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Cellular reprogramming as a tool for harvesting patient specific stem cells

Rishikaysh. V. Pisal. M.Sc.

Extended abstract

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Author: M.Sc. Rishikaysh.V.Pisal
Department of Histology and Embryology

Guide: Prof. MUDr. Jaroslav Mokry
Department of Histology and Embryology

Opponent:

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Prof. MUDr. Jaroslav Mokry. Ph.D
Chairperson of the doctoral dissertation defense commission for doctoral degree program in Anatomy, Histology and Embryology
Guarantor of the study program

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1. Abstract

Cellular reprogramming as a tool for harvesting patient specific stem cells

In the year 2006, Dr. Yamanaka surprised the entire field of medicine, by reporting a technique of inducing pluripotency in somatic cells. In his article, he had displayed that fibroblasts could be reprogrammed to pluripotent stem cell state, by ectopic expression of four transcription factors namely OCT4, SOX2, c-MYC and KLF4. His discovery made a paradigm shift in the field of reprogramming because previous methods of reprogramming were dependent on use of human oocytes and this raised ethical concerns. Moreover, his technique of cellular reprograming broadened the spectrum of application of somatic cells in regenerative medicine.

Objectives of my research were focused on; development of an optimised protocol for detection of mycoplasma that commonly infects animal tissue culture; detailed characterization of reprogrammed clones; targeted differentiation of iPSC towards myogenic lineage, and construction of an expression vector, optimised for miRNA expression.

For detecting mycoplasma infection, we adapted the protocol of Uphoff et al. (2002). By skipping the DNA extraction step (reported in the original protocol) and instead directly using cell culture supernatant and a robust polymerase enzyme for performing PCR, we made the modified protocol more sensitive, robust and rapid.

DPSC isolated from natal tooth produced heterogenous population of cells that expressed haematopoietic stem cell makers CD34 and CD45 in addition to normal DPSC markers.

DPSC derived from male donors displayed higher cell death on transduction of Sendai virus reprogramming vectors compared to DPSC derived from female donors. Furthermore, DPSC derived from female donors expressed higher reprogramming efficiency than two human fibroblasts cell lines.

nDPSC derived iPSC clone closely resembled hESC in terms of morphology, expression of pluripotency markers and gene expression profile. Furthermore, the iPSC clone spontaneously differentiated into derivatives of three embryonic germ layers when cultured as embryonic bodies. In conclusion, the selected iPSC clone passed all the necessary steps included in characterization, thus confirming true induction of pluripotency.

iPSC clones derived from DPSC and WI38, successfully committed to myogenic fate upon treatment with CHIR 99021. Furthermore, among the two iPSC clones, myogenic progenitor specific marker PAX7, was more upregulated in DPSC derived iPSC clone.

Cloning of intronic sequence within DsRed2 reporter gene increased percentage of cells expression the vector by 4 % as compared to native DsRed2 gene.

2. Introduction

2.1. Various cell lines that can be used for reprogramming

Induced pluripotent stem cells have been generated from different cell types isolated from various tissues; but fibroblasts cell line is the most preferred. This is because of easy availability of the biopsy samples from which they are usually isolated, and relatively inexpensive culture requirements. Moreover, Dr. Yamanaka in his proof of principle experiment had also used fibroblasts (Takahashi et al. 2006). Second most commonly used tissue for isolating cells for reprogramming is blood. A single drop of blood was reported to be sufficient for performing cellular reprogramming (Tan et al. 2014). An interesting source of harvesting somatic cells for cellular reprogramming was reported by Xue et al. 2013 and Zhou et al. 2011. In both the articles, authors had harvested renal epithelial cells from normal urine and had subsequently reprogrammed them to. iPSC has also been derived from rare and less frequently used cell lines like pancreatic β-cells (Stadtfeld et al. 2008), stomach cells (Aoi et al. 2008), liver cells (Aoi et al. 2008), keratinocyte (Aasen et al. 2008) etc. Owing to reprogramming conducive environment present in stem cells, many reports have been published where iPSC has been derived from different multipotent stem cells like adipose stem cells (Sun et al. 2009), neural stem cells (Kim et al. 2009a, Kim et al. 2009b), dental pulp stem cells (Yan et al. 2010) etc.

Each cell line has its own pros and cons. Some cell lines can be easily isolated; some might have high reprograming efficiency; while others may require less factors for reprogramming. Thus, the choice of cell line depends upon the type of study that is intended.

2.2. Dental pulp stem cells as a cell source for reprogramming

Dental pulp stem cells (DPSC) are multipotent stem cells that have the capacity to differentiate into chondrocytes, osteoblasts and adipocytes. They are mainly isolated from deciduous teeth and teeth extracted during routine dental procedure. Furthermore, they are relatively easy to isolate, while the tooth extraction procedure is fairly non-invasive and moreover, their use does not attract any ethical concerns. In addition to expressing mesenchymal stem cell markers, DPSC also express pluripotency markers such as OCT4, NANOG, c-MYC, SOX2, stage specific embryonic antigens (SSEA-3, SSEA-4), and tumor recognition antigens (TRA-1–60 and TRA-1–81) (Govindasamy et al. 2010, Kerkis et al. 2006, 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). These features make DPSC highly amenable to reprogramming towards pluripotency.

2.3. Detection of mycoplasma using PCR

Tissue culture products and cell lines are routinely tested for mycoplasma contamination because it significantly alters cell characteristics, thus affecting the reliability of generated data. Several

off the shelf kits and methods are present for detecting mycoplasma. These procedures are based on either of the following techniques; molecular biology, biochemical and radioactive incorporation assays, electron microscopy, etc. A PCR based detection technique reported by (Uphoff et al. 2002) was found to be sensitive, efficient, quick and reproducible. This procedure relies on detecting 16S rRNA gene, which is very well conserved across mycoplasma species. A single run can detect all possible mycoplasma species that commonly infect the animal cell cultures. Few modifications were introduced in the original protocol of (Uphoff et al. 2002) to make it more sensitive, robust and rapid (Pisal et al. 2016).

2.4. Vectors used for delivering reprogramming factors

Ectopic expression of reprogramming factors *OCT4*, *SOX2*, *c-MYC* and *KLF4* requires a vector to introduce them into the cell. In majority of the cases viruses are vectors of choice because of their efficient delivery mechanism and constitutive expression of exogenes (Rao et al. 2012). Based on whether the virus integrate with the host cell or not; it can be categorized as integrative or non-integrative. Viruses that fall under integrative group are retroviruses and lentiviruses; while Sendai and adenovirus fall under non-integrative group (Rao et al. 2012). Reprogramming factors can also be introduced in form of mRNA, proteins or by episomal vector. These are non-viral and non-integrative approaches (Rao et al. 2012).

iPSC generation using Sendai virus (SeV) platform is an ideal vector system for delivering reprogramming factors because of the following reasons: a. the virus replicates in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells and it does not integrate into the host genome (Lamb et al. 1996); b) it efficiently delivers exogenous genes in a wide spectrum of host cell species and tissues (Tokusumi et al. 2002); c) it shows low cytotoxicity upon infection (Tokusumi et al. 2002); and d) allows high-level expression of the delivered exogenous genes (Fusaki et al. 2009).

2.5. Directed differentiation of iPSC towards myogenic lineage

Targeted differentiation of iPSC towards a specific lineage can be beneficial for obtaining renewable source of human cells relevant for cell replacement therapies, drug discovery, toxicology testing, and disease modelling. To this end, we differentiated iPSC towards myogenic lineage using GSK3 inhibitor CHIR99021. After careful analysis of all the procedures available for deriving myogenic lineage, Shelton et al. (2014) protocol was found to be efficient. Additionally, the approach was non-viral and used chemically defined medium. Furthermore, the protocol mentions a provision for enriching and expanding PAX7 positive myogenic progenitor cells by prolonging bFGF treatment. Furthermore, PAX7 positive myogenic progenitor cells possess more regenerative capacity then terminally differentiated myoblasts; hence are interest in the enriching and expanding PAX7 positive cells.

2.6. Constructing vector for miRNA expression

MicroRNAs (miRNA) are a subclass of small non-coding RNAs that fine-tune the regulation of gene expression at the post-transcriptional level. 302/367 miRNA cluster was reported to reprogramme human and murine fibroblasts (Anokye-Danso et al. 2011), while the underlying mechanism associated with reprogramming remained elusive. Hence, with the quest of revealing the true mechanism, it was first decided to construct an optimum vector for miRNA expression. Commonly used expression vectors do not provide optimum environment for their expression. Hence, we decided to construct a vector in which an intron was to be placed within a reporter gene.

It has been hypothesized that cloning of intronic miRNAs within its intrinsic environment i.e. in between the intron will have a positive impact on its expression. With this notion, we designed a vector by cloning an artificial intronic sequence within red fluorescent gene DsRed2. Precise insertion of intron within the reporter gene is essential for spliceosome machinery to accurately splice the intron. Such precise cloning was possible because of cyclic ligation assembly (Pisal et al. 2017).

3. 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.

4. Material and methods

4.1. Isolation of DPSC and human dermal fibroblasts

Isolation and expansion of all DPSC cell lines was done in collaboration with Dr. Jakub Suchanek and Dr. Tomas Soukup. Out of the four DPSC cells, three were isolated from adult donors who were in their early twenties, while natal DPSC was isolated from tooth extracted from an 11-day old baby girl. Out of the two fibroblasts cell lines, one was isolated in-house from skin biopsy sample, while other was commercially available cell line called as WI38 embryonic lung fibroblasts. Prior to reprogramming, all DPSC cell lines were cultured as previously described by Karbanova et al. (2011) while adult human fibroblasts and WI38 lung fibroblasts were cultured in DMEM and MEM basal media respectively with 10% FBS.

4.2. Screening of cell lines for mycoplasma contamination

All the cell lines were cultured for minimum two weeks before harvesting the cell culture supernatant. 1 mL of cell culture supernatant was harvested for mycoplasma detection. Detection was performed using our in-house standardised PCR based screening protocol (Pisal et al. 2016).

4.3. Reprogramming nDPSC, adult fibroblasts and WI38 embryonic lung fibroblasts

CytoTune-iPS 2.0 Sendai Reprogramming Kit (Life Technologies) was used for reprogramming. Cells were processed as per the guidelines and procedures provided by the manufacturer of the kit. For DPSC, few modifications were introduced to the basic protocol.

Isolated reprogrammed clones were cultured on vitronectin coated plates and were regularly passaged using 0.5 mM EDTA.

4.4. Characterization of iPSC clones

iPSC clones were characterized by performing immunocytochemistry, RT-qPCR and embryoid body mediated spontaneous differentiation into three germ layers.

4.5. Targeted differentiation of iPSC towards myogenic lineage

iPSC were differentiated into PAX7 positive muscle progenitor cells (MSC) using GSK3 inhibitor CHIR99021. The MSC were expanded using bFGF to enrich the population of PAX7 positive cells.

At the start of differentiation, iPSC were plated as single cells. The following day they were treated with CHIR99021 for 48 hrs. Following CHIR99021 treatment, cells were cultured in E6 media. On the 8^{th} day of differentiation, E6 medium was supplemented with bFGF. The cells were cultured until day 21.

4.6. Constructing intron cloned DsRed2 miRNA expression vector

Using splice prediction software, sequence of DsRed2 gene was analysed to find an exact location to insert the intron. An artificial intronic sequence was inserted into the DsRed2 gene using cyclic ligation assembly. Successful cloning of intron was confirmed by DNA sequencing. Furthermore, intron recognition and successful splicing was confirmed by checking expression of red fluorescence.

