# A Review on Various Tissue Engineering Techniques to Induce Differentiation of Pluripotent Stem Cells

**Nandita Ghosh[1], Ena Ray Banerjee[2]**

## Introduction:

In 2006, researchers at Kyoto University in Japan identified conditions that would allow specialized adult cells to be genetically "reprogrammed" to assume a stem cell-like state. These adult cells, called induced pluripotent stem cells (iPSCs), were reprogrammed to an embryonic stem cell-like state by introducing genes important for maintaining the essential properties of embryonic stem cells (ESCs). This represents a paradigm shift in our understanding of cellular differentiation and of the plasticity of the differentiated state. Cellular differentiation appears as a unidirectional process, where undifferentiated cells mature to various specialized cell fates, such as neurons, muscle and skin cells. Although much additional research is needed, investigators are beginning to focus on the potential utility of iPSCs as a tool for drug development, modeling of disease, and transplantation medicine. The idea that a patient's tissues could provide him/ her a copious, immune-matched supply of pluripotent cells has captured the imagination of researchers and clinicians worldwide.

During the developmental journey, cells progressively become more restricted in their differentiation potential and as a consequence, they do not retain pluripotency. Most cells mature into fully differentiated cells, although stem cells with limited potency remain in certain locations in the body and serve as a source for cell replacement, for example in the bone marrow, intestine and skin. Differentiated cells are remarkably stable and as a rule they will not shift fate into other types of differentiated cells or revert to the type of undifferentiated cells that can be found in the early embryo. Thus In this review, we summarize the progress that has been made in the iPSC field ,with an emphasis on understanding the mechanisms of cellular reprogramming and its potential applications in cell therapy and also various tissue engineering techniques to induced pluripotent stem cells.

## Reprogramming Cells:

The discovery of induced pluripotency represents the synthesis of scientific principles and technologies that have been developed over the last six decades. As such, the logistical challenges of isolating, culturing, purifying, and differentiating stem cell lines that are extracted from tissues have led researchers to explore options for "creating" pluripotent cells using existing non-pluripotent cells. One strategy to accomplish this goal is nuclear repro-gramming, a technique that involves experimentally inducing a stable change in the nucleus of a mature cell that can then be maintained and replicated as the cell divides through mitosis. Other strategy that has historically been carried out using techniques such as somatic cell nuclear transfer (SCNT), altered nuclear transfer (ANT), and methods to fuse somatic cells with ESCs.

Immunobiology and Regenerative Research Lab, Translational Outcomes Research Group, Department of Zoology, University of Calcutta
[1]PhD, Assistant Professor
[2]PhD, Professor
**Corresponding author:** Ena Ray Banerjee, Immunology and Regenerative Medicine Research Unit, Lab: 214 & 251, 35, Ballygunge Circular Road, Kolkata 700019, E-mail: enaraybanerjee@gmail.com

The development of nuclear reprogramming *in vitro,* the breakthrough method that creates iPSCs. Involves taking mature "somatic" cells from an adult and introducing the genes that encode critical transcription factor proteins, which themselves regulate the function of other genes important for early steps in embryonic development. In the initial 2006 study, it was reported that only four transcription factors *(Oct4, Sox2, Klf4,* and *c-Myc)* were required to reprogram mouse fibroblasts (cells found in the skin and other connective tissue) to an embryonic stem cell-like state by forcing them to express genes important for maintaining the defining skin and other connective tissue) to an embryonic stem cell–like state by forcing them to express genes important for maintaining the defining properties of ESCs. These factors were chosen because they were known to be involved in the maintenance of pluripotency, which is the capability to generate all other cell types of the body. In 2007, two different research groups reached a new milestone by deriving iPSCs from human cells, using either the original four genes9 or a different combination containing *Oct4, Sox2, Nanog,* and *Lin28.* Since then, researchers have reported generating iPSCs from somatic tissues of the monkey and rat.

Several approaches have been investigated to improve reprogramming efficiency and decrease potentially detrimental side effects of the reprogramming process. Subsequent studies have further reduced the number of genes required for reprogramming and researchers continue to identify chemicals that can either substitute for or enhance the efficiency of transcription factors in this process. These breakthroughs continue to inform and to simplify the reprogramming process, thereby advancing the field toward the generation of patient-specific stem cells for clinical application.

## Factors Need to be Considered before Reprogramming Cells:

Reprogramming poses several challenges for researchers who hope to apply it to regenerative medicine. Before reprogramming can be considered for use as a clinical tool, the efficiency of the process must improve substantially. Although researchers have begun to identify the myriad molecular pathways that are implicated in reprogramming somatic cells, much more basic research will be required to identify the full spectrum of events that enable this process. The direct reprogramming of somatic cells to pluripotency accomplished in 2006, when Takahashi and Yamanaka converted adult mouse fibroblasts to iPSCs through ectopic expression of a select group of transcription factors. Subsequent reports optimized this technique, demonstrating that iPSCs were indeed highly similar to ESCs when tested across a rigorous set of assays (Maherali *et al*, 2007; Okita *et al*, 2007; Wernig *et al*, 2007). In 2007, direct reprogramming was achieved in human cells (Takahashi *et al*, 2007b; Yu *et al*, 2007), providing an invaluable contribution to the field of regenerative medicine. While the establishment of iPSC lines is conceptually and technically simple, direct reprogramming is a slow and inefficient process consisting of largely unknown events. Several variables must be considered in order to reproducibly obtain iPSCs, which include (1) the choice of factors used to reprogram cells; (2) the methods used to deliver these factors; (3) the choice of target cell type; (4) the parameters of factor expression, such as timing and levels; (5) the culture conditions used to derive iPSCs; and the methods of (6) identifying and (7) characterizing reprogrammed cells. This review addresses each of these steps in detail and is summarized as an overview in Fig1.

