time (Sheridan et al. 2012). This process is not ideal to obtain a single type of differentiated cell for example cardiomyocytes, hepatocytes or neurons.

Differentiation of iPSC towards neural stem cells, hepatocytes and myogenic progenitor, these three cell types are derivatives of ectoderm, endoderm and mesoderm respectively and will be discussed in detail. Human pluripotent stem cells can be efficiently differentiated in a stepwise manner to dopamine neurons (Gonzalez et al. 2013). This stepwise differentiation protocol traverses through a phase where neural stem cell appears in the culture (Gonzalez et al. 2013). Deriving neural stem cells (NSC) is very advantageous since these stem cells are multipotent and can give rise to various types of neuronal cells, which will be of particular importance in modelling neuronal disease and drug screening. By adding two neural inducers, SB218078 and DMH-1, iPSC or PSC can be differentiated into NSC (Gonzalez et al. 2013). NSC can be further differentiated efficiently to dopaminergic neuron by adding single small molecule guggulsterone (Gonzalez et al. 2013). This reported protocol is efficient, scalable, xeno-free and it produces pure populations of clinical-grade neuronal cells from hPSCs (Gonzalez et al. 2013).

iPSC has been differentiated successfully into almost all possible cell types of the three germ layers. A comprehensive list of all cell types differentiated from iPSC or pluripotent stem cells is in Fig 1. After NSC, next in line are hepatocytes. Siller et al. had reported a stepwise differentiation protocol of pluripotent stem cells into hepatocytes (Siller et al. 2015). The main highlight of the protocol is that it entirely relies on use of small molecules to differentiate pluripotent stem cells to hepatocytes. During differentiation, first stage to appear is formation of definitive endoderm (DE), which is characterized by expression of SOX17 and FOXA2 markers. This differentiation is triggered by short pulse of small molecule CHIR99021. Further differentiation of DE towards hepatic fate is triggered by DMSO (Hay et al. 2008a, Soto-Gutierrez et al. 2007, Sullivan et al. 2010). This treatment causes DE to differentiate into hepatic progenitors, which are identified based on expression of HNF4A and AFP markers. In the final phase, progenitor are differentiated into hepatocyte like cells using combination of three small molecules:

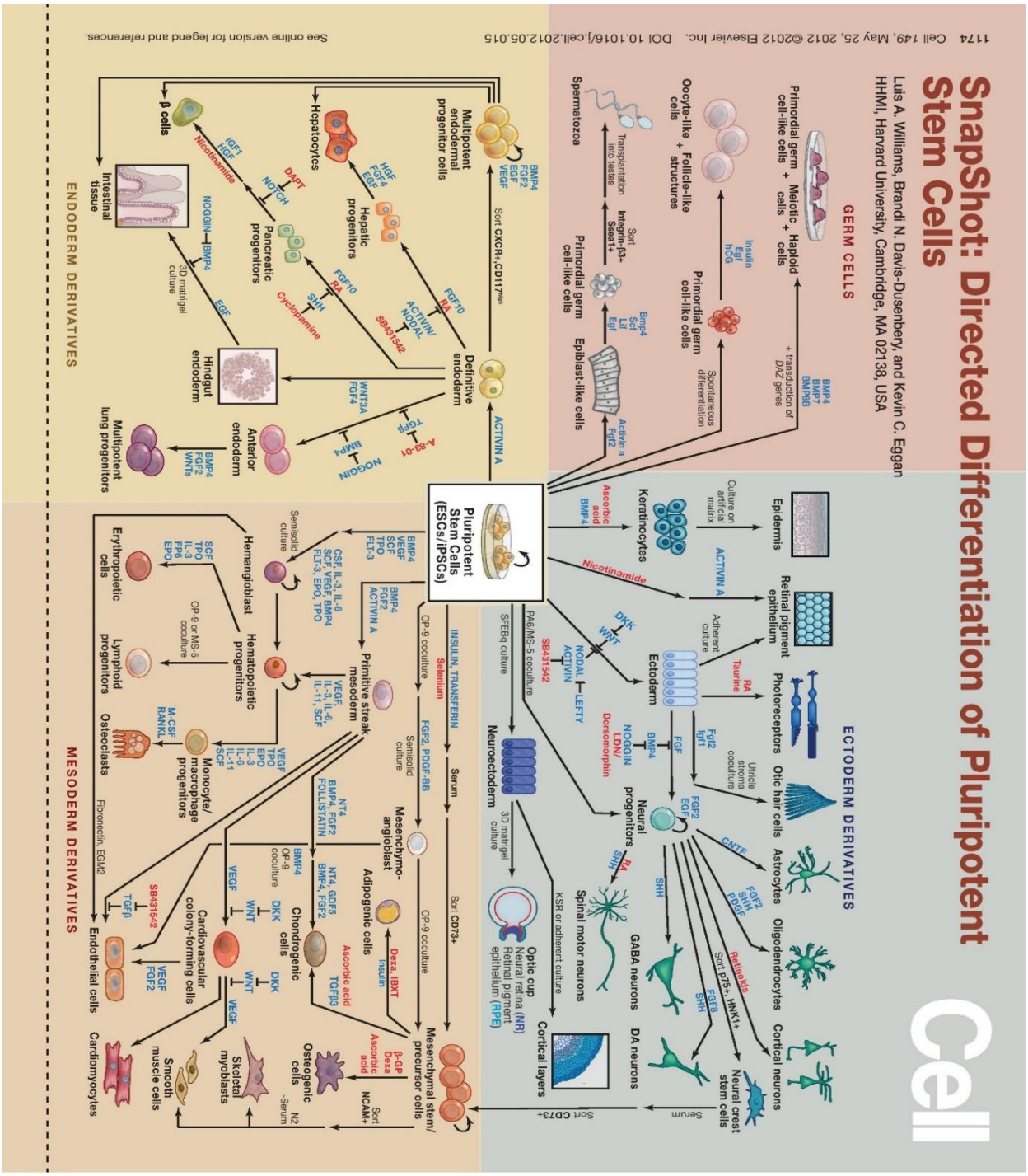

Fig. 1. Directed differentiation of pluripotent stem cells into various cell types. The figure has been adapted from (Williams et al. 2012).

dexamethasone, hydrocortisone-21-hemisuccinate and dihexa (Siller et al. 2015). The final population of cells obtained after completion of differentiation expressed markers characteristic to

adult hepatocytes at the transcriptional and protein levels, as well as key hepatic functions such as serum protein production, glycogen storage, and cytochrome P450 activity. In summary, the authors demonstrated efficient generation of hepatocyte like cells from pluripotent stem cells using small molecules.

Skeletal muscle functioning and its capacity to regenerate declines with progressive aging. This gradually affects the normal routine activities of an individual. Regeneration of skeletal muscle is carried by muscle stem cells. Over the time, regenerative capacity of these stem cells declines. One of the alternative to overcome the declined regenerative capacity of inherent stem cells is to replenish the repertoire with young autologous muscle stem cells. Abundant supply of such stem cells can be obtained from targeted differentiation of hiPSC.

Various protocols are currently available for generating muscle progenitor cells. Direct clinical application of such cells is prohibited due to inefficiency of differentiation and the use of viral vectors and potential insertional mutations (Thomas et al. 2003). Shelton et al. had reported an efficient directed differentiation protocol using serum free and chemically defined conditions (Shelton et al. 2014). This protocol traverses through all the phases of myogenesis which occur during normal embryogenesis.

During embryogenesis, Wnt signalling is critically important for the development of the primitive streak and paraxial mesoderm (Liu et al. 1999), and in the formation of posterior somites and the tail bud (Takada et al. 1994). Protocol of Shelton et al. mainly modulates Wnt signalling. This is achieved by using the GSK3 inhibitor CHIR99021 (CHIR). This small molecule augments mesoderm induction (Tan et al. 2013), leading to myogenesis. Duration of GSK3 inhibition is critical for triggering myogenesis. PAX3/7 stem cells slowly emerge following CHIR99021 mediated GSK3 inhibition. It has been reported that double positive PAX3/7 stem cell population present in the central dermomyotome is maintained throughout embryogenesis and ultimately gives rise to almost all muscles in the body (Buckingham 2007, Kuang et al. 2007).

Stem cells gradually upregulate myogenic regulatory factors to trigger terminal differentiation. This hinders the efficiency of MPC to reconstitute the satellite cell niche during transplantation into muscle (Kuang et al. 2007, Montarras et al. 2005). FGF2 prevents expression of myogenic

regulatory factors (MYF5, MYOD1, and MYOG) during satellite cell activation and enhances proliferation of PAX3/7 expressing stem cells in vitro (Fedorov et al. 1998, Hall et al. 2010).

In summary, the protocol of Shelton et al. is efficient and robust in generating muscle stem cells following CHIR mediated induction of mesoderm. It also mentions provision for expanding stem cell population using FGF2 treatment (Shelton et al. 2014). Other point that needs to be highlighted is that the protocol is chemically defined, serum and transgenic-free yielding a nearly homogeneous myogenic population (Shelton et al. 2014).

1.10. Application of iPSC technology

1.10.1. Disease modelling

iPSC technology can be directly used for disease modelling. Disease specific iPSC cell line can be prepared from various patients. Targeted differentiation of iPSC, back towards the disease specific tissue, will help understand the disease development and progression. This will help in development of specific therapeutics for a particular disease.

Various disease specific iPSC cell lines have been generated for studying disease models and drug discovery. A short list of diseases/disorders that have been successfully modelled using iPSC technology is provided in table 4.

Table 4. Human iPSC lines exhibiting disease phenotypes. Adapted from Siller et al. 2013.

Disease	**Tissue/Cell type affected**	**Drug tests**
Alzheimer's disease	Neurons	γ-secretase inhibitors and modulators have profound effects
Familial dysautonomia	Neurons	Kinetin reduced mutant splice form and increased neuronal differentiation.
Huntington disease (HD)	Neurons	No
Spinal muscular atrophy (SMA)	Neurons	VPA and tobramycin increased *SMN1* protein.
Parkinson's disease (PD)	Dopaminergic neurons	No
LQTS (Type 1 and 2)	Cardiomyocytes	Type 1: None tested. Type 2: Increased sensitivity to

		arrhythmogenic drugs (sotalol), I_{RK} blockers (E-4031), cisapride, nifepidine, pinacidil, ranolazine, nicorandil, isoprenaline, na-dolol, PD-118057.
CPVT1	Cardiomyocytes	Forskolin used to increase cytosolic c-AMP levels and abolish increase of calcium release events; Dantrolene—restored normal Ca^{2+} levels and activity.
Familial dilated cardiomyopathy	Cardiomyocytes	β-adrenergic agonists—cellular stress signs (reduced beating rate, reduced contraction, increase in abnormal α-actin distribution). β-adrenergic blockers rescued the phenotype.
Gaucher's disease	Macrophage (neurons)	Isofagomine partially restored RBC clearance.
Diabetes	Pancreatic cells	No
α1-antitrypsin deficiency	Hepatocytes	No
Familial hypercholesterolemia (FH)	Hepatocytes	Lovaststatin, increased LDL uptake.
Glycogen storage disease type 1a	Hepatocytes	No
Wilson's disease	Hepatocytes (neurons)	Curcumin treatment rescues defect.
Cystic fibrosis (CF)	Airway epithelia lung progenitors	VX-809 treatment results in surface localization of mutant CFTR protein.
Hepatitis C infection	Hepatocytes	Anti-CD81 dose dependently attenuated HCV entry.

1.10.2. Regenerative medicine

Injured or degenerated tissue can be repaired or regenerated using iPSC technology. Cells used for regeneration are autologous in origin; hence will not trigger immune rejection. In addition, other problems like ethical consideration and physiological profile matching can be avoided. Various clinical conditions that can be treated using iPSC are diseases of central nervous system,

cardiovascular diseases, hepatic diseases, hematopoietic disorders, musculoskeletal injury, spinal cord injury (Liu et al. 2011, Nori et al. 2011, Suzuki et al. 2013) Duchenne muscular dystrophy etc. (Kazuki et al. 2010).

Parkinson's disease (PD), a neurodegenerative disease is caused by loss of dopaminergic neurons. Study in rat PD model have shown improvement in behaviour, on transplantation of iPS-derived neurons in the brain (Wernig et al. 2008). In this article, authors have reported an efficient protocol to differentiate iPSC into neural precursor cells. These cells upon further differentiation gave rise to neuronal and glial cells. Upon transplantation into the fetal mouse brain, the cells were able to migrate into various brain regions and differentiate into glia and neurons, including glutamatergic, GABAergic, and catecholaminergic subtypes. Morphological analysis and electrophysiological readings confirmed that the grafted neurons had successfully integrated with the host brain and possessed neuronal activity.

Other in vivo studies have shown that iPS themselves are directly able to repair damaged area of rat cortex (Chen et al. 2010). In this study iPSC mixed with fibrin glue were implanted in subdural cavity of the rat brain subjected to cerebral ischemia. These implanted iPSC efficiently differentiated into astroglial-like and neuron-like cells and displayed functional electrophysiological properties. Transplantation significantly decreased the infarction size and showed improved motor function. They also significantly reduced pro-inflammatory cytokines and simultaneously increased production anti-inflammatory cytokines.

Severe injury or end stage liver disease causes irreversibly damage leading to liver failure. Currently, orthotopic liver transplantation is the most effective therapy for acute and chronic liver failure. However, it is limited by shortage of donors, operative risk, lifelong use of immunosuppressive agents, and very high costs. Cell therapy has been considered as a potential therapeutic alternative to orthotopic liver transplantation (Keeffe 2001, Lee 2001, Ott et al. 2000). Stem cells have gained immense importance in cell therapy because of their potential to supportive tissue regeneration and their ability to generate large amounts of donor cells for transplantation. Hepatocytes can be efficiently derived from iPSC. The iPSC-derived hepatocytes display several hepatic functions, including albumin expression, accumulation of glycogen, metabolism of indocyanine green, accumulation of lipid, active uptake of low-density lipoprotein, synthesis of urea, and express the same hepatocyte mRNA fingerprint.

Red blood cells are depleted due to injury or diseases. Patient's life is at risk if appropriate matching blood group is not available at the time of emergency. Recent advances have shown that iPSC can be used for ex-vivo expansion of various blood components. Currently various techniques are available for differentiation of iPSC to RBCs (Lim et al. 2013). Dias J. et al. reported an efficient differentiation protocol in which iPSC were differentiated to erythroid cells using OP9 stromal cell coculture system (Dias et al. 2011). Differentiation terminated in giving rise to pure population of $CD235a^{+}$ $CD45^{-}$ leukocyte-free RBCs with robust expansion potential and long-life span (up to 90 days). Analysis of globins revealed that iPSC derived RBC predominantly expressed fetal γ and embryonic ε globins. These results open prospect for large-scale production of erythroid cells from somatic cell derived iPSC (Dias et al. 2011).

Most of the prevailing heart diseases can be repaired by iPSC. This can be achieved by replacing the affected cells within the tissue with cells generated from iPSC. Currently iPSC can be efficiently differentiated to cardiac stem cells, cardiomyocytes, cardiac endothelial cells, smooth muscle cells etc (Iacobas et al. 2010, Lian et al. 2013, Skelton et al. 2016, Xie et al. 2009).

1.10.3. Drug discovery and cytotoxicity studies

iPSC technology finds application in identifying new medicines and in toxicity prediction. Accuracy of these studies can be increased by combining iPSC technology and animal model study. Testing systems involving use of animal models or in vitro animal derived cells cannot entirely replicate the exact human physiological conditions and related phenotypic attributions. In some cases, benefits observed in animal models are not observed to be beneficial in humans. In addition, drug toxicity varies with the type of animal model chosen. Finally, before the drug or a therapy is approved it must be tested on human cells or human models. All the above facts limit direct extrapolation of results obtained from animal model study to humans.

iPSC system can be used for identifying new drugs using steps such as identification of a potential drug molecule followed by its synthesis, generation of iPSC, their differentiation to specific somatic cells, and testing for toxic or nontoxic effects of the synthesized drug on the somatic cells. For toxicity studies, iPSC from normal and diseased cells can be used to generate neurons, hepatocytes, cardiomyocytes etc. Molecules showing toxicity and potential side effects on differentiated iPSC are directly rejected without further consideration.

iPSC helps to reduce cost and time required to identify compounds which are toxic or present some side-effects. iPSC system proves to be a better alternative for conventional tests of toxicology and drug research and it has a better safety assessment over conventional testing systems which involves use of animals. For example, iPSC has been used to establish test systems for cytotoxicity–cardiotoxicity, hepatotoxicity, and embryo-toxicity testing (Kimmel et al. 1983, Seiler et al. 2004).

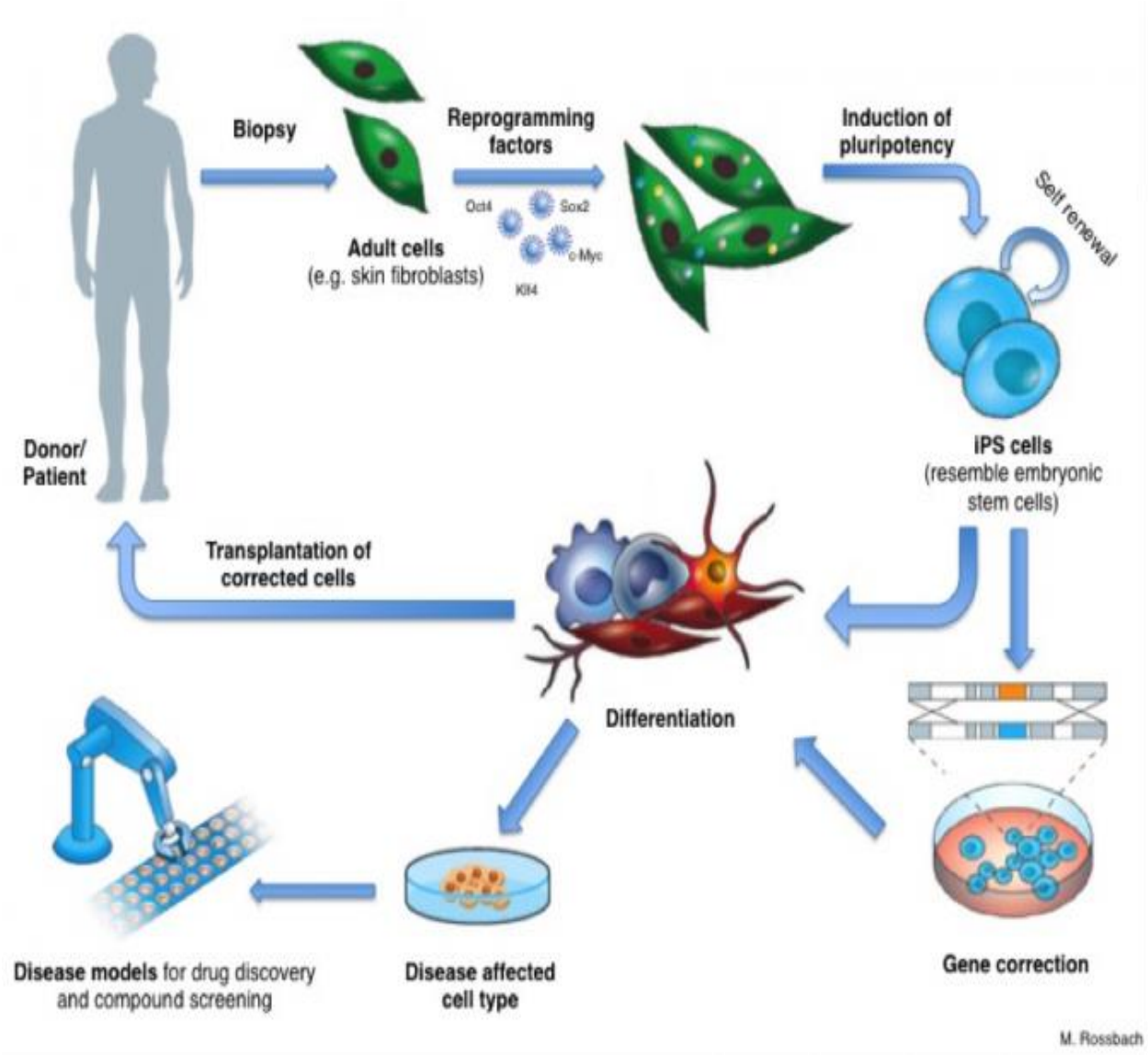

Fig. 2. iPSC derivation and applications: Certain genes can be introduced into adult cells to reprogramme them. The resulting iPS cells resemble embryonic stem cells and can be differentiated into any type of cell to study disease, test drugs or can be used after gene correction to develop future cell therapies. Figure reproduced from eurostemcell.org.

Advantages of iPSC technology can be summarized as follows:

1. Use of iPSC eliminates ethical issues arising due to use of hESC.
2. Reduces chances of immune-rejection.
3. Provides platform for throughput screening for predicting toxicity/therapeutic responses of newly developed drugs.
4. Lowers the overall cost and risk of clinical trials.
5. Provides opportunity for development of a personalized approach for administration of drugs.
6. Can be used for gene targeting and correction technologies (gene therapy).
7. Can provide cells with consistent phenotype essential for disease modelling.

2. Objectives

- Development of efficient and rapid platform for screening mycoplasmas that commonly infect the animal tissue culture.
- Reprogramming DPSC and human dermal fibroblasts into iPSC using OCT4, SOX2, KLF4 and c-MYC transcription factors.
- Characterisation of iPSC clones using immunocytochemistry and real-time PCR.
- Determination of in-vitro differentiation capability of iPSC by forming embryoid bodies.
- Targeted differentiation of iPSC into myogenic progenitor cells.
- Construction expression vector for providing optimum expression condition for miRNA expression.

3. Methodology

For this study, natal tooth was obtained from 11-day-old baby girl. Along with nDPSC, WI38 embryonic lung fibroblasts and human dermal fibroblasts isolated from skin biopsy sample were also reprogrammed. Out of the three fibroblasts cell lines, two are of commercially origin while the third one was isolated from biopsy sample. Informed consent was taken from baby's parents as well as from donor of biopsy sample as per the guidelines of Ethical Committee of the Medical Faculty in Hradec Kralove.

After extraction of dental pulp, nDPSC were expanded followed by extensive characterization. Before performing reprogramming, all cell lines were tested for presence of mycoplasma contamination. Only mycoplasma free cell lines were used for reprogramming. Screening of mycoplasma was performed as per our previously standardized protocol (Pisal et al. 2016). nDPSC were reprogrammed using feeder free protocol. Reprogramming of nDPSC was performed with few modifications in the basic protocol provided by the manufacturer of the reprogramming kit. Following reprogramming, iPSC clones were characterized and their capability to form three germs layers was tested by forming embryoid bodies. After confirmation of pluripotency, one of the clones was selected for targeted differentiation into myogenic progenitors.

The entire reprogramming experiment was divided into four parts

1. Reprogramming of nDPSC and human fibroblasts
2. Characterization of nDPSC and fibroblasts iPSC clones using immunocytochemistry and RT-qPCR
3. Differentiation of natal iPSC clones by embryoid body formation
4. Targeted differentiation of iPSC clone towards myogenic lineage

3.1. Mycoplasma detection using polymerase chain reaction (PCR)

Presence of mycoplasma hampers the cellular reprogramming process. Hence, it is essential that the cell lines used for reprogramming are free of any mycoplasma infection. Various methods are available for detecting mycoplasma. We had chosen PCR method over other methods, because it is sensitive, quick and robust. We had adapted the method as mentioned by Uphoff et al. (Uphoff et al. 2002). We introduced few modifications in the original protocol and enhanced its sensitivity and robustness (Pisal et al. 2016).

Forward primers	**Cell culture mycoplasma species**
CGC CTG AGT AGT ACG TCC GC	*M. fermentans, M. bovis*
CGC CTG AGT AGT ACG TAC GC	*Acholeplasma laidlawii*
TGC CTG GGT AGT ACA TTC GC	*Ureaplasma spp.*
TGC CTG AGT AGT ACA TTC GC	*M. gallisepticum*
CGC CTG AGT AGT ATG CTC GC	*M. arginini, M. hominis, M. hyorhinis, M. orale, M. pneumoniae*
CAC CTG AGT AGT ATG CTC GC	*M. pulmonis*
CGC CTG GGT AGT ACA TTC GC	*M. pirum*
Reverse primers	
GCG GTG TGT ACA AGA CCC GA	*M. arginini, M. bovis, M. fermentans, M. gallisepticum, M. hominis, M. orale, M. pirum, Ureaplasma spp.*
GCG GTG TGT ACA AAA CCC GA	*M. hyorhinis, M. pneumoniae*
GCG GTG TGT ACA AAC CCC GA	*A. laidlawii*

Table 5. Oligonucleotide primers used in PCR for the detection of mycoplasma contamination. Adapted from Uphoff et al. 2002

Protocol is briefly described as follows:

1. 1 mL of cell culture supernatant is collected. Supernatant is either stored at -20°C or is directly used for analysis.
2. Lyophilized primer pairs are reconstituted in nuclease free water. Forward and reverse primers are mixed separately at 5 µM concentration in nuclease free water and are aliquoted into 50 µL volume and stored frozen at –20 °C.

3. PCR is assembled as follows:

PCR components	Concentration 1X / volume
5 μM forward primers	1 μL
5 μM reverse primers	1 μL
Master Mix (Cat. no. M0484S, New England Biolabs)	12.5 μL
Internal control 10 pg/μl	1 μL
Cell culture supernatant	1 μL
Nuclease-free water	8.5 μL

Table 6. List of various components added to the PCR reaction mix.

4. PCR amplification is carried out using following parameters:

Cycle type	Temp.	Time
Denaturation	94 °C	5 min
Cycles	35	
Denaturation	94 °C	30 s
Annealing	55 °C	30 s
Extension	68 °C	60 s
Final Extension	68 °C	5 min
Hold	4 °C	20 min

Table 7. Parameters used for performing PCR.

5. PCR amplified product is electrophoresed using agarose gel.

3.2. Reprogramming of DPSC and fibroblasts

3.2.1. Media and solution preparation

3.2.1.1. Media preparation for expansion of nDPSC

1. Concentration of all the components required to prepare complete nDPSC medium is as follows:

   Alpha MEM (Life Technologies) variable

Fetal calf serum (FCS)	2 %
L-glutamine (Life Technologies)	2 mM
Ascorbic acid 2-phosphate (Sigma)	0.2 mM
Dexamethasone (Sigma)	50 nM
EGF (Peprotech)	10 ng/mL
PDGF-BB (Peprotech)	10 ng/mL
Gentamicin (Life Technologies)	50 µg /mL
ITS* (Life Technologies)	10 µl/ mL

nDPSC medium is prepared without ITS, it is only added to the medium when it is used.

2. Complete medium (without ITS) is stored at 2-8 °C for upto 2 weeks.

3.2.1.2. Media preparation for human fibroblasts

1. 100 mL of fibroblasts medium is prepared by aseptically mixing following components:

MEM with GlutaMAX-I (Life Technologies)	89 mL
ES cell-qualified Fetal Bovine Serum (FBS), (Life Technologies)	10 mL
Penicillin-Streptomycin (Life Technologies)	1 mL

2. Complete medium is stored at 2-8 °C for up to 2 weeks.

3.2.1.3. Essential 8 medium preparation

1. Frozen Essential 8 supplement (Life Technologies) is thawed at 2–8°C overnight before using it to prepare complete medium. **The frozen supplement is thawed at room temperature or overnight at 4 °C. Supplement should not be thawed in the water bath.**
2. The thawed supplement is mixed by gently inverting the vial a couple of times, 10 mL volume is removed from the bottle of Essential 8 basal medium (Life Technologies), and then the entire contents of the Essential 8 supplement is aseptically transferred to the bottle of Essential 8 basal medium. The bottle is swirled to mix to obtain homogenous complete medium.

3. Complete Essential 8 Medium is stored at 2–8°C for up to 2 weeks. Before use, complete medium required for that day is warmed at room temperature until it is no longer cool to the touch. **The medium should not be warmed in the water bath set at 37°C.**

3.2.1.4. Preparation of 0.5 mM EDTA in D-PBS (50 mL)

1. 50 mL of 0.5 mM EDTA is prepared in D-PBS by aseptically mixing the following components in a sterile 50 mL conical tube:

D-PBS (w/o Mg^{2+} and Ca^{2+}) (Life Technologies)	50 mL
0.5 M EDTA (Sigma)	50 µL

2. The solution is filter sterilized and stored at room temperature for up to six months.

3.2.1.5. Preparation of Essential 8 freezing medium

1. 1 mL of freezing medium is prepared by aseptically mixing the following components in a sterile 15 mL conical tube:

Complete Essential 8 medium (Pre-warmed)	0.9 mL
DMSO (Sigma)	0.1 mL

2. Excess freezing medium was discarded.

3.2.1.6. Preparation of Rock inhibitor (Y-27362) stock solution

1. 3122 µL of DMSO is added to 10 mg of Rock inhibitor (Y-27362) RI (Tocris Biosciences) to obtain final concentration of 10 mM. This is the stock solution.
2. 60 aliquots of 50 µL each are prepared and stored at -80°C for long term storage. For short term storage i.e. for 3 months, aliquots are stored at -20°C.
3. 1 µL of RI (10 mM) is added to 1 mL of media. This gives final concentration of 10 µM.
4. Complete Essential 8 medium is filter sterilized after addition of RI.

3.3. Reprogramming timeline

Three cell lines were reprogrammed altogether i.e. human nDPSC, WI-38 (ATCC) human embryonic lung fibroblasts and human fibroblasts (HF) isolated from biopsy sample.

Day –2: nDPSC and human fibroblasts (WI-38 and HF) are separately plated into two wells of a 6-well plate in their respective medium so that they are 30–60% confluent on the day of transduction (Day 0).

Day 0: nDPSC and two fibroblasts cell lines are transduced using the CytoTune 2.0 Sendai reprogramming vectors at the appropriate MOI. The cells are incubated overnight.

Day 1: The medium of all the three cell lines is replaced with their respective fresh complete medium to remove the CytoTune 2.0 Sendai reprogramming vectors (Life Technologies).

Day 2–6: The spent medium is replaced every other day.

Day 7: Transduced cells of all three cell lines are plated on vitronectin-coated culture dishes in their respective medium.

Day 8: Medium of all the three cell lines is changed to complete Essential 8 Medium.

Day 9–28: Spent medium of all three the cell lines is replaced every day and the culture vessels are monitored for the emergence of iPSC colonies. Undifferentiated iPSC are picked and transferred onto fresh culture dishes for expansion.

3.4. Detailed stepwise protocol

1. Two days before transduction, nDPSC and three fibroblast cell lines are plated separately onto two wells of a 6-well plate at the appropriate density to achieve 80% confluency on the day of transduction (Day 0). One of the wells is used to count cells for calculating viral volume.
2. The cells are cultured for two more days to ensure that the cells have fully adhered and extended.
3. On the day of transduction, 1 mL of nDPSC and fibroblast medium is warmed in a water bath for each well to be transduced.
4. Cells from one well of each cell line is harvested to perform a cell count. These cells are not transduced but are used to estimate the cell number in the other well plated in Step 1.
5. Cells are detached from the 6-well plate using 0.5 mL of 0.05% trypsin/EDTA following incubation at room temperature. Upon detachment, 1 mL of medium is added into each well and the cells are collected in a 15 mL conical centrifuge tube.

6. The cells are counted using ViCell automated counter, and the volume is calculated for each virus needed to reach the target multiplicity of infection (MOI) using the live cell count and the titer information given on the certificate of analysis (CoA).
7. Formula for calculating volume of virus is as follows:

$$\text{Volume of virus } (\mu L) = \frac{\text{MOI (CIU/cell)} \times \text{number of cells}}{\text{Titer of virus (CIU/mL)} \times 10\text{–}3 \text{ (mL/}\mu L)}$$

   MOIs recommended for transductions is 5, 5, and 3 (i.e., KOS MOI=5, hc-Myc MOI=5, hKlf4 MOI=3).

   Each tube is thawed one at a time by first immersing the bottom of the tube in a 37°C water bath for 10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, the tube is briefly centrifuged and immediately placed on ice.
8. Calculated volumes from each of the three CytoTune 2.0 Sendai tubes are added to 1 mL of DPSC and fibroblast medium respectively, pre-warmed to 37°C. Solution is thoroughly mixed by pipetting the mixture gently up and down. The next step is completed within 5 minutes.
9. Medium of respective cell lines is aspirated, and the reprogramming virus mixture prepared in Step 8 is added to the well containing the cells. The cells are incubated overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.
10. 24 hours after transduction, the medium from all the three cell lines is replaced with fresh medium.
11. Modification to the original protocol: nDPSC are cultured with growth factors (EGF and PDGF) for 3 days post transduction. Thereafter the cells are cultured in medium without growth factors.
12. All the three cell lines are cultured in their native medium for 4 more days, changing the spent medium with fresh medium every other day.
13. Sufficient number of 100 mm tissue culture dishes are coated with vitronectin. Vitronectin are used at a coating concentration of 0.5 μg/cm^2.

    Procedure for coating culture vessels with vitronectin is as follows:

a. The vial of vitronectin is thawed at room temperature and 60 µL aliquots of vitronectin are prepared in polypropylene tubes. The aliquots are frozen at –80°C or used immediately.
b. To coat 100 mm tissue culture dish, 60 µL aliquot of vitronectin is removed from –80°C storage and thawed at room temperature. One 60 µL aliquot is required for 100 mm dish.
c. 60 µL of thawed vitronectin is added into a 15 mL conical tube containing 6 mL of sterile D-PBS (w/o Mg^{2+} and Ca^{2+}) at room temperature. Vitronectin is gently resuspended by pipetting the dilution up and down.
d. 6 mL of the diluted vitronectin solution is added to a 100 mm dish. (Volumes used for other culture vessels is given in Table 6).

Culture vessel	**Surface area**	**Volume of diluted vitronectin solution**
6-well plate	10 cm^2 per well	1.0 mL/well
12-well plate	4 cm^2 per well	0.4 mL/well
100-mm dish	60 cm^2	6.0 mL

Table 8. Volume of vitronectin required for vessels of different dimensions.

e. The coated plates are incubated at room temperature for 1 hour.

   Note: The culture vessel is either used immediately or stored at 2–8°C for up to a week. Prior to use, the culture vessel is pre-warmed to room temperature for at least 1 hour.
f. The vitronectin solution is aspirated and discarded. The cells are directly passaged onto the vitronectin-coated culture vessels.

14. Seven days after transduction, nDPSC and fibroblast cell lines are transferred on vitronectin-coated culture dishes. The medium from the both cell lines is removed, and the cells are washed once with D-PBS.
15. The cells of all the three cell lines are detached from the 6-well plate by using 0.5 mL of TrypLE Select reagent. The cells are incubated at room temperature until they detach.
16. The cells of all the three cell lines are centrifuged at 200 × g for 4 minutes, the medium is aspirated, and the cells are re-suspended in an appropriate amount of medium.