5. Results

5.1. Screening of cell lines for presence of mycoplasma

Our PCR based mycoplasma detection protocol is a modified version of the original protocol reported by Uphoff et al. 2001. Changes introduced to the original protocol are; direct use of cell culture supernatant for PCR instead of extracting the mycoplasma DNA from the cell culture supernatant; use of robust One Taq DNA polymerase instead of normal polymerase to tolerate inhibitory effect of FBS present in cell culture supernatant. Since direct cell supernatant was used for PCR, initial optimisation was required to find optimum volume of supernatant that could be tolerated by polymerase enzyme.

1 µL volume of cell culture supernatant showed maximum polymerase activity (based on band intensity) while 4 µL was the maximum volume that was tolerable by the polymerase enzyme.

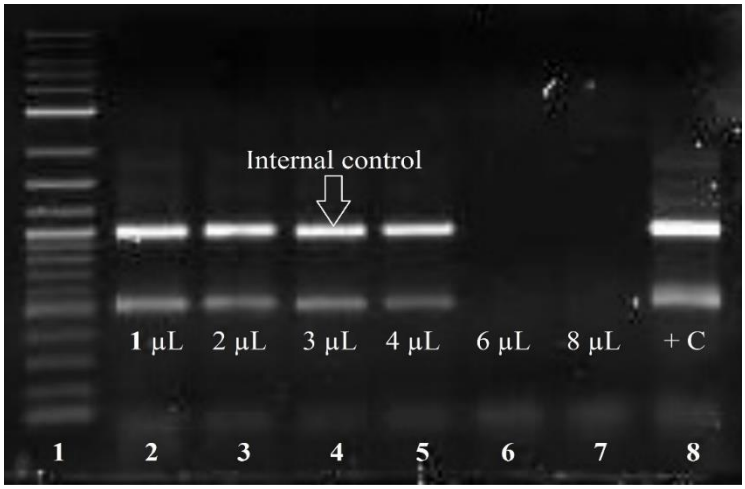

Fig. 1. Determining FBS tolerance limit of One Taq polymerase. Lane 1, DNA ladder; lane 2 -7, different volumes of cell culture supernatants, starting from 1 µL (lane 2) to 8 µL. Lane 8, positive control (+ C). Band corresponding to 968 bp is the internal control while the band below internal control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

After optimising all the parameters, the finalised protocol was used for screening culture supernatants of five cell lines. Appropriate controls were also run along with the test samples to rule out false negative and false positive results. Analysis of the gel electrophoresis image revealed that all the three human fibroblasts cell lines were infected with mycoplasma (Fig. 2).

In conclusion, 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.

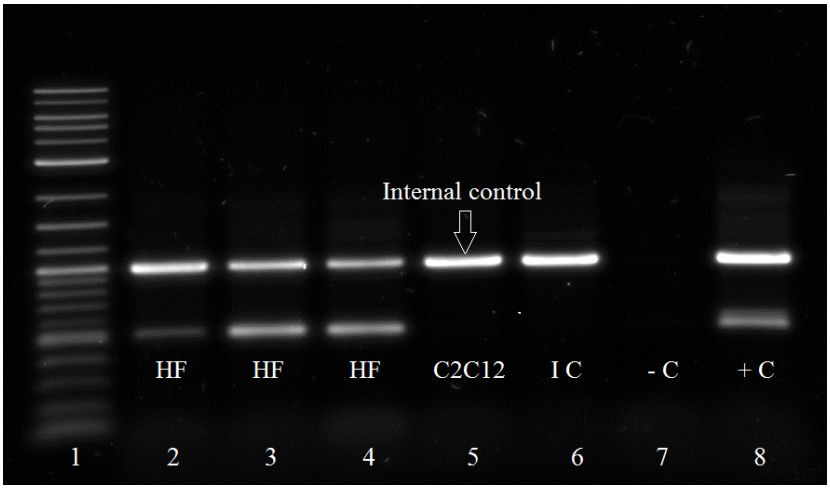

Fig. 2. Analysing various cell culture supernatant for presence of mycoplasma. Lane 1, DNA ladder; lane 2-4, cell culture supernatants from three different human fibroblasts samples; (lane 5) cell culture supernatants from murine myoblasts C2C12; (lane 6) internal control only (IC); (lane 7) negative 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.

5.2. Characterization of dental pulp stem cells

For studying effect of cell type on reprogramming efficiency, it was decided to use dental pulp stem cells (DPSC) and human fibroblasts. All the DPSC cell lines were previously characterised except one that was derived from natal tooth. Like other DPSC cell line, this cell line also grew as heterogeneous population. Moreover, cells displaying fibroblastic morphology were more abundant (Fig. 3).

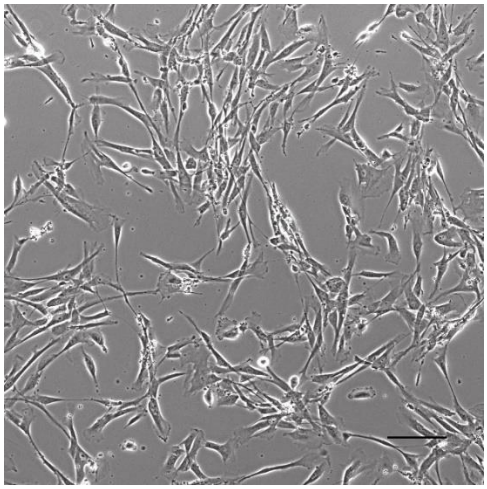

Fig. 3. Bright field image of nDPSC. The cells displaying fibroblastic morphology are the most predominant while few rounded or hexagonal cells are also visible. Scale bar = 200 µm.

Phenotypic analysis of nDPSC using flow cytometry showed positivity for markers like CD34, CD45, CD271, CD71, HLA-DR, CD146, CXCR4, CD29, CD105, CD222, CD166, CD44, CD90, CD10, CD30 and CD73 (Table 1). Out of this group of markers, not all of them are expressed by normal DPSC; these are CD34, CD45, CD271, CD71, CXCR4 and HLA-DR.

Expression of markers, CD34 and CD45 in nDPSC was surprising because these are characteristics markers of haematopoietic stem cells and were not expressed by DPSC previously isolated by us using the same isolation and expansion procedure.

This distinction in expression profile of markers displayed by nDPSC can 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 the cells were 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 1. Panel of markers used for characterizing DPSC. nDPSC expressed 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).

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

Stem cells possess greater differentiation capacity than somatic cells. This property of stem cells is attributed to upregulation of stemness genes. Some of these genes are also part of pluripotency

network. On comparing gene expression levels between hES and nDPSC, it was observed that 17 pluripotency genes were more upregulated in nDPSC than hES. Next, I compared these 17 pluripotency genes between nDPSC and two human fibroblasts cell line. This comparison revealed clear distinction between the two cell types. Furthermore, if DPSC showed higher reprogramming efficiency than fibroblasts, then I could directly correlate this effect to the pluripotency genes that are more upregulated in nDPSC. 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 were used as calibrator to calculate fold change.

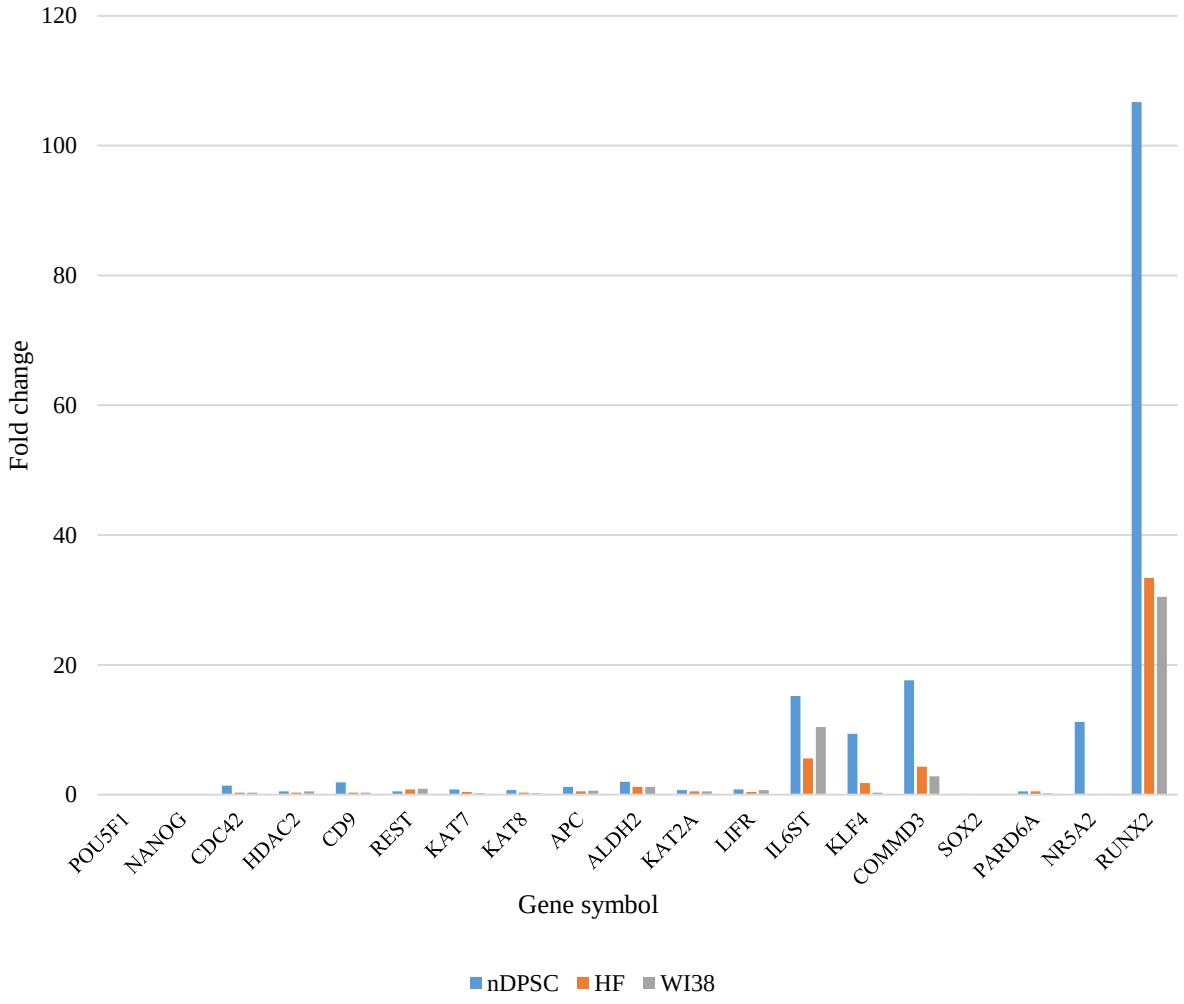

Fig. 4. Graph represents expression of pluripotency genes in non-reprogrammed nDPSC, human fibroblasts and WI38 human embryonic lung fibroblasts. *NR5A2*, *RUNX2*, *COMMD3*, *IL6ST* and *KLF4* are significantly upregulated in nDPSC as compared to two human fibroblasts.

Analysis of this comparison revealed a clear distinction in expression pattern between nDPSC and HF (Fig. 4 and Fig. 5). Out of the 20 set of genes analysed, six genes were highly upregulated in

nDPSC as compared to human fibroblasts. These genes are *KLF4*, *NR5A2*, *RUNX2*, *COMMD3*, *IL6ST* and *TBX3*.