## Choice of Reprogramming Factors:

The four transcription factors, Oct4 (Pou5f1), Sox2,c-Myc, and Klf4, were sufficient to mediate reprogramming (Takahashi and Yamanaka, 2006). This core set of factors has been shown to work across a multitude of mouse cell types (Aoi *et al*, 2008; Eminli *et al*, 2008; Hanna *et al*, 2008; Kim *et al*, 2008; Stadtfeld *et al*, 2008a, 2008c; Wernig *et al*, 2008a), as well as rhesus monkey (Liu *et al*, 2008) and human cells (Park *et al*, 2008a;

Takahashi *et al*, 2007b; Lowry *et al*, 2008. Variations on the four-factor cocktail have been used to successfully reprogram cells. In mouse fibroblasts, Sox1 and Sox3 can replace Sox2, albeit with a decrease in reprogramming efficiency; Klf2 can replace Klf4, and L-Myc and N-Myc can replace c-Myc (Blelloch *et al*, 2007; Nakagawa *et al*, 2008). It has also been reported that a partially different set of factors, OCT4, SOX2, NANOG, and LIN28, is sufficient to reprogram human fibroblasts (Yu *et al*, 2007). While the original suite of four factors remains the standard for direct reprogramming, a handful of small molecules and additional factors have been reported to enhance the reprogramming process and/or functionally replace the role of some of the transcription factors. The identification of such mediators is beginning to yield insight into the mechanisms by which reprogramming occurs, and many

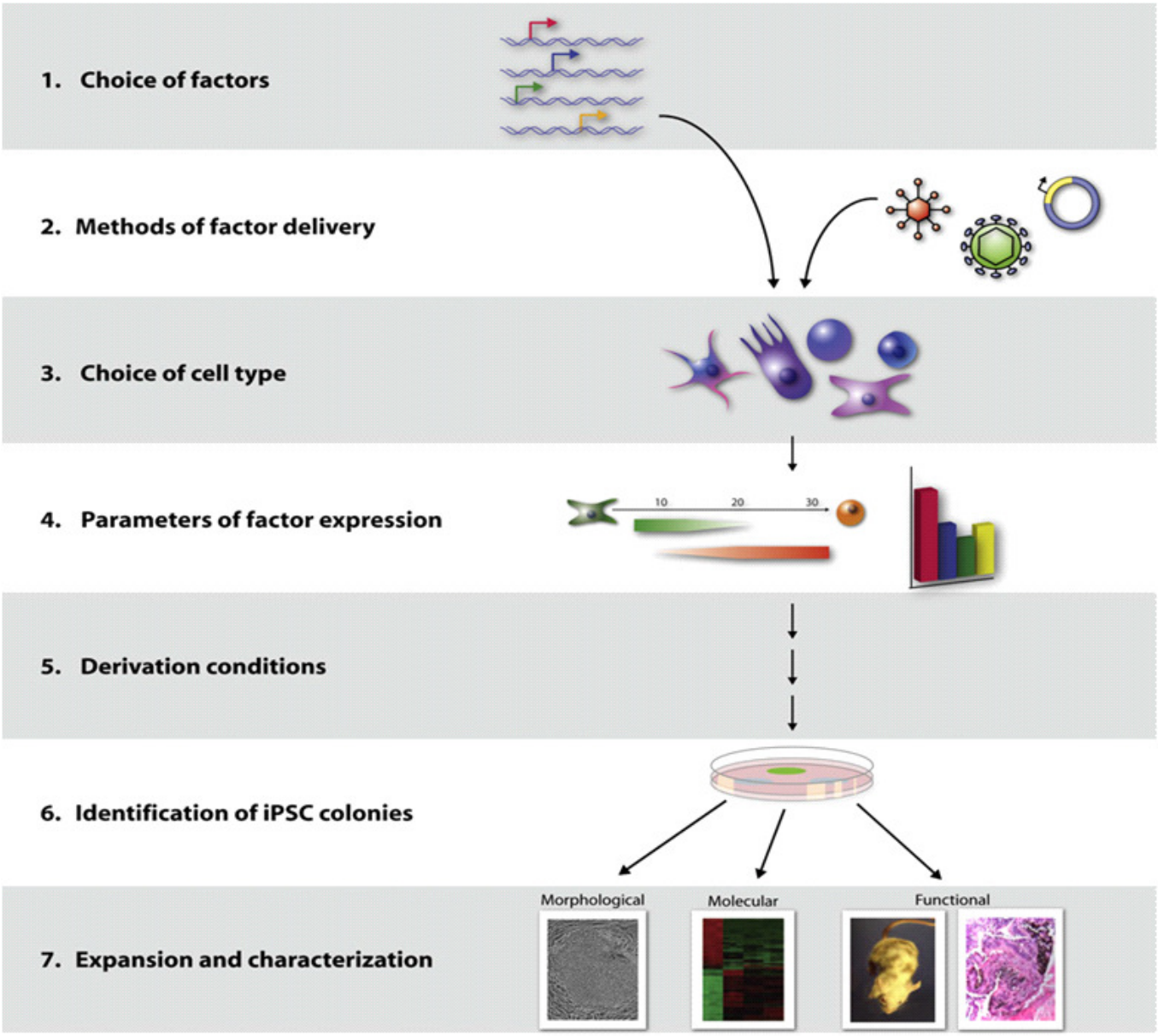


Fig 1– Overview of the iPSC Derivation Process

similar studies are likely to follow.

## Methods of Factor Delivery:

The production of iPSCs has so far been achieved through nucleic- acid-based delivery of the reprogramming factors. Initial generations of mouse and human iPSCs employed retroviral vectors (Takahashi *et al*, 2007b; Takahashi and Yamanaka, 2006) and constitutive lentiviruses (Blelloch *et al*, 2007; Yu *et al*, 2007), while later generations were produced using inducible lentiviruses (Brambrink *et al*, 2008; Hockemeyer *et al*, 2008; Maherali *et al*, 2008; Stadtfeld *et al*, 2008b). Other various methods of factor delivery has also been studied like Moloney-based retrovirus,Transient transfection, Adenovirus, small molecules and Protein transduction.