17. The cells of all the three cell lines are counted using ViCell automated counter. The cells are plated onto at two cell densities i.e. 1.5×10^5 and 5×10^5 cells per 100-mm vitronectin (Life Technologies) coated culture dish. They are incubated overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.
18. 24 hours later, the medium of all the three cell lines is changed to complete Essential 8 Medium, and the spent medium is replaced every day thereafter.
19. Starting on Day 8, the plates are observed every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells.
20. Three weeks after transduction, colonies are grown to an appropriate size for transfer.
21. Undifferentiated iPSC colonies are manually picked and transferred onto vitronectin-coated 12-well culture plates for further expansion.

3.4.1. Picking of iPSC colonies

1. The culture dish containing the reprogrammed cells is placed under an inverted microscope and the colonies are examined under 4× magnification.
2. The colony to be picked is marked on the bottom of the culture dish. Each individual clone is numbered for easy identification.
3. The culture dish is transferred to a sterile cell culture hood (i.e., biosafety cabinet) with an inverted microscope. Before placing the microscope inside the biosafety cabinet, it is disinfected by wiping with 70% ethanol. After disinfection, the microscope is not used immediately, but is allowed to stand in the laminar flow in order to free it from any debris or dust particles.
4. A 25-gauge 2-inch needle attached to an insulin syringe is used to cut the colony to be picked into 3 to 4 pieces in a grid-like pattern. The whole procedure is performed while observing through the microscope.
5. 200 µL pipette adjusted to 100 µL volume is used to aspirate the cut pieces and transfer to a vitronectin-coated 12-well plate containing complete Essential 8 medium.
6. The vitronectin-culture plate containing the picked colonies is incubated in 37°C incubator with a humidified atmosphere of 5% CO_2.

7. After picking the cut pieces, they are cultured in half the volume of media than usual. Next day, remaining half part of the media is added without aspirating the old one. This ensures that sufficient time is given for the colonies to adhere. Thereafter, medium is changed daily.
8. The colonies are passaged once they attain sufficiently large size. The colonies are passaged using 0.5 mM EDTA prepared in D-PBS (w/o Mg^{2+} and Ca^{2+}).
9. The reprogrammed colonies are cultured, expanded, and maintained in complete Essential 8 medium until two wells of a 6-well plate are frozen. Colonies are expanded for minimum five passages before they are cryopreserved.

3.4.2. Passaging iPSC using EDTA

For an established clone, split ratio is usually 1:3. For non-established clones, the split ratio is 1:2 or depending upon the percentage of confluency.

1. Before starting passaging of iPSC colonies, complete Essential 8 medium and vitronectin-coated culture vessels are pre-warmed to room temperature.
2. The spent medium is aspirated from the vessel containing PSCs and the vessel is rinsed twice with 2 mL of D-PBS (w/o Mg^{2+} and Ca^{2+}) per well of a 6-well plate. (Volume for 12 well plate is given below in a separate table).
3. 1mL of 0.5 mM EDTA in D-PBS is added per well of a 6-well plate containing PSCs. The vessel is swirled to coat the entire cell surface. (Volume for 12-well plate is given below in a separate table).
4. The vessel is incubated at 37°C for 3 minutes. After incubation, the plate is observed under inverted microscope. The cells are ready for removal, only when they start to separate and round up, and the colonies appear to have holes in them.
5. The EDTA solution is aspirated, and pre-warmed complete Essential 8 Medium is added to the vessel.
6. The cells are removed from the well by gently squirting medium and pipetting the colonies up.
7. Appropriate volume of pre-warmed complete Essential 8 Medium is added to each well of a vitronectin-coated 6-well plate so that each well contains 2 mL of medium after the cell suspension has been added. (Volume for 12-well plate is given below in table 6).

8. The cells are evenly dispersed across the vessel by performing repeated forward and backward motion in vertical and horizontal position. The vessel is gently placed into the 37°C, 5% CO_2 incubator and the cells are incubated overnight.
9. Medium of iPSC cells is changed after 24 hrs following splitting. Medium is changed daily.

Culture vessel	D-PBS	0.5 mM EDTA in D-PBS	Complete Essential 8 medium
6-well plate	2 mL/well	1 mL/well	2 mL/well
12-well plate	1 mL/well	0.4 mL/well	1 mL/well

Table 9. Volume of EDTA and E8 media required for vessels of different dimensions.

3.4.3. Cryopreserving iPSC clone

1. The spent medium is aspirated from the vessel containing iPSCs and the vessel is rinsed twice with 2 mL of D-PBS (w/o Mg^{2+} and Ca^{2+}) per well of 6-well plate. (Volume for 12 well plate is given below in a separate table).
2. 1 mL of 0.5 mM EDTA in D-PBS is added per well of a 6-well plate containing iPSCs. The vessel is swirled to coat the entire cell surface. (Volume for 12-well plate is given below in a separate table).
3. The vessel is incubated at 37°C for 3 minutes. After incubation, the plate is observed under inverted microscope. The cells are ready for removal, only when they start to separate and round up, and the colonies appear to have holes in them.
4. The EDTA solution is aspirated, and pre-warmed complete Essential 8 Medium is added to the vessel.
5. The cells are removed from the well by gently squirting 1 mL of freezing medium and pipetting the colonies up. The detached cells are transferred into cryovials.
6. The cryovials containing the cells are quickly transferred to a cryo-freezing container to freeze the cells at 1°C per minute and then transferred to –80°C overnight.
7. After overnight storage at –80°C, the cells are transferred to a liquid nitrogen tank vapour phase for long term storage.

3.4.4. Thawing iPSC clone

1. Before thawing iPSC clone, complete Essential 8 medium and vitronectin-coated culture

vessels is pre-warmed to room temperature. iPSC clone is thawed in RI supplemented complete Essential 8 medium.

2. One cryovial containing the desired iPSC clone is removed from vapor phase of liquid nitrogen tank and is placed in cryo-freezing container until further use.
3. Cryovial is thawed in water bath adjusted to 37°C until considerable amount of medium has thawed.
4. Thawed medium along with cells is carefully transferred to 15 mL centrifuge tube.
5. 6 mL of complete Essential 8 medium supplemented with 10 μM of RI is added to the thawed cells in dropwise manner along the walls of the tube.
6. The cells are centrifuged at 120 g for 5 min.
7. Supernatant is carefully aspirated, and the tube is gently tapped to break the cell pellet.
8. The cells are resuspended in 2 mL complete Essential 8 medium supplemented with of RI and the cell suspension is plated in a single well of a vitronectin-coated 6-well plate.
9. The iPSC colonies are cultured with RI for 24 hr.
10. The next day, old medium is aspirated and fresh complete Essential 8 medium without RI is added. Thereafter spent medium is replaced daily.

3.5. Characterization of iPSC clones

iPSC clones were checked for expression of pluripotency markers using immunocytochemistry. Oct4, Sox2, Nanog and SSEA4 markers were used for characterisation. Detailed specification of primary and secondary antibodies used for immunocytochemistry are provided in Table 8.

Antibody name	**Distributor**	**Clone no.**	**Dilution**
Anti-Oct4	Santa Cruz	sc-8629	1:100
Anti-Sox2	Abcam	ab92494	1:100
Anti-Nanog	Life Technologies	PA1-097X	1:100
Anti-SSEA4	Developmental Studies Hybridoma Bank	MC-813-70	1:20
Anti-Rabbit	Jackson Immuno Research	-	1:200
Anti-Goat	Jackson Immuno Research	-	1:200

Table 10. Name of different antibodies used for characterizing nDPSC iPSC clone.

3.5.1. Immunocytochemistry

1. iPSC colonies are grown in vitronectin-coated 12 well plate. They are grown until they attain 60 % confluency.
2. Medium from the wells is aspirated.
3. Colonies are rinsed thrice with 500 µL of D-PBS-T (0.05% Tween 20 (Sigma) in D-PBS (Life Technologies)).
4. Fixation is performed using 500 µL of 4% paraformaldehyde (Lechner) for 10 min.
5. Colonies are rinsed thrice with 500 µL of D-PBS-T (0.05% Tween 20 in D-PBS).
6. 500 µL of Triton X-100 solution (0.5% Triton X-100 in D-PBS) is used for 10 min to permeabilized the cells.
7. Colonies are rinsed thrice with 500 µL of D-PBS-T (0.05% Tween 20 in D-PBS).
8. Antigen blocking is performed using 200 µL of 1:20 diluted donkey (Jackson Immuno Research) or goat serum (Jackson Immuno Research) for 20 min.
9. Without washing primary antibodies are applied to the colonies. Colonies are incubated overnight with 150 µL of 1:100 diluted primary antibodies at 4°C.
10. Colonies are rinsed thrice with 500 µL of D-PBS-T (0.05% Tween 20 in D-PBS).
11. 250 µL of 1:200 diluted secondary antibody is applied to the colonies for 45 min.
12. Colonies are rinsed thrice with 500 µL of D-PBS-T (0.05% Tween 20 in D-PBS).
13. Nucleus is stained using 500 µL of DAPI solution for 10 min
14. Colonies are rinsed thrice with 500 µL of D-PBS-T (0.05% Tween 20 in D-PBS).
15. 100 µL of mounting medium is added to the each well and covered with coverslip.

3.5.2. Alkaline phosphatase staining

1. iPSC colonies are grown in vitronectin-coated 12-well plates. They are grown until they attain 60 % confluency.
2. Medium from the wells is aspirated.
3. Colonies are rinsed thrice with 500 µL of D-PBS-T (0.05% Tween 20 in D-PBS).
4. Fixation is performed using 500 µL of 4% paraformaldehyde for 10 min.
5. Colonies are rinsed thrice with 500 µL of Tris HCl buffer (10mM Tris (Sigma), 0.1% Tween 20. pH adjusted to 8.5).

6. 200 µL of substrate solution is added dropwise to the colonies. Substrate solution is freshly prepared as follows: 50 µL of NBT/BCIP (Roche) (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate respectively) + 10 mL of Tris buffered solution. The solution is filtered before adding to the cells. After adding substrate solution, colonies are incubated in dark for 10 min.
7. Colonies are rinsed thrice with 500 µL of distilled water.
8. 100 µL of mounting medium is added to the each well and covered with coverslip.

3.5.3. Determination of telomere length

1. Genomic DNA is extracted from the cells using the DNeasy Tissue Kit (Qiagen).
2. Telomere length measurement is performed by qPCR according to method described by Cawthon et al. 2002. Relative telomere length is calculated by normalizing the telomere repeat copy number to single-gene copy number and the formula used is $2^{-\Delta Ct}$ (where $\Delta Ct = Ct_{telomere} - Ct_{single\text{-}copy\ gene}$). 36B4, encoding acidic ribosomal phosphoprotein P0, is used as the single copy gene.
3. Telomere and 36B4 gene PCRs are done in separate 96-well plates with each sample run in triplicate in the same well position on an ABI 7500 HT Detection System (Applied Biosystems, USA).
4. Each 20 µL reaction consisted of 20 ng DNA, 1 × SYBR Green master mix (Applied Biosystems), and 200 nM primers. Sequences of primer pairs are given in Table 8:

Primer name	**Sequence of primer**
Telomere forward primer	CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT
Telomere reverse primer	GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT
36B4u	CAG CAA GTG GGA AGG TGT AAT CC
36B4d	CCC ATT CTA TCA TCA ACG GGT ACA A

Table 11. Primer sequences used for determining telomere length.

5. Cycling conditions (for both telomere and 36B4 products) were 10 minutes at 95˚C, followed by 50 cycles of 95˚C for 15 s and 60˚C for 1 minute. Following amplification, a dissociation curve was done to confirm the specificity of the reaction.

3.5.4. Isolation of RNA

RNA is isolated from the cells using Direct-zol RNA MiniPrep RNA isolation kit (Zymo Research).

1. Cells are rinsed twice with D-PBS before the lysis step.
2. Cells are lysed in culture plates by adding 1 mL of TRI Reagent (Sigma) per well of 6-well plate. Thorough lysis is ensured by repeatedly squirting the TRI Reagent solution over the cells.
3. Equal volume of ethanol (95-100%) is added to the sample lysed in TRI Reagent (Sigma) and is mixed thoroughly.
4. The mixture is transferred into a Zymo-Spin IIC Column in a Collection Tube and centrifuged at 16,000 x g for 30 seconds. Samples having volume greater than 700 µL are processed by reloading the column and repeating the Step 4. The column is transferred into a new collection tube and the flow through is discarded.
5. 400 µL RNA Wash Buffer is added to the column and centrifuged at 16,000 x g for 30 seconds.
6. In an RNase-free tube, 5 µL (6 U/µL) of DNase I (Zymo Research) is added to 75 µL of DNA Digestion Buffer and the contents are mixed by gentle inversion. The mix is directly added to the column matrix.
7. DNase I digestion is carried out at room temperature (20-30°C) for 15 minutes.
8. 400 µL of Direct-Zol RNA Pre-Wash is added to the column and centrifuged at 16,000 x g for 30 seconds. The flow-through is discarded and this step is repeated.
9. 700 µL of RNA Wash Buffer is added to the column and centrifuged at 16,000 x g for 2 minutes to ensure complete removal of the wash buffer. The column is carefully transferred into an RNase-free tube.
10. To elute RNA, 50 µL of DNase/RNase-free water is directly added to the column matrix and centrifuged at 16,000 x g for 30 seconds.
11. Isolated RNA is used immediately, or it is stored at -80°C.

3.5.5. Protocol for cDNA synthesis

RNA is reverse transcribed using high capacity cDNA reverse transcription Kit (Life Tech)

1. 10 µL of 2X Reverse Transcription (RT) master mix is pipetted into each 200 µL PCR tube.

   RT Master mix components are thawed on ice and are assembled as shown in Table 10.

Components	Volume per reaction (µL)
10× RT buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe Reverse Transcriptase	1.0
Nuclease free water	4.2
Total	10.0

Table 12. List of various components used for cDNA synthesis and their respective volumes.

2. 10 µL of RNA sample (2 µg/sample) is added into each tube containing the RT master mix. The components are mixed by pipetting up and down two times.
3. The tubes are sealed and centrifuged to spin down the contents and to eliminate any air bubbles.
4. After centrifugation, tubes are loaded onto the thermal cycler.
5. Reverse transcription is performed using thermal cycler conditions as mentioned in Table 11.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Table 13. PCR conditions used for cDNA synthesis.

3.5.5. Protocol for assembling reaction for qPCR

1. cDNA is prepared from 2 µg of RNA in a 20 µL reaction and diluted 1:200 with nuclease free water.
2. qRT-PCR is performed using 7.5 µL of diluted cDNA, along with lyophilized 200 nM of each primer and 7.5 µL of SYBR Select Master Mix in a total reaction volume of 15 µL.

3. Cycling condition for the analysis are 50°C for 2 min (Hold); 95°C for 2 min (Hold); 95°C for 15 sec; 60°C for 40 sec, for 40 cycles. All results are normalized against beta actin gene (ACTB).

All primer pairs are checked for specificity using BLAST analysis and by thermal dissociation curves to ensure amplification of a single product with the appropriate size and melting temperature. For generating heat map ClustVis online software is used (Metsalu et al. 2015). The statistical correlation analysis function was performed using NCSS software. Pairwise matrix of correlation values was generated using heatmapper software (Babicki et al. 2016). All experiments were carried out in duplicates, and the results were presented as means.

3.6. Differentiation of natal iPSC clones by embryoid body formation

3.6.1. Preparation of PVA media

Polyvinyl Alcohol (PVA) powder (Sigma) is added into specific medium (E8 or E6 (Life Technologies) at 4 mg/mL, mixed and stirred for one hour at room temperature, filtered and then stored at 4°C. E8/PVA is used for EB formation (Ng et al. 2008).

3.6.2. Preparation of Poly-HEMA coated Petri dish

1. Poly-HEMA solution is made at 20 mg/mL by dissolving 1g Poly-HEMA (Sigma) in 50 mL 95% ethanol (Penta). The solution is continuously stirred overnight at 37°C.
2. The Petri dishes are coated by pouring the Poly-HEMA/ethanol mixture to cover the dishes and is allowed to dry overnight at room temperature. (Kuroda et al. 2013).

3.6.3. Formation of embryoid bodies

1. iPSC are cultured in E8 medium on vitronectin-coated plates, and medium is changed daily.
2. Two days before EB formation, 60–70% confluent iPSC are passaged onto vitronectin-coated plates with EDTA/D-PBS as previously described (Beers et al. 2012). The passage ratio is usually 1:3, in E8 media with 10 μM RI to increase cell survival.
3. The next day, media is changed to E8 medium without RI.
4. On the day of EB formation, 80% confluent iPSC are washed twice with D-PBS and then they are incubated in 0.5 mM EDTA/D-PBS for 3 min.

5. After incubation, EDTA/D-PBS is aspirated. iPSC colonies are dissociated into small clumps off the plate with 5 mL/dish of E8/PVA with RI.
6. To form self-aggregated EB, cell clumps are suspended into poly-HEMA coated Petri dishes via 1:1 passage (the cells from one well of 6-well plate is suspended in 3 mL E8/PVA media with RI to one poly-HEMA coated 60 mm dish to allow self-aggregation in 37°C incubator for 2 hr.). On the second day, EB should form in various sizes.
7. On the second day, the plate is tilted at 45° angle to allow the EB to gather at the bottom of the plate. Old media is gently aspirated, and the media is changed to Essential 6 medium. The medium is changed every 2 days. EB are cultured for 19 days.
8. Some EB were directly lysed on day 7 and 14 post formation with TRI-Reagent to harvest the RNA.
9. RNA was reverse transcribed and used for quantifying *NES*, *MSI-1* and *PAX6* genes. Primer sequences of the three genes are as follows:

NES-FP- ATGGAGACGTCGCTG; NES-RP- ACAGCCAGCTGGAAC

MSI-1-FP- GACTCCAAAACAATTGACCC; MSI-1-RP- CAAAATATTGCTTCACGTCC

PAX6-FP- AGAGAATACCAACTCCATCAG; PAX6-RP- GATAATGGGTTCTCTCAAACTC

3.6.4. Sectioning of embryoid bodies

1. After 21 days of culturing, embryoid bodies are fixed by adding 10% formalin (Lachner), and after dehydration process they are finally embedded in paraffin. Serial sections (5–6 μm) are cut, mounted on glass slides pre-treated with chrome alum-gelatin, and dried at room temperature overnight.
2. Paraffin-embedded sections are deparaffinized by xylol (Lachner) treatment, hydrated with decreasing concentrations of ethanol (96, 80 and 70%), and then rinsed twice with distilled water.
3. Sectioned embryoid bodies are stained with hematoxylin and eosin to identify various structures formed within embryoid bodies, dehydrated and mounted in DPX (Fluka).

3.6.5. Immunoperoxidase staining of sectioned embryoid bodies

1. For antigen retrieval, deparaffinized sections are exposed to microwaves (700 W) in sodium citrate solution (pH = 6.0) 2 times, for 5 min each.

2. After extensive washing with distilled water, endogenous peroxidase activity is quenched by incubating samples in 3% H_2O_2 (Penta) (3 × 10 min).
3. Sections are then incubated in blocking buffer (5% normal goat serum (Jackson Immuno Research) in PBS) for 20 min and exposed to primary antibody diluted in antibody diluent with background reducing components (Dako) at 4°C overnight. Description of antibodies is as follows:

Antibody name	**Distributor**	**Clone no.**
Anti-βIII Tubulin	Exbio	TU20
Anti-MAP2	Sigma	HM2
Anti-Nestin	Life Technologies	SP103
Anti-Vimentin	Dako	V9
Anti-Pan cytokeratin	Dako	AE1/AE3

Table 14. Name of different antibodies used for determining differentiation capacity of nDPSC derived iPSC clone into three germ layers.

4. After washing with PBS, sections are incubated with reagents from either an anti-mouse or anti-rabbit EnVision peroxidase kit according to the manufacturer's protocol. Colour reactions are performed using the chromogen DAB (3,3′-diaminobenzidine tetrahydrochloride, 2 μg/mL; Fluka, Darmstadt, Germany).
5. After washing with distilled water, DAB precipitate is intensified with 3% $CuSO_4$ solution for 5 min.
6. Sections are then counterstained with Mayer's hematoxylin (Merck) dehydrated and mounted in DPX (Fluka).

3.7. Targeted differentiation of iPSC clone towards myogenic lineage

3.7.1. Media preparation

3.7.1.1. Preparing working stock solution of monothioglycerol (MTG)

1. MTG working stock is prepared by aseptically mixing following components:

MTG stock solution (Sigma)	65 μL
Sterile double distilled water	5 mL

2. Appropriate number of aliquots are prepared and stored at -20 °C

3.7.1.2. Preparation of Neutralizing medium

1. Neutralizing medium is prepared by aseptically mixing following components:

KnockOut Serum Replacement (KOSR) (Life Technologies)	1 mL
KnockOut DMEM (Life Technologies)	5.6 mL

2. The prepared medium is stored at 4 °C for up to 2 weeks.

3.7.1.3. Preparing working stock solution of Holo-Transferrin (2 mg/mL)

1. Holo-Transferrin working stock is prepared by aseptically mixing following components:

Holo-Transferrin (powdered form) (Sigma)	50 mg
Sterile double distilled water	25 mL

2. Appropriate number of aliquots are prepared and stored at -20 °C.

3.7.1.4. Preparing 0.1% Human serum albumin (HSA) and bFGF solution (5 ng/µL)

1. Human serum albumin solution is prepared as follows:

10% HSA (Sigma)	5 µL
PBS w/o Ca^{2+} and Mg^{2+} (Life Technologies)	500 µL

2. To the above solution 25 µL of 100 ng/µL basic fibroblasts growth factor (bFGF) (Peprotech) is added. This gives us final concentration of 5ng/µL.
3. 10 aliquots of 50 µL each are prepared. The aliquots are stored at -20°C until further use.

3.7.1.5. Preparation of StemPro-34 medium

1. Prior to preparing the complete medium, StemPro-34 SFM media and StemPro-34 supplement are warmed to 37°C before combining to achieve uniform mixing. StemPro-34 medium is prepared by aseptically mixing following components:

StemPro-34 SFM (Life Technologies)	50 mL
StemPro-34 supplement (Life Technologies)	1.3 mL
MTG stock solution (Sigma)	150 mL
L-Glutamine (Life Technologies)	500 mL
Transferrin stock solution (Sigma)	267.5 µL

2. The medium stored at 4 °C for up to 2 weeks.

3. Per mL of StemPro-34 media is supplemented with 1 µL of 5 ng/µL bFGF to get final concentration of 5 ng/mL.

3.7.1.6. Preparation of expansion medium for myogenic progenitor cells

1. 50 mL of expansion medium is aseptically prepared by mixing following components:

E6 medium (Life Technologies)	50 mL
bFGF (5 ng/ µL) (Peprotech)	50 µL

2. Media is filtered sterilized after preparation.
3. The medium stored at 4 °C for up to 1 week.

3.7.2. Differentiation of iPSC clone towards myogenic lineage

1. iPSC colonies are maintained in vitronectin-coated 6-well plate.
2. Prior to differentiation, iPSC colonies are split 1:3 and are cultured till they attain 80% confluency.
3. The spent medium is aspirated from the vessel containing iPSC, and the vessel is rinsed twice with 2 mL of D-PBS (w/o Mg^{2+} and Ca^{2+}) per well of 6-well plate
4. 2 mL of fresh E8 medium supplemented with 10 µM RI is added per well of 6-well plate. The cells are incubated for 1 hr in a 37°C, 5% CO_2 incubator.
5. E8 medium supplemented with RI is aspirated and the iPSC colonies are rinsed twice with 2 mL of D-PBS per well of 6-well plate.
6. 0.5 mL of TrypLE solution is added per well of 6-well plate. The colonies are incubated for 5 min in a 37°C, 5% CO_2 incubator.
7. Colonies are triturated using 1000 µL pipette to dissociate the cells.
8. TrypLE solution is neutralized by adding 0.5 mL of neutralizing medium per well of 6-well plate
9. Cells are centrifuged at 200g for 5 min.
10. Supernatant is aspirated, and the cell pellet is resuspended in 3 mL of E8 medium supplemented with 10 µM RI.
11. The cells are counted using ViCell automated counter. 500 µL of cell suspension is used for cell counting.

12. 3.75×10^5 cells are plated per well of vitronectin-coated 6-well plate. The cells are cultured in E8 medium supplemented with 10 μM RI.
13. Old medium is aspirated, and the cells are rinsed twice with D-PBS.
14. 2 mL of E6 medium supplemented with 10 μM GSK3 inhibitor CHIR99021 is added per well of 6-well plate.
15. On Day 1, old media is gently aspirated and fresh E6 medium supplemented with 10 μM GSK3 inhibitor CHIR99021 is added back.
16. On Day 2, old media is aspirated, and the cells are gently rinsed with D-PBS. The cells are cultured in E6 medium without additional supplementation for next 12 days of differentiation. Media is changed daily.
17. On Day 12, old media is aspirated, and the cells are rinsed with D-PBS. 2 mL of StemPro-34 complete medium supplemented with 5 ng/mL FGF2 is added per well of 6-well plate. The cells are cultured in bFGF supplemented StemPro34 medium until Day 20 of differentiation. The media is changed daily.
18. On Day 20, old media is aspirated, and the cells are rinsed with D-PBS. The cells are cultured in E6 medium without additional supplementation for next 15 days. The media is changed daily.
19. The cells are cryopreserved at this stage. Immunostaining and qPCR is performed to detect expression of myogenic markers PAX3, PAX7 and MYF5.

Antibody name	**Distributor**	**Clone /Cat no.**
Anti-PAX3	Developmental Studies Hybridoma Bank	C2
Anti-PAX7	Life Technologies	PA5-23900
Anti-MYF5	Abcam	Ab125078

Table 15. List of different antibodies used to confirm expression myogenic markers by myogenic progenitor cells.

20. Repeated passaging and culturing of differentiated cells in E6 media allows expansion of PAX7 expressing myogenic progenitor cells.

3.7.3. Passaging the myogenic progenitor cells

1. Old medium is aspirated from the plate.

2. The cells are rinsed twice with 2 mL of D-PBS (w/o Ca^{2+} and Mg^{2+}) per well of 6-well plate.
3. 0.5 mL of TrypLE Select is added per well of 6-well plate. The cells are incubated at 37°C until for 5 min.
4. Detached cells are collected by adding 1 mL of neutralizing medium and the contents are transferred to 15 mL centrifuge tube.
5. The cells are centrifuged at 120 × g for 5 min.
6. The supernatant is aspirated, and the pellet is re-suspended in expansion media. The expansion medium is supplemented with RI prior to re-suspending the cells. Final concentration of RI is 10 μM. The media is filter sterilized after addition of RI. Usually 1:6 split ratio is optimum, and cells are 80% confluent in 48 to 72 hrs.
7. The cells are plated on matrigel-coated 6-well plate. The cells are not cultured in RI for more than 24 hrs.
8. Media is changed daily.

3.7.4. Cryopreserving myogenic progenitor cells

1. Old medium is aspirated from the plate.
2. The cells are rinsed twice with 2 mL of D-PBS (w/o Ca^{2+} and Mg^{2+}) per well of 6-well plate.
3. 0.5 mL of TrypLE Select is added per well of 6-well plate. The cells are incubated at 37°C until for 5 min.
4. Detached cells are collected by adding 1 mL of neutralizing medium and the contents are transferred to 15 mL centrifuge tube.
5. The cells are centrifuged at 120 × g for 5 min.
6. The supernatant is aspirated, and the pellet is re-suspended in freezing media (100 μL DMSO + 900 μL complete media).
7. The contents are transferred into cryovial and stored at -80°C.

3.7.5. Thawing myogenic progenitor cells

1. The cryovial is thawed in water bath adjusted to 37°C until a small piece of ice is visible in the cryovial.

2. The cells present in the freezing media are gently aspirated into 15 mL centrifuge tube.
3. Very carefully and in dropwise manner, 6 mL of expansion media is added along the walls of the tube. Expansion medium is supplemented with RI.
4. Media is filter sterilized after addition of RI.
5. The contents are gently mixed by inverting the tube.
6. Centrifuge at 120 × g for 5 min.
7. The supernatant is aspirated, and the pellet is re-suspended in 6 mL of expansion media. The expansion medium is supplemented with RI prior to re-suspending the cells. Final concentration of RI is 10 µM. The media is filter sterilized after addition of RI.
8. The cells are plated on matrigel-coated 6-well plate. The cells are not cultured in RI for more than 24 hrs.
9. Media is changed daily.

3.8. Cloning of synthetic intron within DsRed2 gene

1. DsRed2 gene was PCR amplified as two fragments using native DsRed2 gene as template. First four primers listed in table 15 were used for amplifying the gene.
2. Single stranded artificial intronic sequence was purchased from commercial firm.
3. Single stranded artificial intronic sequence was PCR amplified using primers "Intron FP and Intron RP" mentioned in table 15.
4. Intron was cloned between the two DsRed2 gene fragments using cyclic ligation assembly.
5. Cyclic ligation assembly was performed using two scaffold oligonucleotide connectors (SOC1 and SOC2). Sequence of these connectors is given in table 15. Reaction setup for cyclic ligation assembly is given in table 14. Ligation was performed using thermostable Taq ligase in a thermocycler.

Cycled Ligation Reaction		
Component	**Amount (µL)**	**Final Concentration**
20 nM Insert Mix	2	2 nM
200 nM SOC Mix	1	10 nM
10X Taq Ligase Buffer	2	1X
40 U/µL Taq DNA Ligase	2	80 U
H_2O	13	
Total	20	
Thermocycler Program:		
1. 95ºC 2 Min		

2.	**95ºC**	**30 Sec**
3.	**60ºC**	**2 Min**
4.	**GOTO 2**	**30x**
5.	**55ºC**	**10 Min**
6.	**4ºC**	**HOLD**

Table 16. List of components as well as thermocycler parameters used for cyclic ligation reaction.

6. The final ligation product was PCR amplified using primers DsRed2N1 FP (Hind III RS) and DsRed2N1 RP (XbaI RS) mentioned in the table 15.
7. Following PCR amplification, the amplified product was cloned in an expression plasmid.
8. Plasmid carrying the desired insert was transformed into chemically competent strain of bacteria. This step is used for amplifying the plasmid number.
9. Plasmid carrying desired insert was harvested from the transformed bacteria using commercial available plasmid isolation kit.

Primer name	**Sequence**
FP LF DsRed	5'-ATCTCGAGCTCAAGCTTCGAATTCTG-3'
RP LF DsRed	5'-CTTGTGGGTCTCGCCCTTCA-3'
FP SF DsRed	5'-GCCCTGAAGCTGAAGGAC-3'
RP SF DsRed	5'-CTCTACAAATGTGGTATGGCTG-3'
Intron FP	5'-GTAAGAGTGGTCCGATCGTCGC-3'
Intron RP	5'-CTGCAGGATATCAAAAAAGGGACAGG-3'
DsRed2N1 FP (Hind III RS)	5'-ATGCAAGCTTGCCACCATGGCCTCCTCCGAGAAC-3'
DsRed2N1 RP (XbaI RS)	5'-ATGCTCTAGACGGCCGCTACAGGAACAGGTG-3'
SOC1	5'-TGAAGGGCGAGACCCACAAGGTAAGAGTGGTCCGATCGTC-3'
SOC2	5'-CCTTTTTTGATATCCTGCAGGCCCTGAAGCTGAAGGACGG-3'
Sequence of artificial intron sense strand	5'GTAAGAGTGGTCCGATCGTCGCGACGCGTCATTACTAACTATCAATACTTAATCCTGTCCCTTTTTTGATATCCTGCAG-3'

Table 17. List of primers used for cloning intron within DsRed gene.

4. Results

Cells isolated from various tissues of the body can be used for cellular reprogramming to obtain pluripotent stem cells. However, the reprogramming efficiency varies considerably among the cells isolated from different tissues. Dermal fibroblasts followed by white blood cells are the two most commonly used cell sources for reprogramming. Due to certain shortcomings, like invasive isolation procedure, low reprogramming efficiency or presence of genetic mutation; many cell lines cannot be used for large-scale generation of patient-specific pluripotent stem cells.

Cells have inherent mechanisms hindering the process of reprogramming. These mechanisms are especially prevalent in somatic cells. While the stem cells have a more reprogramming favourable environment and are considered efficient in undergoing reprogramming. In this research, we used dental pulp stem cells (DPSC) and dermal fibroblasts cell lines for reprogramming. DPSC cell lines from different age groups were selected. Out of this selected group, one of the DPSC cell line was isolated from natal tooth. Interesting fact was noted about this cell line when the phenotypic data was compared with the adult DPSC. Natal DPSC expressed certain markers which were not commonly expressed by DPSC. These results prompted us to include this cell line for reprogramming.

Before starting the reprogramming experiment, all the cell lines were screened for mycoplasma. After confirming that the cell lines are free of mycoplasma further experiments were performed. Reprogramming experiment was carried out in serum free, feeder free and in completely defined culture conditions. Culture conditions needed to adapt the DPSC to the Sendai virus reprogramming protocol were optimised.

4.1. Screening of cell lines for presence of mycoplasma

While searching through various methods of mycoplasma screening, I found PCR based detection method to be more sensitive and efficient. Many ready to use kits are available for performing the screening. However, these are expensive and most of the components are proprietary which makes troubleshooting difficult. Considering these facts, I decided to adapt a protocol that was sensitive and easily reproducible. I found these features in Dr. Uphoff protocol (Uphoff et al. 2002). While

performing the screening using Dr. Uphoff's protocol, I found that few modifications could be introduced.

The original protocol consisted of four steps sample collection, DNA isolation, PCR and gel electrophoresis. While following this protocol I noticed that, no mycoplasma DNA was getting isolated from the cell culture supernatant. After careful analysis, it was clear that either the load of mycoplasma infection was low or the volume of collected sample was low. In order to circumvent these problems, I decided to directly perform PCR on cell culture supernatant. This procedure is routinely performed in bacteriology. This method is known as colony PCR; in which a bacterial colony is picked and is placed in PCR mastermix followed by PCR.

Performing colony PCR was the solution but the conditions were not the same as routine colony PCR. Cell culture supernatant contains fetal bovine serum (FBS) which inhibits polymerase activity. Robust polymerase enzyme was needed to overcome the inhibitory effect of FBS. Hence, I used One Taq Polymerase enzyme (New England Biolabs) which was known for its robustness. This was the second modification introduced in the original protocol.

After introducing these two modifications the sensitivity and robustness of the original protocol was enhanced by several folds.