Out of the six genes that were highly upregulated in nDPSC four of them i.e. *KLF4*, *NR5A2*, *IL6ST* and *TBX3* were previously been reported to enhance or influence reprogramming. *KLF4* is one of the reprogramming factors; hence, it would be interesting to eliminate this factor from reprogramming cocktail and study its effect on reprogramming efficiency.

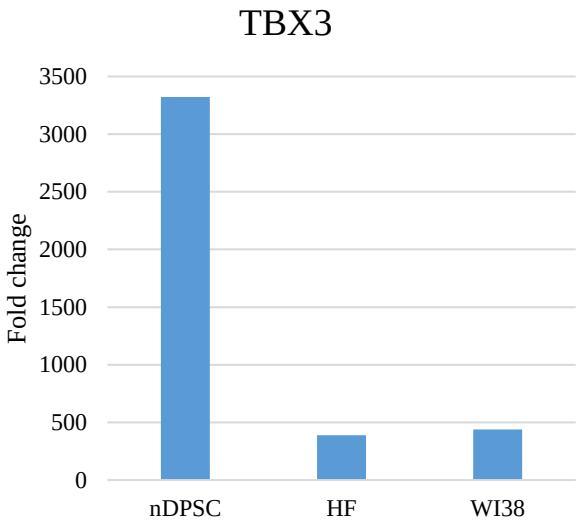

Fig. 5. Graph represents expression of *TBX3* genes in non-reprogrammed nDPSC, human fibroblasts and WI38 human embryonic lung fibroblasts. *TBX3* is significantly upregulated in nDPSC as compared to two human fibroblasts. There is 10-fold difference in the expression between nDPSC and two human fibroblasts.

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

For studying the effect of cell type on reprogramming efficiency, six cell lines were reprogrammed using identical reprogramming conditions. The only condition that differed among the cell lines, was the initial medium in which the cell lines was cultured. Of the six cell lines that were used for reprogramming, four were DPSC; out of which two were derived from female donors, while remaining two were from male donors. The remaining two cell lines were human fibroblasts.

Among DPSC cell lines, male derived ones showed massive cell death on transduction of Sendai virus reprogramming vectors. Due to this initial cell death, very few cells survived to undergo reprogramming; hence these two cell lines recorded the least number of reprogrammed colonies.

Reprogrammed colonies were quantified by detecting alkaline phosphatase (AP) activity using NBT-BCIP substrate. AP positive colonies stain violet on addition of the chromogenic substrate (Fig. 6). Reprogramming efficiency is determined by counting the number of violet stained

colonies and dividing it with 75, 000 (total number of transduced cells plated). The answer is then multiplied by 100 to get the percentage.

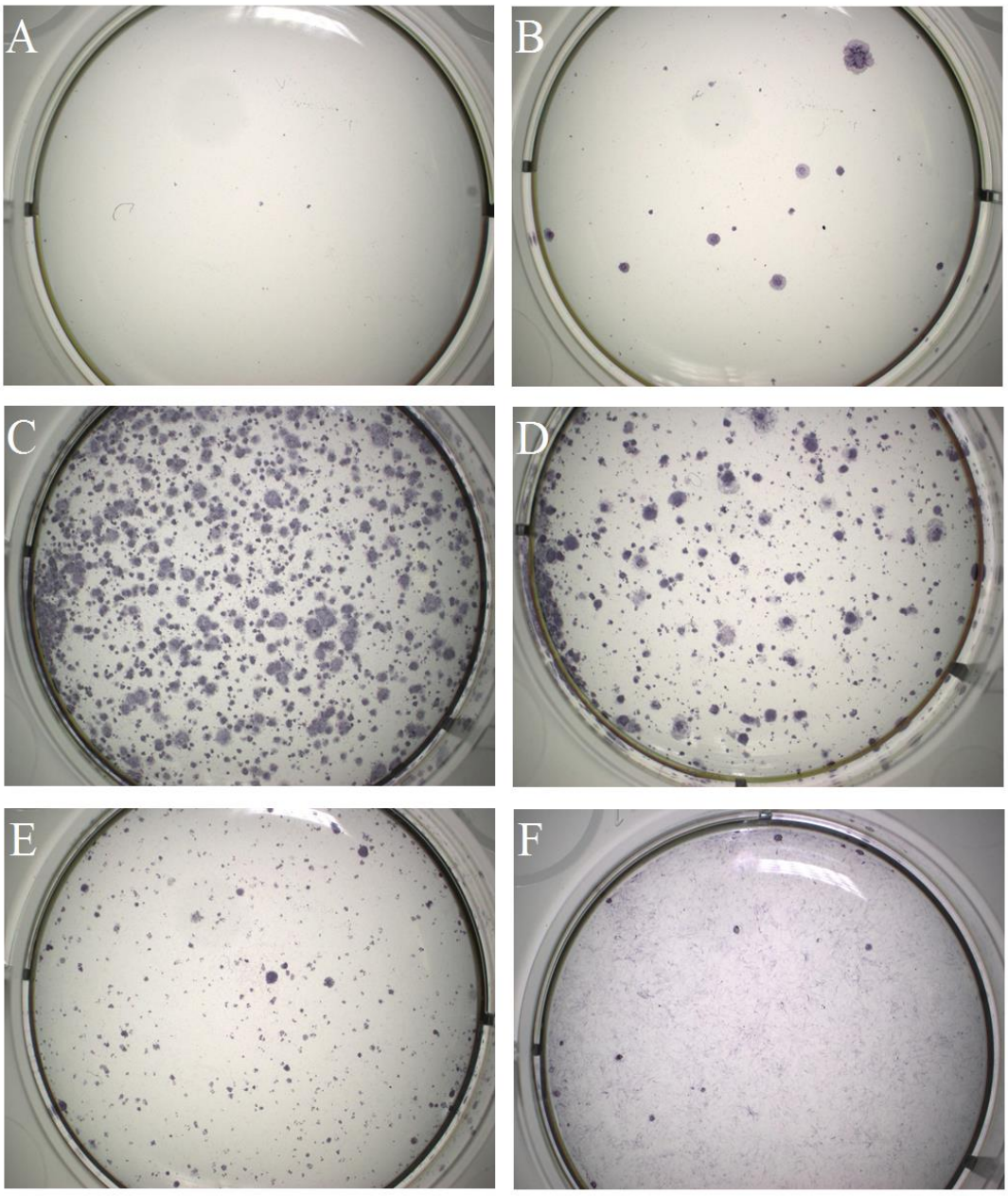

Fig. 6. Alkaline phosphatase staining of reprogrammed colonies using NBT-BCIP solution. Images A and B are from adult male DPSC, C and D are from adult female DPSC and nDPSC respectively and E and F from HF and WI38 respectively. Adult male DPSC showed lowest reprogramming efficiency (A and B) followed by human fibroblasts (E and F). Adult female DPSC showed maximum reprogramming followed by nDPSC (C and D).

Percentage of reprogramming efficiencies displayed by all the cell lines used for reprogramming are: adult female DPSC >4 % (Fig. 6C), nDPSC 1.3% (Fig. 6D), in-house human fibroblasts 0.75

% (Fig. 6E), adult male DPSC 0.016 % (Fig. 6B), WI38 0.016 % (Fig. 6F) and adult male DPSC 0.0106 (Fig. 6A). Another important point to mention was that reprogrammed colonies appeared earlier in DPSC than the fibroblasts.

5.5. Characterisation of iPSC clones

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. 7A and Fig. 8C and F). nDPSC derived iPSC clone

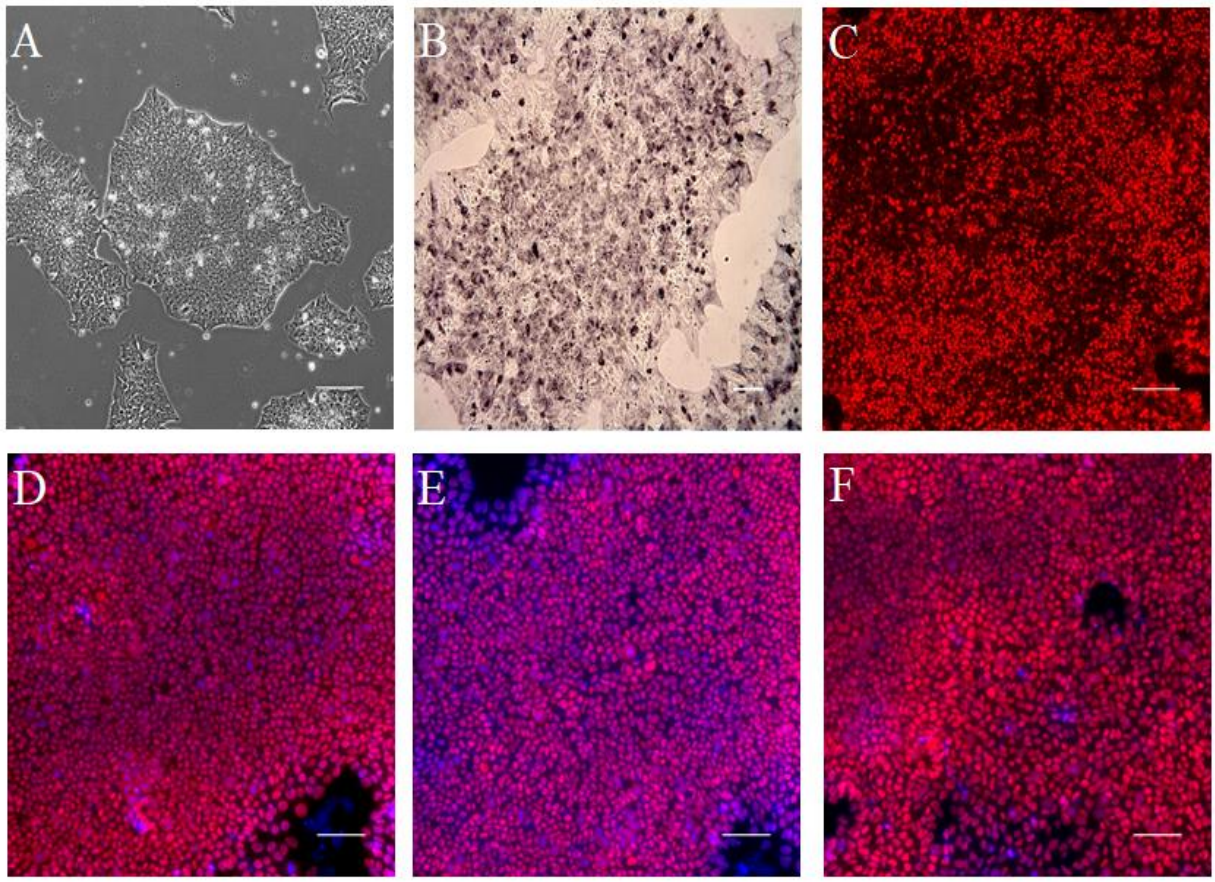

Fig. 7. Characterization of iPSC clone derived from nDPSC. Reprogrammed cells proliferated as tightly packed colony; which is identical to that observed in ES cells (A). Colonies were intensely positive for cell surface markers, alkaline phosphatase and SSEA 4 respectively (B and C). Core pluripotency 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.

showed strong positivity for cell surface markers like (SSEA-4) (Fig. 7C) and alkaline phosphatase (Fig. 7B). Moreover, the clone expressed strong positivity for core pluripotency transcription factors OCT4 (Fig. 7D), SOX2 (Fig. 7E) and NANOG (Fig. 7F). HF and WI38 fibroblasts derived

iPSC clones expressed strong positivity for two pluripotency factors OCT4 (Fig. 8A and 8D) and SOX2 (Fig. 8B and 8E).