## Choice of Cell Types:

For the first reprogramming attempts in both mouse and human, fibroblasts were used as the starting cell population. Adult fibroblasts have been previously shown to be amenable to reprogramming by nuclear transfer in mouse (Wakayama *et al*, 1998) and cell fusion in both mouse and human (Cowan *et al*, 2005; Tada *et al*, 2001). Since the success of fibroblast reprogramming, a multitude of mouse cell types, including stomach cells (Aoi *et al*, 2008), liver cells (Aoi *et al*, 2008; Stadtfeld *et al*, 2008c), pancreatic b cells (Stadtfeld *et al*, 2008a), lymphocytes (Hanna *et al*, 2008), and neural progenitor cells (Eminli *et al*, 2008; Kim *et al*, 2008), as well as human keratinocytes (Aasen *et al*, 2008; Maherali *et al*, 2008), have been reprogrammed. Several factors must therefore be considered in determining the optimal cell type for a given application: (1) the ease at which reprogramming factors can be introduced, which varies both by cell type and delivery approach; (2) the availability and ease of derivation of the given cell type; and (3) the age and source of the cell.

## Parameters of Factor Expression:

To improve the process of iPSC derivation, there are certain parameters of factor expression. The length of time required for cells to become independent of factor expression has been addressed using doxycycline-inducible systems, use of separately delivered reporter constructs, such as GFP-encoding vectors. For viral-based methods, titers are influenced by the gene of interest, as the gene product is expressed at high levels during packaging and can potentially alter the function of the packaging cells (Tiscornia *et al*, 2006). The best method for quantification is a direct analysis of expression in the cell type of interest; this assessment can be accomplished by using a reporter-linked construct, such as IRES-GFP, or through immunostaining, which permits analysis at a single-cell level. For a more accurate measure of factor delivery, one can also assess co infectivity to determine the percentage of cells receiving all factors.

## Culture and Derivation Condition:

Both mouse and human iPSC derivation proceed under the same culture conditions used for ESC maintenance (Akutsu *et al*, 2006; Cowan *et al*, 2004; Lerou *et al*, 2008; Nagy *et al*, 2003), and it is important to ensure that the selected conditions support ESC growth. The use of knockout serum replacement provides an alternative culture condition for the reprogramming of various cell types for which standard serum is unsuitable. A key aspect for creating favorable derivation conditions is to achieve an optimal cell density.

## Identification of iPSCs Colonies:

The identification of iPSC colonies based solely upon morphological criteria requires a considerable degree of ESC expertise. In general, mouse ESC colonies can be distinguished by their refractive, or ‘‘shiny,’’ appearance and tight, well-defined borders, while human ESC colonies display a cobblestone appearance with prominent nucleoli and pronounced individual cell borders. The stepwise morphological changes that occur during reprogramming have been depicted in both systems (Fig 2).

## Expansion and Characterization of Cells:

The steps involved in taking a new colony to a fully established iPSC line are identical to those for ESC derivation, which have been described in detail elsewhere (Akutsu *et al*, 2006; Lerou *et al*, 2008; Nagy *et al*, 2003. Several criteria have been set forth to ascertain whether a fully reprogrammed state has been achieved, which include an array of unique features associated with pluripotency, encompassing morphological, molecular, and functional attributes (Fig 3). On a molecular level, iPSCs must display gene expression profiles that areindistinguishable from ESCs, which extends to the display of other associated features, including (1) protein-level expression of key pluripotency factors (eg, Oct4, Nanog) and ESC-specific surface antigens; (2) functional telomerase expression; and (3) expression of genes involved in retroviral silencing, such as de novo methyl transferases and Trim28 (Lei *et al*, 1996; Wolf and Goff, 2007). At a functional level, iPSCs must demonstratethe ability to differentiate into lineages from all three embryonic germ layers. A hierarchy of criteria has been put forth, and in order of increasing levels of stringency, these include:(1) in vitro differentiation, (2) teratoma formation, (3) chimera contribution, (4) germline transmission, and (5) tetraploid complementation (direct generation of entirely ESC/iPSC-derivedmice) (Jaenisch and Young, 2008).As performing all available assays for the demonstration of pluripotency is infeasible, a suggested minimal set of criteria should be fulfilled in order to ascertain that a genuine iPSC hasbeen obtained. Accordingly, these include (1) all morphological attributes, including unlimited self-renewal; (2) expression of key pluripotency genes with a concomitant downregulation oflineage-specific genes associated with the cell of origin; (3)transgene independence; and (4) proof of functional differentiation through the highest-stringency test acceptable.

## Generation of Induced Pluripotent Stem Cells Using Drosophila as a Model:

The arthropod *Drosophila melanogaster* is an attractive genetic model due to the short life span, large number of offspring, and applicability of many genetic techniques (van Ham *et al, 2009*). Drosophila have been used to model Parkinson's, Huntington's, and Prion disease. Unfortunately, production of non-mammalian stem cells has been limited, due to problematic or unknown isolation procedures, and insufficient maintenance methods (Lavial and Pain, 2010). For these reasons, there has been a desire to generate stem cells for these species, allowing disease and mechanistic models to be explored, and possibly transgenic animals to be generated. Induced stem cells could provide such a model.

## The Adult Drosophila Malpighian Tubules Are Maintained by Multipotent Stem Cells:

All animals must excrete the waste products of metabolism. Excretion is performed by the kidney in vertebrates and by the Malpighian tubules in Drosophila. The mammalian kidney has an inherent ability for recovery and regeneration after ischemic injury. Stem cells and progenitor cells have been proposed to be responsible for repair and regeneration of injured renal tissue. In Drosophila, the Malpighian tubules are thought to be very stable and no stem cells have been identified. This study has identified multipotent stem cells in the region of lower tubules and ureters of the Malpighian tubules. Using lineage tracing and molecular marker labeling, it was demonstrated that several differentiated cells in the Malpighian tubules arise from the stem cells and an autocrine JAK-STAT signaling regulates the stem cells' self-renewal. Identifying adult kidney stem cells in Drosophila may provide important clues for understanding mammalian kidney repair and regeneration during injury (Singh, 2008).