For performing PCR combination of seven forward and three reverse primers were used. These sequences are same as those mentioned by Dr. Uphoff in his original protocol. Even though One Taq polymerase is robust it can tolerate FBS upto a certain limit. I optimised the concentration of cell culture supernatant and found out the range of cell culture supernatant volume that can be tolerated by the polymerase enzyme (Fig. 3).

Interpretation of gel electrophoresis results. Presence of band around 500 bp mark indicates mycoplasma contamination while the band corresponding to 1000 bp is the internal control. A lane with two bands, one at 500 bp and other at 1000 bp indicates positive sample, while presence of only one band at 1000 bp indicates negative sample. If no band is visible, it indicates false negative result, which might be due to presence of inhibitors in the cell culture supernatant.

For finding the optimum concentration of cell culture supernatant, different volumes like 1 µL (lane 2), 2 µL (lane 3), 3 µL (lane 4), 4 µL (lane 5), 6 µL (lane 6) and 8 µL (lane 7) were selected

(Fig. 3). Amplified PCR product was detected until 4 µL of cell culture supernatant, this limit can be regarded as the peak threshold of tolerance. Careful observation of the gel electrophoresis image revealed that 1 µL volume of cell culture supernatant had minimum inhibitory effect. Moreover, the band intensity was also the brightest indicating maximum enzyme activity.

In summary, we can say that the modifications applied to the original protocol were not only able to retain the detection capability of the original protocol but also enhanced its sensitivity, and robustness. In addition, direct use of cell culture instead of DNA isolation reduced the overall time required for performing the screening.

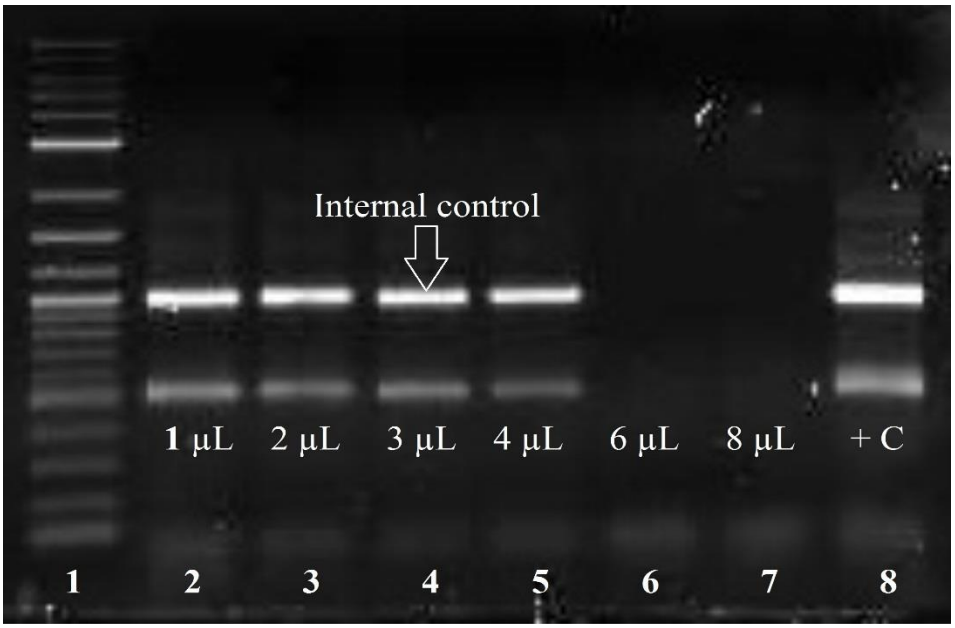

Fig. 3. Determining FBS tolerance limit of One Taq polymerase. Lane 1, DNA ladder; lane 2 -7, different volumes of cell culture supernatants, starting from 1 µL (lane 2) to 8 µL. Lane 8, positive control (+ C). Band corresponding to 968 bp is the internal control while the band below internal control corresponding to 510 bp depicts presence of mycoplasma.

After optimising all the parameters, the finalised protocol was put to test. Total five cell lines were chosen for analysis. Appropriate controls were also run along with the test samples; these include only internal control, water control and positive control. Out of the five samples, three cell lines were of human origin (human fibroblasts) while one was of murine origin (Fig. 4). All three human fibroblasts cell lines used were isolated in house from biopsy samples. Presence of band at 500 bp position in the positive control lane (lane 8) confirmed working of optimised conditions. Analysis

of the gel electrophoresis image further revealed that all the three human fibroblasts cell lines were infected with mycoplasma (Fig. 4). Band intensity gives us an idea about the level of contamination. Lane 3 and 4 represents heavily contaminated cell lines while the load of contamination is low in lane 2.

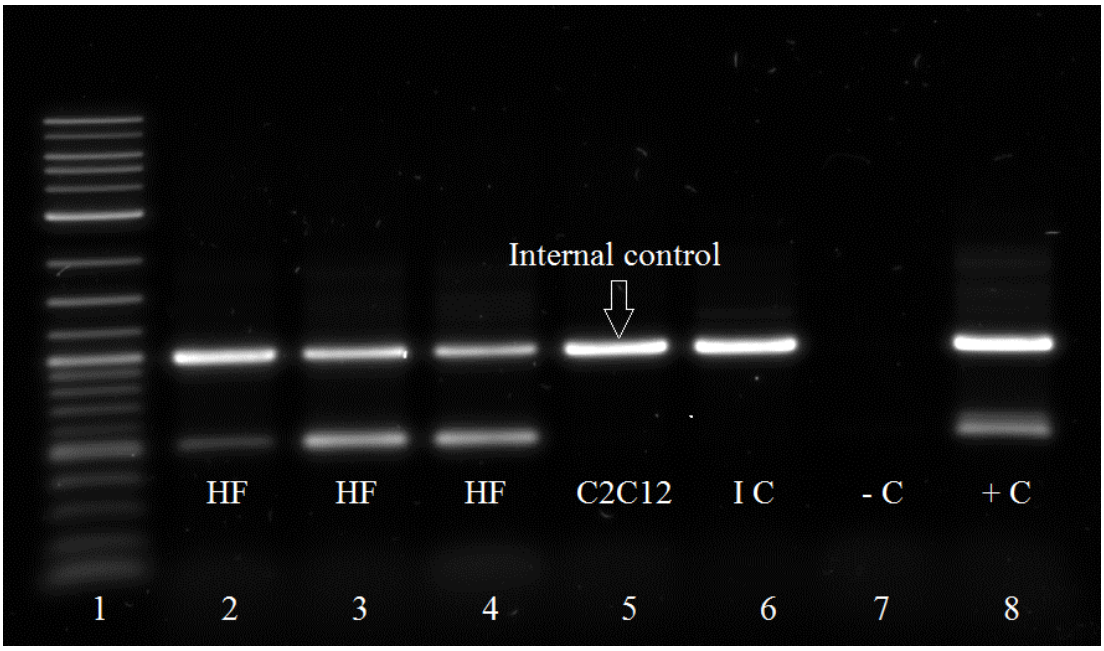

Fig. 4. Analysing various cell culture supernatant to ascertain whether the standardised parameters are functioning. Lane 1, DNA ladder; lane 2-4, cell culture supernatants from three different human fibroblasts samples; (lane 5) cell culture supernatants from murine myoblasts C2C12; (lane 6) internal control only (IC); (lane 7) negative control (- C) and (lane 8) positive control (+ C). 1 µL of cell culture supernatant was used for analysis. Band corresponding to 968 bp is the internal control while the band below internal control corresponding to 510 bp depicts presence of mycoplasma.

As previously mentioned in the introduction, screening of cell lines prior to reprogramming is essential. I choose to screen all the cell lines routinely used in our lab. This is to be sure that all the cell lines currently been used are mycoplasma free in order to eliminate the possibility of accidently introducing the mycoplasma infection in the middle of the reprogramming experiment.

For reprogramming experiment, seven cell lines were screened. As mentioned in the above paragraph, optimised volume of cell culture supernatant (1 µL) and PCR conditions (mentioned in materials and method) were used. The result of mycoplasma detection is presented in Fig. 5. In

order to rule out false negative results, an internal control was introduced. Internal control band amplifies at ~ 1000 bp while mycoplasma specific band appears at ~ 500 bp. Since, there was no mycoplasma specific band visible at around 500 bp, the cell lines were considered free of any mycoplasma infection.

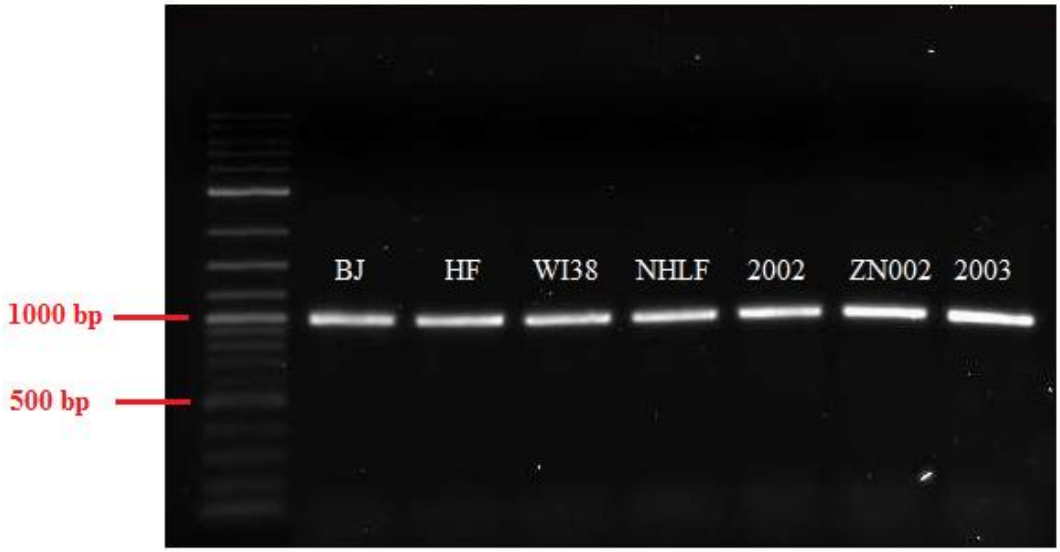

Fig. 5. Screening of cell lines for detection of mycoplasma contamination. Cell culture supernatant was directly added to PCR mastermix and the template amplification is performed followed by gel electrophoresis.

4.2. Reprogramming of DPSC and human fibroblasts cell lines to induced pluripotent stem cells

After confirming absence of mycoplasma in all the cell lines, each cell line was passaged at least twice before transducing with Sendai reprogramming viruses. This ensured that the cells were stable and actively proliferating. Cell lines from early passages were chosen, so that majority of the cells were in log phase and possibility of chromosome aberration was low. Except for WI38 human fibroblasts, all the rest of the cell lines i.e. natal DPSC (nDPSC), three adult DPSC and HF were actively proliferating, and their doubling time was in between 18 to 24 hours. Actively proliferating cells yield good reprogramming efficiency because each cell division involves disassembly and assembly of chromatin, which makes it easier for the reprogramming factors to bind the promoters of endogenous genes *OCT4*, *SOX2* and *NANOG;* activation of these genes is essential to attain pluripotency.

Isolation and characterization of DPSC was done in collaboration with Dr. Jakub Suchanek and Dr. Tomas Soukup. Each newly isolated DPSC cell line is first expanded to sufficiently large population of cells. Alongside, population-doubling time is also simultaneously determined. Phenotypic characterization is done at the last using standardised panel of markers. Phenotypic characterization is important because it helps to ascertain the type of cell population that has been enriched during the course of expansion. This is especially important in case of DPSC because they are isolated from dental pulp, which houses various type of cell population.

4.2.1. Characterization of dental pulp stem cells

Visual inspection through microscope reveals a heterogeneous population of cells within the DPSC culture with cells expressing fibroblastic morphology more prevalent (Fig. 6). Occurrence of tooth at birth is a very rare event and occurs 1 in 3500 births. Considering this rarity, we thought of comparing population doubling and phenotypic characters of natal DPSC (nDPSC) with their adult counterparts. For the comparative study, three adult DPSC cell lines were chosen; average age of the donor was in between 16 to 20 years. Surprisingly, rate of doubling was slightly different for nDPSC during initial passages (i.e. from 2 to 6) as compared to adult DPSC during the same passages (Fig. 7). Doubling time ranged from 21 to 25 hr for nDPSC while adult DPSC had high variation and ranged from 22 to 58 hr.

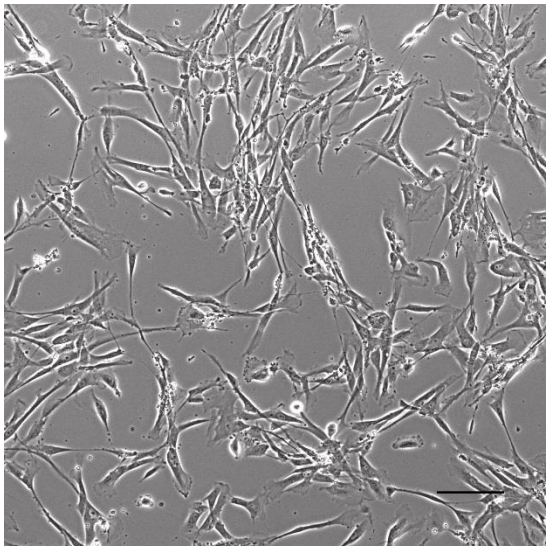

Fig. 6. Bright field image of nDPSC. The cells expressing fibroblastic morphology are the most predominant while few rounded or hexagonal cells are also visible. Scale bar = 200 μm.

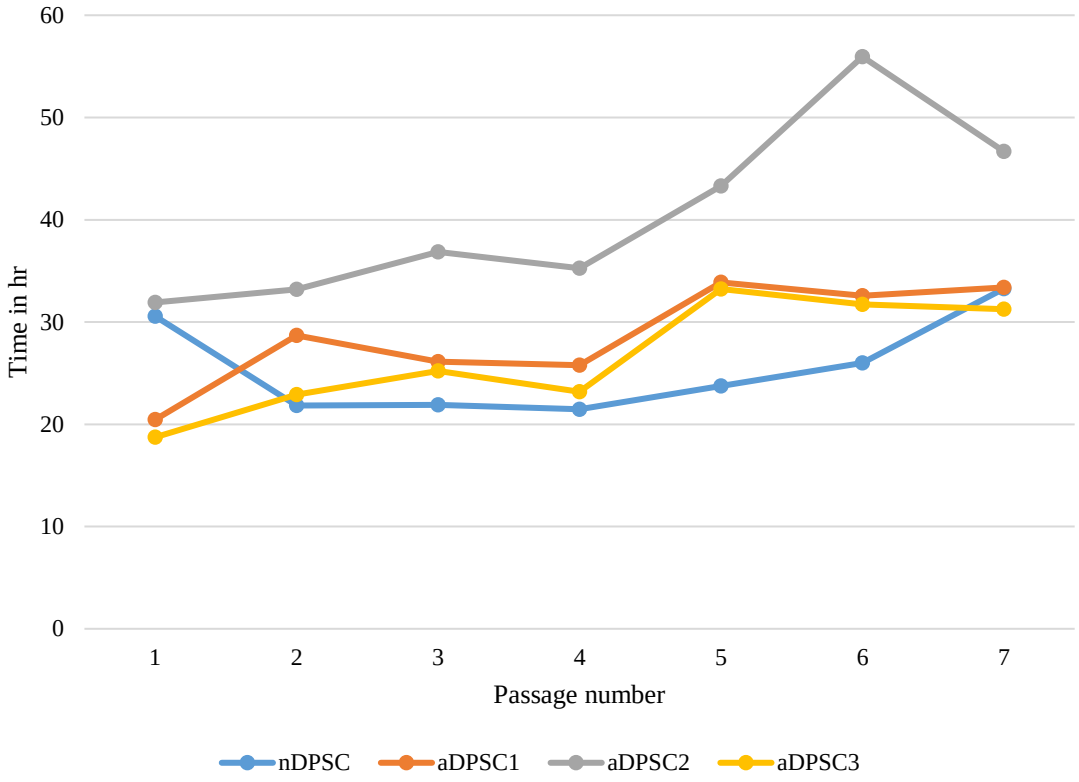

Fig. 7. Graph representing doubling time of nDPSC and adult DPSC measured at different passages. nDPSC had lower doubling time from passage 2 to 6; as compared to three adult DPSC.

Phenotypic characterization was done using flow cytometry (FC). Majority of these are cell surface markers. Based on our research and data published elsewhere, DPSC are positive for CD73, CD90, CD105, CD13, CD29, CD44 and HLA-A, B and C while characteristically negative for CD45 and CD34 (Table 1). Other less prominent markers that DPSC do not express are CD271, CD71, CXCR4 and HLA-DR.

Upon comparing the results of nDPSC and adult DPSC, some interesting and surprising observations were noted. Apart from expressing characteristics DPSC markers, nDPSC showed positive for CD34 and CD45 (Table 18); these markers are characteristic of haematopoietic stem cells. This observation might be explained by the fact that the culture is heterogeneous in nature and the cells were isolated from very young age of development, due to which they are very immature, hence expressing broad spectrum of markers.

Markers	nDPSC	adult DPSC
CD34	+++	-
CD45	+++	-
CD271	+++	+
CD71	+++	+
HLA-DR	+++	-
CD146	+++	-
CXCR4	+++	++
CD29	+++	+++
CD105	+++	+++
CD222	+++	+++
CD166	+++	+++
CD44	+++	+++
CD90	+++	+++
CD10	+++	+++
CD13	+++	+++
CD73	+++	+++

Table 18. Standard panel of markers used for characterizing DPSC. nDPSC express markers characteristic of DPSC. In addition, they also express hematopoietic stem cell markers CD34 and CD45, which are normally not expressed by DPSC. Adult DPSC column has been compiled using combination of our experimental data as well as from data published elsewhere. This table is taken from my article Pisal et al. 2018.

4.2.2. Comparing expression of pluripotent genes between human fibroblasts and nDPSC

Pluripotency genes are expressed at the basal level by all somatic cells. Before proceeding for reprogramming we thought of analysing expression of pluripotency genes in few cell lines. This would help to draw rough conclusion as to whether expression of certain pluripotency genes before the start of reprogramming has any impact on reprogramming efficiency. For this study, expression of 17 pluripotency genes was compared between nDPSC and two human fibroblasts (HF) (Fig. 8 and 9). Relative quantification method was the method of choice and $2^{-\Delta\Delta CT}$ formula was used to calculate the fold change. Beta actin was used to normalise the expression data and hESC was used as calibrator to calculate fold change.

Analysis of this comparison revealed a clear distinction in expression pattern between nDPSC and HF (Fig. 8). Within this set of 20 genes, *OCT4*, *SOX2* and *KLF4* were included; these are three out of four factors that were used for reprogramming. Expression of *OCT4* and *SOX2* was similar but expression of *KLF4* was significantly higher in nDPSC. Another important group of genes is the core pluripotency group, which comprises of *OCT4*, *SOX2* and *NANOG*. These genes were expressed at the basal level in both the cell lines. Continuing with remaining 16 genes; *TBX3* (Fig. 9), *NR5A2* and *RUNX2* were significantly upregulated in nDPSC while *COMMD3* and *IL6ST* were upregulated by more than 3 times with the exception of *IL6ST* in WI38. Rest of the genes were expressed at similar levels across nDPSC and two HF.

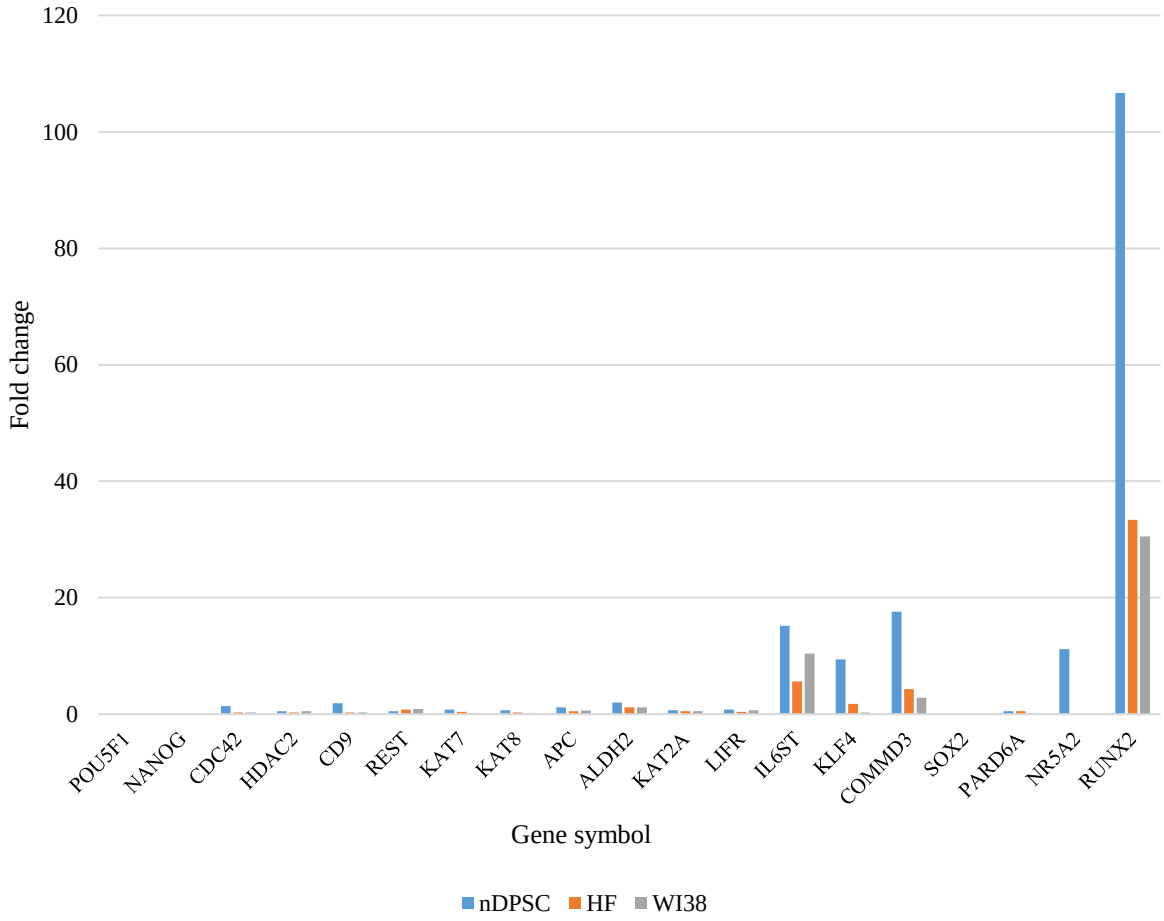

Fig. 8. Graph represents expression of pluripotency genes in non-reprogrammed nDPSC, human fibroblasts and WI38 human embryonic lung fibroblasts. NR5A2, RUNX2 and COMMD3 are significantly upregulated in nDPSC as compared to two human fibroblasts. Moreover, KLF4 (one of the factors used for reprogramming) is also upregulated in nDPSC.

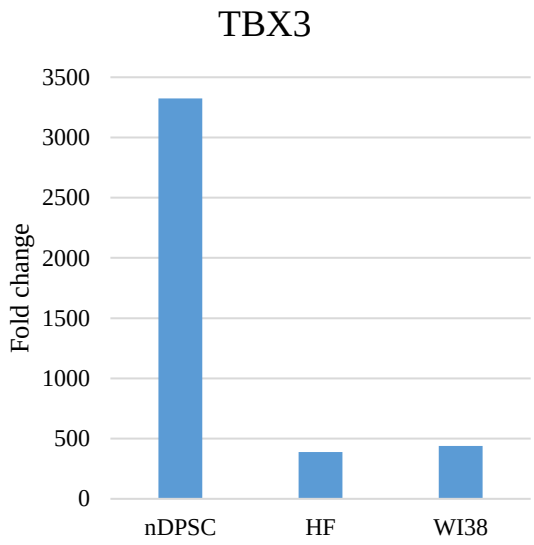

Fig. 9. Graph represents expression of TBX3 genes in non-reprogrammed nDPSC, human fibroblasts and WI38 human embryonic lung fibroblasts. TBX3 is significantly upregulated in nDPSC as compared to two human fibroblasts. There is 10-fold difference in the expression between nDPSC and two human fibroblasts.

4.2.3. Effect of Sendai virus transduction on nDPSC, adult DPSC and human fibroblasts

Total six cell lines i.e. nDPSC, three adult DPSC and two human fibroblasts were used for reprogramming. These cell lines were cultured in their respective medium until specific time point; there after Essential 8 medium was used for propagation and expansion of reprogrammed cells. These details of specific time points and culture conditions are mentioned in Materials and methods.

Cells were transduced with Sendai virus reprogramming vectors after the cells had reached approximately 80% confluency. Out of the four DPSC cell lines two cell lines were derived from female donors (nDPSC and one adult DPSC) (Fig. 10C and D) and two from male donors (Fig. 10A and B). Varying degree of cytotoxicity was observed amongst the cell lines after virus transduction. Adult male DPSC cell lines showed massive cell death and the cells were not able to revive from this cytotoxic shock. Hence, only handful of cells survived in these two cell lines. While in female DPSC cell lines, the severity of cell death was comparatively less, and remarkably high number of cells survived. Between the two human fibroblasts, the commercially available

cell line WI38 (Fig. 10F) showed least cell death while high cell death was observed in in-house isolated human fibroblasts (HF) (Fig. 10E).

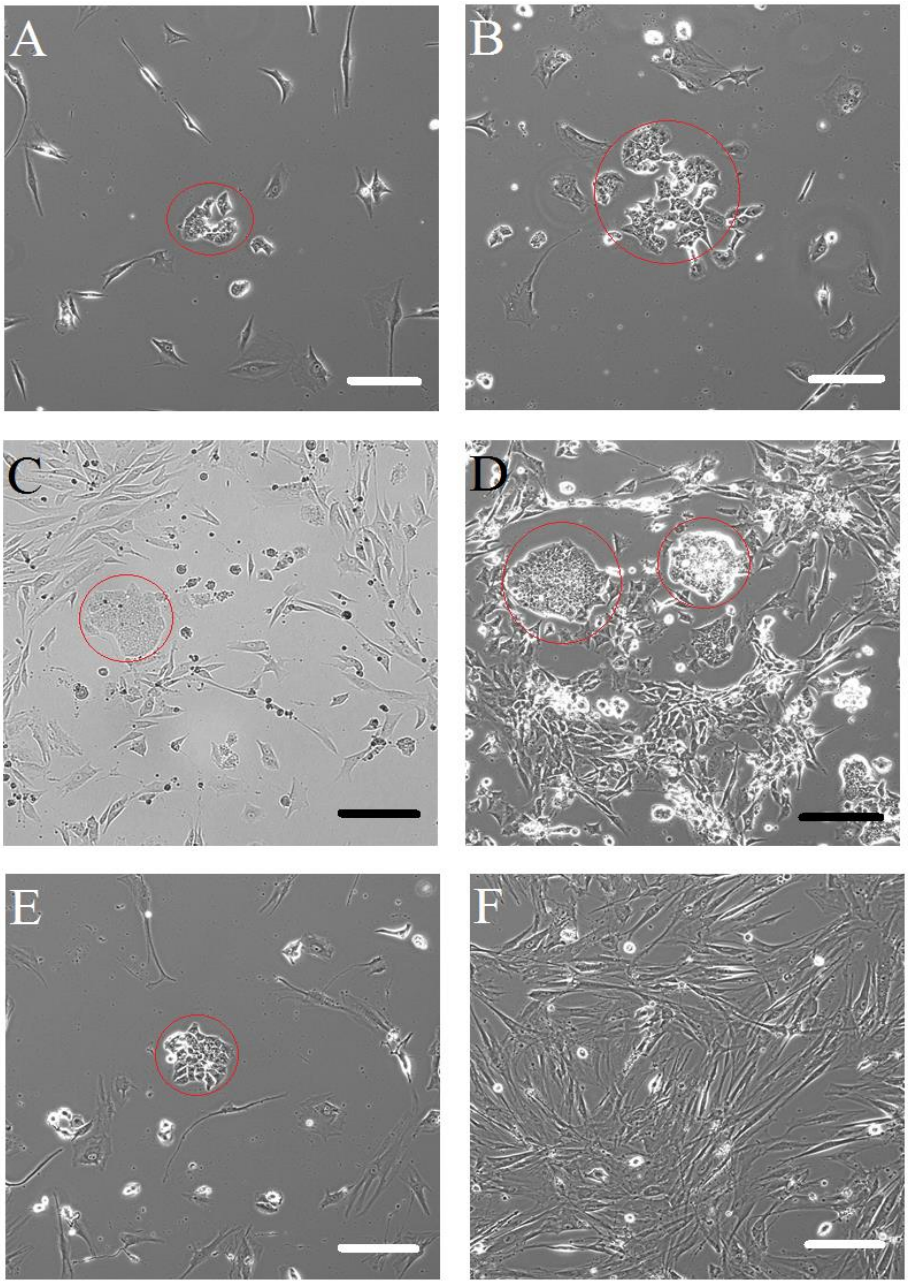

Fig. 10. Brightfield images of nDPSC, adult DPSC and human fibroblasts 14 days post transduction. (A and B) are of male adult DPSC, (C) of female nDPSC, (D) of female adult DPSC, (E) of in-house isolated human fibroblasts (HF) and (F) of WI38 human fibroblasts. Cell lines depicted in Fig. C, D and F had higher survivability as compared to Fig. A, B and E. Red circle is used for highlighting emergence of induced pluripotent stem cells colonies. Scale bars = 200 µm.

4.2.4. Reprogramming efficiency of nDPSC, adult DPSC and human fibroblasts

Each cell line has a unique combination of chromatin signature and inhibitory factors, which significantly influences the ability of the cell to undergo reprogramming from differentiated state to pluripotent state. The results that will be presented below will truly reflect this fact because each cell line which underwent reprogramming showed significant variation in the reprogramming efficiencies.

Reprogrammed colonies start emerging at approximately 12 days post transduction. Preliminary sign of reprogrammed cells is seen in form of closely packed cluster of rounded cells Fig. 10 A-E. Alkaline phosphatase (AP) is one of the early markers expressed by reprogrammed cells. This marker is commonly used for determining reprogramming efficiency. Colorimetric and immunostaining are two available staining methods for detecting AP. Colorimetric method is based of addition of chromogenic substrate, which reacts to produce an insoluble precipitate. Depending on the type of substrate used, colonies stain either red (Naphthol AS-MX) or purple (NCP-BCIP). Reprogramming efficiency is determined by using following formula:

$$\frac{\text{Number of Alkaline phosphatase positive colonies}}{\text{Total number of transduced cells plated}} \times 100$$

NBT-BCIP substrate was used for staining AP. For determining reprogramming efficiency, 75,000 cells (Sendai virus transduced) of each cell line were plated in a 12-well plate. Two adult male DPSC cell lines, which had shown severe cell death, showed the lowest reprogramming efficiency (Fig. 11A and B). Hardly eight very tiny AP positive colonies appeared in one of adult male DPSC cell line (Fig. 11A); while twelve clearly visible colonies were observed in other adult male DPSC cell line (Fig. 11B). Adult female DPSC cell line (Fig. 11C) displayed more than 3000 AP positive colonies. Out of the six cell lines used for reprogramming this particular cell line displayed maximum number of reprogrammed colonies. nDPSC grabbed the second position by displaying 966 AP positive colonies (Fig. 11D).

In-house isolated human fibroblasts showed 550 AP positive colonies (Fig. 11E). Embryonic lung human fibroblast cell line WI38 showed only 12 AP colonies (Fig. 11F). I expected this cell line to show higher number of reprogrammed colonies because this cell line showed negligible cytotoxicity upon Sendai virus transduction; also, the cell line better adapted to media transition

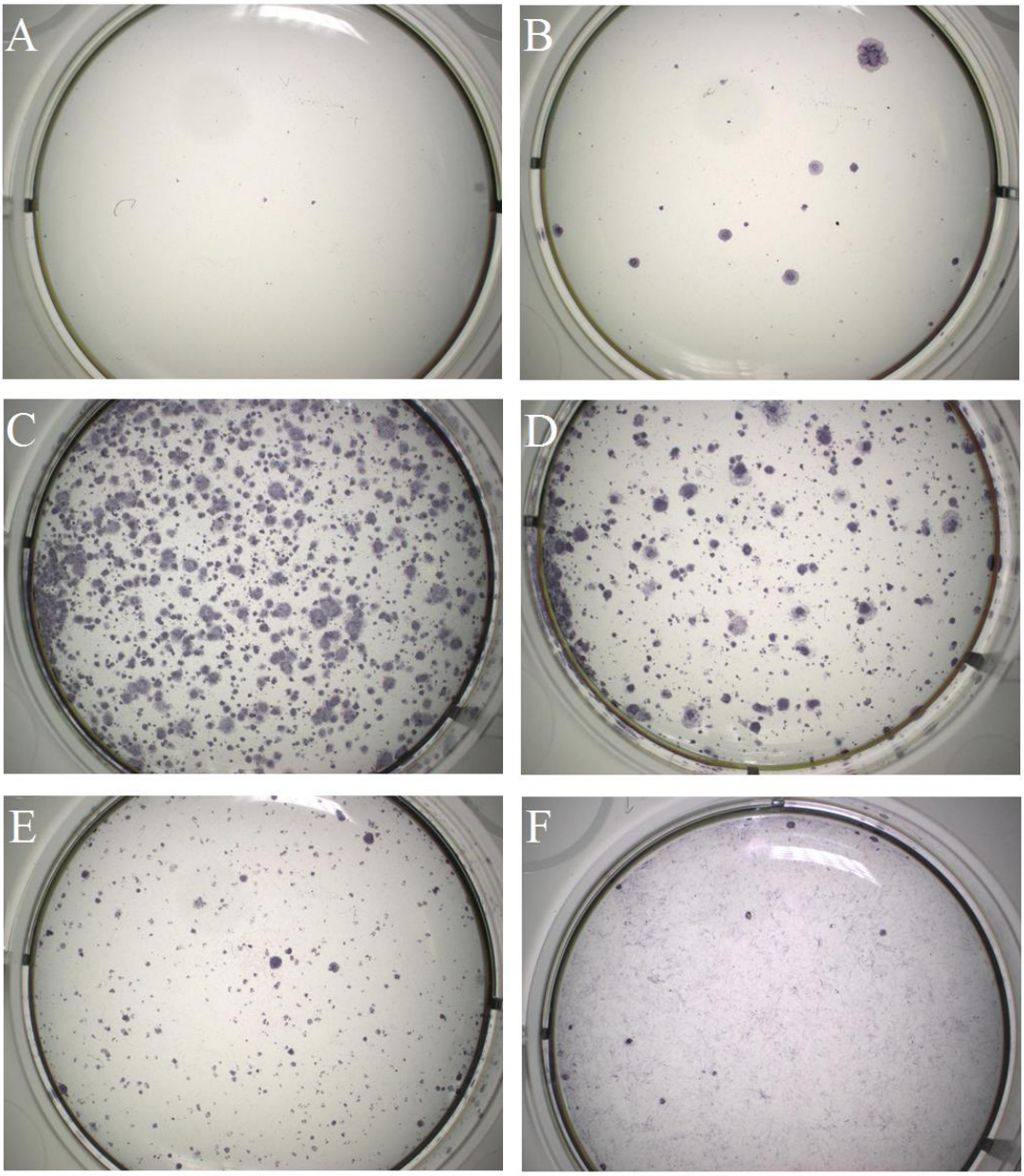

Fig. 11. Alkaline phosphatase staining of reprogrammed colonies using NBT-BCIP solution. Images A and B are from adult male DPSC, C and D are from adult female DPSC and nDPSC respectively and E and F from HF and WI38 respectively. Adult male DPSC showed lowest reprogramming efficiency (A and B) followed by human fibroblasts (E and F). Adult female DPSC showed maximum reprogramming followed by nDPSC (C and D).