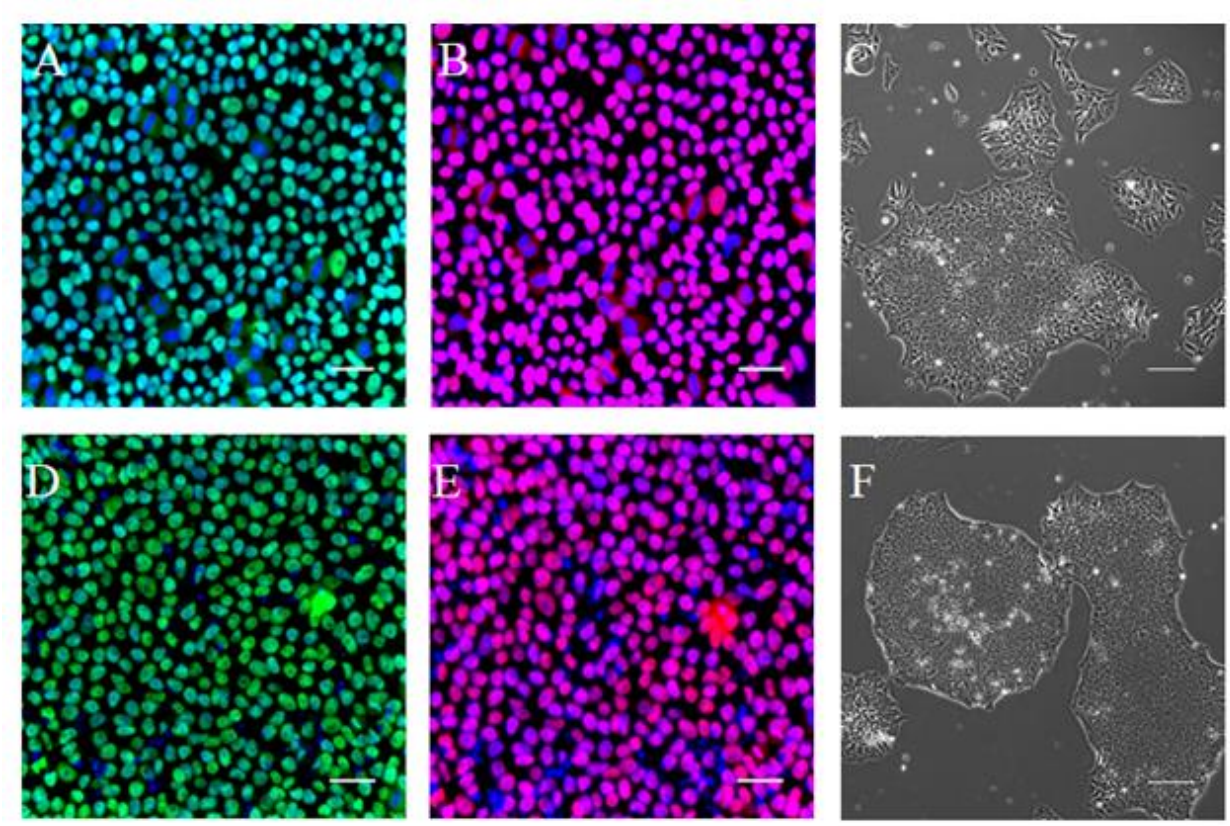

Fig. 8. Characterization of iPSC clones from HF and WI38 human fibroblasts. Images of HF and WI38 derived induced pluripotent stem cells displayed morphology typical to hESC colony (C and F). Immunocytochemistry of HF derived iPSC showed strong positivity for OCT4 and SOX2 (A-B). Similar results were 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.

5.6. Comprehensive characterisation of iPSC clones using expression analysis

Following preliminary characterisation, each iPSC clone was subjected to comprehensive gene expression analysis. In total 83 set of genes was 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 is presented as $\log_2$ value of fold change in the form of continuous line graph. The 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.

The set of 83 genes was divided into three groups; pluripotency genes, early differentiation genes and somatic cell specific genes. Pluripotency group comprised of 52 genes. First set of 26 pluripotency genes is displayed in the Fig. 9. Fold change within +/- 5 was considered as non-significant difference. Trend of gene expression for the first set of pluripotency genes was similar between the three iPSC clones that were analysed (Fig. 9). Relative to hES, all the three iPSC clones showed more than 5-fold upregulation of *COL2A1* gene, while simultaneous down regulation of more than 5-fold of *NODAL* gens.

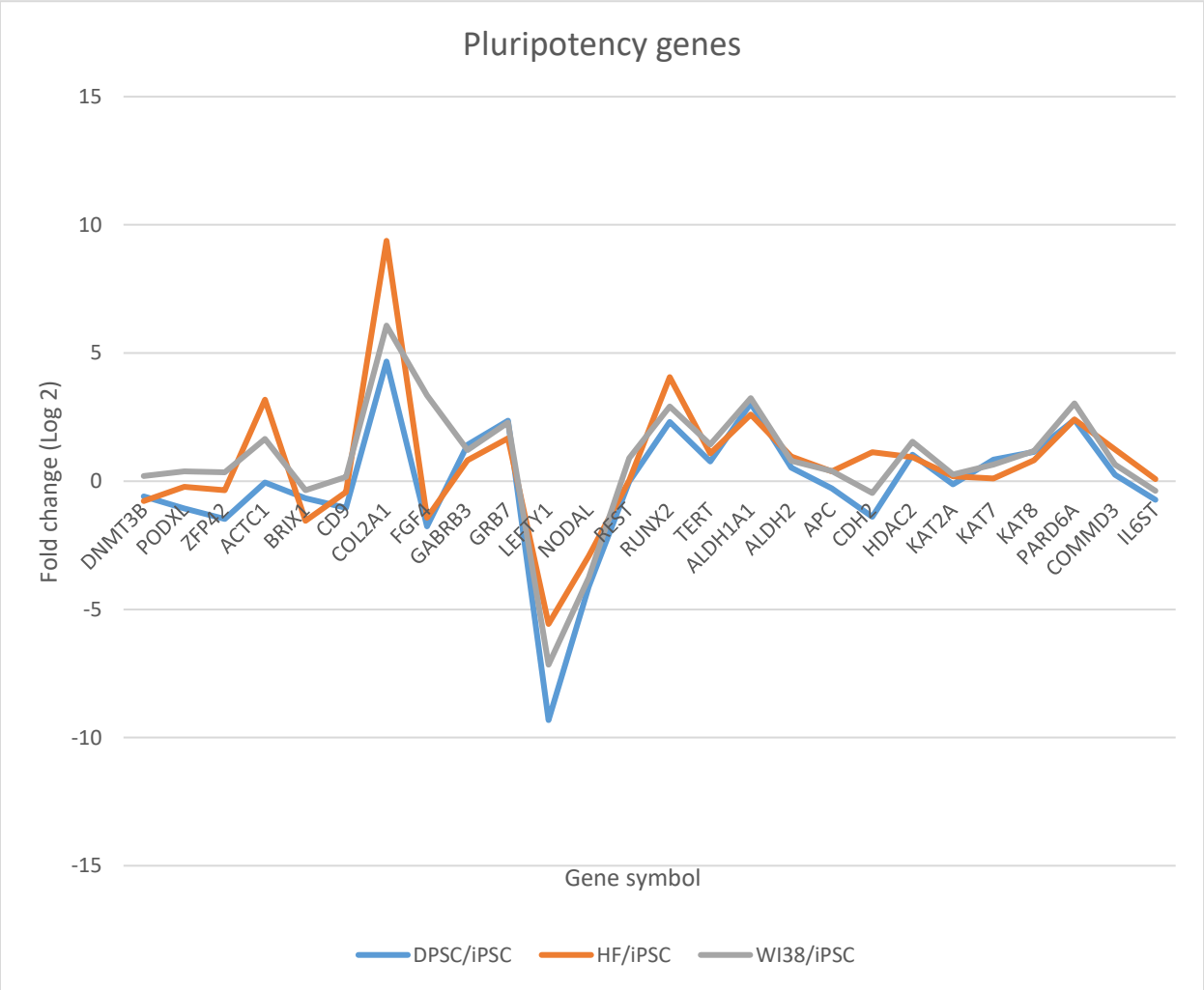

Fig. 9. The graph represents expression of first 26 set of pluripotency genes out of 52 genes in nDPSC/iPSC, HF/iPSC and WI38/iPSC relative to hES. Data is presented as $\log_2$ values of fold change. Positive value indicates up expression as compared to hES while negative values indicated down regulation. 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 10.

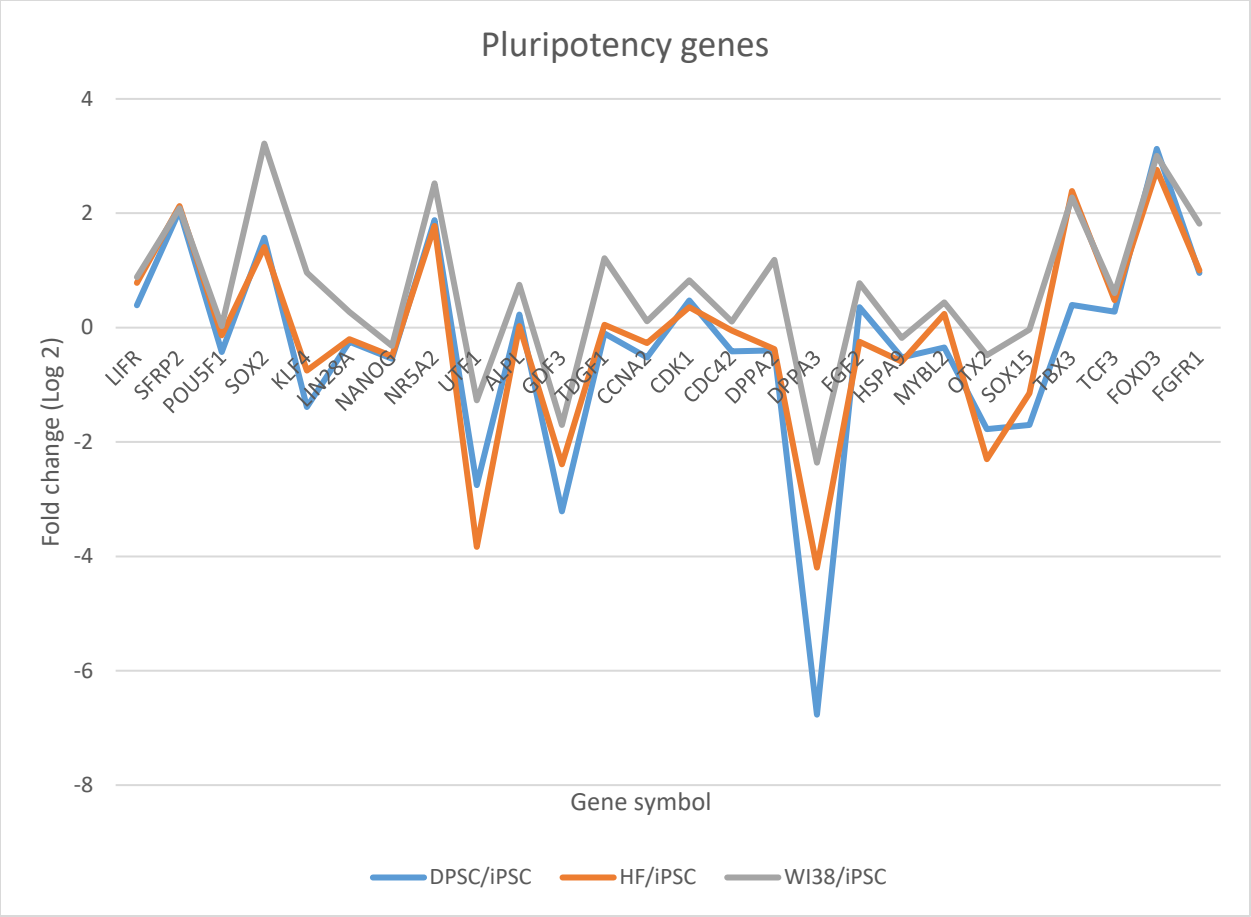

Fig.10. The graph represents expression of last 26 set of pluripotency genes in nDPSC/iPSC, HF/iPSC and WI38/iPSC relative to hES. Data is presented as $\log_2$ values of fold change. Positive value indicates up expression as compared to hES while negative values indicated down regulation. Across all the iPSC clones derived from different cell types, the trend of gene expression is very similar.