The regenerating renal cells may come from one of the three possible sources, based on previous studies. First, the circulating blood contains bone marrow-derived stem cells able to differentiate into non-haematopoietic cells, such as cells of the kidney. Second, the differentiated glomerular and tubular cells may also be able to dedifferentiate into

stem-like cells to repair the damaged tissues. Third, large numbers of slowly cycling cells have recently been identified in the mouse renal papilla region; these cells may be adult kidney stem cells and may participate in renal regeneration after ischemic injury. Further, the ureter and the renal collecting ducts were formed from the epithelium originating from the ureteric bud, and the nephrons and glomeruli were formed from the metanephric mesoderm-derived portion during kidney development. Two distinguished stem cell types have been proposed as responsible for repairing the renal collecting tubules and the nephrons. This study identified a type of pluripotent stem cells (RNSCs) in the Drosophila renal organ. The stem cells are able to generate all cell types of the adult fly MTs. In the region of lower tubules and ureters, autocrine JAK-STAT signaling regulates the stem cell self-renewal. Weak JAK-STAT signaling may convert an RNSC into a renalblast (RB), which will differentiate into an RC in the region of lower tubules and ureters, and a type I or type II cell in the upper tubules. These data indicate that only one type of stem cell may be responsible for repair and regeneration of the whole damaged tissues in mammalian kidney (Singh, 2008).

The Drosophila RNSCs represent a unique model to study the molecular mechanisms that regulate stem cell or cancer stem cell behavior. In most of the stem cell systems that has been well characterized to date, stem cells always reside in a specialized microenvironment, called a niche. A niche is a subset of neighboring stromal cells and has a fixed anatomical location. The stromal cells often secrete growth factors to regulate stem cell behavior. The stem cell niche plays an essential role in maintaining stem cells, and stem cells will lose stem cell status once they are detached from the niche. The niche often provides the balanced (proliferation-inhibiting and proliferation-stimulating) signals that keep the stem cells dividing slowly. The inhibitory signals keep the stem cell quiescent most of the time while the stimulating signals promote stem cell division, to replenish lost differentiated cells. Maintaining the balance between proliferation-inhibiting and proliferation-stimulating signals is the key to maintaining tissue homeostasis (Singh, 2008).

Drosophila RNSCs are controlled differently. This study has demonstrated that the JAK-STAT signaling regulates the stem cell self-renewal. Both the ligand Upd and the receptor Dome are expressed in the RNSCs and the autocrine JAK-STAT signaling regulates the stem cell self-renewal; thus, the self-sufficient stem cells control their self-renewal or differentiation and do not need to constrained to a fixed niche. However, the RNSCs are still confined to the region of lower tubules and ureters even in the Upd overexpressed flies, suggesting that some other factors besides the JAK-STAT signaling may restrict the RNSCs to the region of the lower tubules and ureters (Singh, 2008).

Recent studies also suggest that tumors may arise from small populations of so-called cancer stem cells (CSCs). The CSCs probably have arisen from mutations that dysregulate normal stem cell self-renewal. For example, mutations that block the proliferation-inhibiting signals or promote the proliferation-stimulating signals can convert the normal stem cells into CSCs. This study demonstrates that amplifying the JAK-STAT signaling by overexpressing its ligand Upd stimulates the RNSCs to proliferate and also to differentiate into RC, which results in tumorous overgrowth in the MT. Therefore, the Drosophila RNSC system may also be a valuable in vivo system in which to study CSC regulation (Singh, 2008).

The RNSCs are located in the region of the lower tubules and ureter of the MTs, while ISCs are located at the posterior midgut. The MTs' ureters connect to the posterior midgut. The two types of stem cells are at close anatomical locations in the adult fly digestion system and also share some properties. For example, both of them are small nuclear cells, Arm-positive, and express *esg*. However, RNSCs and ISCs produce distinctly different progenies. ISCs produce progenies that include either Su(H)GBE-lacZ- or Pros-positive cells, which are not among the progenies of RNSCs because Su(H)GBE-lacZ and Pros

are not expressed in the MTs. RNSCs produce progenies that include Cut- or TSH-positive cells, which are not among the progenies of ISCs because Cut and TSH are not expressed in the posterior midgut. One possibility for this difference is that, although RNSCs and ISCs originate from the same stem cell pool, their particular environments restrict their differentiation patterns. Future experiments, such as transferring RNSCs to the posterior midgut and vice versa, should be able to test this model (Singh, 2008).

The JAK-STAT signaling regulates self-renewal of the male germline, the male somatic, female escort stem cells in fly. The signaling also regulates self-renewal and maintenance of mammalian embryonic stem cells. This study reports that the JAK-STAT signaling regulates self-renewal of RNSCs. The JAK-STAT signaling may be a general stem cell signaling and also regulate stem cell self-renewal in other, un-characterized stem cell systems (Singh, 2008). *esg* has been used as a marker of both male germline stem cells. This study has demonstrated that the *esg*-Gal4. UAS-GFP transgene is specifically expressed in RNSCs. The function of the *esg* gene is to maintain cells as diploid in Drosophila imaginal cells. Stem cells may have to be diploid, and *esg* may be a general stem cell factor. Identifying a stem cell signaling pathway (such as the JAK-STAT signal transduction pathway) and a stem cell factor (such as *esg*) will provide useful tools for identifying stem cells in other systems and for understanding stem cell regulation in general (Singh, 2008).