(i.e. media change from regular culture media to Essential 8 media) which was not the case with other cell lines. In other cell lines, non-reprogrammed cells started slowly dying after media transition.

To summarise, the percentage of reprogramming efficiencies displayed by all the cell lines used for reprogramming are adult female DPSC >4 % (Fig. 11C), nDPSC 1.3% (Fig. 11D), in-house human fibroblasts 0.75 % (Fig. 11E), adult male DPSC 0.016 % (Fig. 11B), WI38 0.016 % (Fig. 11F) and adult male DPSC 0.0106 (Fig. 11A). Another important point to mention was that reprogrammed colonies appeared earlier in DPSC than the fibroblasts.

4.3. Characterization of reprogrammed iPSC clones

Characterization of iPSC colonies is an essential step because not all colonies undergo complete reprogramming. Many colonies displaying human embryonic stem cell (hES) morphology are partially reprogrammed because they fail to activate the entire pluripotency network; which directly influences their differentiation ability into three germ layers. Detection of pluripotency markers and trigeminal differentiation capacity are two methods used for characterizing newly isolated iPSC clones. OCT4, SOX2, NANOG (core pluripotency factors), TRA 1-81 and SSEA-4 are the basic pluripotency markers that are initially examined. Expression of these markers is done at the RNA (gene expression analysis) and protein level. Quantitative real time PCR is commonly used method for studying gene expression analysis while immunostaining and western blotting for proteins. On passing the preliminary screening, iPSC clones are subjected to three germ layer differentiation capacity. Embryoid body differentiation, targeted differentiation and teratoma formation are the methods of choices. The former two are done in-vitro while the later requires injection of iPSC into mice (immunocompromised or nude mice in case of human iPSC).

In my experiment, reprogramming was carried out in feeder free and serum free conditions. Recombinant vitronectin was used for coating the culture dishes while Essential 8 medium was used for expansion and propagation of iPSC clones. For characterization, few clones from nDPSC, in-house isolated human fibroblasts (HF) and WI38 human fibroblasts were chosen. As mentioned above, all the clones were first screened for basic pluripotency markers. Following which, gene expression of 83 carefully selected genes, which included pluripotency genes, early differentiation genes and somatic cell specific genes were analysed. One of nDPSC derived iPSC clone was subjected to embryoid body mediated spontaneous differentiation. Targeted differentiation into hepatic endoderm, cardiomyocytes and neuro-epithelium was performed in iPSC clones derived from nDPSC, HF and WI38.

From each reprogrammed cell line, maximum possible number of clones was picked. Forty clones were picked from nDPSC, twenty from HF and nine from WI38. nDPSC yielded numerous iPSC colonies, but only select few clones were picked because the process of colony expansion and characterization is expensive, time consuming and laborious. HF also yielded many colonies but only few survived; most of them spontaneously differentiated and had to be discarded. WI38 being least efficient in reprogramming formed only nine colonies. Before characterization, each clone was expanded for minimum five passages to ensure complete activation of pluripotency network; this yields a stable clone and reduces the possibility of spontaneous differentiation.

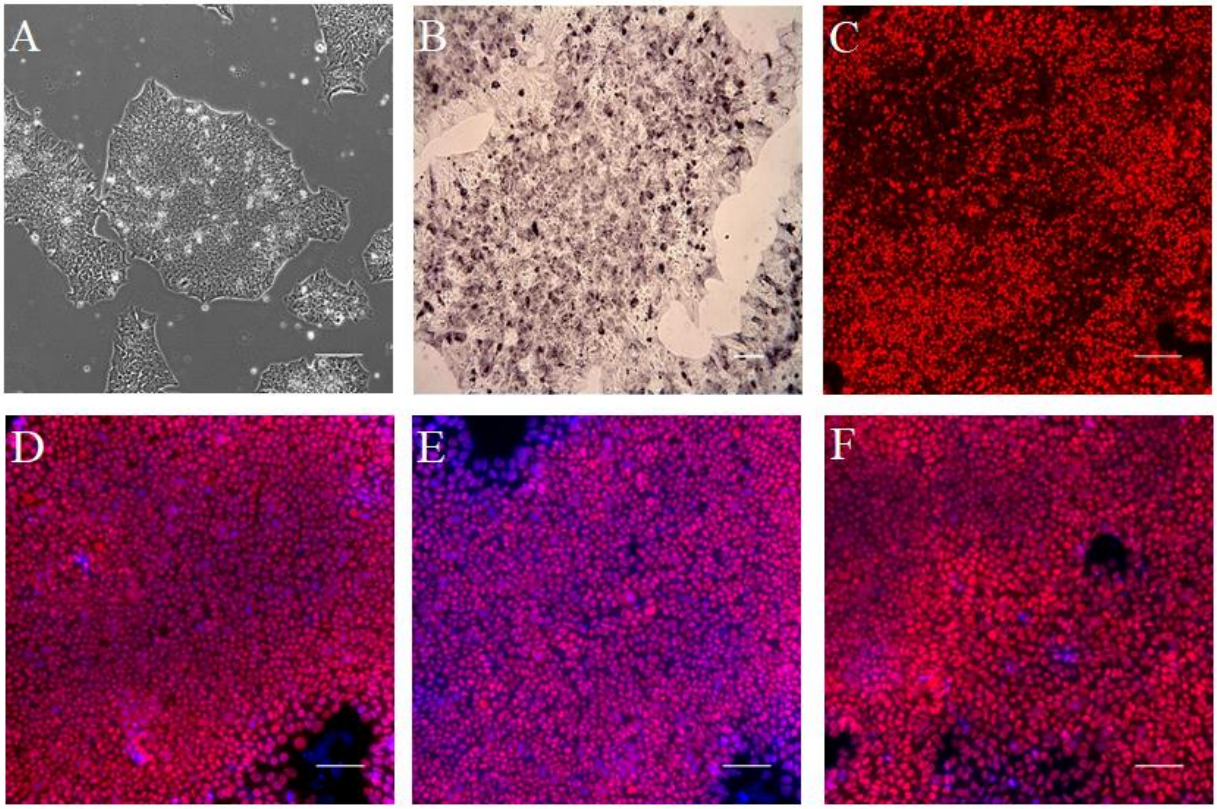

Fig. 12. Characterization of iPSC clone derived from nDPSC. Reprogrammed cells proliferated as tightly packed colony; which is identical to that observed in ES cells (A). Colonies were intensely positive for cell surface markers, alkaline phosphatase and SSEA 4 respectively (B and C). Core pluripotency factors (OCT4, SOX2 and NANOG) were highly expressed and were localised to the nucleus (D-F). Nuclei were counterstained with DAPI. Images are shown as overlap of two channels (D-F). Scale bar = 200 µm.

For detail characterization one clone each from nDPSC, HF and WI38 cell lines was selected. All the selected clones formed colonies of tightly packed cells which resembled the characteristic

morphology expressed by hES cells (Fig. 12A and Fig. 13C and F). Embryonic stem cells (ES) are the natural counterparts of induced pluripotent stem cells; moreover, they are used as gold standard for confirming pluripotency in iPSC. Stage specific embryonic antigen 4 (SSEA-4), a cell surface marker expressed only by non-differentiated human pluripotent stem cells was highly expressed in nDPSC iPSC clone (Fig. 12C); based on the results of immunocytochemistry. Moreover, immunostaining for anti-OCT4, anti-SOX2 and anti-NANOG in nDPSC iPSC clone resulted in strong nuclear localised signal (Fig. 12D-F); nuclear localised isoforms of OCT4 SOX2 and NANOG are the isoforms required for maintaining pluripotency. This indicates that appropriate isoforms of OCT4, SOX2 and NANOG are expressed in nDPSC iPSC clone. In HF and WI38 only OCT4 and SOX2 markers were assessed. Like nDPSC iPSC clone they also expressed strong nuclear localised signal (Fig. 13A-B and D-E).

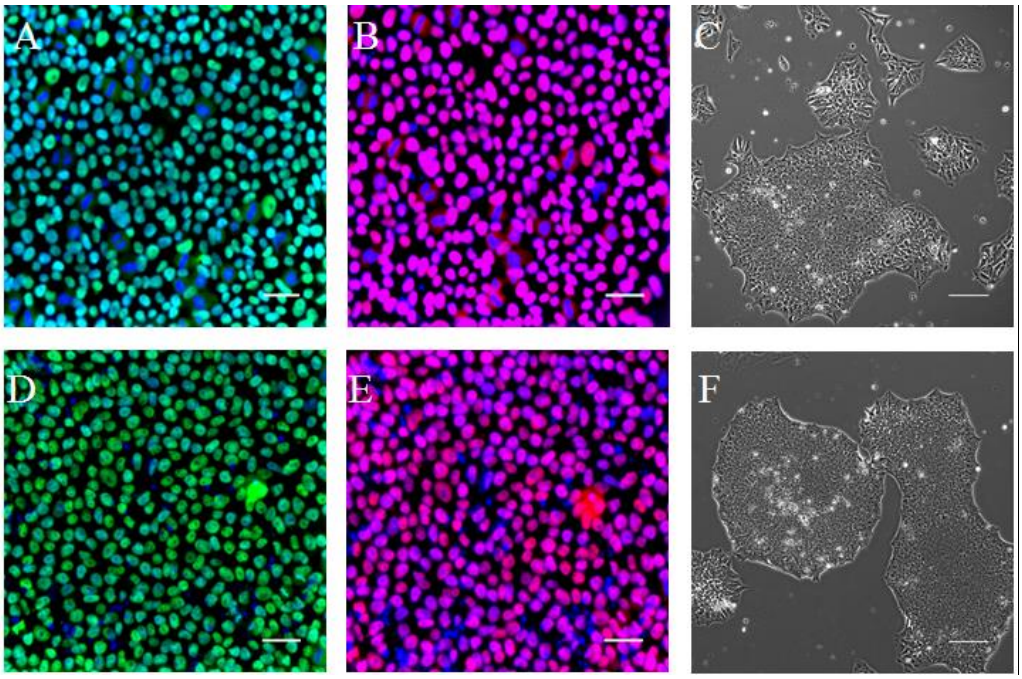

Fig. 13. Characterization of iPSC clones from HF and WI38 human fibroblasts. Images of HF and WI38 derived induced pluripotent stem cells displayed morphology typical to hESC colony (C and F). Immunocytochemistry of HF derived iPSC showed strong positivity for OCT4 and SOX2 (A-B). Similar results were observed for WI38 derived iPSC for OCT4 and SOX2 (D-E). Nuclei were counterstained with DAPI. Images are shown as overlap of two channels (A-B and D-E). Scale bar = 200 μm.

After preliminary characterisation, each clone was subjected to comprehensive gene expression analysis. As mentioned above, 83 genes were analysed. The analysis was performed by

quantitative PCR using SYBR detection platform. Relative quantification method was chosen for analysing qPCR data and within relative quantification ΔΔ Ct method was used. $2^{-\Delta\Delta Ct}$ formulae was used for calculating fold change. ACTB (beta actin) gene was used for normalising the data while hES was used as calibrator sample. The data was presented as $\log_2$ value of fold change. Data is represented in form of continuous line graph depicting the trends observed in gene expression across the three clones. Value close to zero indicates very subtle difference in gene expression between iPSC clone and hES. Positive values indicate overexpression and negative value indicates under expression. These values are relative to values expressed by hES.

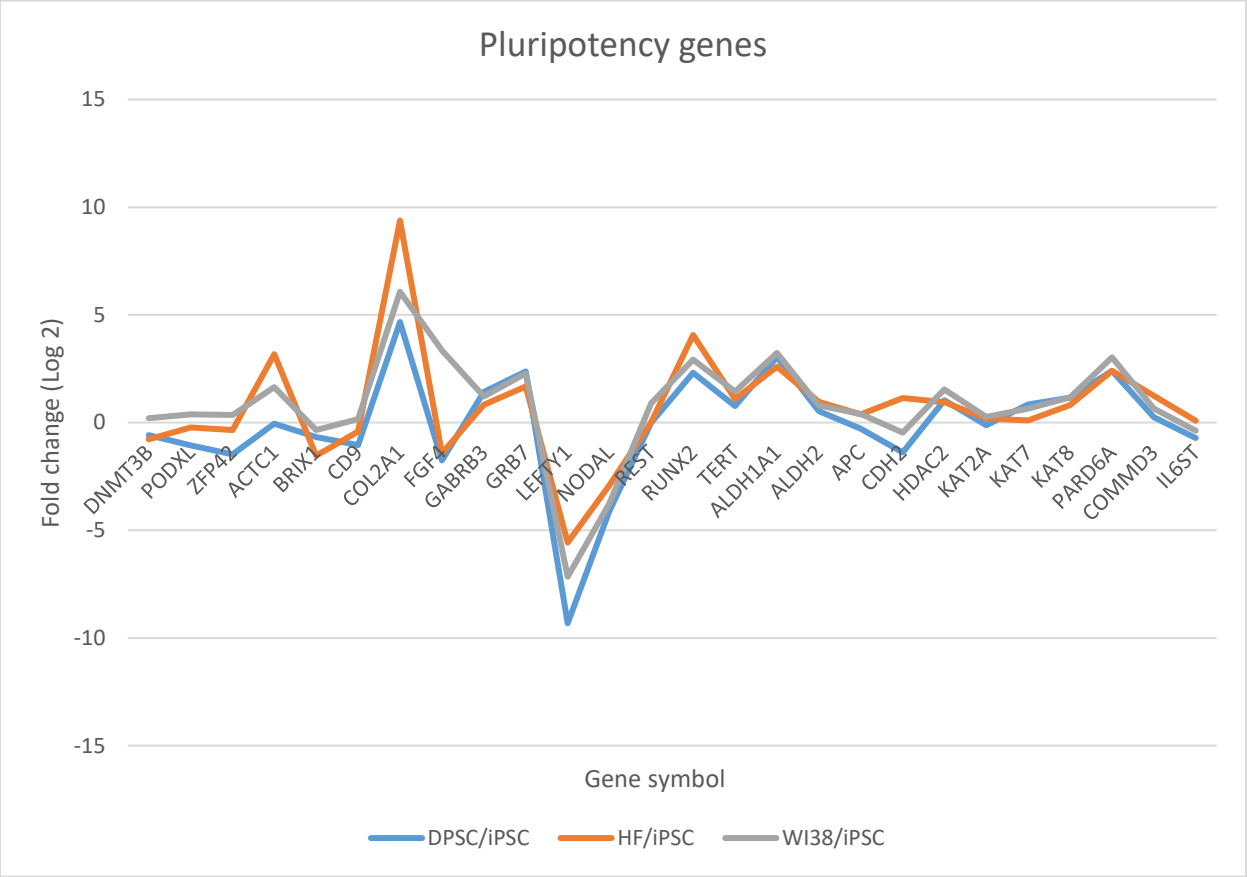

Fig. 14. The graph represents expression of first 26 set of pluripotency genes out of 52 genes in nDPSC/iPSC, HF/iPSC and WI38/iPSC relative to hES. Data is presented as $\log_2$ values of fold change. Positive value indicates up expression as compared to hES while negative values indicated down regulation. Across all the iPSC clones derived from different cell types, the trend of gene expression is very similar. Out of 52 pluripotency genes, 26 are presented in this graph while remaining 26 are presented in the Fig 15.

The trend of graph clearly indicates that expression of pluripotency genes starting from *DNMT3B* until *COMMD3* (Fig. 14) is very similar across all the three clones derived from three different cell types. Fold change of 5 whether positive or negative is considered as the tolerance limit (Takahashi et. al. 2007). The expression falling within this range is considered as similar or comparable to that observed in hES. Any value falling out of this range is either considered as up regulated (if it is positive value) or down regulated (if it is negative value) depending upon the sign it bears. *COL2A1* is up-regulated in iPSC clones while *NODAL* is down regulated relative to hES (Fig. 14).

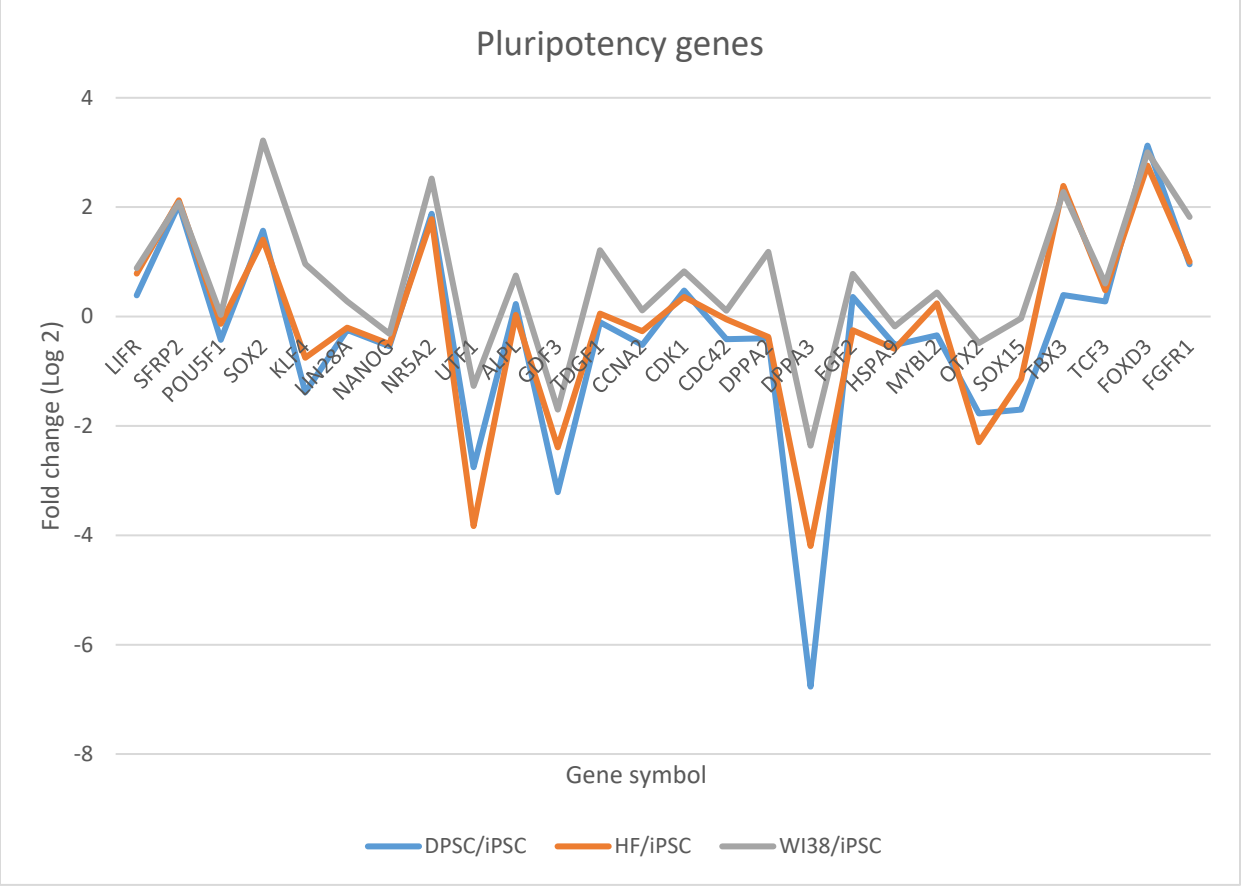

Fig.15. The graph represents expression of last 26 set of pluripotency genes in nDPSC/iPSC, HF/iPSC and WI38/iPSC relative to hES. Data is presented as $\log_2$ values of fold change. Positive value indicates up expression as compared to hES while negative values indicated down regulation. Across all the iPSC clones derived from different cell types, the trend of gene expression is very similar.

Like the above graph, the trend of remaining 25 set of pluripotency genes is very similar between the clones except for *FGF2* in nDPSC iPSC clone; in which the gene is down regulated by more

than 5 folds (Fig. 15). *POU5F1* (*OCT4*) and *NANOG* expression values are very close to zero and the lines representing different clones almost overlap (Fig. 15). This signifies that expression of these two factors is almost identical to each other as well as to hES. Expression of *SOX2* is slightly higher in iPSC clones as compared to hES; while within the clones, expression is highest in WI38 iPSC clone. Levels of core pluripotency factors (*OCT4*, *SOX2* and *NANOG*) expressed by the clones is closely comparable to that of hESC. This is important because these factors together activate remaining factors of pluripotency network which is essential for maintaining pluripotency. Relative to hES, expression of *UTF1*, *GDF3*, *DPPA3* and *SOX15* genes is down regulated in all three iPSC clones (Fig. 15).

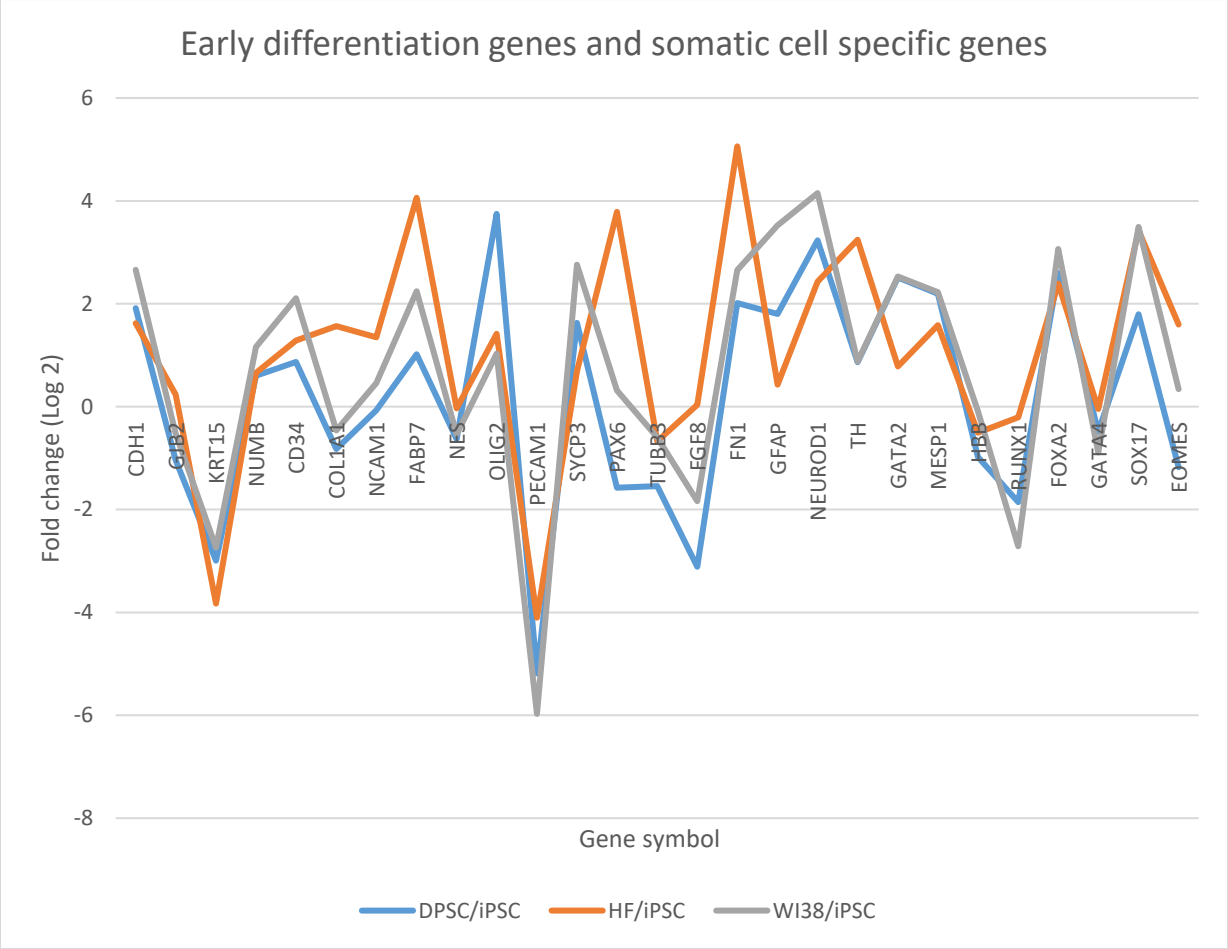

Fig. 16. The graph represents expression of somatic cell specific genes and early differentiation genes in nDPSC/iPSC, HF/iPSC and WI38/iPSC relative to hES. Data is presented as $\log_2$ values of fold change. Genes from CDH1 to SYCP3 are somatic cell specific genes while the remaining are early differentiation specific genes. Positive value indicates up expression as compared to hES while negative values indicated

down regulation. Across all the iPSC clones derived from different cell types, the trend of gene expression is very similar.

The trend of gene expression between the different iPSC clones is also similar in Fig. 16. From the graph it is clearly observed that most of the genes specific to somatic cells (i.e. genes from *CDH1* to *SYCP3*) are slightly up-regulated in iPSC clones relative to hES (Fig. 16). Moreover, similar up-regulation is also seen for early differentiation genes (genes from *PAX6* to *EOMES*) (Fig. 16). Genes belonging to somatic cells i.e. *KRT15* and *PECAM1* are down regulated in iPSC clones and similar trend is observed in two of early differentiation genes i.e. *FGF8* and RUNX1 (Fig. 16). Slight up regulation of somatic cell specific genes and early differentiation genes might be indicative of the fact that the iPSC clones might still be retaining basal level of epigenetic memory of the parental cell line from which they were derived.

In conclusion we can say that gene expression pattern between iPSC clones derived from different cell lines is very closely associated to each other than to ES. However, the fold change of various genes between ES cells and iPSC clones is very low which indicates that iPSC clones are pluripotent, and the gene expression levels observed in iPSC clones was comparable to that observed in hES cells.

4.4. Spontaneous differentiation of iPSC clones to determine their invitro differentiation capacity

Apart from checking expression of pluripotency markers it is essential to check whether iPSC are able to differentiate into different cells types each representing one of the three germ layers. This confirmatory test validates the pluripotent nature of iPSC. Keeping in mind 3R principal (refine, reduce and replace), I opted for embryoid body mediated differentiation over teratoma formation.

iPSC colonies were dissociated into small aggregates and were maintained as suspension culture. TGF-β and bFGF growth factors were withdrawn from the basal media to initiate differentiation. Embryoid bodies were maintained in culture for a sufficient period to observe cell types representing ectoderm, mesoderm and endoderm. Maximum period for which embryoid bodies (EB) were cultured was 21 days. Apart from this maximum span, EB were also harvested at 9 days and 14 days. EB were cultured in suspension till the end of the experiment and were not

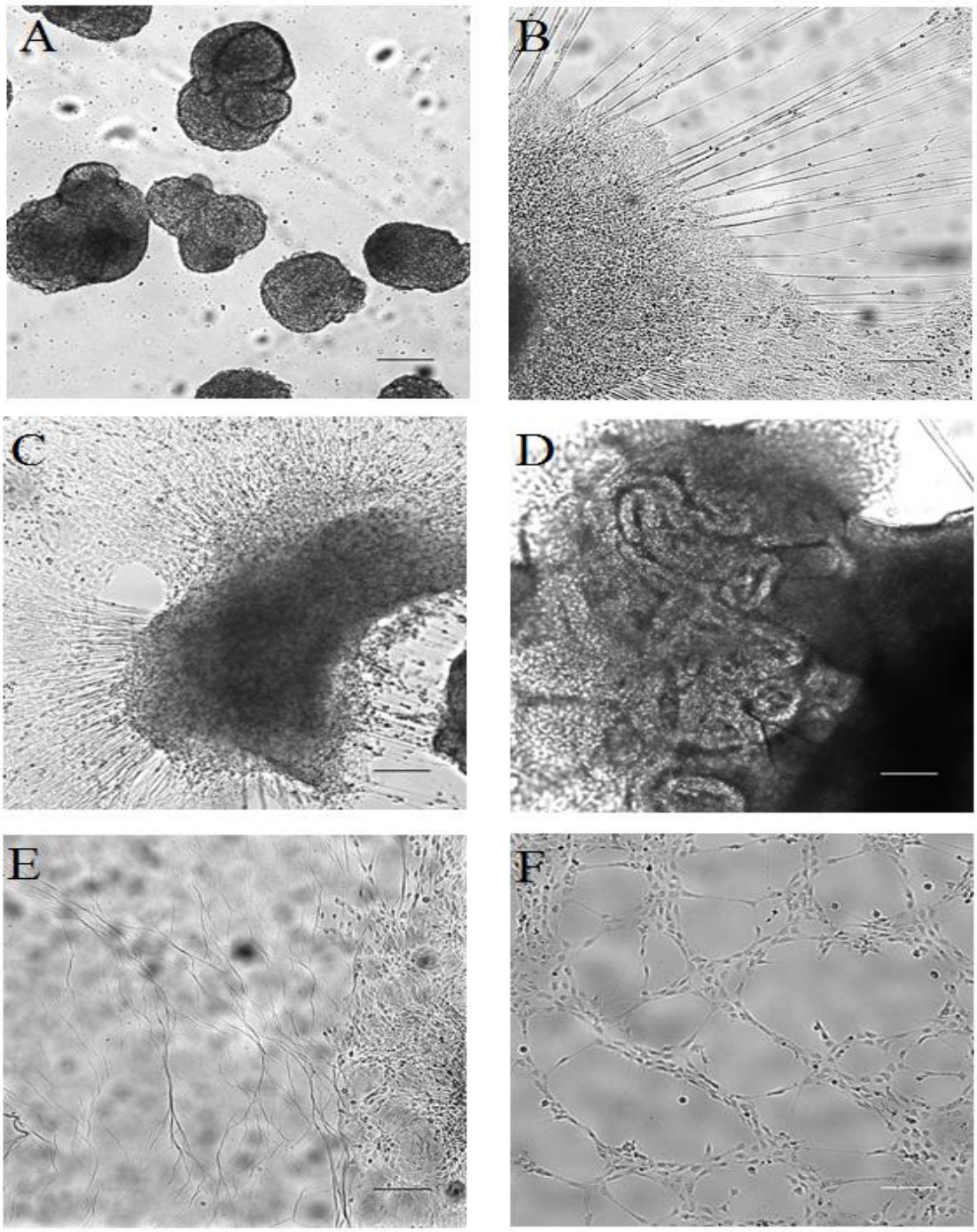

Fig. 17. Spontaneous differentiation of iPSC using embryoid body formation. The figure displays images characteristic to different cell types, structures and cell arrangements formed during spontaneous differentiation of iPSC, when they are cultured as embryoid bodies. (A) Spherical structures representing embryoid bodies. Numerous axons emerging from dense cluster of cells (B and C). (Fig. D) Coiled tube like structure representing primitive gut tube like structure. (E and F) various cell phenotypes with distinct morphological structures. Scale bars = 200 μm.

allowed to adhere to bottom of the culture plate. During culturing, the EB displayed various structures which resembled neuronal network (Fig. 17B and C), gut like tube (Fig. 17D), and various other morphological distinct structures (Fig. 17E and F).

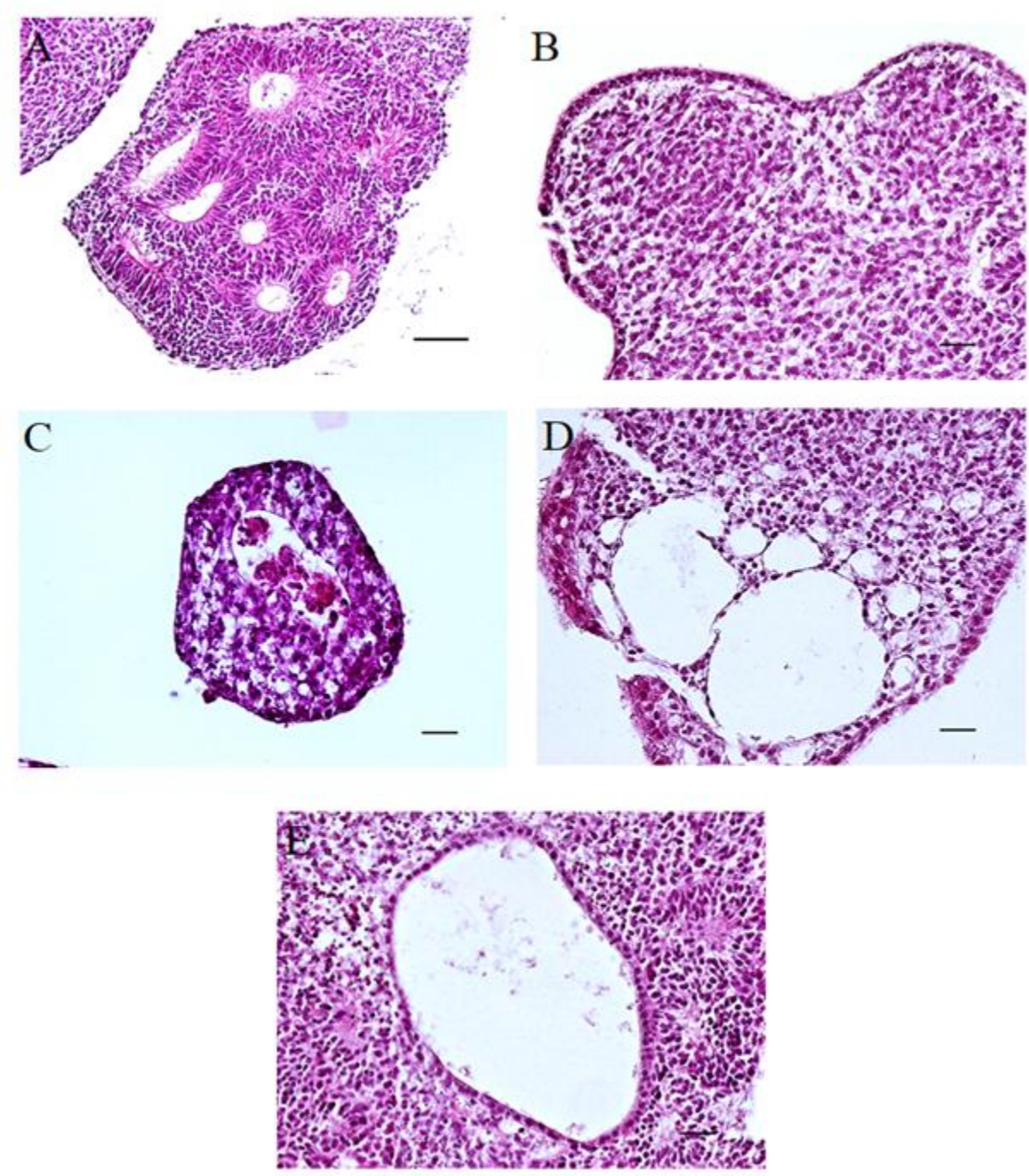

Fig. 18. Staining of sectioned embryoid bodies with haematoxylin and eosin. During the period of culturing, the EB formed various structures that were clearly visible in sections stained with haematoxylin eosin.