In the remaining set of pluripotency genes, the trend of gene expression is very similar across the three iPSC clones (Fig. 11). Gene expression levels of nDPSC iPSC closely overlap with that of HF iPSC while slight upregulation is observed in WI38 iPSC for few genes (Fig. 11). Relative to hES, expression of only *DPPA3* was downregulated by more than 5-fold in nDPSC derived iPSC clone (Fig. 11).

Like the previous two graphs, the trend is similar among the three iPSC clones in this graph also (Fig. 11). Relative to hES, only *PECAM1* gene appears to be downregulated by more than 5-fold in iPSC clones (Fig. 11). After careful observation of the graph, we can notice that some of the early differentiation genes and somatic cell specific genes are slightly more upregulated in iPSC clones compared to hES. This might be because of the presence of residual epigenetic memory of their respective parental cell line that was not completely erased during reprogramming.

In conclusion, the gene expression pattern between iPSC clones derived from different cell lines was very closely associated to each other than to ES. However, the fold change of various genes

between ES cells and iPSC clones was very low which indicated that iPSC clones were pluripotent, and the gene expression levels observed in iPSC clones was comparable to that observed in hES cells.

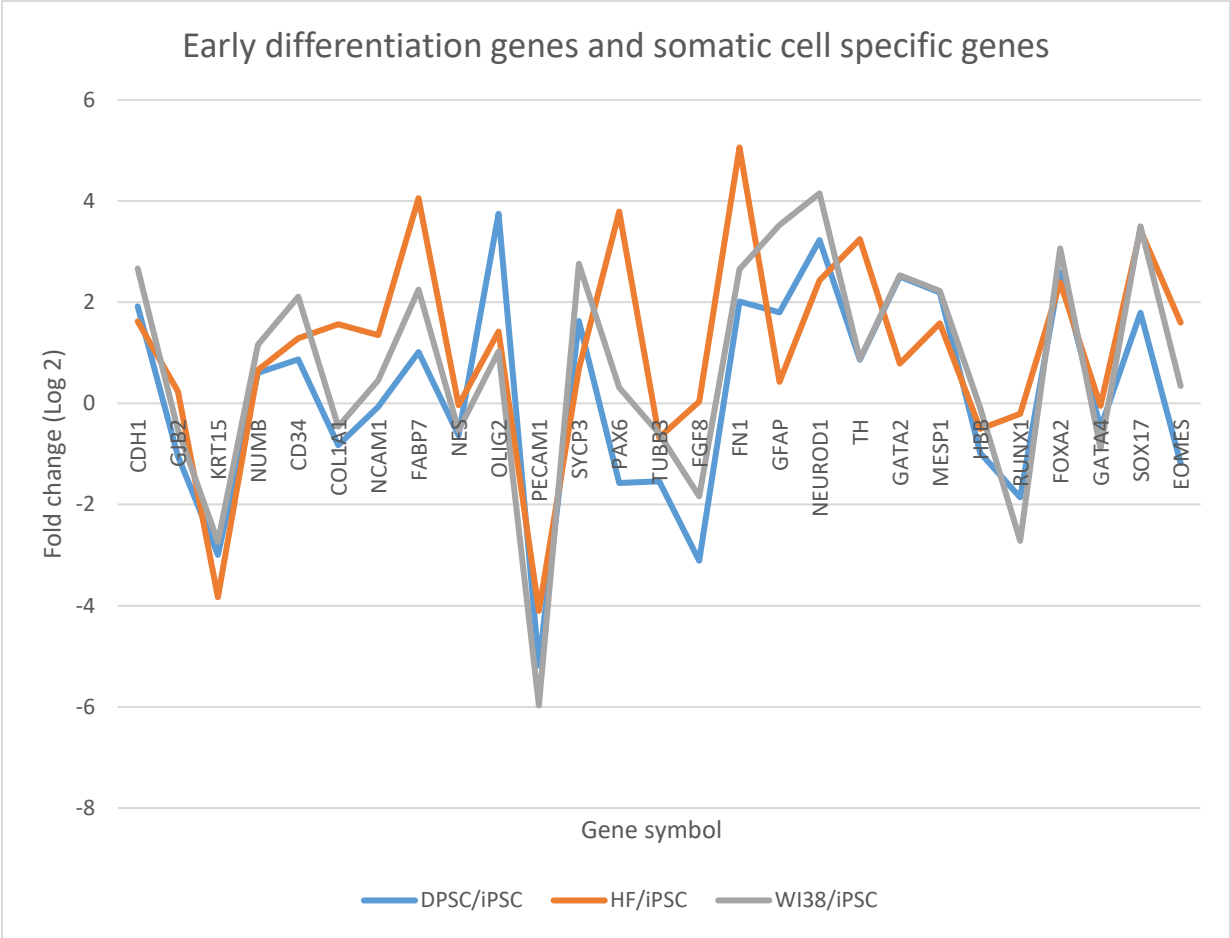

Fig. 11. The graph represents expression of somatic cell specific genes and early differentiation genes in nDPSC/iPSC, HF/iPSC and WI38/iPSC relative to hES. Data is presented as $\log_2$ values of fold change. Genes from CDH1 to SYCP3 are somatic cell specific genes while the remaining are early differentiation 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.

5.7. Spontaneous differentiation of iPSC clone using embryonic bodies (EB)

Pluripotency of nDPSC iPSC clone was confirmed by checking its capacity to differentiate into derivatives of three embryonic germ layers. Differentiation was triggered by culturing the iPSC as embryoid bodies (EB). EB were cultured for 19 days, following which, they were processed for paraffin embedded sectioning. Sectioned EB were stained with the haematoxylin and eosin to observe different morphological structures within the bodies. In addition, the EB were stained against markers of three germ layers.

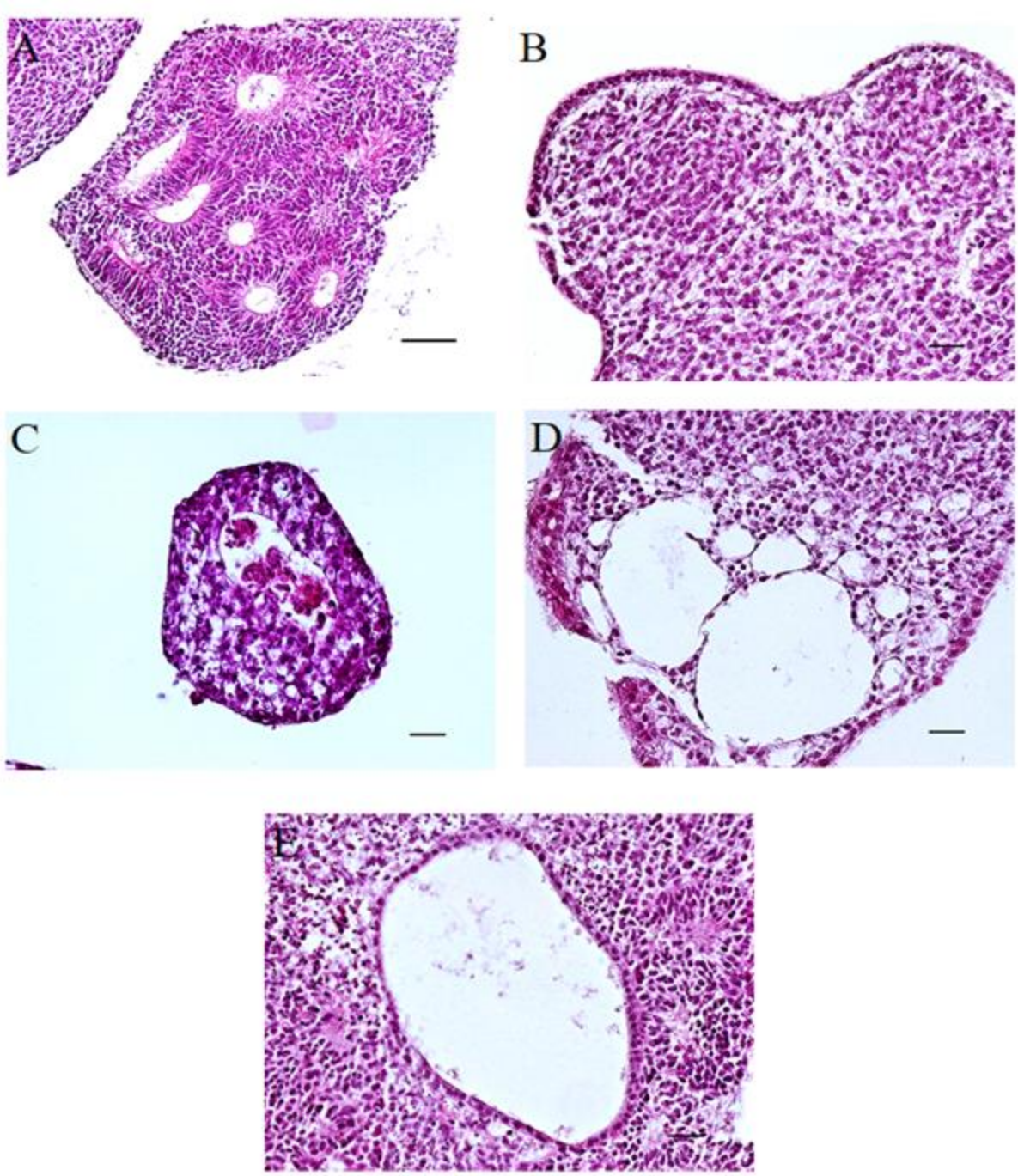

Fig. 12. Staining of sectioned embryoid bodies with haematoxylin and eosin. During the period of culturing the EB formed various structures that were clearly visible on performing haematoxylin eosin staining. Neural rosette (ectoderm) was the most predominant structure (Fig. A). Covering epithelium (ectoderm) like structure (Fig. B). (Fig. C-D) Blood islands and fat cells like morphology (mesoderm) and single cell thick, simple columnar epithelium representing primitive gut like epithelium (endoderm) (Fig. E). (Fig. F) Lymph node like structure lined by sinus. Scale bars = 200 μm.