## Techniques Involved in Tissue Engineering to Induced Differentiation of Pluripotent Stem Cells:

1. Morphological analysis
   -Alkaline phosphatase staining
2. Molecular assays:
   -Analysis of pluripotency gene expression
   (a) RT-PCR (b) Immunostaining (c) Pluripotency marker
   -DNA methylation analysis
   (a) Bisulfite sequencing
   -Transcriptional profiling
   -Karyotyping
   -ESC-like histone modifications (ChIP)
   - X chromosome reactivation (FISH)
   - Retroviral silencing
3. Functional character
   -Analyze in vitro differentiation
   -Analyze in vivo differentiation (Teratomas, Chimeras, Tetraploid complementation)
   -Mate to test germline transmission

***(1) Morphology –*** The first indication that a differentiated cell has been reprogrammed into an iPSCs is a change in morphology of the cells growing in the petri dish. For example, skin cells grow as flattened cells in a dish; however, as they become reprogrammed the iPSCs grow in round clumps known as colonies. The colonies are visible under a microscope, and can be picked using careful techniques in the laboratory. Once colonies are picked, they can be expanded to generate a clonal iPSC population.

***(2) Expression of pluripotency markers –*** During reprogramming, the differentiated cells turn off genes that are expressed in a differentiated state, and turn on the expression of genes that are uniquely expressed in an undifferentiated state (pluripotent stem cells). Because these genes are only expressed in pluripotent stem cells and not in other cell types, they are referred to as pluripotency markers. Expression of pluripotency markers is like a molecular signature that lets scientists know that the cells have been reprogrammed to a pluripotent state.

***(3) Cell culture –*** For *Drosophila*, Schindler's cell line (S2), an epithelial-like cell line, was purchased (ATCC, CRL 1963) and passaged (1:10) and maintained per supplier's specifications in *Drosophila* complete medium. BG2 cells were purchased from the *Drosophila* Genome Research Center (ML-dmBG2; number 51), and main-tained with growth culture conditions provided by the center. Mouse embryonic stem cells (ESCs; line R1 [Nagy *et al,* 1993]) were cultured using standard conditions

(Joyner, 1999). Chicken ESCs (25th passage) were provided by Dr Bertrand Pain (Clermont University, France) and cultured according to their protocol (Lavial *et al,* 2007). Adult cell lines for mouse were either generated or purchased.

***(4) Vectors –*** Lentiviral vectors were generated in human embryonic kidney (HEK) 293T cells (Cell Biolabs, San Diego, CA, Cat # LTV-100), using a third-generation lentiviral system, following a previously described protocol (*Cockrell and Kafri, 2007*). Prior to transfection, the cells were plated on 10 cm collagen coated plates at a density that resulted in 60–70% confluency at the time of transfection. A transfection mix was prepared with either 5, 10, or 15 $\mu$g of DNA of the STEMCCA vector or control GFP lentiviral vectors (EF1$\alpha$-GFP; both kindly provided by Dr Gustavo Mostoslavsky), packaging cassette (REV and Gag/Pol, 10 $\mu$g) and the VSV-G (5$\mu$g) envelope expression cassette, respectively. The cells were then transfected with the mix, using 40 $\mu$l of Lipofectamine (Invitrogen, Carlsbad, CA) per plate. 8 hr after the addition of DNA, the transfected cells were washed with PBS and fresh complete media as used for mouse cells. Media with viral particles were collected every 24 hr for the next 48 hr and stored at 4°C until complete. Viral particles were separated from cellular debris by centrifugation at 4000g for 5 min followed by filtration through a 0.45-micron filter. The titer was measured using Quick-Titer (Cell Biolabs Inc, Cat # VPK-112) and promptly stored at "80°C. If necessary, titer concentrations were increased by ultracentrifugation (SW-29 rotor) at 50,000g for 2 hr, followed by re-suspension in PBS (pH = 7.2). A commercially available human stem cell cassette with GFP (Biosettia, cat# iPSC-p4F01) on the avian cells was used. We established DNA preps and lentiviral vectors as above. Maximum titer was significantly less than with the STEMCCA cassette (2.5 × 108 U/ml). For *Drosophila* transductions, we also generated a plasmid with the Metallothionein inducible promoter from the vector pMT/ BiP/ V5-His A (Invitrogen). The four transcription factors in the STEMMCA cassette described above were cloned into pMT/BiP/V5-His A in two steps: first, the Oct-4 and Klf-4 segment, followed by the Sox-2, c-myc segment. The cloning was confirmed by sequencing using plasmid and gene specific primers.

***(5) Alkaline phosphatase staining –*** Alkaline phosphatase (AP) is a universal pluripotent marker for all types of pluripotent stem cells including embryonic stem cells, embryonic germ cells, and induced pluripotent stem cells. The pluripotent status of stem cells can be characterized by a high level of AP expression, along with the expression of multiple pluripotency markers including the transcription factors Nanog, Oct4, Sox2 stage specific embryonic antigens, SSEA-1, -3, -4, and tumor related an tigens, TRA-1- 60, TRA-1-81.Alkaline phosphatase (ALP) activity was measured using the STEMTAG Immunohistochemical Kit (Cat# CBA-300, Cell Biolabs), following the manufacturer's protocol. Control fibroblasts, ESCs, and iPSCs were washed with PBS, and fixed with either 4% paraformaldehyde or the kit's fixing solution for 10 min at room temperature. The fixing solution was then aspirated, the staining solution was placed in each well for 30 min and stored in the dark at room temperature. The wells were washed with $dH_20$ 3 times and images were taken immediately under a stereomicroscope without coverslipping. A dark blue/purple color product indicates the presence of ALP enzymatic activity normally found in stem cells, whereas differentiated cells will not stain. The same protocol was also employed, in some instances, with Vector Red as an indicator (Vector Laboratories, inc, Burlingame, CA).