Neural rosette (ectoderm) was the most predominant structure (A). Covering epithelium (ectoderm) like structure (B). (C) Blood islands (mesoderm). (D) Pseudo cyst like morphology. (E) Single cell thick, simple columnar epithelium representing primitive gut like epithelium (endoderm). Scale bars = 200 μm.

Experiment was terminated by harvesting EB and processing them for paraffin embedded sectioning. Sectioned bodies were stained with the haematoxylin and eosin to identify different morphologies, while immunostaining was performed to detect expression of specific markers.

Blood island like structures were observed in the EB harvested on 9^{th} day post EB formation (Fig. 18C). The structures contained bunch of cells in the middle lined by empty space. In all the EB that were stained and observed, presence of neural rosettes was predominant (Fig. 18A and Fig. 18A-C). EB harvested close to 21 days displayed formation of covering epithelium (Fig. 18B). In addition, simple columnar cells forming characteristic tube-like structure (gut like epithelium) was observed (Fig. 18E). These tube/gut like structures were very rare and were limited to just few EB. Haematoxylin eosin staining helped in identifying different structures which together represented the three germ layers.

Expression of specific markers were detected using immunoperoxidase staining. Nestin, β III tubulin, MAP2, Vimentin and Pan cytokeratin are the differentiation markers chosen for detection. Nestin, β III tubulin, MAP2 are neuro epithelial markers; these markers represent ectoderm. While vimentin represents mesoderm and Pan cytokeratin identifies endoderm specific markers. Neuro-epithelial markers were positive in many sections (Fig. 19A-C) followed by vimentin (Fig. 19D). Pancytokeratin positivity was only restricted to the structures resembling gut like epithelium and gland like structure (Fig. 19E and F). The above presented data and figures were obtained from a single iPSC clone derived from nDPSC.

The selected iPSC clone not only displayed different structures each representing one of the three germ layers but also expressed specific markers associated with the derivatives of three-germ layer. One of the clone derived from nDPSC had attained true pluripotency because it showed positivity for markers associated with pluripotency (Fig. 12), expressed pluripotency genes at the level comparable to hES (Fig. 14 and Fig. 15) and last but not the least, spontaneously differentiated into all the germ layers (Fig. 17, Fig. 18 and Fig. 19).

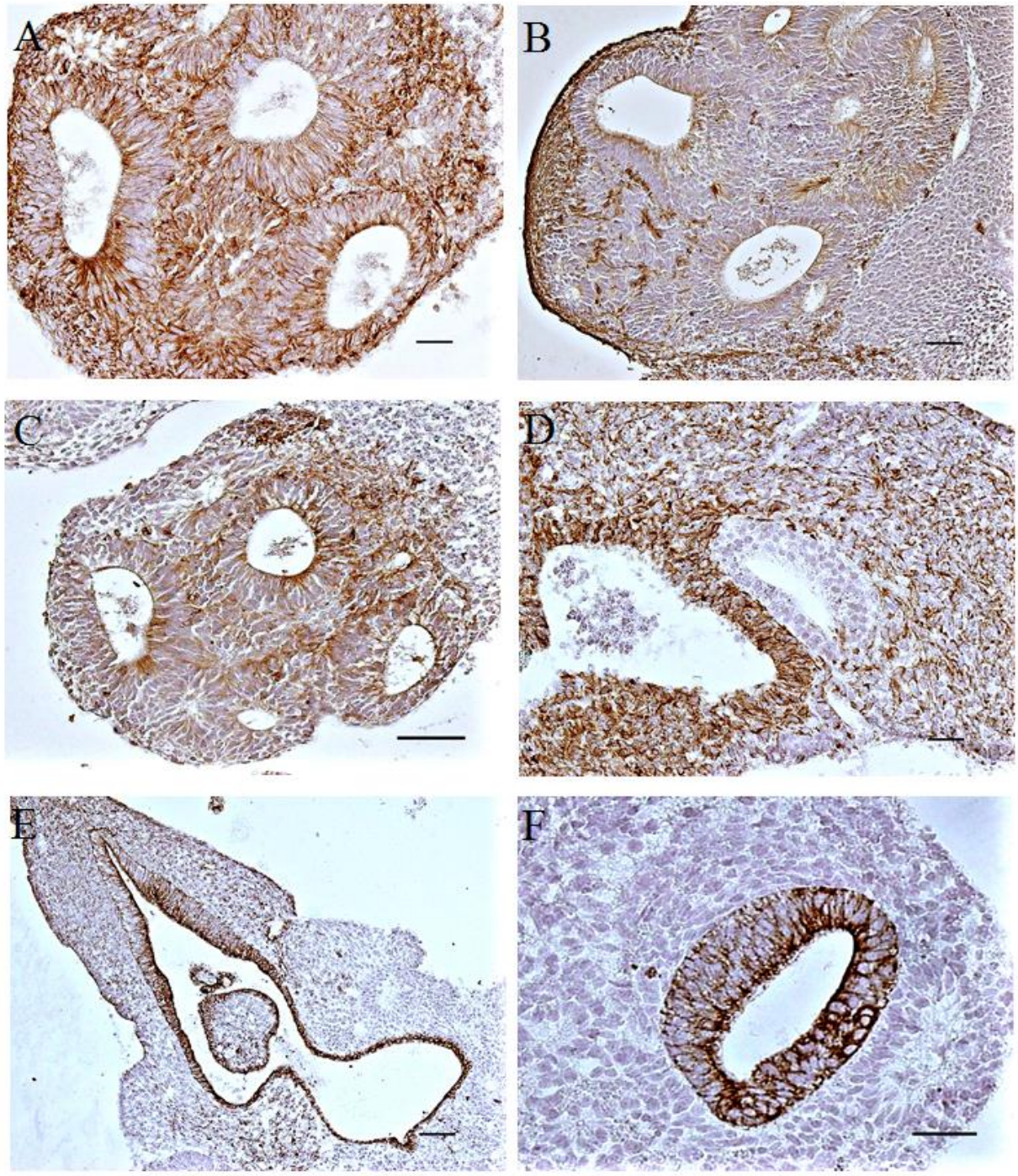

Fig. 19. Detection of germ layer specific markers. Neural rosettes showed positivity for nestin, MAP2 and β III tubulin (A-C). These markers are specifically expressed by cells which originate from ectoderm. Some of the structures within EB were vimentin positive (D). Vimentin was chosen as mesoderm specific marker. (E and F) Gut-like epithelium and gland like structure were Pan cytokeratin positive; an endoderm specific marker. Scale bars = 200 μm.

4.5. Tracking expression of neuronal markers at different time points in embryoid bodies

Appearance of neural rosettes and neurons were predominant in EB sections stained with haematoxylin eosin and bright field microscopy respectively. These observations prompted me to investigate the type of neuronal markers that were expressed in EB. I also analysed the expression pattern of these markers i.e. whether they are up regulated or down regulated as the EB get older. For the experiment, EB isolated at two-time point (7th and 14th day post formation) were compared to each other.

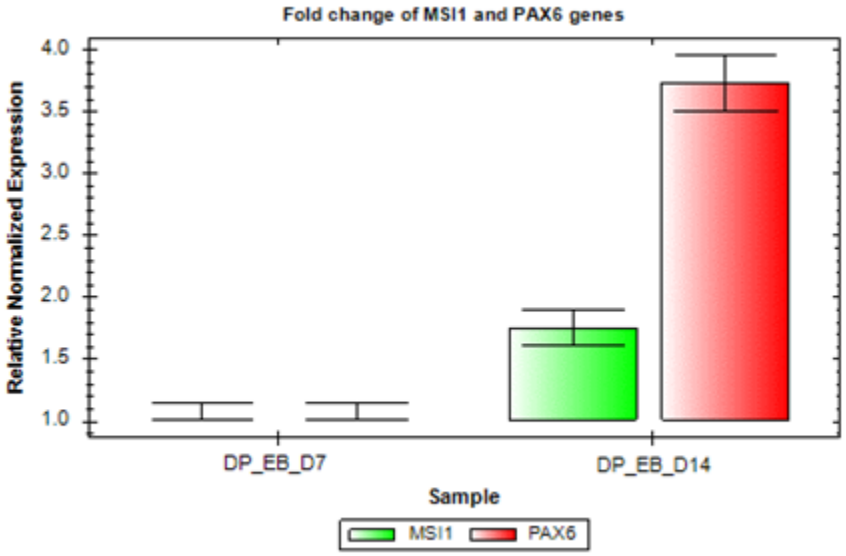

Fig. 20. Expression of *MSI-1* and *PAX6* by nDPSC derived EB. Gene expression values of EB harvested on 7th day were used as calibrator. EB cultured for longer period showed higher expression. Between *PAX6* and *MSI-1*, expression of *PAX6* was higher by approximately two-fold.

PAX6, *MSI-1* and *NES* (nestin) were the three genes selected for analysing RNA extracted from EB. These three genes are the early differentiation markers linked to neurogenesis. Surprisingly, expression of *NES* was not detectable in EB derived from nDPSC iPSC as well as WI38 iPSC. *NES* gene has many transcript variants and each variant is expressed exclusively in a specific cell type. It might be the case, that the gene probes, which we used, detected a variant of *NES* which is not expressed by early neural precursor cells present in EB. In EB derived from nDPSC iPSC, *PAX6* and *MSI-1* expression got upregulated as the EB got older (Fig. 20). In both the figures (Fig. 20 and Fig. 21), expression values of genes in EB isolated on 7th day are presented as zero because these values were used as calibrator values to calculate fold change. Relative to EB cultured for 7

days, EB cultured for 14 days showed up regulation of 1.7 and 3.7 folds for MSI-1 and PAX6 genes respectively (Fig. 20). Same as nDPSC derived EB, expression of *PAX6* and *MSI-1* increased in WI38 derived EB as they grew older (Fig. 21). Relative to EB cultured for 7 days, EB cultured for 14 days showed up regulation of 1.95 folds for both *MSI-1* and *PAX6* genes (Fig. 21). Level of up-regulation for both the genes on 14th day of culture, was approximately same for WI38 derived EB (Fig. 21). On comparing data from the two cell lines, it was observed that the expression of *PAX6* was higher in nDPSC derived EB than WI38 derived EB.

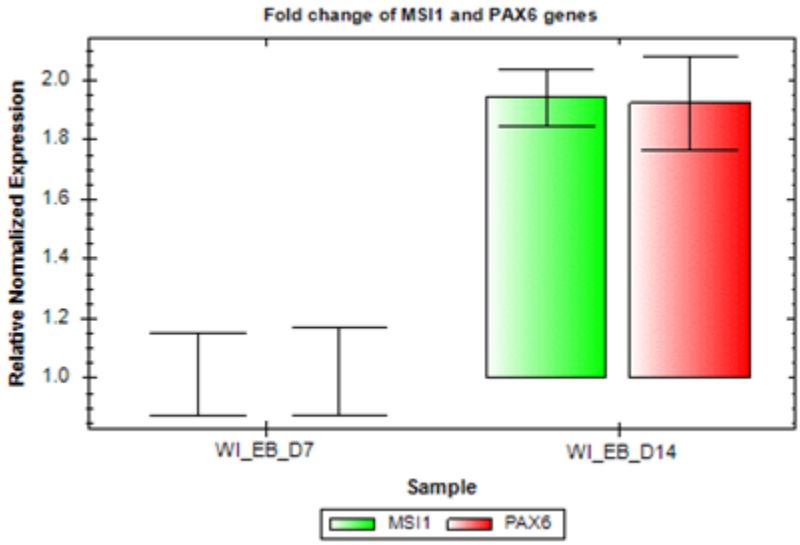

Fig. 21. Expression of *MSI-1* and *PAX6* by WI38 derived EB. Gene expression values of EB harvested on 7th day were used as calibrator. EB cultured for longer period showed higher expression and both the genes were expressed at approximately same level.

From the above data, it can be concluded that expression of *MSI-1* and *PAX6* is up regulated at 14th day relative to the expression observed on 7th day.

4.6. Assessing telomere length in reprogrammed iPSC and comparing with parental cell line

We adapted the method presented by Cawthon et al. (2002) to determine the relative telomere length. His method is based on quantitative PCR in which the relative telomere length is presented as a numerical factor by which the test DNA sample differs from the reference DNA sample in its ratio of telomere repeat copy number to single copy gene copy number.

Relative telomere length of three nDPSC derived iPSC clones was compared to that of parental cell line. All the three iPSC clones showed more than three-fold increase in telomere length relative to the parental cell line (Fig. 22). Out of the three selected clones, fold change expressed by two clones was approximately the nearly identical while that of the third was slightly higher. At the time of analysis, the later clone was on seventh passage while the former were on twelfth passage. This difference in telomere length within the clones might be due to difference in the passage number.

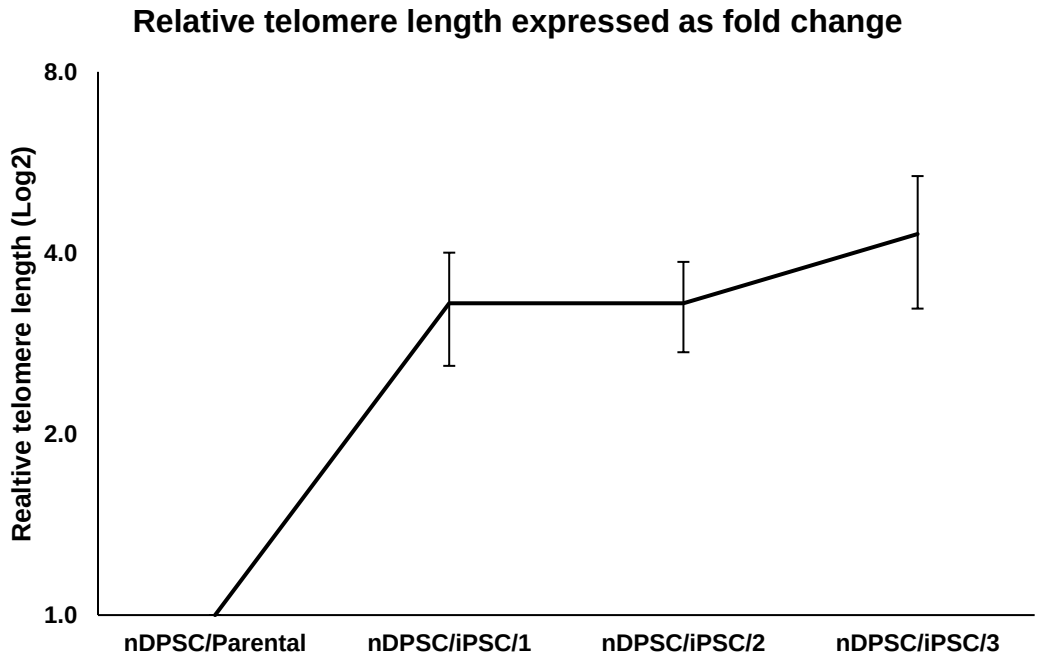

Fig. 22. The graph represents telomere length expressed by three nDPSC iPSC clones relative to their parental cell line. In all the three iPSC clones, telomeres were elongated by more than three-fold. Between the three iPSC clones, clone number three expressed higher fold change while the remaining had nearly identical length. Data presented as the average +/- standard deviation; technical triplicates.

This result clearly reflects that during reprogramming, telomeres are elongated so that the reprogrammed cells can undergo indefinite divisions without the consequence of telomere attrition. Other information that became known from this experiment was that, the clones that are at lower passage number display elongated telomere as compared to clones that are at higher passage number; this indicates that telomere length decreases as the clones are passaged but it stabilises after certain decrease.

4.7. Directed differentiation of iPSC to myogenic progenitors using small molecule inhibitor

In our department, one of the group focuses on studying recellularisation of naturally derived ECM scaffolds obtained from murine muscle tissues. They are trying to repopulate the decellarised scaffold with myogenic cells of different hierarchy. Stem cells display better regeneration capacity than differentiated cells; bearing this property in mind, we thought early myogenic progenitor would prove to have better scaffold reseeding capacity than myoblasts.

For differentiating iPSC to myogenic progenitors, I adapted protocol of Shelton et al. (2014). Myogenesis is triggered using a small molecule inhibitor CHIR 99021, which specifically inhibits GSK-3 enzyme. This inhibition activates the Wnt signalling pathway. Prolonged inhibition of GSK-3 for 48 hr commits the differentiating cells towards myogenic lineage. The committed cells were treated with FGF2 to enrich the population of myogenic progenitors. This treatment also helps in partially blocking differentiation of progenitor cells.

iPSC clones derived from nDPSC and WI38 were chosen for differentiation. GSK-3 inhibition by CHIR 99021 resulted in massive cell death in both iPSC clones (Fig. 23B and Fig. 24B). Cell death occurs in those cells that fail to commit to myogenic fate. Cell death observed in nDPSC iPSC clone was too massive and close to 85 % of population perished (Fig. 23B). In both the clones, the surviving cells quickly repopulated the empty spaces left behind by initial cell death (Fig. 23C and Fig. 24C). Both the iPSC clones gradually changed their morphology from tightly packed colony of spherical cells to individual spindle shaped, fibroblasts like cells (Fig. 23C-E and Fig. 24C-D). Differentiation was terminated on emergence of muscle progenitor cells and to enrich their population they were repeated passaged in medium supplemented with FGF2. Newly derived cells were analysed for expression of PAX7 using immunostaining and Real-Time PCR. Anti-PAX7 immunostaining showed strong positivity in both the clones (Fig. 23F and Fig. 25A) and Real-Time PCR also reflected strong up regulation at the gene level.

Real-Time PCR was performed to analyse two genes, *PAX3* and PAX7 (Fig. 26). *PAX3* is an initial marker which is used for assessing myogenic commitment of cells while *PAX7* is used for identifying emergence and presence of myogenic progenitor cells. Between the two genes, expression of *PAX3* was higher as compared to *PAX7* in nDPSC derived iPSC while exactly opposite expression was observed in WI38 derived iPSC (Fig. 26).

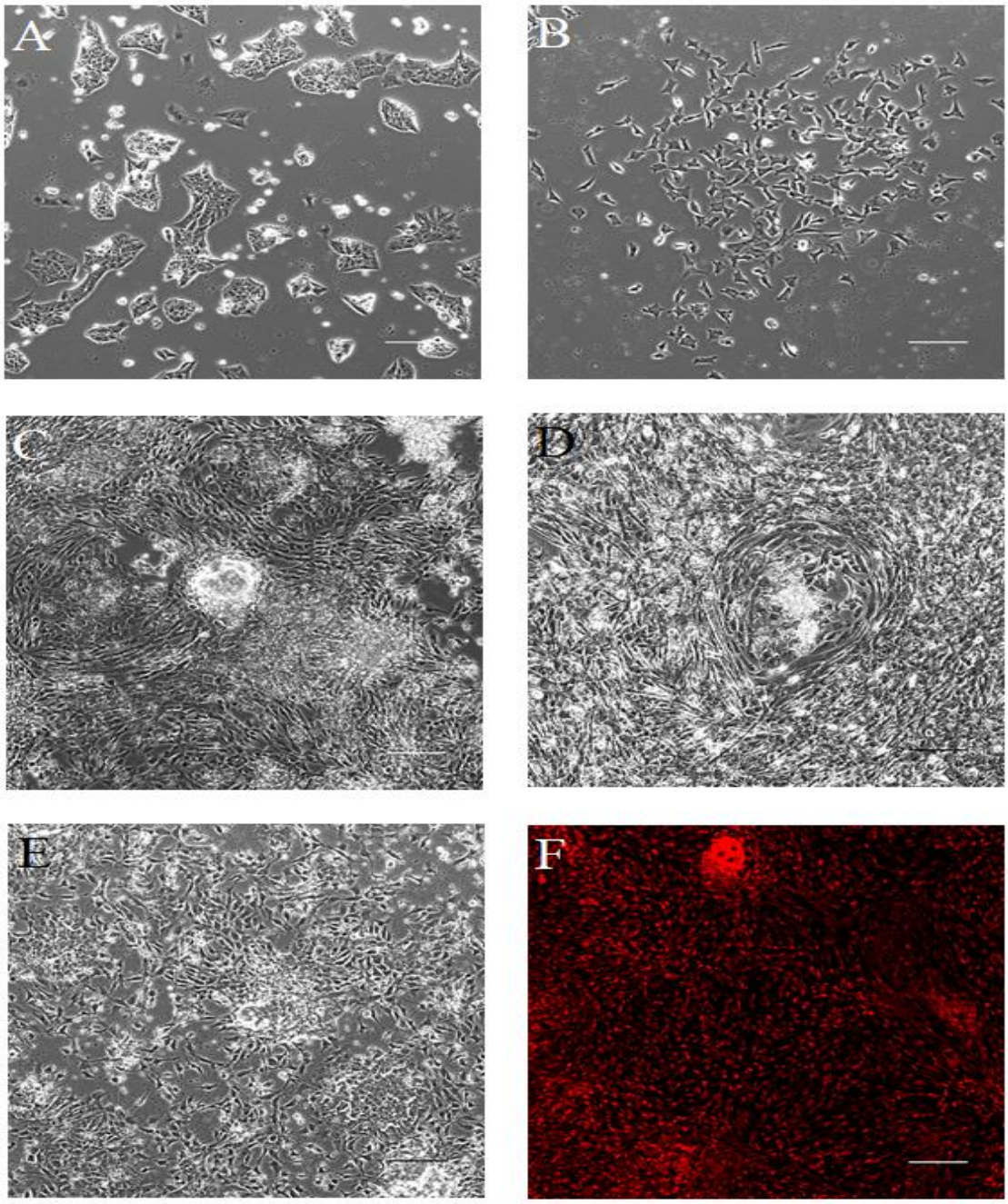

Fig. 23. Directed differentiation of nDPSC derived iPSC towards myogenesis. During the process of differentiation iPSC changed their morphology from tightly packed colonies to individual spindle cells and densely packed cluster of cells. Phase contrast images captured at different time points; Day 0 (A); Day 3 (B) nDPSC derived iPSC showed massive cell death on CHIR 99021 treatment; Day 12 (C); Day 21 (D). Differentiation was terminated on day 21 following which cells were passaged. Morphology of cells after

first passage is shown in Fig. E. myogenic progenitor after first passage showed strong positivity for PAX7 (F). Scale bars = 200 μm.

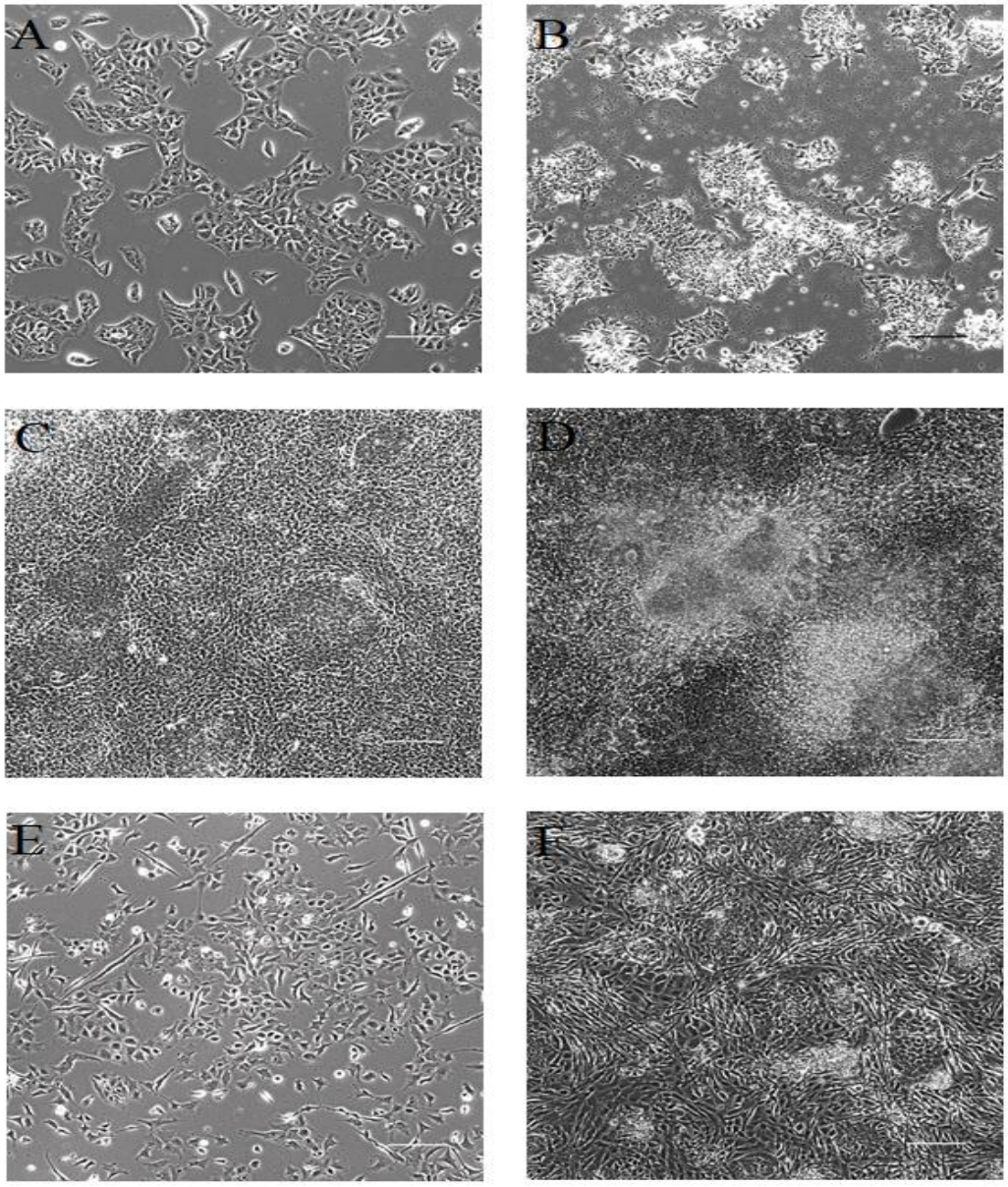

Fig. 24. Directed differentiation of WI38 derived iPSC towards myogenesis. As compared to nDPSC these cells showed relatively cell death on CHIR 99021 treatment. Hence, surviving cells quickly populated the vacant spaces and the culture became over confluent by day 5 (C). Phase contrast images captured at different time points; Day 0 (A); Day 3 (B); Day 5 (C); Day 11 (D). After day 11, the cells started forming very dense clusters. Like nDPSC, differentiation was terminated on day 21 following which cells were passaged. Morphology of cells after first passage is shown in Fig. E. Heterogeneous population of cells is

observed after first passage with few myotube like structures (E). Morphology of cells on fifth passage (F). Cells rapidly proliferative and form dense clusters within very short time (F). Scale bars = 200 µm.

Myogenic progenitor derived from WI38 were also analysed for MYF5 and MYOG markers (Fig. 25B and C). Immunostaining for both these markers yielded strong signal indicating high expression.

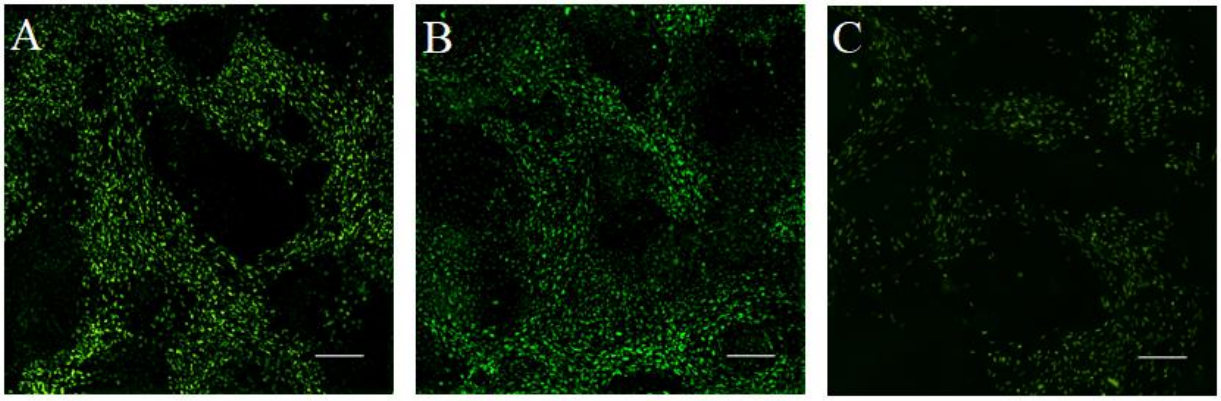

Fig. 25. Immunostaining of myogenic progenitors derived from WI38 derived iPSC. Immunostaining was performed after first passage following differentiation. Myogenic progenitors showed strong reactivity for anti-PAX7 (A), anti-MYF5 (B) and anti-MYOG (C) antibodies. Unstained empty patches might be those cells which failed to commit to myogenic lineage. Scale bars = 200 µm.

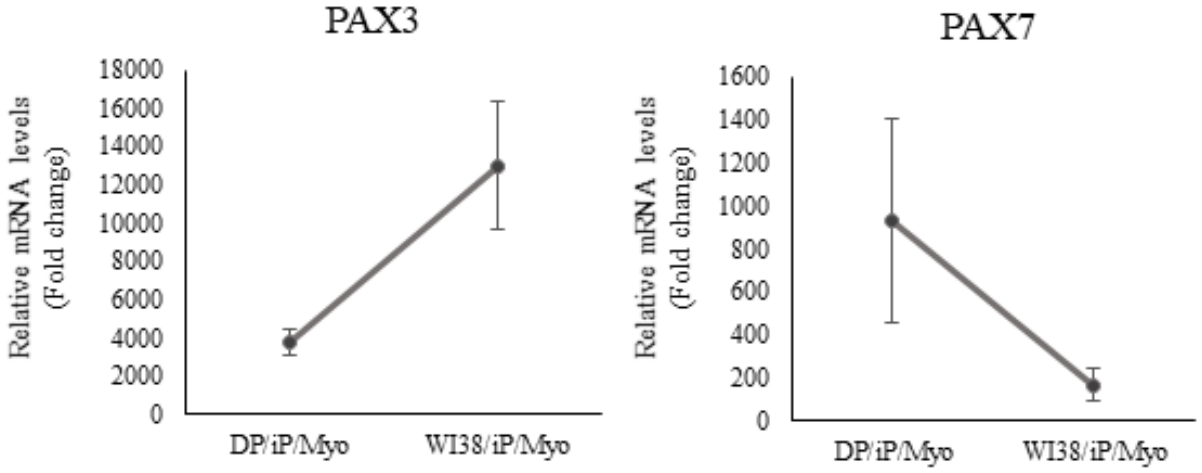

Fig. 26. Real-Time quantification of *PAX3* and *PAX7* genes by iPSC derived from nDPSC and WI38. Expression of *PAX3* was higher than *PAX7*. Expression of PAX3 was higher in WI38 derived iPSC while PAX7 expression was higher in nDPSC derived iPSC. Data presented as the average +/- standard deviation; technical triplicates.

Both the iPSC clones were able to differentiate into myogenic progenitors but nDPSC derived iPSC can be considered to contain higher population of myogenic progenitor cells. This conclusion can be made because expression of *PAX7* directly correlates to presence of myogenic progenitor cells; moreover, my result also shows six-fold up regulation in nDPSC derived iPSC.

4.8. Cloning of intron within open reading frame of dsRED2 gene

Along with classical approach of reprogramming, we also tried reprogramming cells using miRNA 302/367 cluster. For this approach, it was decided to first construct an expression vector in which an intron would be cloned within the open reading frame of the reporter gene DsRed2. miRNA 302/367 cluster is an intronic cluster, hence to provide optimum processing environment, an intron was cloned within the reporter gene DsRed2.

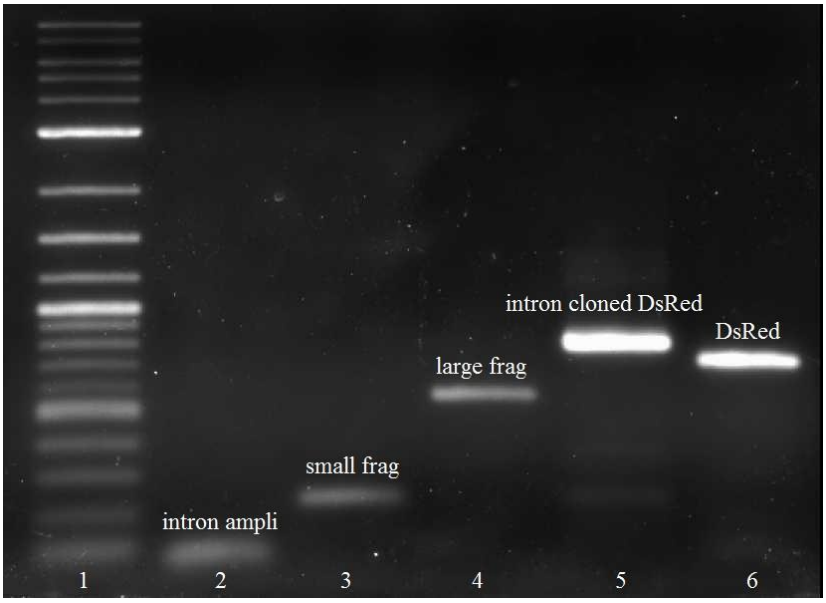

Fig. 27. Gel electrophoresis image of different PCR amplified products used for constructing intron cloned DsRed2 gene. Intron was cloned into DsRed by ligating three fragments together i.e. PCR amplified intronic sequence (lane 2) and two PCR amplified fragments of DsRed2 gene (lane 2 and 3). Successful cloning of intron was verified by comparing positions of intron cloned DsRed band (lane 5) with native DsRed (lane 6) band. Intron cloned DsRed was slight above the native DsRed, which confirmed insertion.