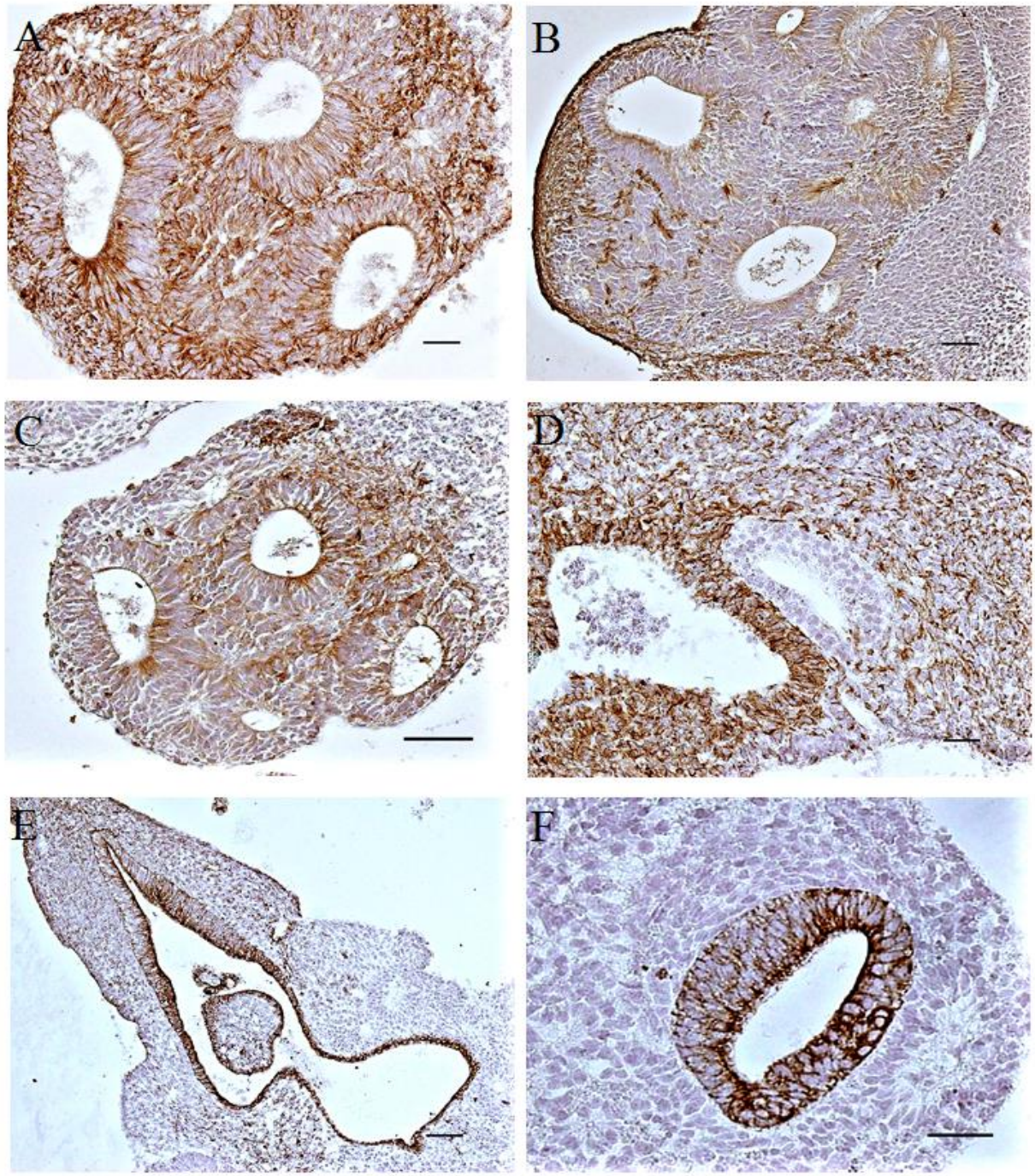

Fig. 13. Detection of germ layer specific markers. Neural rosettes showed positivity for Nestin, MAP2 and β III tubulin (Fig. A-C). These markers are specifically expressed by cells which originate from ectoderm. Some of the structures within EB were Vimentin positive (Fig. D). Vimentin was chosen as mesoderm specific marker. Gut like epithelium and gland like structure were pancytokeratin positive; an endoderm specific marker. Scale bars = 200 μm.

Few structures observed in haematoxylin eosin stained sections were neural rosettes (ectoderm) (Fig. 12A), neuroepithelial lining (ectoderm) (Fig. 12B), blood island like structure (mesoderm) (Fig. 12C), cyst like structure (Fig. 12D) and gut like epithelium (endoderm) (Fig. 12E).

Five markers were selected for identifying derivatives of three embryonic germ layers. These are nestin, β III tubulin and MAP2 for ectoderm, vimentin for mesoderm and pancytokeratin for endoderm. Cytoplasmic positivity was seen for nestin (Fig. 13A) and β III tubulin markers (Fig. 13C) within neural rosettes, while neuroepithelium lining like structure displayed strong MAP2 (Fig. 13B) positivity. Vimentin displayed wide spread positivity in the observed section except in the middle of the image where gland like structure is present (Fig. 13D). Gut like epithelium (Fig. 13E) and simple gland (Fig. 13F) like structure showed strong positivity for pancytokeratin.

In conclusion, the selected iPSC clone was able to form structures representing all the three embryonic germ layers. Moreover, marker specific immunoperoxidase staining confirmed presence of derivatives of all the three germ layers. Taking into consideration all the results, it would be safe to say that the selected nDPSC iPSC clone has attained true pluripotency.

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

Relative telomere length was determined using the method presented by Cawthon et al. (2002). Relative telomere length of three nDPSC derived iPSC clones were compared to that of their parental cell line. All the three iPSC clones showed more than three-fold increase in telomere length relative to the parental cell line (Fig. 14). Out of the three selected clones, fold change expressed by two clones was nearly identical while that of the third was slight higher. At the time of analysis, the later clone was on seventh passage while the former ones were on twelfth passage. This difference in telomere length within the clones might be due to difference in the passage number.

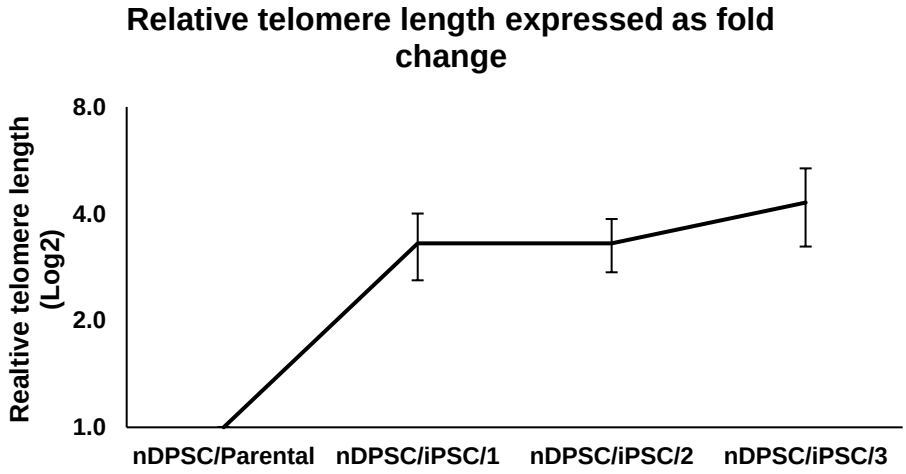

Fig. 14. The graph represents telomere length expressed by three nDPSC iPSC clones relative to their parental cell line. In all the three iPSC clones, telomeres were elongated by more than three-fold. Between

the three iPSC clones, clone number three expressed higher fold change while the remaining had nearly identical length.

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

iPSC clones were forced to differentiate into myogenic lineage by activating Wnt signalling pathway; which is possible by using a small molecule inhibitor called CHIR 99021. This molecule specifically inhibits GSK-3 enzyme which naturally blocks Wnt signalling. In short CHIR99021 activates Wnt signalling by specifically blocking GSK-3 enzyme.

Two iPSC clones, one from nDPSC, and the other from WI38, were used for directed differentiation. Both the clones were treated with the small molecule CHIR 99021 to differentiate them towards myogenic fate. Differentiation was terminated at a stage where myogenic progenitors appear in the culture and population of these cells was expanded by repeatedly passaging and culturing the cells in FGF2 supplemented medium.

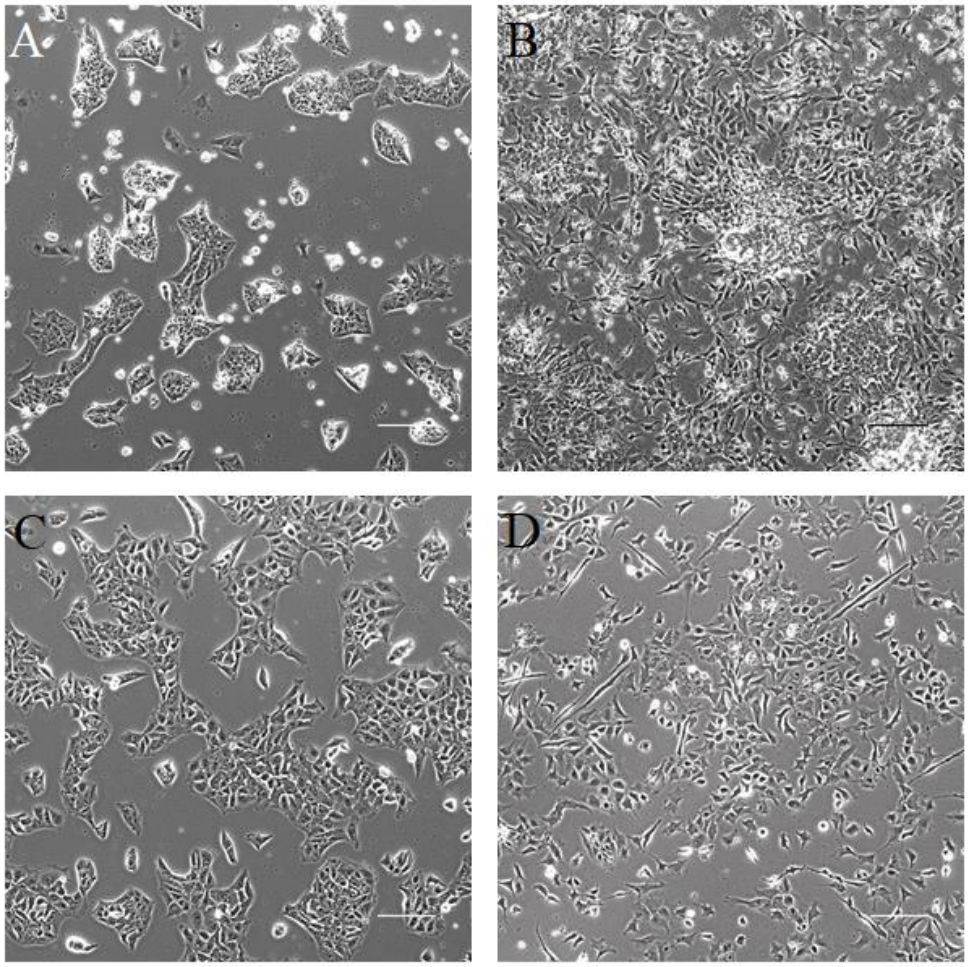

Fig. 15. Directed differentiation of iPSC clones derived from nDPSC and WI38 cell lines, towards myogenesis. (A) nDPSC derived iPSC at start of differentiation i.e. Day 0; (B) nDPSC derived iPSC on 22nd day of differentiation. (C) WI38 derived iPSC at start of differentiation i.e. Day 0; (D) WI38 derived

iPSC on 22nd day of differentiation. Differentiated cells (B and D) display clear transformation in their morphology compared to their iPSC state. When propagated as iPSC, the cells grow as tightly packed clusters (A and C), this morphology is lost on differentiation (B and D) and more individualistic cells with spindle shaped morphology appear. Some myotube like cells are also visible in Fig. D. Scale bars= 200µM.