***(6) Transduction of cells and iPSC culture –*** Transduction was performed using the ViraDuctin system, as per supplier's protocol (Cell Biolabs, Cat # LTV-201) in complete media. Before transduction, cells were thawed and cultured in complete media until 80% confluent. After transduction, cells were grown for 5 days (2 days for *Drosophila*), then passaged (first passage), and let to grow for approximately 20 days (8 days for *Drosophila*) in 3i

Media (Stem Cell Sciences, UK, SCS-SF-ES-01) or our mouse stem cell media for mouse cells (Ying *et al.,* 2008), and *Drosophila* stem cell media. *Drosophila* cells grew faster than the vertebrate fibroblasts, and thus, markers were observable at earlier time points. Cells were then subsequently passaged when cultures reached con-fluency, which was every 3 days for *Drosophila* cells, and divided 1:10 (*Drosophila*, due to more rapid growth). Before we performed detailed analyses on multiple transfections, viral transduction efficiency values were assessed at three different STEMCCA concentrations in 48 well plates and cell colony forming units quantified in the vertebrate species. We measured 11 independently transduced plates, and analyzed differences based on titer and species. Based on these initial transduction experi-ments, most subsequent transductions were performed at 108 U/ml for mouse and 9.5 × 109 U/ml for fly to achieve similar colony forming unit levels as starting points for our analyses. For subsequent analysis, in order to achieve statistical confidence, we transfected 12 to 30 wells seeded with primary cells, in seven different independent experiments. Each well was inde-pendently transfected. Samples of the cells were then extracted at various time points, to identify the presence of exogenous or endogenous genes and proteins, via RT-PCR and immunocytochemistry, respectively. For all species, negative control groups were conducted utilizing fibroblasts transduced with a GFP containing lentivirus and grown in the stem cell media. For in vivo pluripotency experiments, both fibroblasts and iPSC-like cells were first transduced with the GFP lentiviral vector (titer 108), following the same transfection protocol. We also performed post induction GFP transfection on the *Drosophila* cells, although these were not used for in vivo studies. To transduce S2 cells with the Metallothione in inducible promoter plasmid, we used a previously described protocol (Santos *et al,* 2007). To induce expression of the transcription factors, 1–2 days after transfection, copper sulfate was added to the medium to a final concentration of 500 $\mu$M (5 $\mu$l of a 100 mM CuSO4 stock). Colonies were observed after around the 7th day, but they numbered less than with cells transduced with the mouse genes. These colonies showed alkaline phosphatase staining and formed embryoid bodies.

***(7) qRT-PCR –*** Cells or embryoid bodies were spun down and RNA isolated using a standard kit (Promega SV total RNA isolation system, Z3105). RNA was quantified using a NanoDrop 2000c (Thermo Scientific, Waltham, MA) and then stored in "80°C. Complementary DNA (cDNA) was produced by reverse transcription (RT) in a 20 $\mu$l reaction using the supplier's protocol (10 $\mu$l of 2X RT buffer and 1 $\mu$l of 20X Superscript II enzyme; Applied Biosystems). The cDNA was then used as a template to perform PCR gene expression assays in 20 $\mu$l reactions containing 1 $\mu$l template (2 $\mu$g/$\mu$l), 10 $\mu$l 2X Gene Expression Master Mix (BioRad, Hercules, CA) and forward and reverse TaqMan primer probes (Generated by Applied Biosystems) [Developmental biology and stem cells Rosselló *et al.* eLife 2013;2:e00036. DOI: 10.7554/eLife.00036] or in 20 $\mu$l reactions containing the same reagents, but in place of TaqMan primers, custom PCR primers and 1 $\mu$l SYBR green (BioRad). To discriminate between endogenous and exogenous expression of the stem cell genes across species, different primers were generated for mouse and the non-mammalian species, using non-overlapping sequences. To discriminate between mouse exogenous and endogenous genes, primers to the WPRE region of the vector were used. Using this strategy, the estimated relative amount of endogenous expression was calculated as the expression level of the WPRE segment subtracted from the total RNA of the mouse specific transcription factors. The reactions were performed in a Cx96 real-time machine (BioRad). Cycling conditions were 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. No-template controls were run for each primer set and probe. 18S rRNA endogenous control was run for each sample using TaqMan primers that recognized the RNA in all species tested (Cat# Eukaryotic 18S RNA HS99999901_S1; Applied Biosystems). The results were normalized to the endogenous 18S expression and to the gene expression level of the control fibroblast/primary cell groups using the AA CT method common for RT-PCR analyses. All primers showed efficiency levels above 90%,

using the protocol in the MIQE guidelines (minimal information for publication of real-time PCR experiments)