For intron cloning, cyclic ligation assembly method (CLA) was used. DsRed2 gene was amplified as two fragments using PCR. An artificial single stranded intronic sequence was custom synthesized from commercial firm. This single stranded intronic sequence, was PCR amplified.

Finally, intron was cloned between two DsRed2 fragments using two scaffold oligonucleotide connectors and Taq ligase (Takahashi et al. 1984). The final ligation product was PCR amplified and cloned in an expression plasmid. Intron was cloned between 233 bp small DsRed2 fragment and 556 bp large DsRed2 fragment using cyclic ligation assembly (Fig. 27; lane 3 and 4). After intron cloning, the intron cloned DsRed2 band appeared slightly above native DsRed2 band, this indicated that intronic sequence was successfully cloned within the two DsRed2 fragments. Further confirmation of intron cloning was obtained from DNA sequencing (Fig. 27; lane 5). The sequencing results confirmed precise insertion of intron within the DsRed2 (Fig. 28).

CATGKGAAYWTATAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACSAYT
YACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGCCACCATGGCCTCCTCCGAGAACGTCATCAC
CGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCACCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGC
GAGGGCCGCCCCTACGAGGGCCACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTG
GGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACATCCCCGACTACAAG
AAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGCGACCGTGAC
CCAGGACTCCTCCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGACGGC
CCCGTGATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAA
GGGCGAGACCCACAAGGTAAGAGTGGTCCGATCGTCGCGACGCGTCATTACTAACTATCAATACTTAATCCTGTCC
CTTTTTTGATATCCTGCAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACATG
GCCAAGAAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACGCCAAGCTGGACATCACCTCCCACAACGAGGAC
TACACCATCGTGGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCGTCTAGAGGGCCC
GTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCC
TTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAATAGGT
GTCATTCTATTCTTGGGGGGGTGGGGTGGGGCAGGAAAACCAAGGGG

Fig. 28. DNA sequence of DsRed2 gene after insertion of artificial plasmid. Intron was precisely cloned within the DsRed gene. Green font represents the sequence of entire DsRed2 gene, red and underlined font depicts intronic sequence and font in black is the sequence outside the DsRed2 gene.

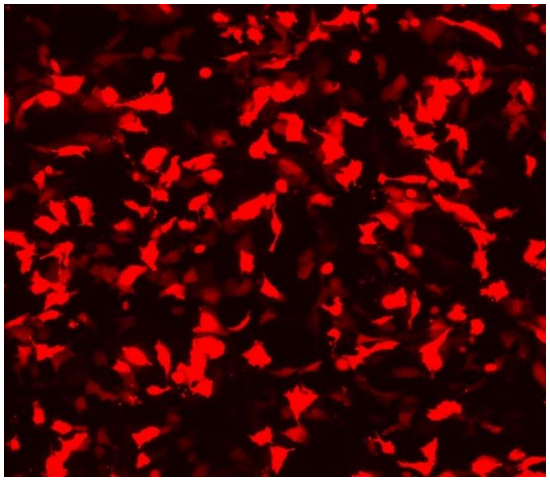

Fig. 29. Expression of red fluorescence by HeLa cells on transfection of intron cloned DsRed2. Expression of red fluorescence confirms recognition and splicing of artificial intron cloned within DsRed2 gene.

Intron recognition and splicing was confirmed by transient transfection of HeLa cells with the intron cloned DsRed2 plasmid. HeLa cells expressed the red fluorescence and the peak of expression was seen 48 hr. post transfection (Fig. 29).

Surprisingly, cloning of intron increased the number of cells expressing the red fluorescent protein as compared to native DsRed2 (Fig. 30). There was an overall four percent increase. Exact mechanism behind this increase is unknown. DsRed2 protein has been shown to aggregates and has been associated with aggregation-mediated toxicity. It might be possible that intron increased aggregation time or reduced protein aggregation; thus, increasing the number of cells expressing red fluorescent protein.

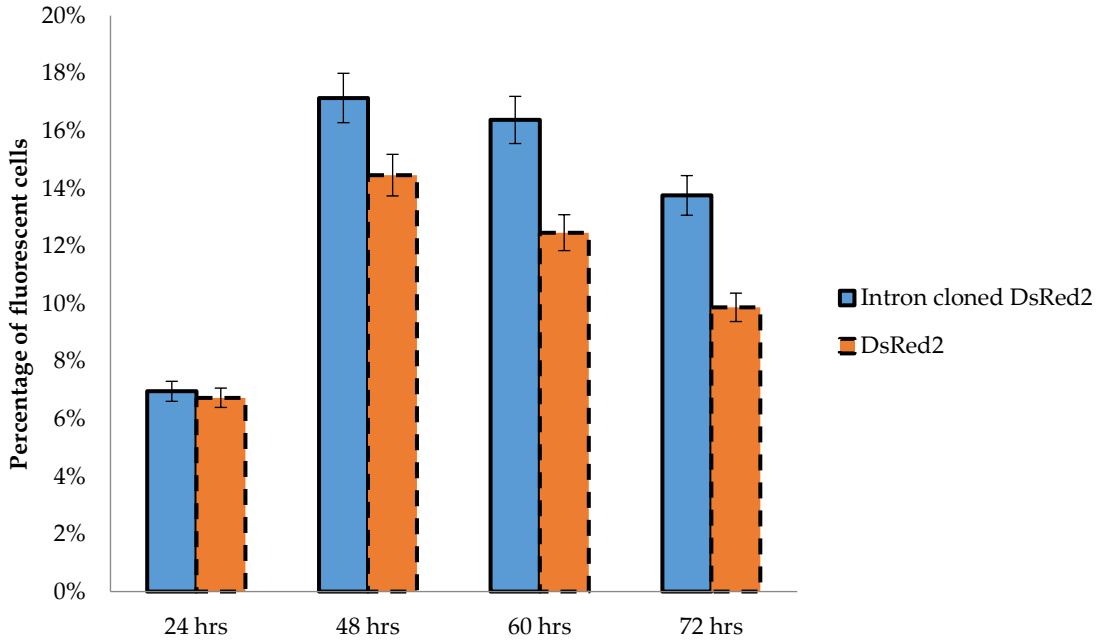

Fig. 30. Comparative analysis of number of cells expressing red fluorescence when transfected with native DsRed2 gene and intron cloned DsRed2 gene.

5. Discussion

First successful culture of pluripotent stem cells isolated from embryoblasts of murine embryos was reported in 1981 (Evans et al. 1981). After a gap of 17 years from first successful isolation of murine ES cells, isolation of human embryonic stem cells was reported from human blastocysts in the year 1998 by Thomson et al. (1998). These two discoveries laid the foundation for future pluripotent stem cell research. An important breakthrough in the field of reprogramming was reported by Wilmut et al. (1997) when his group successfully cloned a sheep by somatic cell nuclear transfer (SCNT); one of the three reprogramming approaches. Surprisingly, this research was reported one year before the first report of culturing hES cells. In SCNT, fertilized oocyte is enucleated and replaced by nucleus from somatic cell. At the time of discovery, the exact mechanism of reprogramming was unknown, but it was apparent that some factors were present in the cytoplasm of enucleated oocyte that changed the epigenetics of somatic nucleus. Second approach of reprogramming was reported by Tada et al. 2001 where they fused somatic cell with embryonic stem cell. This fusion erased somatic identity of the epigenome and instead imparted epigenomic signature similar to ES cells. Both these reprogramming approaches induced totipotent stem cell state to somatic cells. While studying the reprogramming mechanism Tada et al. (2001) highlighted that expression of *OCT4* gene was activated 48 hrs upon fusion of somatic cell with embryonic stem cell. This discovery was the first to emphasize the importance of *OCT4* gene in reprogramming. Dr. Yamanaka discovered an entirely new approach for reprogramming somatic cells which did not rely on fusion or enucleation of ES cells (Takahashi et al. 2006). He identified four transcription factors which when forcefully expressed would reprogramming somatic cells to pluripotent stem cells over a span of few weeks. These transcription factors were OCT4, SOX2, c-MYC and KLF4.

After its first discovery in 2006 by Dr. Yamanaka, this method of reprogramming has been the choice for generating large number of pluripotent stem cells in most laboratories. This is because the other two methods of reprogramming are dependent entirely on donation of oocytes from young women which raises ethical issues as human embryo is destroyed during the process (Brouwer et al. 2015). Furthermore, large number of oocytes would be required for generating patient specific pluripotent stem cell lines for disease modelling studies (Brouwer et al. 2015)

which makes its use impractical. Moreover, both the approaches are sophisticated and require very experienced hands to carry out the procedure (Brouwer et al. 2015).

Induced pluripotent stem cells (iPSC) obtained from different cell types display variation in reprogramming efficiency. Each cell line has a unique set of enhancers and barriers that directly influences reprogramming efficiency. Cell line expressing maximum number of enhancers with minimum or no barriers could be considered ideal for reprogramming. Fibroblasts are the most widely used cell type because most proof of principle experiments like SCNT (Wilmut et al. 1997) and iPSC (Takahashi et al. 2006, Takahashi et al. 2007) used fibroblasts for generating the pluripotent stem cells. In addition, they are easily derived by simple technique (Park et al. 2008) and are relatively inexpensive to culture. Range of cell types that have been successfully reprogrammed to iPSC has increased during the years following the report of iPSC generation in 2006. Examples of few are human peripheral blood (Tan et al. 2014), B-lymphocytes (Hanna et al. 2008), pancreatic β-cells (Aoi et al. 2008), keratinocytes (Aasen et al. 2008), human cord blood (Giorgetti et al. 2009), neural stem cells (Kim et al. 2009c), adipocyte stem cells (Sun et al. 2009), hematopoietic stem cells (Eminli et al. 2009), dental pulp stem cells (Yan et al. 2010), stomach cells (Aoi et al. 2008), hepatocytes (Stadtfeld et al. 2008a) etc.

Fibroblasts and peripheral blood cells are the most common cell types used for reprogramming; owing to its easy availability and relatively less invasive procedure for procurement. Both these cell types are differentiated cells and exhibit limited plasticity. Compared to fibroblasts and peripheral blood cells, tissue specific stem cells are relatively undifferentiated and display higher plasticity (Ebrahimi et al. 2011, Korbling et al. 2003). These two features make stem cells more amenable and efficient in reprogramming as compared to differentiated cells (Eminli et al. 2009, Giorgetti et al. 2009, Kim et al. 2009c, Niibe et al. 2011, Wang et al. 2013, Yulin et al. 2012). In addition, stem cells display open chromatin state as well as higher level of chromatin modellers (e.g. KDM2B) (Vidal et al. 2014) which together makes the chromatin more accessible to reprogramming factors. Apart from these reprogramming enhancers, stem cells have reduced levels of reprogramming barriers like TGF-β and MAP kinase pathways (Vidal et al. 2014). In short, stem cells possess more reprogramming enhancers than barriers which makes them better candidate for reprogramming compared to differentiated cells like fibroblasts and peripheral blood cells.

Cells extracted from early developmental tissues harbour less genetic mutation because exposure to external mutagens is minimum as compared to their adult counterparts. Human cord blood is one of the perfect examples. Another example is natal tooth. Occurrence of natal tooth is very rare event, with just one baby born with a tooth for every 3,500 births (Dyment et al. 2005). This makes it very rare sample, hence will be used seldom for performing any experiment. Luckily, we got this rare opportunity to isolate cells from natal tooth. After initial expansion, cells were characterised by analysing their cell surface markers. Characterization revealed that these cells express markers characteristic to DPSC like CD29, CD105, CD222, CD166, CD44, CD90, CD10, CD13 and CD73 as well as two markers characteristic to haematopoietic stem cells (CD34 and CD45) (Pisal et al. 2018). From our data as well as data published by other groups, it is an established fact that DPSC are negative for CD34 and CD45 markers (Atari et al. 2012, Karbanova et al. 2011, Ferro et al. 2012, Alvarez et al. 2015, Suchanek et al. 2009). Even previous article that described isolation of natal DPSC confirmed this fact (Akpinar et al. 2014). In order to find relevant explanation to our observed results, we searched previous published articles on DPSC and found few articles, which provided plausible explanation. As per the articles, dental pulp harbours two types of cell population. In the first type, cells do not express haematopoietic markers (CD34, CD45 and CD14) but express mesenchymal specific markers (STRO-1, CD29, CD44 and CD13) (Pisciotta et al. 2015); while the second type express haematopoietic marker CD34 but are negative for CD45. Search for articles describing double positivity for CD34 and CD45 markers in DPSC failed to yield any results. Hence, we thought of comparing our media composition to that of previous article describing natal DPSC isolation (Akpinar et al. 2014). Surprisingly, the media composition differed significantly. This difference in media composition might have selectively expanded unique population of cells that might explain the ambiguities observed in our results.

After phenotypic characterization of nDPSC, I compared expression of pluripotency genes between nDPSC and fibroblasts. From the selected pluripotency genes, expression of *NR5A2*, *RUNX2*, *COMMD3*, *KLF4*, *IL6ST* and *TBX3* genes were found to be highly upregulated in nDPSC as compared to fibroblasts. *NR5A2* gene has been described to enhance kinetics of *OCT4*, *SOX2*, *c-MYC* and *KLF4* mediated reprogramming (Heng et al. 2010). Upregulation of *KLF4* gene is highly encouraging because cells can undergo complete reprogramming only when the endogenous counterparts of ectopically expressed reprogramming factors are activated; since *KLF4* gene is naturally upregulated in nDPSC, it will definitely enhance reprograming efficiency.

Furthermore, it would be interesting to eliminate *KLF4* from the reprogramming cocktail and study its effect on reprogramming kinetics and efficiency. *IL6ST* is an interleukin 6 transducer and has been known to activate STAT3 pathway, which in turn has been linked to enhance proliferation and stemness in mesenchymal stem cells (Kishimoto 2005, Hossain et al. 2015). *TBX3* gene, which was up regulated by 10-fold in nDPSC, had been shown to improve quality of iPSC; in addition, it was also associated in regulating pluripotency factors (Han et al. 2010). Moreover, it was proved that iPSC generated from OCT4, SOX2, KLF4 and TBX3 displayed superior germ cell contribution and germ line transmission frequency (Han et al. 2010). The above discussion concludes that nDPSC display reprogramming conducive environment which might positively influence the reprogramming efficiency.

Mycoplasma infected cell lines display altered growth characteristics, morphology, metabolism, antigenicity and chromosomal aberrations (Drexler et al. 2002, Razin et al. 1998, Rottem 2003). Since mycoplasma significantly alters the cell characteristics, there is high risk of obtaining skewed results if mycoplasma infected cell lines are used for experiments. Markoullis et al. 2009 had studied the effect of mycoplasma infection on murine ES cells. The study revealed that infected ES cell line shows marked decrease in OCT4 expressing cells as well as increase in chromosomal aberrations (Markoullis et al. 2009). Furthermore, infected ES cells showed reduced formation of germline chimeras and the pups generated from the infected cells were born unhealthy (Markoullis et al. 2009). The above results can be extrapolated to cells undergoing reprogramming. If mycoplasma infected cells are used for reprogramming, then there is high probability that the cell line will show significantly reduction in reprogramming efficiency or the reprogramming process might get completely disrupted. Several kits are commercially available for mycoplasma detection, but I decided to adapt protocol of Uphoff et al. (2002) for screening the cells. While replicating the procedure, I had trouble in extracting mycoplasma DNA from infected cell culture supernatant. Hence, instead of performing DNA extraction step, I directly used the cell culture supernatant for performing PCR. In addition, I used robust polymerase enzyme for PCR. By introducing these two modifications, I made the original protocol more sensitive, efficient and rapid (Pisal et al. 2016).

Prof. Shinya Yamanaka made a path-breaking discovery by reprogramming somatic cells to pluripotent stem cells (Takahashi et al. 2006). Moreover, he showed that just by ectopic expression of four transcription factors this dramatic change was possible (Takahashi et al. 2006). These

factors were OCT4, SOX2, c-MYC and KLF4; OSKM is the common abbreviation used for these factors (Takahashi et al. 2006). The task of finding this minimal set of factors was difficult. Initially Dr. Yamanaka started by analysing all the genes that were up regulated in embryonic stem cells. Following which he narrowed down his search to 24 potential genes. After trying various combinations and exclusion on these 24 genes, he finally arrived at four essential factors i.e. OCT4, SOX2, c-MYC and KLF4. Apart from original combination of OSKM, other combinations that were reported are OCT4, SOX2, KLF4, GLIS1 (Maekawa et al. 2011); OCT4, SOX2, NANOG, LIN28 (Yu et al. 2007); OCT4, SOX2, c-MYC, KLF4, LIN28 (Warren et al. 2010); OCT4, SOX2, c-MYC, KLF4, Zic3 (Declercq et al. 2013); OCT4, SOX2, KLF4, TBX3 (Han et al. 2010) etc. Even though many combinations of reprogramming factors are published, still most labs prefer to use the canonical combination of OSKM. Hou et al. (2013) reported a gene free approach for generating iPSC using combination of seven small molecules. Many research groups thrived hard to reduce the number of genes required for reprogramming and instead use small molecules to decrease the risk of cancer. In most of the cases OCT4 was found to be indispensable. Hou et al. (2013) had reported a modest reprogramming efficiency of 0.2 % in murine fibroblasts. This approach named as chemically induced pluripotent stem cells was restricted to murine cells only no further research was done to extend the findings to human cells. Anokye-Danso et al. (2011) reported a non-canonical approach of reprogramming using micro RNA (miRNA). In this study miRNA cluster 302/367 was used to reprogram murine and human fibroblasts. This approach remained restricted to human fibroblasts and no articles were published using this approach on any other human cell line.

Many vector platforms are available for introducing reprogramming factors into the cells. Majority of the vectors are virus based for example retrovirus, lentivirus, adenovirus, Sendai virus etc. Dr. Yamanaka in his pioneering article Yamanaka et al. (2006) had used retroviral vectors to deliver the reprogramming factors. Retrovirus and lentivirus are the most commonly used vectors for gene delivery. These RNA viruses need to integrate with the host genome to express their genes. This insertional mutagenesis increases the chances of cancer, which presents an obstacle to therapeutic use of iPSC generated using such vectors. Furthermore, the reprogramming efficiency reported using these vectors is also very low (Rao et al. 2012). Excisable version of lentivirus is available (PiggyBac) which erases the virus footprint from the host cell, but the problem of insertional mutagenesis still persists (Soldner et al. 2009). Adenoviruses are non-integrative

viruses that were used for delivering reprogramming factors. Unfortunately, these viruses presented very low reprogramming efficiency, which restricted their use (Rao et al. 2012). Other approaches that are virus free like episomes, minicircles, recombinant proteins, transposons etc. were used but the reprogramming efficiency reported by these methods were exceptionally low (Rao et al. 2012). Transfection of reprogramming factors in form of synthetic mRNA was reported to have high reprogramming efficiency (Rao et al. 2012, Warren et al. 2010). Down side of this approach is that it is very labour intensive, expensive and reprogramming capacity of this method is limited to few cell lines only (Rao et al. 2012). Sendai virus mediated delivery of reprogramming factors displayed relatively high reprogramming efficiency as compared to retroviral and lentiviral vectors (Fusaki et al. 2009b). Sendai virus vector is a negative sense single stranded RNA virus that does not rely on host genome integration for its propagation (Lamb et al. 1996). Furthermore, the virus replicates in the host cytoplasm with minimal cytotoxicity and can infect wide range of host cell species and tissues (Tokusumi et al. 2002). Last but not the least the vector permits high level of exogenous gene expression without any hindrance (Fusaki et al. 2009a). Commercial standardised format of Sendai virus reprogramming kit is available with many published results using different type of cell lines from different species. Moreover, specific mutation introduced in the virus genome (virus available from the commercial vendor) allows complete removal of virus footprints within short time span (Ban et al. 2011). IPSC generated using this reprogramming platform can be used for therapeutic purposes. All the above-mentioned advantages makes this vector platform highly preferred among most of the research labs to generate new iPSC cell lines.

In the current research one of the important objectives was to study the effect of starting cell line on reprogramming efficiency. For this study I compared the reprogramming efficiencies of four DPSC cell lines with two fibroblasts cell lines. Out of the four DPSC cell lines, two cell lines were derived from male donors and remaining two were from female donors, while both the fibroblasts cell lines were derived from female donors. Upon transduction of Sendai virus reprogramming vectors, two male DPSC cell lines showed massive cell death following which 90 % of the plated cells were washed off. The other cell lines also displayed cell death, but the extent was significantly lower. One of the female DPSC cell line displayed numerous reprogrammed colonies, which was difficult to quantify (Fig. 11C). This cell line had the highest reprogramming efficiency followed by another female DPSC cell line (Fig. 11D). The cell line which displayed highest reprogramming efficiency was derived from adult female while the second highest was derived from 11-day old

baby girl. The only physiological distinction between these two DPSC cell lines was that the former one expressed low transduction mediated toxicity and higher proliferation rate as compared to later. Remaining two DPSC cell lines showed very few reprogrammed colonies because of massive cell death at the start of the experiment. Out of the two fibroblasts cell lines one was from adult and other was of embryonic origin. Adult fibroblasts displayed third highest reprogramming efficiency while WI38 was fourth highest. The difference between the number of colonies observed between the two fibroblasts was greater than 10-fold. WI38 was of embryonic origin, hence it was assumed to have more reprogramming favourable environment and therefore higher reprogramming efficiency. However, the results revealed exactly opposite picture. Among the different cell lines, reprogrammed colonies appeared earlier in DPSC cell lines compared to fibroblasts. In DPSC, small colonies were visible by day 12 post transduction while in fibroblasts it was 18 days post transduction.

Some interesting facts noticed after comparing the reprogramming efficiency results across all the cell lines. WI38 showed lowest cytotoxicity as well as lowest reprogramming efficiency; reprogramming efficiency of female derived DPSC was higher than their male counterpart; reprogramming efficiency of adult female DPSC was higher than natal female DPSC; overall reprogramming efficiency of adult female DPSC was higher than fibroblasts. These results clearly indicate that reprogramming is a complex process and many unknown factors play role during reprogramming that significantly affects stoichiometry of reprogramming.

Characterization of iPSC clones is important to confirm true attainment of pluripotency. First step of characterization begins by checking the morphological change and expression of pluripotency markers (Marti et al. 2013, Baghbaderani et al. 2016). This preliminary characterization is followed by differentiation test, where the iPSC clones are differentiated by either EB formation or teratoma formation (Marti et al. 2013, Baghbaderani et al. 2016). In this test, iPSC clones are checked for their capacity to generate derivatives of three germ layers. Other test included in characterization are telomere length analysis and karyotyping.

Apart from analysing expression of few pluripotency genes, I analysed expression of 83 set of genes, which comprised of pluripotency genes, early differentiation genes and somatic cell specific genes. The idea behind this comprehensive analysis was to get a quick overview of the

pluripotency status of the clone under study. By comparing gene expression results of the new clone with established iPSC clone or ES cells, I could comment whether the gene expression results are comparable or not. Furthermore, close analysis of pluripotency genes helped to ascertain whether crucial set of genes necessary for establishing pluripotency are activated to appropriate levels or not. A reprogrammed clone should ideally up-regulate all pluripotency associated genes with simultaneous down regulation of somatic cell specific genes. While comparing gene expression results of three iPSC clones derived from three different cell lines to each other and to hES, I found that gene expression pattern was closely related between the iPSC clones than to hES. These observations are in accordance with the results published by Chin et al. 2009. Outwardly, iPSC are indistinguishable from ES but a close look at gene expression profile will reveal subtle difference. Most of these differences dissolve as iPSC become more and more established but certain degree of distinction always prevails (Chin et al. 2009).

For confirming the trilineage differentiation capacity, I used EB mediated spontaneous differentiation and targeted differentiation. In the past, teratoma assay was considered as gold standard for confirming trilineage differentiation capacity (O'Connor et al. 2011, Ungrin et al. 2007). In this procedure, iPSC cells are injected in richly vascularised tissues of an immunodeficient mouse model like SCID mice. Over few weeks, iPSC cells differentiate to form teratoma comprised of cells from all three germ layers (Kooreman et al. 2010). However, current research has found limitation in this approach. Major limitations are that it lacks sensitivity and is labour intensive. Moreover, it only provides qualitative results meaning it only confirms whether certain cell types are present but does not speak about the relative ratios of cells that have differentiated. Furthermore, this approach does not comply with 3R (Replacement, Reduction and Refinement) principles to reduce animal sufferings. Hence, I have chosen EB mediated differentiation over teratoma assay; and to further confirm the pluripotency, the cells were differentiated into derivatives of three germ layers using directed differentiation approach.

Pluripotent stem cells (ES or iPSC) are virtually immortal i.e. they can proliferate indefinitely if spontaneously differentiation is prevented. To maintain indefinite proliferation, pluripotent stem cells elongate their telomeres using telomerase enzyme. In addition, telomerase enzyme plays crucial role in reprogramming and self-renewal of iPSC (Wang et al. 2012). Furthermore, induction of true pluripotency requires maintenance of telomere length by telomerase (Wang et al.

2012). While comparing telomere data it was evident that iPSC clones had 3-fold increase in their telomere length relative to the parental cell line. Out of the three clones used for analysis, two were cultured for extended period i.e. they were from high passage number while one clone was cultured for relatively short period i.e. it was from lower passage number. On comparing data within these three clones, it was seen that the clone, which was having lower passage number, displayed higher telomere compared to the other two clones having higher passage number. Suhr et al. (2009) reported similar findings where they have shown that early passage iPSC clones exhibit elongated telomere length. It has been speculated that shortening of telomere length could limit the scalability, safety and therapeutic efficacy of iPSC (Wang et al. 2012).

It is essential to study genomic integrity because variation in copy number could potentially affect cellular function. Genomic stability of somatic and iPS cells is verified by studying R and G band karyotype analysis. It is important to include karyotyping in iPSC characterisation because it allows detection of duplicate copies of genes that could potentially affect pluripotency and differentiation potential of iPSC (Maitra et al. 2005, Spits et al. 2008, Wu et al. 2008). Karyotype analysis of two iPSC clones had normal karyotype of 46 XX without aneuploidy or polyploidy. One of the advantages of using Sendai virus reprogramming vectors is that the chances of genomic instability potentially arising due to viral insertion in host genome is nil.

iPSC technology is useful in making advancement in the field of disease modelling, cell transplantation and clinical trial. After the first report of reprogramming human fibroblasts by Yamanaka et al. 2007, study of disease modelling commenced by reprogramming somatic cells from aged patients (Dimos et al. 2008). A comprehensive list of diseases that have been successfully modelled using iPSC technology has been thoroughly complied in review article of Inoue et al. (2011). Disease modelling begins with isolation of disease specific somatic cells followed by generation of corresponding iPS cell line. This disease specific iPS cell line is differentiated back to the cell type displaying the disease phenotype; this approach helps to understand progression of the disease and can also be used to appropriately target the causative factors. Cell transplantation is another branch where iPSC technology has ventured and provided new hope to patients who cannot be easily treated using conventional medicine. Prominent advantages of iPSC are as follows: patient's own cells can be reprogrammed and used for

treatment, such autologous cells suppress the risk of immune rejection and infection; certain diseases caused by gene defect can be corrected using gene editing tools and allogenic cells from healthy donors can be used for treating certain diseases where it is not possible to use patients own cells (Inoue et al. 2014). First direct use of iPSC in regenerative medicine was reported in 2014 in Japan where scientists transplanted autologous iPSC-derived retinal cells into eye of a patient suffering from age-related macular degeneration. Follow up post one year showed that the transplanted cells not only survived but also helped to improve vision and supress vision degeneration. Furthermore, cells did not evoke any immune response, nor did they show adverse proliferation (Mandai et al. 2017). Proof of concept for use of gene edited cells for disease treatment was successfully proved in mouse model of sickle-cell anaemia (Hanna et al. 2007). In this study mutant iPS cell line was edited using homologous recombination to replace the mutant gene with healthy gene. iPS cells carrying the healthy gene were differentiated and subsequently transplanted back into the mice to cure the disease. Concept of treating diseases using autologous iPSC seems promising but the approach seems to be impractical for wide spread use because of the high cost associated with the whole process. Moreover, it takes more than three months to generate, characterise and differentiate iPSC which is not optimum for treating certain disorders like spinal cord injury (Nakamura et al. 2012, Takahashi et al. 2013), severe myocardial infarction etc. Since use of autologous iPSC seems less likely, scientist have come up with idea of using allogenic iPSC for transplantation therapy. Immunogenic compatibility is essential for the success of allogenic cell therapy. With iPS cell lines it is possible to preselect the donor genotype; this possibility opens new avenue for generating various types of immune compatible cells for vast population from a small group of donors. Clinical grade iPS cell line can be generated from diverse group of donors with verified health conditions and different types of human leucocyte antigen (HLA) (Takahashi et al. 2013). Transplanted cells or organs are rejected by the recipient's immune system mainly because of mismatch of carbohydrate blood group antigens such as ABO or due to HLA incompatibility (Takahashi et al. 2013). Furthermore, donor recipient mismatch at minor HLA loci or major histocompatibility complex can also lead to immune rejection (Takahashi et al. 2013). In case of iPSC, the cells normally do not express any immunogenic antigens but in rare case they can become immunogenic due to antigenic arrangement arising from derivation and culture condition (Takahashi et al. 2013). Future of iPSC-based therapy will be based on collecting iPSC stocks derived from various HLA homozygous donors (Takahashi et al. 2013, Nakajima et

al. 2007, Okita et al. 2011). Scientists based in UK hypothesized that 10 cell lines homozygous for common HLA types selected from 10,000 individuals could provide complete HLA antigen match for 37.7% of UK population while a potentially beneficial match for 67.4% population (Taylor et al. 2012). Among Japanese population, it is estimated that 30 homozygous cell lines isolated from 15,000 individuals would match 82.2% of populations at HLA antigen level for HLA-A, HLA-B and HLA-DR while 50 homozygous cell lines isolated from 24,000 individuals would match 90.7% of population (Nakatsuji et al. 2008). Foreseeing the prospect in allogenic iPSC, vision of Dr. Yamanaka and other prominent scientists in the field is to develop network of HLA homozygous haplobanks of iPS cell lines.

nDPSC have vast regenerative capacity since they express osteogenic, chondrogenic, adipogenic, myogenic and neurogenic markers under basal conditions and they possess the capacity to differentiate into mature adipocytes, myoblasts, neuro-glial cells, chondroblasts and osteoblasts in vitro under permissive differentiation conditions (Karaoz et al. 2010). By reprogramming nDPSC to iPSC, we have broadened the spectrum of their application in regenerative medicine.

Extracted teeth are traditionally thought to be medical waste. Unlike hESC, which involve the destruction of human embryos, dental stem cells are readily accessible and most importantly, there are no ethical considerations. The therapeutic capability and clinical benefits of dental stem cells are not limited to dental use but can also be used for regenerative medicine (Huang et al. 2010).

From our research as well as research done by other groups, it can be highlighted that natal DPSC as well as adult DPSC hold immense regenerative potential. This potential can be significantly broadened by reprogramming DPSC to pluripotent stem cells. To harness this benefit, it is essential to preserve the tooth. Like cord blood banking, tooth banking is a new concept which is emerging. The first commercial tooth bank was established at National Hiroshima University in Japan in 2004. One of the essential steps for banking any tissues is availability of viable cryopreservation method. Tissues from tooth pulp can be stored for long-term by using Cell Alive System (CAS) (Huang et al. 2010). This method of cryopreservation maintains good cell viability and differentiation capability after cryopreservation (Huang et al. 2010). Moreover, it is possible to cryopreserve human pulpal tissues by encompassing the entire pulp with cryoprotectant (Huang et al. 2010). Thus, by using appropriate cryopreservation processes, tooth storage and banking will

provide significant contributions to clinical auto-transplantation and regenerative medicine (Huang et al. 2010).

In 2011 Danso et al. (Anokye-Danso et al. 2011) published an article proving that forced expression of 302/367 miRNA cluster can reprogram somatic cells to pluripotent state without OCT4, SOX2, c-MYC and KLF4. For using this approach of reprogramming, an expression vector was constructed by cloning artificial intron within the open reading frame of reporter gene DsRed2. This approach was not only helpful in providing optimum expression conditions for miRNA but also in confirming intron splicing (by detecting red fluorescence). Surprisingly, intron cloning increased number of cell expressing red fluorescent protein by four percent as compared to native DsRed2 gene (Pisal et al. 2017). This cloning experiment helped us to understand and practically apply cyclic ligation assembly (CLA) for assembling DNA fragments (Roth et al. 2014). Improvement in number of cell expressing DsRed2 protein will help in widening scope of DsRed2, which is currently limited because of its cytotoxic effect caused by oligomerization and aggregation (Strongin et al. 2007).

6. Conclusion

To date, nobody has reported derivation of iPSC from natal dental pulp stem cells. In addition, the nDPSC which we had expanded displayed unique marker expression profile which was not previously reported (Pisal et al. 2018). The cells expressed hematopoietic and pluripotent stem cell markers in addition to mesenchymal stem cell markers (Pisal et al. 2018). This signifies that the cells are relatively immature and might harbour conducive environment for reprogramming.

Before reprogramming, all the cell lines were screened for mycoplasma to ensure they are free of mycoplasma infection. PCR based protocol which was adapted for screening the cell lines was modified by introducing two changes; directly performing PCR on cell culture supernatant instead of DNA extraction and replacing normal polymerase enzyme with robust enzyme. These two modifications made the new protocol more sensitive, robust and rapid (Pisal et al. 2016).

Along with nDPSC, I successfully reprogrammed two fibroblasts cell lines. Few selected clones from each cell line were thoroughly characterised using immunocytochemistry, real-time PCR and in vitro differentiation. Instead of characterising the iPSC clones by analysing expression of core pluripotency factors (*OCT4*, *SOX2* and *NANOG*) alone, I chose to comprehensively characterise the clones by analysing expression of 84 genes in total genes. Comparative analysis of these genes was helpful in determining the closeness of the iPSC clones with hES cells.

Natal DPSC (nDPSC) yielded very robust and numerous iPSC colonies on transduction of Sendai virus reprogramming vectors. nDPSC derived induced pluripotent stem cells (nDPSC-iPSC) closely resembled hESC in terms of morphology, expression of pluripotency markers and gene expression profile. Furthermore, nDPSC-iPSC spontaneously differentiated into derivatives of three embryonic germ layers when cultured as embryonic bodies (Pisal et al. 2018). In addition, pluripotency of iPSC was further cemented by differentiating the cells into hepatic endoderm, beating cardiomyocytes and covering epithelium using directed differentiation approach (Pisal et al. 2018). Relative telomere length increased more than three folds in nDPSC iPSC clones as compared to parental cell line; moreover, they also displayed normal karyotype of 46 XY with no aneuploidy or polyploidy at 12^{th} passage (Pisal et al. 2018). The above presented characterisation results confirm induction of true pluripotency in the nDPSC derived iPSC.