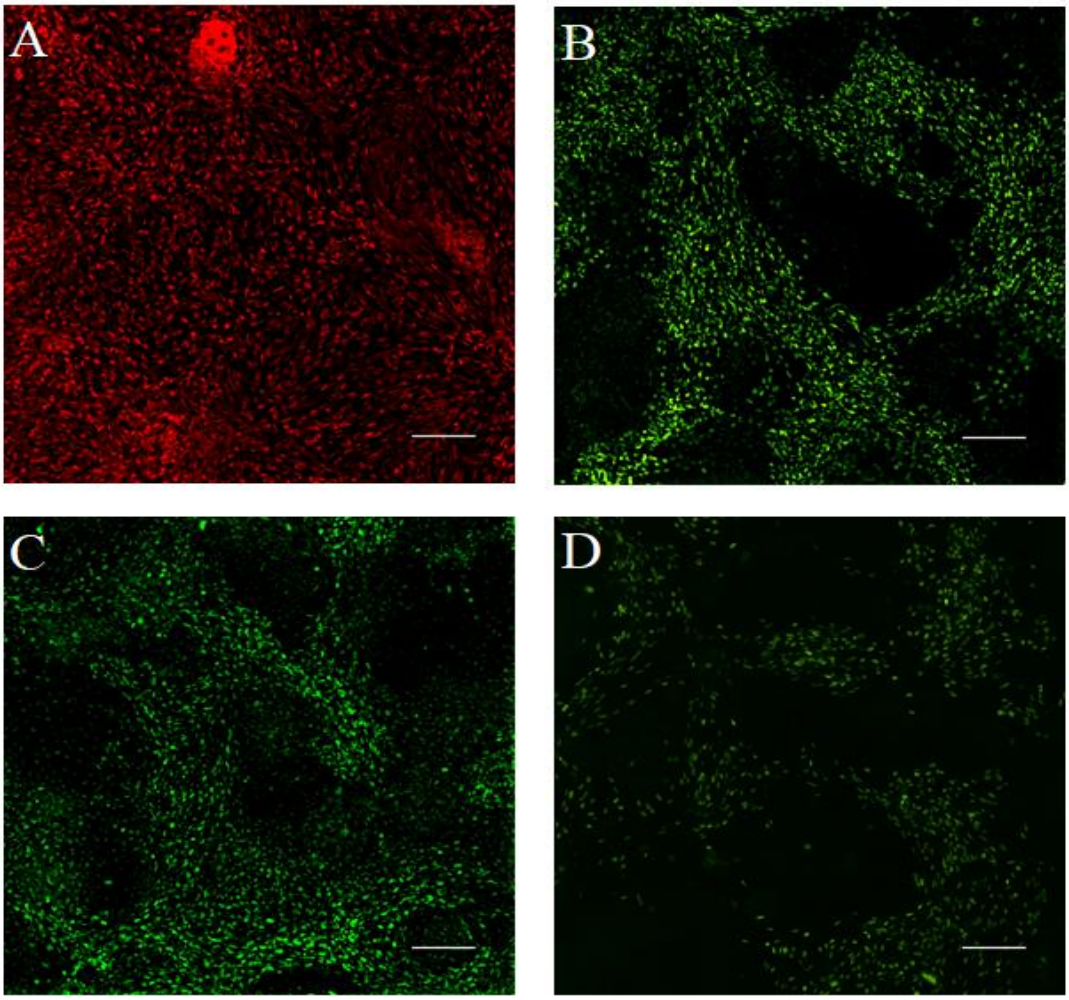

Fig. 16. Immunostaining of myogenic progenitors derived from iPSC clones of nDPSC and WI38 cell lines. Immunostaining was performed after first passage following differentiation. Myogenic progenitors derived from nDPSC and WI38 showed strong reactivity for anti-PAX7 (A and B) antibody. In addition, WI38 derived myogenic progenitors showed strong reactivity for anti-MYF5 (C) and anti-MYOG (D) antibodies. Scale bars = 200 µm.

On 21st day of differentiation cell were passaged, and the cells were cultured in FGF2 supplemented medium to enrich the population of myogenic progenitors. After first passage, immunostaining was performed to detect expression of myogenic markers. Differentiated cells derived from iPSC of nDPSC and WI38 cell lines, showed strong positivity for myogenic marker PAX7 (Fig. 16A and B). Expression of other myogenic markers like MYF5 and MYOG were also analysed in WI38 derived cells and were positive for both the markers. Myogenic progenitors of both the cell lineages expressed initial myogenic marker PAX3 as well as progenitor specific

marker PAX7. Among the two cell lineages used for differentiation, expression of PAX3 was higher in WI38 while PAX7 was higher in nDPSC.

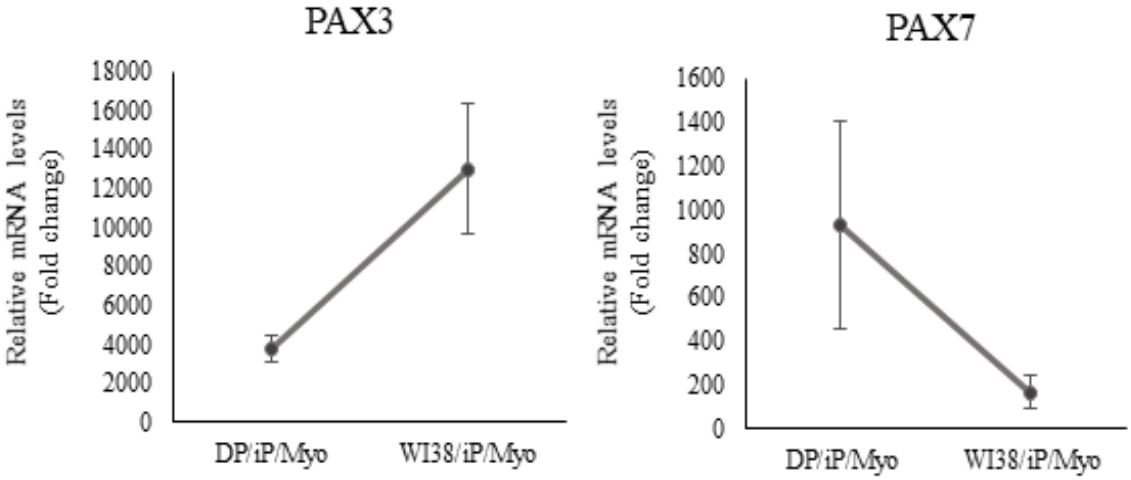

Fig. 17. Real-Time quantification of *PAX3* and *PAX7* genes in differentiated cells derived from nDPSC and WI38. Expression of *PAX3* was higher than *PAX7*. Expression of PAX3 was higher in WI38 derived iPSC while PAX7 expression was higher in nDPSC derived iPSC. Data presented as the average +/- standard deviation; technical triplicates.

5.10. Cloning of intron within open reading frame of DsRed2 gene

An artificial intron sequence was cloned within DsRed2 gene using cyclic ligation assembly. Cloning of intron was confirmed by performing DNA sequencing (Fig. 18), while intron splicing was verified by observing red fluorescence (Fig. 19).

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

Fig. 18. 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.

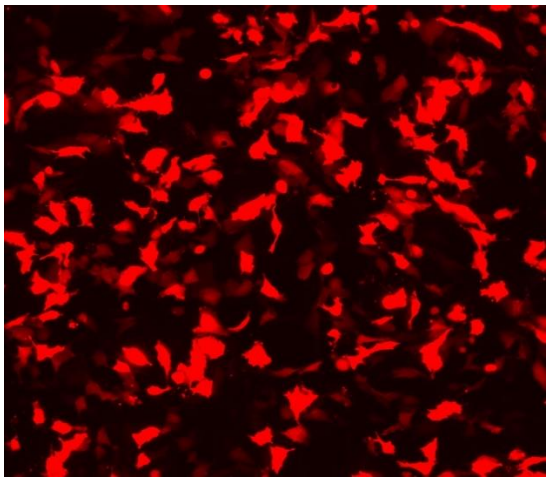

Fig. 19. Expression of red fluorescence by HeLa cells on transfection of intron cloned DsRed2. Expression of red fluorescence confirms recognition and splicing of artificial intron cloned within DsRed2 gene.

Cloning of intron increased number of cells expressing the new construct by four percent, compared to the native DsRed2 gene (Fig. 20).

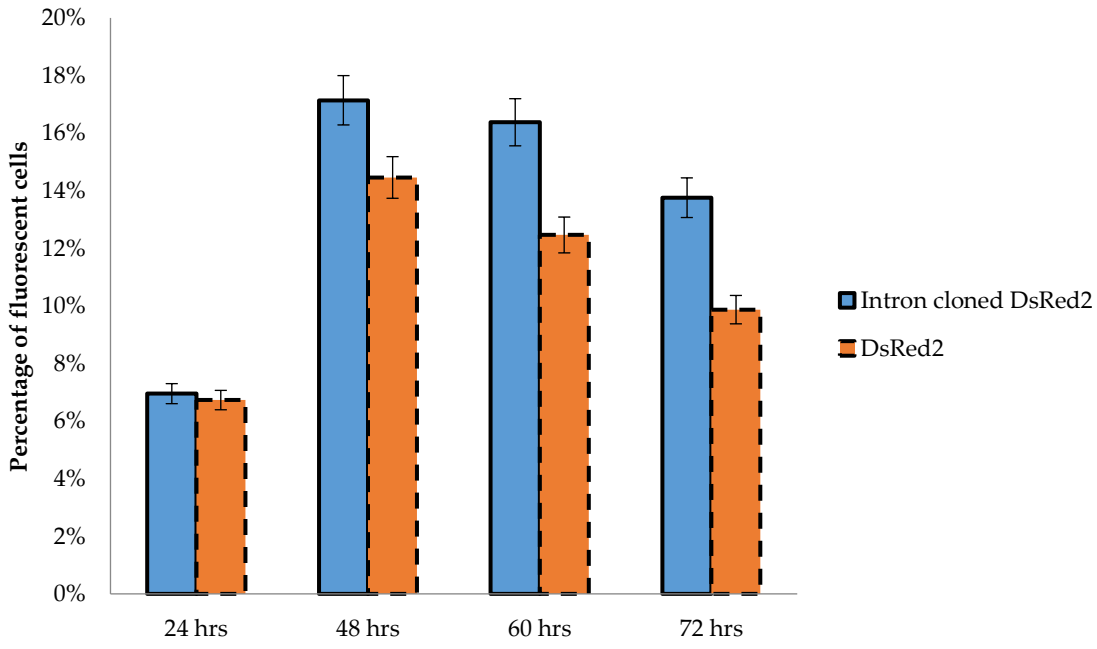

Fig. 20. Comparative analysis of number of cells expressing red fluorescence when transfected with native DsRed2 gene and intron cloned DsRed2 gene.