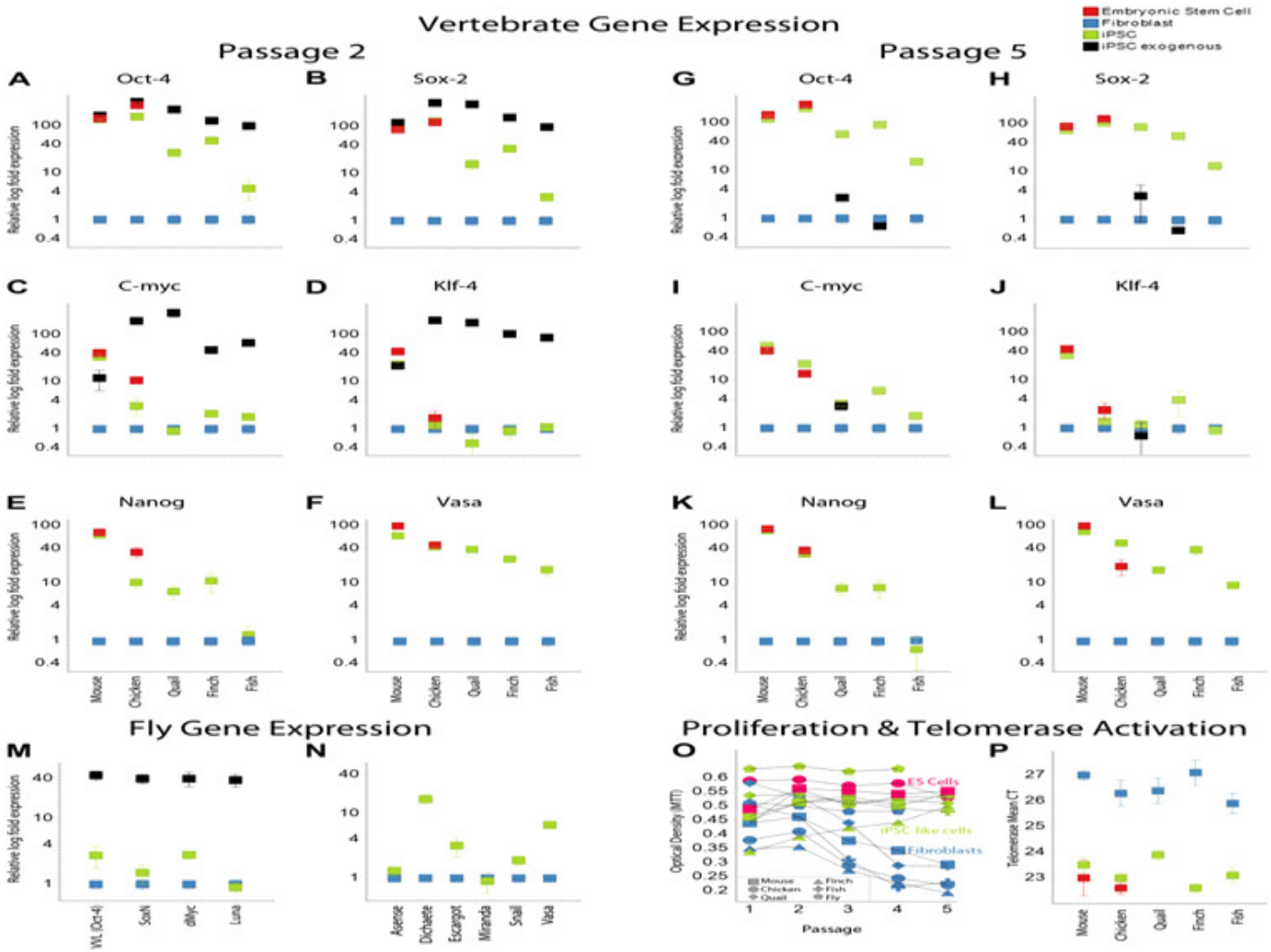


Fig 2 – Upregulation of Stem Cell Genes in Mouse, Birds, Fish, and *Drosophila* by Mouse Transcription Factors. (A–D) qRT-PCR of Exogenous (Black) Mouse and Endogenous (Green) Species-specific Expression of *Oct-4* (A), *Sox-2* (B), *c-myc* (C), and *Klf-4* (D) in iPSC-like Cells of Each Species after the Second Passage Relative to (Normalized) Non-transduced Fibroblast Controls (Blue). Mouse and Chicken ESCs Were Included as Positive Controls (Red).

(Bustin *et al.,* 2009). For statistical analysis, 2-way ANOVAs were performed on two factors (genes and cell types [iPSC, fibroblast, ESC, EB]) on n = 5 independently transduced lines (replicates) for each of the vertebrate species or n = 3 independent lines for the *Drosophila* cells.

***(8) MTT (proliferation) assay –*** To assess proliferation, we used the MTT (3-[4,5-Dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) Quantitative Cell Proliferation Assay (Cat# 30-1010K; ATCC). Tetrazolium salts are reduced metabol-ically by the cells, resulting in a colorimetric change. The resulting intracellular purple formazan is solubilized and quantified spectrophotometrically (at 570 nm). Cells (induced and controls) for all species were plated at 10,000 cells/well (in quintuplets, from independently transduced cells) and incubated for 24 hr. 10 $\mu$l of the MTT reaction solution was added to each

plate and incubated for 3 hr. 100 $\mu$l of detergent was added to each plate, stored for 2 hr in the dark (room temperature), and the absorbance was measured at 570 nm using a Molecular Devices Emax Microplate Reader.