Non-canonical approach of reprogramming using miRNA cluster was reported in 2011 but the accompanying mechanism remained unknown. In this quest of understanding the mechanism, I first decided to construct a vector for expressing the miRNA cluster. An expression vector was constructed by cloning artificial intronic sequence between two fragments of DsRed2 gene. Upon transfection of this newly constructed vector into HeLa cells, the cells not only spliced the cloned intron but, in the process, also increased the number of cells expressing the vector (observed by checking expression of red fluorescence). Compared to native gene, engineered gene showed four percent increase in number of cells expressing red fluorescent protein (Pisal et al. 2017).

7. References

AASEN, T., RAYA, A., BARRERO, M.J., GARRETA, E., CONSIGLIO, A., GONZALEZ, F., VASSENA, R., BILIC, J., PEKARIK, V., TISCORNIA, G., EDEL, M., BOUE, S., IZPISUA BELMONTE, J.C. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat. Biotechnol., 2008, vol. 26, no. 11. pp. 1276-1284.

AKPINAR, G., KASAP, M., AKSOY, A., DURUKSU, G., GACAR, G., KARAOZ, E. Phenotypic and proteomic characteristics of human dental pulp derived mesenchymal stem cells from a natal, an exfoliated deciduous, and an impacted third molar tooth. Stem Cells Int., 2014, vol. 2014. pp. 457059.

AL-HABIB, M., YU, Z., HUANG, G.T.J. Small molecules affect human dental pulp stem cell properties via multiple signaling pathways. Stem Cells Dev., 2013, vol. 22, no. 17. pp. 2402-2413.

ALVAREZ, R., LEE, H.L., HONG, C., WANG, C.Y. Single CD271 marker isolates mesenchymal stem cells from human dental pulp. Int. J. Oral Sci., 2015, vol. 7, no. 4. pp. 205-212.

ANOKYE-DANSO, F., TRIVEDI, C.M., JUHR, D., GUPTA, M., CUI, Z., TIAN, Y., ZHANG, Y., YANG, W., GRUBER, P.J., EPSTEIN, J.A., MORRISEY, E.E. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell. Stem Cell., 2011, vol. 8, no. 4. pp. 376-388.

AOI, T., YAE, K., NAKAGAWA, M., ICHISAKA, T., OKITA, K., TAKAHASHI, K., CHIBA, T., YAMANAKA, S. Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science, 2008, vol. 321, no. 5889. pp. 699-702.

ARTHUR, A., RYCHKOV, G., SHI, S., KOBLAR, S.A., GRONTHOS, S. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells, 2008, vol. 26, no. 7. pp. 1787-1795.

ATARI, M., GIL-RECIO, C., FABREGAT, M., GARCIA-FERNANDEZ, D., BARAJAS, M., CARRASCO, M.A., JUNG, H.S., ALFARO, F.H., CASALS, N., PROSPER, F., FERRES-PADRO, E., GINER, L. Dental pulp of the third molar: A new source of pluripotent-like stem cells. J. Cell. Sci., 2012, vol. 125, no. 14. pp. 3343-3356.

AUGELLO, A., KURTH, T.B., DE BARI, C. Mesenchymal stem cells: A perspective from in vitro cultures to in vivo migration and niches. Eur. Cell. Mater., 2010, vol. 20. pp. 121-133.

BABICKI, S., ARNDT, D., MARCU, A., LIANG, Y., GRANT, J.R., MACIEJEWSKI, A., WISHART, D.S. Heatmapper: Web-enabled heat mapping for all. Nucleic Acids Res., 2016, vol. 44, no. 1. pp. 147-53.

BAGHBADERANI, B.A., SYAMA, A., SIVAPATHAM, R., PEI, Y., MUKHERJEE, O., FELLNER, T., ZENG, X., RAO, M.S. Detailed characterization of human induced pluripotent stem cells manufactured for therapeutic applications. Stem Cell. Rev., 2016, vol. 12. pp. 394-420.

BAN, H., NISHISHITA, N., FUSAKI, N., TABATA, T., SAEKI, K., SHIKAMURA, M., TAKADA, N., INOUE, M., HASEGAWA, M., KAWAMATA, S., NISHIKAWA, S. Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive sendai virus vectors. Proc. Natl. Acad. Sci. U. S. A., 2011, vol. 108, no. 34. pp. 14234-14239.

BANITO, A., GIL, J. Induced pluripotent stem cells and senescence: Learning the biology to improve the technology. EMBO Rep., 2010, vol. 11, no. 5. pp. 353-359.

BEERS, J., GULBRANSON, D.R., GEORGE, N., SINISCALCHI, L.I., JONES, J., THOMSON, J.A., CHEN, G. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. Nat. Protoc., 2012, vol. 7, no. 11. pp. 2029-2040.

BELTRAO-BRAGA, P.C., PIGNATARI, G.C., MAIORKA, P.C., OLIVEIRA, N.A., LIZIER, N.F., WENCESLAU, C.V., MIGLINO, M.A., MUOTRI, A.R., KERKIS, I. Feeder-free derivation of induced pluripotent stem cells from human immature dental pulp stem cells. Cell Transplant., 2011, vol. 20, no. 11-12. pp. 1707-1719.

BELTRAO-BRAGA, P.C., PIGNATARI, G.C., MAIORKA, P.C., OLIVEIRA, N.A., LIZIER, N.F., WENCESLAU, C.V., MIGLINO, M.A., MUOTRI, A.R., KERKIS, I. Feeder-free derivation of induced pluripotent stem cells from human immature dental pulp stem cells. Cell Transplant., 2011, vol. 20, no. 11-12. pp. 1707-1719.

BRAMBRINK, T., FOREMAN, R., WELSTEAD, G.G., LENGNER, C.J., WERNIG, M., SUH, H., JAENISCH, R. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell. Stem Cell., 2008, vol. 2, no. 2. pp. 151-159.

BROUWER, M., ZHOU, H., NADIF KASRI, N. Choices for induction of pluripotency: Recent developments in human induced pluripotent stem cell reprogramming strategies. Stem Cell Rev., 2015, vol. 12. pp. 54-72.

BUCKINGHAM, M. Skeletal muscle progenitor cells and the role of pax genes. C. R. Biol., 2007, vol. 330, no. 6-7. pp. 530-533.

BUNNELL, B.A., FLAAT, M., GAGLIARDI, C., PATEL, B., RIPOLL, C. Adipose-derived stem cells: Isolation, expansion and differentiation. Methods, 2008, vol. 45, no. 2. pp. 115-120.

CACCHIARELLI, D., TRAPNELL, C., ZILLER, M.J., SOUMILLON, M., CESANA, M., KARNIK, R., DONAGHEY, J., SMITH, Z.D., RATANASIRINTRAWOOT, S., ZHANG, X., HO SUI, S.J., WU, Z., AKOPIAN, V., GIFFORD, C.A., DOENCH, J., RINN, J.L., DALEY, G.Q., MEISSNER, A., LANDER, E.S., MIKKELSEN, T.S. Integrative analyses of human reprogramming reveal dynamic nature of induced pluripotency. Cell, 2015, vol. 162, no. 2. pp. 412-424.

CAHAN, P., LI, H., MORRIS, S.A., LUMMERTZ DA ROCHA, E., DALEY, G.Q., COLLINS, J.J. CellNet: Network biology applied to stem cell engineering. Cell, 2014, vol. 158, no. 4. pp. 903-915.

CASHEN, A.F., LAZARUS, H.M., DEVINE, S.M. Mobilizing stem cells from normal donors: Is it possible to improve upon G-CSF? Bone Marrow Transplant., 2007, vol. 39, no. 10. pp. 577-588.

CAWTHON, R.M. Telomere measurement by quantitative PCR. Nucleic Acids Research, 2002, vol. 30, no. 10. pp. e47-e47.

CHEN, H., AKSOY, I., GONNOT, F., OSTEIL, P., AUBRY, M., HAMELA, C., ROGNARD, C., HOCHARD, A., VOISIN, S., FONTAINE, E., MURE, M., AFANASSIEFF, M., CLEROUX, E., GUIBERT, S., CHEN, J., VALLOT, C., ACLOQUE, H., GENTHON, C., DONNADIEU, C., DE VOS, J.,

SANLAVILLE, D., GUERIN, J.F., WEBER, M., STANTON, L.W., ROUGEULLE, C., PAIN, B., BOURILLOT, P.Y., SAVATIER, P. Reinforcement of STAT3 activity reprogrammes human embryonic stem cells to naive-like pluripotency. Nat. Commun., 2015, vol. 6. pp. 7095.

CHEN, H.C., LEE, Y.S., SIEBER, M., LU, H.T., WEI, P.C., WANG, C.N., PENG, H.H., CHAO, A.S., CHENG, P.J., CHANG, S.D., CHEN, S.J., WANG, T.H. MicroRNA and messenger RNA analyses of mesenchymal stem cells derived from teeth and the wharton jelly of umbilical cord. Stem Cells Dev., 2012, vol. 21, no. 6. pp. 911-922.

CHEN, S.J., CHANG, C.M., TSAI, S.K., CHANG, Y.L., CHOU, S.J., HUANG, S.S., TAI, L.K., CHEN, Y.C., KU, H.H., LI, H.Y., CHIOU, S.H. Functional improvement of focal cerebral ischemia injury by subdural transplantation of induced pluripotent stem cells with fibrin glue. Stem Cells Dev., 2010, vol. 19, no. 11. pp. 1757-1767.

CHIN, M.H., MASON, M.J., XIE, W., VOLINIA, S., SINGER, M., PETERSON, C., AMBARTSUMYAN, G., AIMIUWU, O., RICHTER, L., ZHANG, J., KHVOROSTOV, I., OTT, V., GRUNSTEIN, M., LAVON, N., BENVENISTY, N., CROCE, C.M., CLARK, A.T., BAXTER, T., PYLE, A.D., TEITELL, M.A., PELEGRINI, M., PLATH, K., LOWRY, W.E. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell. Stem Cell., 2009, vol. 5, no. 1. pp. 111-123.

CORDEIRO, M.M., DONG, Z., KANEKO, T., ZHANG, Z., MIYAZAWA, M., SHI, S., SMITH, A.J., NOR, J.E. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. J. Endod., 2008, vol. 34, no. 8. pp. 962-969.

COUBLE, M.L., FARGES, J.C., BLEICHER, F., PERRAT-MABILLON, B., BOUDEULLE, M., MAGLOIRE, H. Odontoblast differentiation of human dental pulp cells in explant cultures. Calcif. Tissue Int., 2000, vol. 66, no. 2. pp. 129-138.

D'AQUINO, R., GRAZIANO, A., SAMPAOLESI, M., LAINO, G., PIROZZI, G., DE ROSA, A., PAPACCIO, G. Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: A pivotal synergy leading to adult bone tissue formation. Cell Death Differ., 2007, vol. 14, no. 6. pp. 1162-1171.

D'AQUINO, R., TIRINO, V., DESIDERIO, V., STUDER, M., DE ANGELIS, G.C., LAINO, L., DE ROSA, A., DI NUCCI, D., MARTINO, S., PAINO, F., SAMPAOLESI, M., PAPACCIO, G. Human neural crest-derived postnatal cells exhibit remarkable embryonic attributes either in vitro or in vivo. Eur. Cell. Mater., 2011, vol. 21. pp. 304-316.

DE MIGUEL, M.P., FUENTES-JULIAN, S., ALCAINA, Y. Pluripotent stem cells: Origin, maintenance and induction. Stem Cell. Rev., 2010, vol. 6, no. 4. pp. 633-649.

DECLERCQ, J., SHESHADRI, P., VERFAILLIE, C.M., KUMAR, A. Zic3 enhances the generation of mouse induced pluripotent stem cells. Stem Cells Dev., 2013, vol. 22, no. 14. pp. 2017-2025.

DIAS, J., GUMENYUK, M., KANG, H.J., VODYANIK, M., YU, J., THOMSON, J.A., SLUKVIN, I.I. Generation of red blood cells from human induced pluripotent stem cells. Stem Cells Dev., 2011, vol. 20, no. 9. pp. 1639-1647.

DIMOS, J.T., RODOLFA, K.T., NIAKAN, K.K., WEISENTHAL, L.M., MITSUMOTO, H., CHUNG, W., CROFT, G.F., SAPHIER, G., LEIBEL, R., GOLAND, R., WICHTERLE, H., HENDERSON, C.E., EGGAN, K. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science, 2008, vol. 321, no. 5893. pp. 1218-1221.

DREXLER, H.G.; and UPHOFF, C.C. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology, 2002, vol. 39, no. 2. pp. 75-90.

DYMENT, H., ANDERSON, R., HUMPHREY, J., CHASE, I. Residual neonatal teeth: A case report. J. Can. Dent. Assoc., 2005, vol. 71, no. 6. pp. 394-397.

EBRAHIMI, B. Reprogramming barriers and enhancers: Strategies to enhance the efficiency and kinetics of induced pluripotency. Cell. Regen., 2015, vol. 4. pp. 10-015-0024-9.

EBRAHIMI, B., YAGHOOBI, M., KAMAL-ABADI, A., RAOOF, M. Human dental pulp stem cells express many pluripotency regulators and differentiate into neuronal cells. Neural Regen. Res., 2011, vol. 6, no. 34. pp. 2666-2672.

BRAHIMI, B. Reprogramming of adult stem/progenitor cells into iPSCs without reprogramming factors. J. Med. Hypo. and Ideas, 2015, vol. 9, no. 2. pp. 99-103.

ELLIS, P., FAGAN, B.M., MAGNESS, S.T., HUTTON, S., TARANOVA, O., HAYASHI, S., MCMAHON, A., RAO, M., PEVNY, L. SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. Dev. Neurosci., 2004, vol. 26, no. 2-4. pp. 148-165.

EMINLI, S., FOUDI, A., STADTFELD, M., MAHERALI, N., AHFELDT, T., MOSTOSLAVSKY, G., HOCK, H., HOCHEDLINGER, K. Differentiation stage determines reprogramming potential of hematopoietic cells into iPS cells. Nat. Genet., 2009, vol. 41, no. 9. pp. 968-976.

EVANS, M.J.; and KAUFMAN, M.H. Establishment in culture of pluripotential cells from mouse embryos. Nature, 1981, vol. 292, no. 5819. pp. 154-156.

FEDOROV, Y.V., JONES, N.C., OLWIN, B.B. Regulation of myogenesis by fibroblast growth factors requires beta-gamma subunits of pertussis toxin-sensitive G proteins. Mol. Cell. Biol., 1998, vol. 18, no. 10. pp. 5780-5787.

FERRO, F., SPELAT, R., BELTRAMI, A.P., CESSELLI, D., CURCIO, F. Isolation and characterization of human dental pulp derived stem cells by using media containing low human serum percentage as clinical grade substitutes for bovine serum. PLoS One, 2012, vol. 7, no. 11. pp. e48945.

FUSAKI, N., BAN, H., NISHIYAMA, A., SAEKI, K., HASEGAWA, M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on sendai virus, an RNA virus that does not integrate into the host genome. Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci., 2009, vol. 85, no. 8. pp. 348-362.

GALAN-CARIDAD, J.M., HAREL, S., ARENZANA, T.L., HOU, Z.E., DOETSCH, F.K., MIRNY, L.A., REIZIS, B. Zfx controls the self-renewal of embryonic and hematopoietic stem cells. Cell, 2007, vol. 129, no. 2. pp. 345-357.

GANDARILLAS, A.; and WATT, F.M. c-myc promotes differentiation of human epidermal stem cells. Genes Dev., 1997, vol. 11, no. 21. pp. 2869-2882.

GANDIA, C., ARMINAN, A., GARCIA-VERDUGO, J.M., LLEDO, E., RUIZ, A., MINANA, M.D., SANCHEZ-TORRIJOS, J., PAYA, R., MIRABET, V., CARBONELL-UBEROS, F., LLOP, M., MONTERO, J.A., SEPULVEDA, P. Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. Stem Cells, 2008, vol. 26, no. 3. pp. 638-645.

GIORGETTI, A., MONTSERRAT, N., AASEN, T., GONZALEZ, F., RODRIGUEZ-PIZA, I., VASSENA, R., RAYA, A., BOUE, S., BARRERO, M.J., CORBELLA, B.A., TORRABADELLA, M., VEIGA, A., IZPISUA BELMONTE, J.C. Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. Cell. Stem Cell., 2009, vol. 5, no. 4. pp. 353-357.

GONZALEZ, R., GARITAONANDIA, I., ABRAMIHINA, T., WAMBUA, G.K., OSTROWSKA, A., BROCK, M., NOSKOV, A., BOSCOLO, F.S., CRAW, J.S., LAURENT, L.C., SNYDER, E.Y., SEMECHKIN, R.A. Deriving dopaminergic neurons for clinical use. A practical approach. Sci. Rep., 2013, vol. 3. pp. 1463.

GOVINDASAMY, V., ABDULLAH, A.N., RONALD, V.S., MUSA, S., AB AZIZ, Z.A., ZAIN, R.B., TOTEY, S., BHONDE, R.R., ABU KASIM, N.H. Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. J. Endod., 2010, vol. 36, no. 9. pp. 1504-1515.

GRECO, S.J., LIU, K., RAMESHWAR, P. Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells. Stem Cells, 2007, vol. 25, no. 12. pp. 3143-3154.

GRONTHOS, S., BRAHIM, J., LI, W., FISHER, L.W., CHERMAN, N., BOYDE, A., DENBESTEN, P., ROBEY, P.G., SHI, S. Stem cell properties of human dental pulp stem cells. J. Dent. Res., 2002, vol. 81, no. 8. pp. 531-535.

GRONTHOS, S., MANKANI, M., BRAHIM, J., ROBEY, P.G., SHI, S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc. Natl. Acad. Sci. U. S. A., 2000, vol. 97, no. 25. pp. 13625-13630.

GUILAK, F., LOTT, K.E., AWAD, H.A., CAO, Q., HICOK, K.C., FERMOR, B., GIMBLE, J.M. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J. Cell. Physiol., 2006, vol. 206, no. 1. pp. 229-237.

HAASE, A., OLMER, R., SCHWANKE, K., WUNDERLICH, S., MERKERT, S., HESS, C., ZWEIGERDT, R., GRUH, I., MEYER, J., WAGNER, S., MAIER, L.S., HAN, D.W., GLAGE, S., MILLER, K., FISCHER, P., SCHOLER, H.R., MARTIN, U. Generation of induced pluripotent stem cells from human cord blood. Cell. Stem Cell., 2009, vol. 5, no. 4. pp. 434-441.

HALL, J.K., BANKS, G.B., CHAMBERLAIN, J.S., OLWIN, B.B. Prevention of muscle aging by myofiber-associated satellite cell transplantation. Sci. Transl. Med., 2010, vol. 2, no. 57. pp. 57-83.

HAN, J., YUAN, P., YANG, H., ZHANG, J., SOH, B.S., LI, P., LIM, S.L., CAO, S., TAY, J., ORLOV, Y.L., LUFKIN, T., NG, H.H., TAM, W.L., LIM, B. Tbx3 improves the germ-line competency of induced pluripotent stem cells. Nature, 2010, vol. 463, no. 7284. pp. 1096-1100.

HANNA, J., WERNIG, M., MARKOULAKI, S., SUN, C.W., MEISSNER, A., CASSADY, J.P., BEARD, C., BRAMBRINK, T., WU, L.C., TOWNES, T.M., JAENISCH, R. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science, 2007, vol. 318, no. 5858. pp. 1920-1923.

HANNA, J., MARKOULAKI, S., SCHORDERET, P., CAREY, B.W., BEARD, C., WERNIG, M., CREYGHTON, M.P., STEINE, E.J., CASSADY, J.P., FOREMAN, R., LENGNER, C.J., DAUSMAN, J.A., JAENISCH., R. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell, 2008, vol. 133, no. 2. pp. 250-264.

HAY, D.C., FLETCHER, J., PAYNE, C., TERRACE, J.D., GALLAGHER, R.C., SNOEYS, J., BLACK, J.R., WOJTACHA, D., SAMUEL, K., HANNOUN, Z., PRYDE, A., FILIPPI, C., CURRIE, I.S., FORBES, S.J., ROSS, J.A., NEWSOME, P.N., IREDALE, J.P. Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. Proc. Natl. Acad. Sci. U. S. A., 2008, vol. 105, no. 34. pp. 12301-12306.

HENG, J.C., FENG, B., HAN, J., JIANG, J., KRAUS, P., NG, J.H., ORLOV, Y.L., HUSS, M., YANG, L., LUFKIN, T., LIM, B., NG, H.H. The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell. Stem Cell., 2010, vol. 6, no. 2. pp. 167-174.

HOCHEDLINGER, K.; and PLATH, K. Epigenetic reprogramming and induced pluripotency. Development, 2009, vol. 136, no. 4. pp. 509-523.

HOSSAIN, A., GUMIN, J., GAO, F., FIGUEROA, J., SHINOJIMA, N., TAKEZAKI, T., PRIEBE, W., VILLARREAL, D., KANG, S.G., JOYCE, C., SULMAN, E., WANG, Q., MARINI, F.C., ANDREEFF, M., COLMAN, H., LANG, F.F. Mesenchymal stem cells isolated from human gliomas increase proliferation and maintain stemness of glioma stem cells through the IL-6/gp130/STAT3 pathway. Stem Cells, 2015, vol. 33, no. 8. pp. 2400-2415.

HOU, P., LI, Y., ZHANG, X., LIU, C., GUAN, J., LI, H., ZHAO, T., YE, J., YANG, W., LIU, K., GE, J., XU, J., ZHANG, Q., ZHAO, Y., DENG, H. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science, 2013, vol. 341, no. 6146. pp. 651.

HUANG, Y., YANG, J., WANG, C., LEE, S. Dental stem cells and tooth banking for regenerative medicine. J. Exp. Clin. Med., 2010, vol. 2, no. 3. pp. 111.

IACOBAS, I., VATS, A., HIRSCHI, K.K. Vascular potential of human pluripotent stem cells. Arterioscler. Thromb. Vasc. Biol., 2010, vol. 30, no. 6. pp. 1110-1117.

INOUE, H., NAGATA, N., KUROKAWA, H., YAMANAKA, S. iPS cells: A game changer for future medicine. EMBO J., 2014, vol. 33, no. 5. pp. 409.

INOUE, H.; and YAMANAKA, S. The use of induced pluripotent stem cells in drug development. Clin. Pharmacol. Ther., 2011, vol. 89, no. 5. pp. 655-661.

INOUE, M., TOKUSUMI, Y., BAN, H., KANAYA, T., TOKUSUMI, T., NAGAI, Y., IIDA, A., HASEGAWA, M. Nontransmissible virus-like particle formation by F-deficient sendai virus is temperature sensitive and reduced by mutations in M and HN proteins. J. Virol., 2003, vol. 77, no. 5. pp. 3238-3246.

JO, Y.Y., LEE, H.J., KOOK, S.Y., CHOUNG, H.W., PARK, J.Y., CHUNG, J.H., CHOUNG, Y.H., KIM, E.S., YANG, H.C., CHOUNG, P.H. Isolation and characterization of postnatal stem cells from human dental tissues. Tissue Eng., 2007, vol. 13, no. 4. pp. 767-773.

KADAR, K., KIRALY, M., PORCSALMY, B., MOLNAR, B., RACZ, G.Z., BLAZSEK, J., KALLO, K., SZABO, E.L., GERA, I., GERBER, G., VARGA, G. Differentiation potential of stem cells from human dental origin - promise for tissue engineering. J. Physiol. Pharmacol., 2009, vol. 60 Suppl 7. pp. 167-175.

KARAOZ, E., DOGAN, B.N., AKSOY, A., GACAR, G., AKYUZ, S., AYHAN, S., GENC, Z.S., YURUKER, S., DURUKSU, G., DEMIRCAN, P.C., SARIBOYACI, A.E. Isolation and in vitro characterisation of dental pulp stem cells from natal teeth. Histochem. Cell Biol., 2010, vol. 133, no. 1. pp. 95-112.

KARBANOVA, J., SOUKUP, T., SUCHANEK, J., PYTLIK, R., CORBEIL, D., MOKRY, J. Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium. Cells Tissues Organs, 2011, vol. 193, no. 6. pp. 344-365.

KAZUKI, Y., HIRATSUKA, M., TAKIGUCHI, M., OSAKI, M., KAJITANI, N., HOSHIYA, H., HIRAMATSU, K., YOSHINO, T., KAZUKI, K., ISHIHARA, C., TAKEHARA, S., HIGAKI, K., NAKAGAWA, M., TAKAHASHI, K., YAMANAKA, S., OSHIMURA, M. Complete genetic correction of ips cells from duchenne muscular dystrophy. Mol. Ther., 2010, vol. 18, no. 2. pp. 386-393.

KEEFFE, E.B. Liver transplantation: Current status and novel approaches to liver replacement. Gastroenterology, 2001, vol. 120, no. 3. pp. 749-762.

KERKIS, I., KERKIS, A., DOZORTSEV, D., STUKART-PARSONS, G.C., GOMES MASSIRONI, S.M., PEREIRA, L.V., CAPLAN, A.I., CERRUTI, H.F. Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells Tissues Organs, 2006, vol. 184, no. 3-4. pp. 105-116.

KERKIS, I.; and CAPLAN, A.I. Stem cells in dental pulp of deciduous teeth. Tissue Eng. Part B. Rev., 2012, vol. 18, no. 2. pp. 129-138.

KIM, C.F., JACKSON, E.L., WOOLFENDEN, A.E., LAWRENCE, S., BABAR, I., VOGEL, S., CROWLEY, D., BRONSON, R.T., JACKS, T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell, 2005, vol. 121, no. 6. pp. 823-835.

KIM, D., KIM, C.H., MOON, J.I., CHUNG, Y.G., CHANG, M.Y., HAN, B.S., KO, S., YANG, E., CHA, K.Y., LANZA, R., KIM, K.S. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell. Stem Cell., 2009, vol. 4, no. 6. pp. 472-476.

KIM, J.B., GREBER, B., ARAUZO-BRAVO, M.J., MEYER, J., PARK, K.I., ZAEHRES, H., SCHOLER, H.R. Direct reprogramming of human neural stem cells by OCT4. Nature, 2009, vol. 461, no. 7264. pp. 649-643.

KIM, J.B., SEBASTIANO, V., WU, G., ARAUZO-BRAVO, M.J., SASSE, P., GENTILE, L., KO, K., RUAU, D., EHRICH, M., VAN DEN BOOM, D., MEYER, J., HUBNER, K., BERNEMANN, C., ORTMEIER, C., ZENKE, M., FLEISCHMANN, B.K., ZAEHRES, H., SCHOLER, H.R. Oct4-induced pluripotency in adult neural stem cells. Cell, 2009, vol. 136, no. 3. pp. 411-419.

KIM, J.B., ZAEHRES, H., WU, G., GENTILE, L., KO, K., SEBASTIANO, V., ARAUZO-BRAVO, M.J., RUAU, D., HAN, D.W., ZENKE, M., SCHOLER, H.R. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature, 2008, vol. 454, no. 7204. pp. 646-650.

KIMMEL, C.A.; and YOUNG, J.F. Correlating pharmacokinetics and teratogenic endpoints. Fundam. Appl. Toxicol., 1983, vol. 3, no. 4. pp. 250-255.

KIRALY, M., KADAR, K., HORVATHY, D.B., NARDAI, P., RACZ, G.Z., LACZA, Z., VARGA, G., GERBER, G. Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo. Neurochem. Int., 2011, vol. 59, no. 3. pp. 371-381.

KISHIMOTO, T. Interleukin-6: From basic science to medicine--40 years in immunology. Annu. Rev. Immunol., 2005, vol. 23. pp. 1-21.

KOOREMAN, N.G.; and WU, J.C. Tumorigenicity of pluripotent stem cells: Biological insights from molecular imaging. J. R. Soc. Interface, 2010, vol. 7, no. 6. pp. S753-63.

KORBLING, M., ESTROV, Z., CHAMPLIN, R. Adult stem cells and tissue repair. Bone Marrow Transplant., 2003, vol. 32, no. 1. pp. S23-4.

KUANG, S., KURODA, K., LE GRAND, F., RUDNICKI, M.A. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell, 2007, vol. 129, no. 5. pp. 999-1010.

KUCIA, M., HALASA, M., WYSOCZYNSKI, M., BASKIEWICZ-MASIUK, M., MOLDENHAWER, S., ZUBA-SURMA, E., CZAJKA, R., WOJAKOWSKI, W., MACHALINSKI, B., RATAJCZAK, M.Z. Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ oct-4+ very small embryonic-like cells purified from human cord blood: Preliminary report. Leukemia, 2007, vol. 21, no. 2. pp. 297-303.

KUMADA, Y.; and ZHANG, S. Significant type I and type III collagen production from human periodontal ligament fibroblasts in 3D peptide scaffolds without extra growth factors. PLoS One, 2010, vol. 5, no. 4. pp. e10305.

KURODA, Y., WAKAO, S., KITADA, M., MURAKAMI, T., NOJIMA, M., DEZAWA, M. Isolation, culture and evaluation of multilineage-differentiating stress-enduring (muse) cells. Nat. Protoc., 2013, vol. 8, no. 7. pp. 1391-1415.

LAINO, G., D'AQUINO, R., GRAZIANO, A., LANZA, V., CARINCI, F., NARO, F., PIROZZI, G., PAPACCIO, G. A new population of human adult dental pulp stem cells: A useful source of living autologous fibrous bone tissue (LAB). J. Bone Miner. Res., 2005, vol. 20, no. 8. pp. 1394-1402.

LAMB, R.A., KOLAKOFSKY, D. Paramyxoviridae: The Viruses and their Replication. FIELDS, B. N., KNIPE, D.M., HOWLEY, P.M (Eds). Fields Virology. Lippincott-Raven Press, 1996.

LARUE, L., ANTOS, C., BUTZ, S., HUBER, O., DELMAS, V., DOMINIS, M., KEMLER, R. A role for cadherins in tissue formation. Development, 1996, vol. 122, no. 10. pp. 3185-3194.

LEE, L.A. Advances in hepatocyte transplantation: A myth becomes reality. J. Clin. Invest., 2001, vol. 108, no. 3. pp. 367-369.

LENGNER, C.J., CAMARGO, F.D., HOCHEDLINGER, K., WELSTEAD, G.G., ZAIDI, S., GOKHALE, S., SCHOLER, H.R., TOMILIN, A., JAENISCH, R. Oct4 expression is not required for mouse somatic stem cell self-renewal. Cell. Stem Cell., 2007, vol. 1, no. 4. pp. 403-415.

LI, H.O., ZHU, Y.F., ASAKAWA, M., KUMA, H., HIRATA, T., UEDA, Y., LEE, Y.S., FUKUMURA, M., IIDA, A., KATO, A., NAGAI, Y., HASEGAWA, M. A cytoplasmic RNA vector derived from nontransmissible sendai virus with efficient gene transfer and expression. J. Virol., 2000, vol. 74, no. 14. pp. 6564-6569.

LI, R., LIANG, J., NI, S., ZHOU, T., QING, X., LI, H., HE, W., CHEN, J., LI, F., ZHUANG, Q., QIN, B., XU, J., LI, W., YANG, J., GAN, Y., QIN, D., FENG, S., SONG, H., YANG, D., ZHANG, B., ZENG, L., LAI, L., ESTEBAN, M.A., PEI, D. A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. Cell. Stem Cell., 2010, vol. 7, no. 1. pp. 51-63.

LIAN, X., ZHANG, J., AZARIN, S.M., ZHU, K., HAZELTINE, L.B., BAO, X., HSIAO, C., KAMP, T.J., PALECEK, S.P. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. Nat. Protoc., 2013, vol. 8, no. 1. pp. 162-175.

LIANG, J., WAN, M., ZHANG, Y., GU, P., XIN, H., JUNG, S.Y., QIN, J., WONG, J., COONEY, A.J., LIU, D., SONGYANG, Z. Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. Nat. Cell Biol., 2008, vol. 10, no. 6. pp. 731-739.

LIM, W.F., INOUE-YOKOO, T., TAN, K.S., LAI, M.I., SUGIYAMA, D. Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells. Stem Cell. Res. Ther., 2013, vol. 4, no. 3. pp. 71.

LIU, H., GRONTHOS, S., SHI, S. Dental pulp stem cells. Methods Enzymol., 2006, vol. 419. pp. 99-113.

LIU, H., KIM, Y., SHARKIS, S., MARCHIONNI, L., JANG, Y.Y. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. Sci. Transl. Med., 2011, vol. 3, no. 82. pp. 82ra39.

LIU, P., WAKAMIYA, M., SHEA, M.J., ALBRECHT, U., BEHRINGER, R.R., BRADLEY, A. Requirement for Wnt3 in vertebrate axis formation. Nat. Genet., 1999, vol. 22, no. 4. pp. 361-365.

LOH, Y.H., AGARWAL, S., PARK, I.H., URBACH, A., HUO, H., HEFFNER, G.C., KIM, K., MILLER, J.D., NG, K., DALEY, G.Q. Generation of induced pluripotent stem cells from human blood. Blood, 2009, vol. 113, no. 22. pp. 5476-5479.

LOWRY, W.E., RICHTER, L., YACHECHKO, R., PYLE, A.D., TCHIEU, J., SRIDHARAN, R., CLARK, A.T., PLATH, K. Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc. Natl. Acad. Sci. U. S. A., 2008, vol. 105, no. 8. pp. 2883-2888.

LUDWIG, T.E., BERGENDAHL, V., LEVENSTEIN, M.E., YU, J., PROBASCO, M.D., THOMSON, J.A. Feeder-independent culture of human embryonic stem cells. Nat. Methods, 2006, vol. 3, no. 8. pp. 637-646.

MAEKAWA, M., YAMAGUCHI, K., NAKAMURA, T., SHIBUKAWA, R., KODANAKA, I., ICHISAKA, T., KAWAMURA, Y., MOCHIZUKI, H., GOSHIMA, N., YAMANAKA, S. Direct

reprogramming of somatic cells is promoted by maternal transcription factor Glis1. Nature, 2011, vol. 474, no. 7350. pp. 225-229.

MAITRA, A., ARKING, D.E., SHIVAPURKAR, N., IKEDA, M., STASTNY, V., KASSAUEI, K., SUI, G., CUTLER, D.J., LIU, Y., BRIMBLE, S.N., NOAKSSON, K., HYLLNER, J., SCHULZ, T.C., ZENG, X., FREED, W.J., CROOK, J., ABRAHAM, S., COLMAN, A., SARTIPY, P., MATSUI, S., CARPENTER, M., GAZDAR, A.F., RAO, M., CHAKRAVARTI, A. Genomic alterations in cultured human embryonic stem cells. Nat. Genet., 2005, vol. 37, no. 10. pp. 1099-1103.