6. Discussion

Induced pluripotent stem cells have immense application potential in the field of regenerative medicine (Hirschi et al. 2014). During systemic screening of pluripotency genes expressed in embryonic stem cells, Prof Yamanaka found that, a set of four factors such as *OCT4*, *SOX2*, *c-MYC* and *KLF4* when ectopically expressed were able to dedifferentiate somatic cells to pluripotent stem cells (Takahashi et al. 2006). This discovery lead to paradigm shift from use of embryonic stem cells to induced pluripotent stem cells. Over the years, profound improvement has been made in the reprogramming technology, which has facilitated generation of virus-free iPSCs (Rao et al. 2012), thus eliminating the potential risk of insertional mutagenesis and virus induced tumorigenesis. Moreover, iPSC has been derived from almost all possible cell types present (Rao et al. 2012); out of which fibroblasts (Park et al. 2011) and blood cells (Tan et al. 2014) are among the favourites of the researchers due to easy accessibility of tissue biopsy and blood samples; and relatively easy culturing conditions.

In this study, one of our objective was to study effect of starting cell type on reprogramming efficiency. Other objectives were; to test our modified version of mycoplasma detection protocol which was based on previously reported protocol of Uphoff et al. (2002); comprehensively characterize newly generated iPSC clones; use non-canonical approach of reprogramming by using micro RNA 302/367 cluster.

Mycoplasma severely hampers cellular characteristics and genome of cultured cells (Drexler et al. 2002, Razin et al. 1998, Rottem 2003). Mycoplasma has been suspected in hindering the reprogramming process. Furthermore, it has been reported to causes significant reduction in number of embryonic stem cells expressing OCT4 and other pluripotency genes (Markoullis et al. 2009). Bearing in mind these effects, we screened all our cell lines for mycoplasma. For this, we used gold standard PCR based protocol previously reported by (Uphoff et al. 2002). We introduced few modifications to the original protocol and increased its efficacy and robustness while simultaneously reducing the time required to perform the analysis (Pisal et al. 2016).

Among the four DPSC cell lines that were used for reprogramming, one was derived from natal tooth. While characterising the nDPSC, it was found that they not only expressed common DPSC markers like CD29, CD105, CD222, CD166, CD44, CD90, CD10, CD13 and CD73 (Karbanova et al. 2011, Atari et al. 2012, Ferro et al. 2012, Alvarez et al. 2015, Suchanek et al. 2009) but also haematopoietic stem cell markers CD34 and CD45 (Pisal et al. 2018); these two markers are not commonly expressed by DPSC.

DPSC were expanded in low serum medium containing EGF and PDGF growth factors. It was crucial to stop supplementing the medium with these growth factors at the right time point during reprogramming. It was identified that, discontinuing the two growth factors, two days post viral transduction yielded good number of iPSC colonies.

Reprogramming efficiencies of human DPSC were compared to human fibroblasts. Since DPSC are stem cells in nature they were predicted to have more reprogramming conducive nature than fibroblasts. Stem cells are hierarchically above fibroblasts, they are relatively undifferentiated and display higher plasticity (Ebrahimi et al. 2011, Korbling et al. 2003). Moreover, these two characteristics render stem cells more amenable and efficient in reprogramming (Kim et al. 2009b, Eminli et al. 2009, Giorgetti et al. 2009, Niibe et al. 2011, Wang et al. 2013, Yulin et al. 2012). In addition, stem cells express more reprogramming enhancers like e.g. KDM2B chromatin remodellers and reduced levels of barriers like TGF-β and MAP kinase pathways (Vidal et al. 2014). On calculating reprogramming efficiencies, overall, DPSC showed higher reprogramming efficiency confirming the fact that stem cells are more amenable to reprogramming than fibroblasts. An important point to highlight is that, out of the four DPSC cell line chosen for reprogramming two cell lines which were derived from male donor displayed massive cell death after transduction of Sendai virus reprogramming vectors. Owing to which these two cell lines displayed least reprogrammed colonies as fewer cells survived to undergo reprogramming, while the remaining two DPSC cell lines were derived from female donors. These two female cell lines had higher reprogramming efficiency than fibroblasts. From these results, it was clear that male DPSC were more sensitive to Sendai virus compared to their female counterpart. However, more sample number comprising of both female and male derived DPSC needs to be included in the analysis, to truly verify the relationship between gender and Sendai virus cytotoxicity.

iPSC clones derived from all the cell lines were characterized to confirm induction of pluripotency. In addition to checking the expression of few pluripotency genes like OCT4, SOX2 and NANOG, we chose to characterize each clone using 80 additional set of genes. This set of genes comprised of pluripotency genes, early differentiation genes and somatic cell specific genes. By comparing the gene expression analysis of the newly isolated iPSC clone with embryonic stem cell, it was possible to confirm whether the expression levels of all the genes were comparable or not. This would help to ascertain whether the new iPSC clone has attained pluripotency equivalent to embryonic stem cell or not.

All reprogrammed iPSC clones were manually isolated and expanded. Few clones from each cell line were used for characterization. All selected iPSC clones closely resembled hESC in terms of their morphology, expression of pluripotency markers and gene expression profiles. Ability of an iPSC clone to differentiate into derivatives of three layers is essential to confirm true pluripotency (Baghbaderani et al. 2016, Marti et al. 2013). This can be achieved using either EB mediated spontaneous differentiation or by directed differentiation. nDPSC derived iPSC clone was subjected to EB mediated differentiation; while one clone each from nDPSC and two fibroblasts cell lines were differentiated into neuroepithelium, beating cardiomyocytes and hepatic endoderm using directed differentiation (Pisal et al. 2018). All characterization results cumulatively confirmed that the iPSC clones had attained true pluripotency. In addition, iPSC clones from nDPSC were also analysed for telomere elongation. Analysis confirmed that the iPSC clones had undergone three-fold increase in telomere length as compared to the parental cell line.

Furthermore, it was also seen that the iPSC clones that were cultured for extended period had slightly shorter telomere length compared to ones cultured for shorter period. Few articles have rightly pointed out this distinction and have provided plausible reasons (Suhr et al. 2009, Wang et al. 2012). Karyotyping of nDPSC derived iPSC and WI38 derived iPSC displayed normal karyotype of 46 XX, with no aneuploidy or polyploidy.

A non-canonical approach of reprogramming involving forced expression of micro RNA cluster 302/367 was shown to reprogramme murine and human cells (Anokye-Danso et al. 2011). Highlight of this approach was that it could perform the reprogramming process all by itself without any assistance of OCT4, SOX2, c-MYC or KLF4 transcription factors. To study the mechanism linked to micro RNA mediated reprogramming, we initially constructed a vector comprising of a reporter gene with an intron cloned within its open reading frame. After careful analysis, we found out that the intron cloning had increased the number of cells expressing the reporter gene. To our surprise, in addition to developing an expression vector for intronic miRNA we had also increased the number of cells expressing the reporter gene, which was previously reported to be low (Pisal et al. 2017).

7. Conclusion

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

Mycoplasma detection protocol reported by Uphoff et al. (2002) was based on extracting mycoplasma DNA from the cell culture supernatant. While adapting this protocol it was found that the DNA extraction step was unable to isolated sufficient quantity of mycoplasma DNA for performing PCR. Hence, the extraction step was omitted and instead cell culture supernatant was directly used for PCR. In addition, normal polymerase enzyme was replaced with robust enzyme. These two modifications made the revised protocol more sensitive, robust and rapid (Pisal et al. 2016).

iPSC clones isolated from all the cell lines i.e. four human DPSC and two human fibroblasts closely resembled hESC in terms of morphology, expression of pluripotency markers and gene expression profile. Furthermore, nDPSC derived iPSC were spontaneously differentiated into derivatives of three germ layer using embryonic bodies. In addition, iPSC clones from nDPSC and two fibroblasts cell lines were able to differentiate into neuroepithelium, beating cardiomyocytes and hepatic endoderm using directed differentiation. Relative telomere length increased more than three folds in nDPSC iPSC clones as compared to parental cell line. Characterisation results confirmed that the all iPSC clones considered for characterisation were induced with true pluripotency.

Vector constructed for expressing miRNA cluster contained an intron cloned within the reporter gene DsRed2. The intron was placed within the gene by using cyclic ligation assembly. Surprisingly, the newly engineered DsRed2 gene showed an unexpected increase in number of cells expressing the red fluorescence as compared to the native DsRed2.

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9. Overview of published work

Original research articles in scientific journals with impact factor

1. **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. (IF 4.259)
2. **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. (IF 0.894)
3. **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. (IF 0,939)
4. HREBIKOVA, H., BEZNOSKA, P., BEZROUK, A., CHVATALOVA, J., **PISAL, R**., MOKRY, J. Decellularized skeletal muscle: A promising biologic scaffold for tissue engineering. J Biomater. Tissue Eng., 2017, vol. 7, no. 6. pp. 491-498. (IF 1.383)

Original research articles in scientific journals without impact factor

1. HREBIKOVA, H., CIZKOVA, D., CHVATALOVA, J., **PISAL, R.**, ADAMCIK, R., BEZNOSKA, P., DIAZ-GARCIA, D., MOKRY, J. Cell stratification, spheroid formation and bioscaffolds used to grow cells in three dimensional cultures. Acta Medica (Hradec Kralove), 2015, vol. 58, no. 3. pp. 79-85.

Original review article in impact factor journal

1. **RISHIKAYSH, P.**, DEV, K., DIAZ, D., QURESHI, W.M.S., FILIP, S., MOKRY, J. Signaling involved in hair follicle morphogenesis and development. Int. J. Mol. Sci., 2014, vol. 15, no. 1. pp. 1647-1670. (IF 3.226)

Original chapter in a book

1. MOKRY, J., **PISAL, R**. Stem cell biology and tissue engineering in dental sciences. VISHWAKARMA, A., et al eds., Boston: Academic Press, 2015. Chapter 17 - The basic principles of stem cells, pp. 237. ISBN 978-0-12-397157-9.

Attendance at conferences and seminars

1. **PISAL, R**., MOHIUDDIN, W., HREBIKOVA, H., MOKRY J. Nestin Expression in large blood vessels. Proc. of Intermediate Filaments in Health and Disease, p. 95. 55th Symposium of the Society for Histochemistry, 11-14 June 2013, Prague.

2. **PISAL, R.**, HREBIKOVA, H., CHVATALOVA, J., DIAZ, D., KUNKE, D., SOUKUP, T., MOKRY, J. Cloning of synthetic intron into coding sequence of red fluorescent gene for confirmatory expression of micro RNA cloned within the intron. p. 31-32. 158 Morphology, 6-8 Sept 2015, Olomouc.

3. DEV, K., MOKRY, J., DIAZ, D., **PISAL R**. Amniotic fluid stem cells and their clinical implication. p. 22. Proc. of the 9th Symposium and Workshop on Molecular Pathology and Histo(cyto)chemistry, April 26-27, 2013, Olomouc.

4. HREBIKOVA, H., ADAMCIK, R., **PISAL, R.**, DIAZ, D., DEV, K., MOKRY, J. Decellularized Matrix of Skeletal Muscle Seeded with Murine Myoblasts as a Tissue-Engineering Approach (preliminary data). Proc. of Intermediate Filaments in Health and Disease, p. 92. 55th Symposium of the Society for Histochemistry, 11-14 June 2013, Prague.

5. HREBIKOVA, H., **PISAL, R.**, DIAZ, D., MOKRY, J. Decellularized skeletal muscle scaffold as a suitable pro-myogenic environment. Tissue Engineering Congress 2014. 4th June 2014, United Kingdom.

6. CHVATALOVA, J., MOKRY, J., HREBIKOVA, H., **PISAL, R.** Colonization of muscle scaffolds by different cell types. p. 14. 158 Morphology, 6-8 Sept 2015, Olomouc.