MAJO, F., ROCHAT, A., NICOLAS, M., JAOUDE, G.A., BARRANDON, Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. Nature, 2008, vol. 456, no. 7219. pp. 250-254.

MANDAI, M., WATANABE, A., KURIMOTO, Y., HIRAMI, Y., MORINAGA, C., DAIMON, T., FUJIHARA, M., AKIMARU, H., SAKAI, N., SHIBATA, Y., TERADA, M., NOMIYA, Y., TANISHIMA, S., NAKAMURA, M., KAMAO, H., SUGITA, S., ONISHI, A., ITO, T., FUJITA, K., KAWAMATA, S., GO, M.J., SHINOHARA, C., HATA, K., SAWADA, M., YAMAMOTO, M., OHTA, S., OHARA, Y., YOSHIDA, K., KUWAHARA, J., KITANO, Y., AMANO, N., UMEKAGE, M., KITAOKA, F., TANAKA, A., OKADA, C., TAKASU, N., OGAWA, S., YAMANAKA, S., TAKAHASHI, M. Autologous induced stem-cells derived retinal cells for macular degeneration. N. Engl. J. Med., 2017, vol. 376, no. 11. pp. 1038-1046.

MARKOULLIS, K., BULIAN, D., HOLZLWIMMER, G., QUINTANILLA-MARTINEZ, L., HEILIGER, K.J., ZITZELSBERGER, H., SCHERB, H., MYSLIWIETZ, J., UPHOFF, C.C., DREXLER, H.G., ADLER, T., BUSCH, D.H., SCHMIDT, J., MAHABIR, E. Mycoplasma contamination of murine embryonic stem cells affects cell parameters, germline transmission and chimeric progeny. Transgenic Res., 2009, vol. 18, no. 1. pp. 71-87.

MAROOF, A.M., KEROS, S., TYSON, J.A., YING, S.W., GANAT, Y.M., MERKLE, F.T., LIU, B., GOULBURN, A., STANLEY, E.G., ELEFANTY, A.G., WIDMER, H.R., EGGAN, K., GOLDSTEIN, P.A., ANDERSON, S.A., STUDER, L. Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. Cell. Stem Cell., 2013, vol. 12, no. 5. pp. 559-572.

MARTI, M., MULERO, L., PARDO, C., MORERA, C., CARRIO, M., LARICCHIA-ROBBIO, L., ESTEBAN, C.R., IZPISUA BELMONTE, J.C. Characterization of pluripotent stem cells. Nat. Protoc., 2013, vol. 8, no. 2. pp. 223-253.

MATHAPATI, S., SILLER, R., IMPELLIZZERI, A.A., LYCKE, M., VEGHEIM, K., ALMAAS, R., SULLIVAN, G.J. Small-molecule-directed hepatocyte-like cell differentiation of human pluripotent stem cells. Curr. Protoc. Stem Cell. Biol., 2016, vol. 38. pp. 1G.6.1-1G.6.18.

METSALU, T.; and VILO, J. ClustVis: A web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. Nucleic Acids Res., 2015, vol. 43, no. 1. pp. 566-570.

MIN, J.H., KO, S.Y., CHO, Y.B., RYU, C.J., JANG, Y.J. Dentinogenic potential of human adult dental pulp cells during the extended primary culture. Hum. Cell, 2011, vol. 24, no. 1. pp. 43-50.

MIURA, M., GRONTHOS, S., ZHAO, M., LU, B., FISHER, L.W., ROBEY, P.G., SHI, S. SHED: Stem cells from human exfoliated deciduous teeth. Proc. Natl. Acad. Sci. U. S. A., 2003, vol. 100, no. 10. pp. 5807-5812.

MOKRY, J., KARBANOVA, J., FILIP, S. Differentiation potential of murine neural stem cells in vitro and after transplantation. Transplant Proc. 2005, vol. 37, pp. 268-272.

MOKRY, J., KARBANOVA, J., FILIP, S., CIZKOVÁ, D., PAZOUR, J., ENGLISH, D. Phenotypic and morphological characterization of in vitro oligodendrogliogenesis. Stem Cells Dev. 2008, vol. 17, no. 2, pp. 333-342.

MOKRY, J., KARBANOVA, J., OSTERREICHER, J. Experimental brain injury induces activation of neural stem cells in the forebrain subependymal. Appl. Immunohistochem. Mol. Morphol. 2003, vol. 11, no. 2, pp. 161-167.

MOKRY, J., SUBRTOVA, D., NEMECEK, S. Microstructure of nonpassaged spheroids formed by EGF-responsive neural precursor cells in vitro. Elec. J. Pathol. Histol. 1999, vol. 5, pp. 992-1006.

MOKRY, J., SUBRTOVA, D., NEMECEK, S. Cultivation of neural EGF-responsive precursor cells. Sbor Věd Prací LF UK Hradec Králové. 1995, vol. 38, no. 4, pp. 167-174.

MOKRY, J., SUBRTOVA, D., NEMECEK, S. Differentiation of epidermal growth factor-responsive neural precursor cells within neurospheres. Acta Med (Hradec Králové). 1996, vol. 39, pp. 7-20.

MONTARRAS, D., MORGAN, J., COLLINS, C., RELAIX, F., ZAFFRAN, S., CUMANO, A., PARTRIDGE, T., BUCKINGHAM, M. Direct isolation of satellite cells for skeletal muscle regeneration. Science, 2005, vol. 309, no. 5743. pp. 2064-2067.

MORRIS, S.A., CAHAN, P., LI, H., ZHAO, A.M., SAN ROMAN, A.K., SHIVDASANI, R.A., COLLINS, J.J., DALEY, G.Q. Dissecting engineered cell types and enhancing cell fate conversion via CellNet. Cell, 2014, vol. 158, no. 4. pp. 889-902.

NAKAJIMA, F., TOKUNAGA, K., NAKATSUJI, N. Human leukocyte antigen matching estimations in a hypothetical bank of human embryonic stem cell lines in the japanese population for use in cell transplantation therapy. Stem Cells, 2007, vol. 25, no. 4. pp. 983-985.

NAKAMURA, M.; and OKANO, H. Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells. Cell Res., 2012, vol. 23. pp. 70.

NAKAMURA, S., YAMADA, Y., KATAGIRI, W., SUGITO, T., ITO, K., UEDA, M. Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. J. Endod., 2009, vol. 35, no. 11. pp. 1536-1542.

NAKATSUJI, N., NAKAJIMA, F., TOKUNAGA, K. HLA-haplotype banking and iPS cells. Nat. Biotechnol., 2008, vol. 26, no. 7. pp. 739-740.

NG, E.S., DAVIS, R., STANLEY, E.G., ELEFANTY, A.G. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat. Protoc., 2008, vol. 3, no. 5. pp. 768-776.

NIIBE, K., KAWAMURA, Y., ARAKI, D., MORIKAWA, S., MIURA, K., SUZUKI, S., SHIMMURA, S., SUNABORI, T., MABUCHI, Y., NAGAI, Y., NAKAGAWA, T., OKANO, H., MATSUZAKI, Y. Purified mesenchymal stem cells are an efficient source for iPS cell induction. PLoS One, 2011, vol. 6, no. 3. pp. e17610.

NIKOLOVA, T., WU, M., BRUMBAROV, K., ALT, R., OPITZ, H., BOHELER, K.R., CROSS, M., WOBUS, A.M. WNT-conditioned media differentially affect the proliferation and differentiation of cord blood-derived CD133+ cells in vitro. Differentiation, 2007, vol. 75, no. 2. pp. 100-111.

NORI, S., OKADA, Y., YASUDA, A., TSUJI, O., TAKAHASHI, Y., KOBAYASHI, Y., FUJIYOSHI, K., KOIKE, M., UCHIYAMA, Y., IKEDA, E., TOYAMA, Y., YAMANAKA, S., NAKAMURA, M., OKANO, H. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proc. Natl. Acad. Sci. U. S. A., 2011, vol. 108, no. 40. pp. 16825-16830.

NOURBAKHSH, N., SOLEIMANI, M., TAGHIPOUR, Z., KARBALAIE, K., MOUSAVI, S.B., TALEBI, A., NADALI, F., TANHAEI, S., KIYANI, G.A., NEMATOLLAHI, M., RABIEI, F., MARDANI, M., BAHRAMIYAN, H., TORABINEJAD, M., NASR-ESFAHANI, M.H., BAHARVAND, H. Induced in vitro differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells. Int. J. Dev. Biol., 2011, vol. 55, no. 2. pp. 189-195.

O'CONNOR, M.D., KARDEL, M.D., EAVES, C.J. Functional assays for human embryonic stem cell pluripotency. Methods Mol. Biol., 2011, vol. 690. pp. 67-80.

OKITA, K., MATSUMURA, Y., SATO, Y., OKADA, A., MORIZANE, A., OKAMOTO, S., HONG, H., NAKAGAWA, M., TANABE, K., TEZUKA, K., SHIBATA, T., KUNISADA, T., TAKAHASHI, M., TAKAHASHI, J., SAJI, H., YAMANAKA, S. A more efficient method to generate integration-free human iPS cells. Nat. Methods, 2011, vol. 8, no. 5. pp. 409-412.

OKTAR, P.A., YILDIRIM, S., BALCI, D., CAN, A. Continual expression throughout the cell cycle and downregulation upon adipogenic differentiation makes nucleostemin a vital human MSC proliferation marker. Stem Cell. Rev., 2011, vol. 7, no. 2. pp. 413-424.

OSATHANON, T., NOWWAROTE, N., PAVASANT, P. Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLCgamma signaling pathway. J. Cell. Biochem., 2011, vol. 112, no. 7. pp. 1807-1816.

OTT, M., SCHMIDT, H.H., CICHON, G., MANNS, M.P. Emerging therapies in hepatology: Liver-directed gene transfer and hepatocyte transplantation. Cells Tissues Organs, 2000, vol. 167, no. 2-3. pp. 81-87.

PAINO, F., RICCI, G., DE ROSA, A., D'AQUINO, R., LAINO, L., PIROZZI, G., TIRINO, V., PAPACCIO, G. Ecto-mesenchymal stem cells from dental pulp are committed to differentiate into active melanocytes. Eur. Cell. Mater., 2010, vol. 20. pp. 295-305.

PAPP, B.; and PLATH, K. Reprogramming to pluripotency: Stepwise resetting of the epigenetic landscape. Cell Res., 2011, vol. 21, no. 3. pp. 486-501.

PARK, I.H., LEROU, P.H., ZHAO, R., HUO, H., DALEY, G.Q. Generation of human-induced pluripotent stem cells. Nat. Protoc., 2008, vol. 3, no. 7. pp. 1180-1186.

PARK, J.H., DAHERON, L., KANTARCI, S., LEE, B.S., TEIXEIRA, J.M. Human endometrial cells express elevated levels of pluripotent factors and are more amenable to reprogramming into induced pluripotent stem cells. Endocrinology, 2011, vol. 152, no. 3. pp. 1080-1089.

PFEIFFER, T., SCHUSTER, S., BONHOEFFER, S. Cooperation and competition in the evolution of ATP-producing pathways. Science, 2001, vol. 292, no. 5516. pp. 504-507.

PISAL, R.V., HREBIKOVA, H., CHVATALOVA, J., KUNKE, D., FILIP, S., MOKRY, J. Detection of mycoplasma contamination directly from culture supernatant using polymerase chain reaction. Folia Biol. (Praha), 2016, vol. 62, no. 5. pp. 203-206.

PISAL, R.V., HREBIKOVA, H., CHVATALOVA, J., SOUKUP, T., STANISLAV, F., MOKRY, J. Cloning of intronic sequence within DsRed2 increased the number of cells expressing red fluorescent protein. Biomed. Pap., 2017, vol. 161, no. 4. pp. 354-359.

PISAL, R.V., SUCHANEK, J., SILLER, R., SOUKUP, T., HREBIKOVA, H., BEZROUK, A., KUNKE, D., MICUDA, S., FILIP, S., SULLIVAN, G., MOKRY, J. Directed reprogramming of comprehensively characterized dental pulp stem cells extracted from natal tooth. Sci. Rep., 2018, vol. 8, no. 1. pp. 6168.

PISCIOTTA, A., CARNEVALE, G., MELONI, S., RICCIO, M., DE BIASI, S., GIBELLINI, L., FERRARI, A., BRUZZESI, G., DE POL, A. Human dental pulp stem cells (hDPSCs): Isolation, enrichment and comparative differentiation of two sub-populations. BMC Dev. Biol., 2015, vol. 15. pp. 10.1186/s12861-015-0065-x.

PROCKOP, D.J. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science, 1997, vol. 276, no. 5309. pp. 71-74.

RAO, M.S.; and MALIK, N. Assessing iPSC reprogramming methods for their suitability in translational medicine. J. Cell. Biochem., 2012, vol. 113, no. 10. pp. 3061-3068.

RAZIN, S., YOGEV, D., NAOT, Y. Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev., 1998, vol. 62, no. 4. pp. 1094-1156.

REYNOLDS, B.A., TETZLAFF, W., WEISS, S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci., 1992, vol. 12, no. 11. pp. 4565-4574.

REYNOLDS, B.A.; and WEISS, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science, 1992, vol. 255, no. 5052. pp. 1707-1710.

RIEKSTINA, U., CAKSTINA, I., PARFEJEVS, V., HOOGDUIJN, M., JANKOVSKIS, G., MUIZNIEKS, I., MUCENIECE, R., ANCANS, J. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. Stem Cell. Rev., 2009, vol. 5, no. 4. pp. 378-386.

RODRIGUEZ, A.M., ELABD, C., AMRI, E.Z., AILHAUD, G., DANI, C. The human adipose tissue is a source of multipotent stem cells. Biochimie, 2005, vol. 87, no. 1. pp. 125-128.

ROSSANT, J. Stem cells from the mammalian blastocyst. Stem Cells, 2001, vol. 19, no. 6. pp. 477-482.

ROTH, T.L., MILENKOVIC, L., SCOTT, M.P. A rapid and simple method for DNA engineering using cycled ligation assembly. PLoS One, 2014, vol. 9, no. 9. pp. e107329.

ROTTEM, S. Interaction of mycoplasmas with host cells. Physiol. Rev., 2003, vol. 83, no. 2. pp. 417-432.

SALAZAR, K.D., LANKFORD, S.M., BRODY, A.R. Mesenchymal stem cells produce wnt isoforms and TGF-beta1 that mediate proliferation and procollagen expression by lung fibroblasts. Am. J. Physiol. Lung Cell. Mol. Physiol., 2009, vol. 297, no. 5. pp. L1002-11.

SAMAVARCHI-TEHRANI, P., GOLIPOUR, A., DAVID, L., SUNG, H.K., BEYER, T.A., DATTI, A., WOLTJEN, K., NAGY, A., WRANA, J.L. Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell. Stem Cell., 2010, vol. 7, no. 1. pp. 64-77.

SASAKI, R., AOKI, S., YAMATO, M., UCHIYAMA, H., WADA, K., OKANO, T., OGIUCHI, H. Neurosphere generation from dental pulp of adult rat incisor. Eur. J. Neurosci., 2008, vol. 27, no. 3. pp. 538-548.

SAXENA, A., FISH, J.E., WHITE, M.D., YU, S., SMYTH, J.W., SHAW, R.M., DIMAIO, J.M., SRIVASTAVA, D. Stromal cell-derived factor-1alpha is cardioprotective after myocardial infarction. Circulation, 2008, vol. 117, no. 17. pp. 2224-2231.

SCHMITTGEN, T.D.; and LIVAK, K.J. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc., 2008, vol. 3, no. 6. pp. 1101-1108.

SEGRE, J.A., BAUER, C., FUCHS, E. Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat. Genet., 1999, vol. 22, no. 4. pp. 356-360.

SEILER, A., VISAN, A., BUESEN, R., GENSCHOW, E., SPIELMANN, H. Improvement of an in vitro stem cell assay for developmental toxicity: The use of molecular endpoints in the embryonic stem cell test. Reprod. Toxicol., 2004, vol. 18, no. 2. pp. 231-240.

SERWOLD, T., HOCHEDLINGER, K., INLAY, M.A., JAENISCH, R., WEISSMAN, I.L. Early TCR expression and aberrant T cell development in mice with endogenous prerearranged T cell receptor genes. J. Immunol., 2007, vol. 179, no. 2. pp. 928-938.

SHELTON, M., METZ, J., LIU, J., CARPENEDO, R.L., DEMERS, S.P., STANFORD, W.L., SKERJANC, I.S. Derivation and expansion of PAX7-positive muscle progenitors from human and mouse embryonic stem cells. Stem Cell. Reports, 2014, vol. 3, no. 3. pp. 516-529.

SHERIDAN, S.D., SURAMPUDI, V., RAO, R.R. Analysis of embryoid bodies derived from human induced pluripotent stem cells as a means to assess pluripotency. Stem Cells Int., 2012, vol. 2012. pp. 738910.

SILLER, R., GREENHOUGH, S., NAUMOVSKA, E., SULLIVAN, G.J. Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. Stem Cell. Reports, 2015, vol. 4, no. 5. pp. 939-952.

SILLER, R., GREENHOUGH, S., PARK, I.H., SULLIVAN, G.J. Modelling human disease with pluripotent stem cells. Curr. Gene Ther., 2013, vol. 13, no. 2. pp. 99-110.

SILLER, R., NAUMOVSKA, E., MATHAPATI, S., LYCKE, M., GREENHOUGH, S., SULLIVAN, G.J. Development of a rapid screen for the endodermal differentiation potential of human pluripotent stem cell lines. Sci. Rep., 2016, vol. 6. pp. 37178.

SILLER, R.; and SULLIVAN, G.J. Rapid screening of the endodermal differentiation potential of human pluripotent stem cells. Curr. Protoc. Stem Cell. Biol., 2017, vol. 43. pp. 1G.7.1-1G.7.23.

SIQUEIRA DA FONSECA, S.A., ABDELMASSIH, S., DE MELLO CINTRA LAVAGNOLLI, T., SERAFIM, R.C., CLEMENTE SANTOS, E.J., MOTA MENDES, C., DE SOUZA PEREIRA, V., AMBROSIO, C.E., MIGLINO, M.A., VISINTIN, J.A., ABDELMASSIH, R., KERKIS, A., KERKIS, I. Human immature dental pulp stem cells' contribution to developing mouse embryos: Production of human/mouse preterm chimaeras. Cell Prolif., 2009, vol. 42, no. 2. pp. 132-140.

SKELTON, R.J., BRADY, B., KHOJA, S., SAHOO, D., ENGEL, J., ARASARATNAM, D., SALEH, K.K., ABILEZ, O.J., ZHAO, P., STANLEY, E.G., ELEFANTY, A.G., KWON, M., ELLIOTT, D.A., ARDEHALI, R. CD13 and ROR2 permit isolation of highly enriched cardiac mesoderm from differentiating human embryonic stem cells. Stem Cell. Reports, 2016, vol. 6, no. 1. pp. 95-108.

SOLDNER, F., HOCKEMEYER, D., BEARD, C., GAO, Q., BELL, G.W., COOK, E.G., HARGUS, G., BLAK, A., COOPER, O., MITALIPOVA, M., ISACSON, O., JAENISCH, R. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell, 2009, vol. 136, no. 5. pp. 964-977.

SOTO-GUTIERREZ, A., NAVARRO-ALVAREZ, N., ZHAO, D., RIVAS-CARRILLO, J.D., LEBKOWSKI, J., TANAKA, N., FOX, I.J., KOBAYASHI, N. Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines. Nat. Protoc., 2007, vol. 2, no. 2. pp. 347-356.

SPITS, C., MATEIZEL, I., GEENS, M., MERTZANIDOU, A., STAESSEN, C., VANDESKELDE, Y., VAN DER ELST, J., LIEBAERS, I., SERMON, K. Recurrent chromosomal abnormalities in human embryonic stem cells. Nat. Biotechnol., 2008, vol. 26, no. 12. pp. 1361-1363.

STADTFELD, M., BRENNAND, K., HOCHEDLINGER, K. Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr. Biol., 2008, vol. 18, no. 12. pp. 890-894.

STADTFELD, M., MAHERALI, N., BREAULT, D.T., HOCHEDLINGER, K. Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell. Stem Cell., 2008, vol. 2, no. 3. pp. 230-240.

STAERK, J., DAWLATY, M.M., GAO, Q., MAETZEL, D., HANNA, J., SOMMER, C.A., MOSTOSLAVSKY, G., JAENISCH, R. Reprogramming of human peripheral blood cells to induced pluripotent stem cells. Cell. Stem Cell., 2010, vol. 7, no. 1. pp. 20-24.

STEVENS, A., ZULIANI, T., OLEJNIK, C., LEROY, H., OBRIOT, H., KERR-CONTE, J., FORMSTECHER, P., BAILLIEZ, Y., POLAKOWSKA, R.R. Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. Stem Cells Dev., 2008, vol. 17, no. 6. pp. 1175-1184.

STRECKFUSS-BOMEKE, K., WOLF, F., AZIZIAN, A., STAUSKE, M., TIBURCY, M., WAGNER, S., HUBSCHER, D., DRESSEL, R., CHEN, S., JENDE, J., WULF, G., LORENZ, V., SCHON, M.P., MAIER, L.S., ZIMMERMANN, W.H., HASENFUSS, G., GUAN, K. Comparative study of human-induced pluripotent stem cells derived from bone marrow cells, hair keratinocytes, and skin fibroblasts. Eur. Heart J., 2013, vol. 34, no. 33. pp. 2618-2629.

STRONGIN, D.E., BEVIS, B., KHUONG, N., DOWNING, M.E., STRACK, R.L., SUNDARAM, K., GLICK, B.S., KEENAN, R.J. Structural rearrangements near the chromophore influence the maturation speed and brightness of DsRed variants. Protein Eng. Des. Sel., 2007, vol. 20, no. 11. pp. 525-534.

SUCHANEK, J., SOUKUP, T., VISEK, B., IVANCAKOVA, R., KUCEROVA, L., MOKRY, J. Dental pulp stem cells and their characterization. Biomed. Pap., 2009, vol. 153, no. 1. pp. 31-35.

SUCHANEK, J., SUCHANKOVA KLEPLOVA, T., REHACEK, V., BROWNE, K.Z., SOUKUP, T. Proliferative capacity and phenotypical alteration of multipotent ecto-mesenchymal stem cells from human exfoliated deciduous teeth cultured in xenogeneic and allogeneic media. Folia Biol. (Praha), 2016, vol. 62, no. 1. pp. 1-14.

SUHR, S.T., CHANG, E.A., RODRIGUEZ, R.M., WANG, K., ROSS, P.J., BEYHAN, Z., MURTHY, S., CIBELLI, J.B. Telomere dynamics in human cells reprogrammed to pluripotency. PLoS One, 2009, vol. 4, no. 12. pp. e8124.

SULLIVAN, G.J., HAY, D.C., PARK, I.H., FLETCHER, J., HANNOUN, Z., PAYNE, C.M., DALGETTY, D., BLACK, J.R., ROSS, J.A., SAMUEL, K., WANG, G., DALEY, G.Q., LEE, J.H., CHURCH, G.M., FORBES, S.J., IREDALE, J.P., WILMUT, I. Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology, 2010, vol. 51, no. 1. pp. 329-335.

SUN, N., PANETTA, N.J., GUPTA, D.M., WILSON, K.D., LEE, A., JIA, F., HU, S., CHERRY, A.M., ROBBINS, R.C., LONGAKER, M.T., WU, J.C. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proc. Natl. Acad. Sci. U. S. A., 2009, vol. 106, no. 37. pp. 15720-15725.

SUZUKI, N., YAMAZAKI, S., YAMAGUCHI, T., OKABE, M., MASAKI, H., TAKAKI, S., OTSU, M., NAKAUCHI, H. Generation of engraftable hematopoietic stem cells from induced pluripotent stem cells by way of teratoma formation. Mol. Ther., 2013, vol. 21, no. 7. pp. 1424-1431.

TADA, M., TAKAHAMA, Y., ABE, K., NAKATSUJI, N., TADA, T. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr. Biol., vol. 11, no. 19. pp. 1553-1558.

TAKADA, S., STARK, K.L., SHEA, M.J., VASSILEVA, G., MCMAHON, J.A., MCMAHON, A.P. Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev., 1994, vol. 8, no. 2. pp. 174-189.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K., YAMANAKA, S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 2007, vol. 131, no. 5. pp. 861-872.

TAKAHASHI, K.; and YAMANAKA, S. A decade of transcription factor-mediated reprogramming to pluripotency. Nat. Rev. Mol. Cell Biol., 2016, vol. 17, no. 3. pp. 183-193.

TAKAHASHI, K.; and YAMANAKA, S. Induced pluripotent stem cells in medicine and biology. Development, 2013, vol. 140, no. 12. pp. 2457-2461.

TAKAHASHI, K.; and YAMANAKA, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 2006, vol. 126, no. 4. pp. 663-676.

TAKAHASHI, M., YAMAGUCHI, E., UCHIDA, T. Thermophilic DNA ligase. purification and properties of the enzyme from thermus thermophilus HB8. J. Biol. Chem., 1984, vol. 259, no. 16. pp. 10041-10047.

TAN, H.K., TOH, C.X., MA, D., YANG, B., LIU, T.M., LU, J., WONG, C.W., TAN, T.K., LI, H., SYN, C., TAN, E.L., LIM, B., LIM, Y.P., COOK, S.A., LOH, Y.H. Human finger-prick induced pluripotent stem cells facilitate the development of stem cell banking. Stem Cells Transl. Med., 2014, vol. 3, no. 5. pp. 586-598.

TAN, J.Y., SRIRAM, G., RUFAIHAH, A.J., NEOH, K.G., CAO, T. Efficient derivation of lateral plate and paraxial mesoderm subtypes from human embryonic stem cells through GSKi-mediated differentiation. Stem Cells Dev., 2013, vol. 22, no. 13. pp. 1893-1906.

TAYLOR, C.J., PEACOCK, S., CHAUDHRY, A.N., BRADLEY, J.A., BOLTON, E.M. Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient HLA types. Cell. Stem Cell., 2012, vol. 11, no. 2. pp. 147-152.

THOMAS, C.E., EHRHARDT, A., KAY, M.A. Progress and problems with the use of viral vectors for gene therapy. Nat. Rev. Genet., 2003, vol. 4, no. 5. pp. 346-358.

THOMSON, J.A., ITSKOVITZ-ELDOR, J., SHAPIRO, S.S., WAKNITZ, M.A., SWIERGIEL, J.J., MARSHALL, V.S., JONES, J.M. Embryonic stem cell lines derived from human blastocysts. Science, 1998, vol. 282, no. 5391. pp. 1145-1147.

TOKUSUMI, T., IIDA, A., HIRATA, T., KATO, A., NAGAI, Y., HASEGAWA, M. Recombinant sendai viruses expressing different levels of a foreign reporter gene. Virus Res., 2002, vol. 86, no. 1-2. pp. 33-38.

TOMARU, Y., HASEGAWA, R., SUZUKI, T., SATO, T., KUBOSAKI, A., SUZUKI, M., KAWAJI, H., FORREST, A.R., HAYASHIZAKI, Y., FANTOM Consortium, SHIN, J.W., SUZUKI, H. A transient disruption of fibroblastic transcriptional regulatory network facilitates trans-differentiation. Nucleic Acids Res., 2014, vol. 42, no. 14. pp. 8905-8913.

UNGRIN, M., O'CONNOR, M., EAVES, C., ZANDSTRA, P.W. Phenotypic analysis of human embryonic stem cells. Curr. Protoc. Stem Cell. Biol., 2007, vol. Chapter 1. pp. Unit 1B.3.

UPHOFF, C.C.; and DREXLER, H.G. Comparative PCR analysis for detection of mycoplasma infections in continuous cell lines. In Vitro Cell. Dev. Biol. Anim., 2002, vol. 38, no. 2. pp. 79-85.

VAZIRI, H., CHAPMAN, K.B., GUIGOVA, A., TEICHROEB, J., LACHER, M.D., STERNBERG, H., SINGEC, I., BRIGGS, L., WHEELER, J., SAMPATHKUMAR, J., GONZALEZ, R., LAROCCA, D., MURAI, J., SNYDER, E., ANDREWS, W.H., FUNK, W.D., WEST, M.D. Spontaneous reversal of the developmental aging of normal human cells following transcriptional reprogramming. Regen. Med., 2010, vol. 5, no. 3. pp. 345-363.

VIDAL, S., AMLANI, B., CHEN, T., TSIRIGOS, A., STADTFELD, M. Combinatorial modulation of signaling pathways reveals cell-type-specific requirements for highly efficient and synchronous iPSC reprogramming. Stem Cell. Reports, 2014, vol. 3, no. 4. pp. 574-584.

WANG, F., YIN, Y., YE, X., LIU, K., ZHU, H., WANG, L., CHIOUREA, M., OKUKA, M., JI, G., DAN, J., ZUO, B., LI, M., ZHANG, Q., LIU, N., CHEN, L., PAN, X., GAGOS, S., KEEFE, D.L., LIU, L.

Molecular insights into the heterogeneity of telomere reprogramming in induced pluripotent stem cells. Cell Res., 2012, vol. 22, no. 4. pp. 757-768.

WANG, J., GU, Q., HAO, J., BAI, D., LIU, L., ZHAO, X., LIU, Z., WANG, L., ZHOU, Q. Generation of induced pluripotent stem cells with high efficiency from human umbilical cord blood mononuclear cells. Genomics, Proteomics & Bioinformatics, 2013, vol. 11, no. 5. pp. 304.

WANG, Z., ORON, E., NELSON, B., RAZIS, S., IVANOVA, N. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell. Stem Cell., 2012, vol. 10, no. 4. pp. 440-454.

WARREN, L., MANOS, P.D., AHFELDT, T., LOH, Y., LI, H., LAU, F., EBINA, W., MANDAL, P.K., SMITH, Z.D., MEISSNER, A., DALEY, G.Q., BRACK, A.S., COLLINS, J.J., COWAN, C., SCHLAEGER, T.M., ROSSI, D.J. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell, 2010, vol.7, no.5, 618-630.

WERNIG, M., ZHAO, J.P., PRUSZAK, J., HEDLUND, E., FU, D., SOLDNER, F., BROCCOLI, V., CONSTANTINE-PATON, M., ISACSON, O., JAENISCH, R. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with parkinson's disease. Proc. Natl. Acad. Sci. U. S. A., 2008, vol. 105, no. 15. pp. 5856-5861.

WILLIAMS, L.A., DAVIS-DUSENBERY, B., EGGAN, K.C. SnapShot: Directed differentiation of pluripotent stem cells. Cell, vol. 149, no. 5. pp. 1174-1174.e1.

WILMUT, I., SCHNIEKE, A.E., MCWHIR, J., KIND, A.J., CAMPBELL, K.H.S. Viable offspring derived from fetal and adult mammalian cells. Nature, 1997, vol. 385. pp. 810.

WU, H., KIM, K.J., MEHTA, K., PAXIA, S., SUNDSTROM, A., ANANTHARAMAN, T., KURAISHY, A.I., DOAN, T., GHOSH, J., PYLE, A.D., CLARK, A., LOWRY, W., FAN, G., BAXTER, T., MISHRA, B., SUN, Y., TEITELL, M.A. Copy number variant analysis of human embryonic stem cells. Stem Cells, 2008, vol. 26, no. 6. pp. 1484-1489.

XIE, C.Q., HUANG, H., WEI, S., SONG, L.S., ZHANG, J., RITCHIE, R.P., CHEN, L., ZHANG, M., CHEN, Y.E. A comparison of murine smooth muscle cells generated from embryonic versus induced pluripotent stem cells. Stem Cells Dev., 2009, vol. 18, no. 5. pp. 741-748.

XUE, Y., CAI, X., WANG, L., LIAO, B., ZHANG, H., SHAN, Y., CHEN, Q., ZHOU, T., LI, X., HOU, J., CHEN, S., LUO, R., QIN, D., PEI, D., PAN, G. Generating a non-integrating human induced pluripotent stem cell bank from urine-derived cells. PLoS One, 2013, vol. 8, no. 8. pp. e70573.

YAMADA, Y., NAKAMURA, S., ITO, K., SUGITO, T., YOSHIMI, R., NAGASAKA, T., UEDA, M. A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology. Tissue Eng. Part A., 2010, vol. 16, no. 6. pp. 1891-1900.

YAN, X., QIN, H., QU, C., TUAN, R.S., SHI, S., HUANG, G.T. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev., 2010, vol. 19, no. 4. pp. 469-480.

YAO, S., CHEN, S., CLARK, J., HAO, E., BEATTIE, G.M., HAYEK, A., DING, S. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc. Natl. Acad. Sci. U. S. A., 2006, vol. 103, no. 18. pp. 6907-6912.

YU, J., VODYANIK, M.A., SMUGA-OTTO, K., ANTOSIEWICZ-BOURGET, J., FRANE, J.L., TIAN, S., NIE, J., JONSDOTTIR, G.A., RUOTTI, V., STEWART, R., SLUKVIN, I.I., THOMSON, J.A. Induced pluripotent stem cell lines derived from human somatic cells. Science, 2007, vol. 318, no. 5858. pp. 1917-1920.

YULIN, X., LIZHEN, L., LIFEI, Z., SHAN, F., RU, L., KAIMIN, H., HUANG, H. Efficient generation of induced pluripotent stem cells from human bone marrow mesenchymal stem cells. Folia Biol. (Praha), 2012, vol. 58, no. 6. pp. 221-230.

ZHANG, J., NUEBEL, E., DALEY, G.Q., KOEHLER, C.M., TEITELL, M.A. Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. Cell. Stem Cell., 2012, vol. 11, no. 5. pp. 589-595.

ZHAO, Y., WANG, H., MAZZONE, T. Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics. Exp. Cell Res., 2006, vol. 312, no. 13. pp. 2454-2464.

ZHOU, T., BENDA, C., DUZINGER, S., HUANG, Y., LI, X., LI, Y., GUO, X., CAO, G., CHEN, S., HAO, L., CHAN, Y.C., NG, K.M., HO, J.C., WIESER, M., WU, J., REDL, H., TSE, H.F., GRILLARI, J., GRILLARI-VOGLAUER, R., PEI, D., ESTEBAN, M.A. Generation of induced pluripotent stem cells from urine. J. Am. Soc. Nephrol., 2011, vol. 22, no. 7. pp. 1221-1228.

ZUK, P.A., ZHU, M., ASHJIAN, P., DE UGARTE, D.A., HUANG, J.I., MIZUNO, H., ALFONSO, Z.C., FRASER, J.K., BENHAIM, P., HEDRICK, M.H. Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell, 2002, vol. 13, no. 12. pp. 4279-4295.