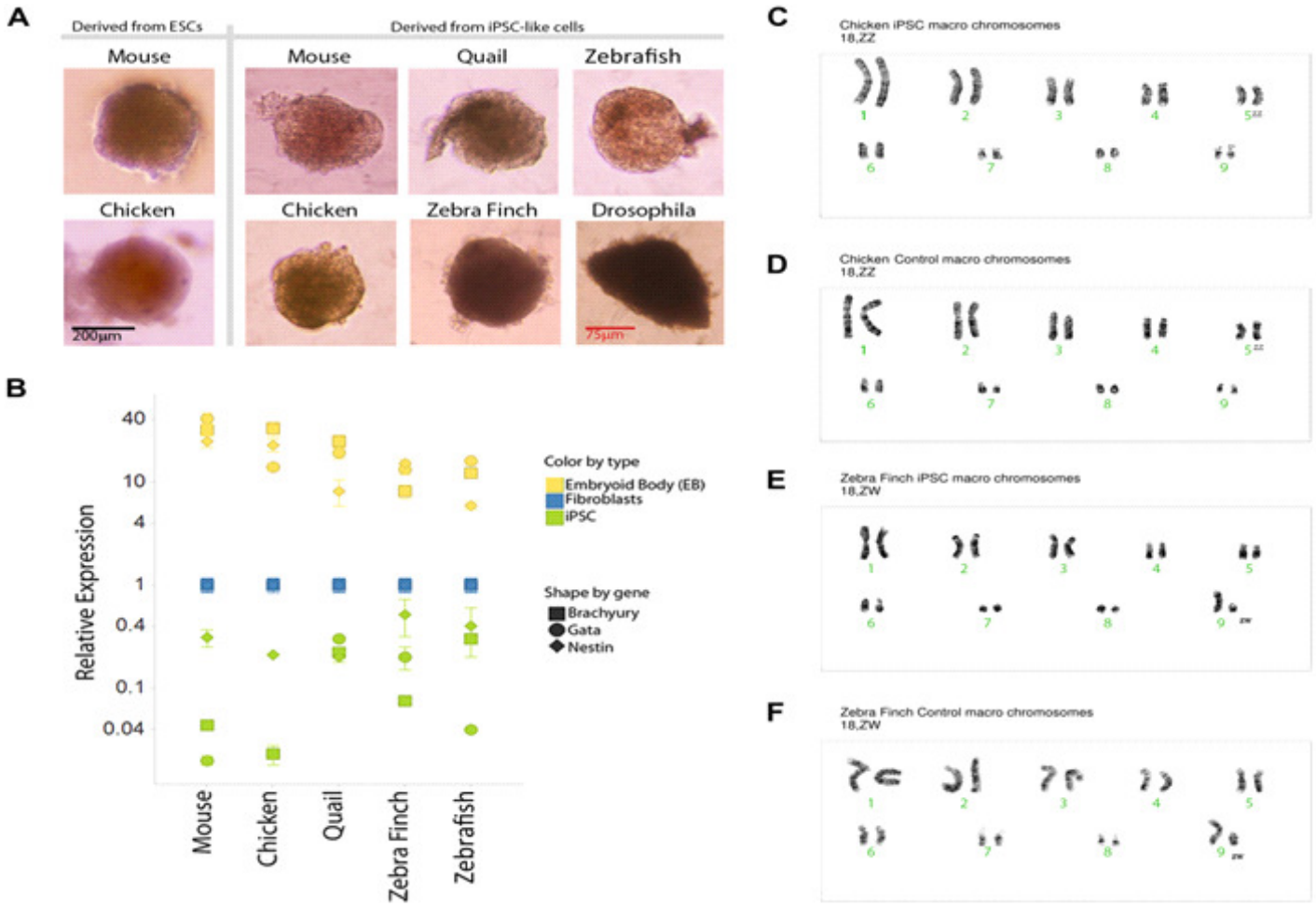


Fig 3 – Karyotyping and in vitro Pluripotency of iPSC-like Cells. (A) Embryoid Bodies (EB) from iPSC-like Cells in Differentiation Media. (B) qRT-PCR Gene Expression Analyses of Nestin (Ectoderm Marker), Brachyury (Mesoderm), and Gata-4 (Endoderm) Homologs in Undifferentiated iPSC-like Cells (Green) and in EBs (Yellow) from Mouse, Bird and Fish Relative to Their Control Fibroblasts (Normalized; Blue). Error bars, S.E.M. (n = 5 Replicates of Independently Generated Cell Lines or EBs)

ANOVA was performed to test for differences between cell types and species (n = 5 independent lines, per species). Statistical significance was considered at $p<0.05$.

***(9) Telomerase activity –*** Telomerase expression is low or absent in most somatic tissues, but not in germ cells, stem cells, and tumors (Meyne *et al,* 1989). The telomerase binds to a particular repeat sequence TTAGGG present at the ends of chromosomes of most eukaryotic species and extends them during cell replication. Telomerase enzymatic activity was determined using the Quantitative Telomerase Detection Kit (BioMax, USA, MT3012), following the manufacturer's protocol. Cell extracts containing proteins and RNA were generated from the ESC, iPSC, and control fibroblast, and then telomerase activity was measured. If telomerase is present, it adds nucleotide repeats to the end of an oligonucleotide substrate

of the kit, which is subsequently amplified by real time qPCR. Quantitation was carried out by the PCR software of the BioRad Cx96 system. Positive control (template provided with kit) and negative control (heat inactivated samples) reactions were performed. Cycling conditions for the BioRad Cx96 real-time machine were as follows: 48°C for 10 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/ extension). All reactions were performed in quintuplets. Paired *t*-tests were performed to test for differences of telomerase in the iPSC-like and control fibroblasts of each cell line. Statistical significance was considered at $p<0.05$.

***(10) Karyotyping –*** Karyotyping was performed as previously described (Bangs and Donlon, 2005), by Karyologic, inc. Briefly, cells were seeded in t-25 tissue culture flasks, and allowed to grow. Colchicine (Colcemid, Invitrogen 15210-040) was added to each flask (0.25 ml/ 5 ml media) and incubated at 37°C, 5% CO2 for 12 hr. Cells were then trypsinized, transferred to 15 ml tubes and spun down at 1200 RPM, for 8 min. Cells were then resuspended in 0.0075 KCL and incubated at room temperature (6 min) before being spun down again. Cells were then fixed with Methanol/Acetic acid fixative (3:1) and stored overnight. Cell suspensions were then dropped into cold slides, dried and baked for 20 hr at 65°C. In order to assess the banding of the chromosomes, slides were treated with 0.05% trypsin 0.02 EDTA at room temper-ature for 12 s, rinsed quickly in 100% ethanol and then in Gurr's phosphate buffer (pH 6.8, Invitrogen #10582-013). Slides were then stained with Karyomax Giemsa (Invitrogen #10092-013), per manufacturer instructions. To assess the chromosomes, Applied Imaging Genus Cytovision Software (v2.8) was used.

***(11) Embryoid body formation –*** In order to form embryoid bodies (EBs), the hanging drop method was used (Keller, 1995). After harvesting the iPSCs or control fibroblasts (or S2 cells) directly from culture on the stem cell media, they were washed with PBS (pH7.4; Gibco) to remove any LIF and resuspended in 'differentiation media' which is complete media for each species excluding LIF, cytokines, chemical inhibitors and mercaptoethanol. The cells were then micropipetted in 20 $\mu$l drops containing <"500 cells each on the lids of bacteriological plates (Sigma, 100 mm). The lids were inverted over a dish filled with 10 ml PBS and incubated for 2–3 days. After the embryoid bodies had formed from the iPSC-like cells, the drops were flushed from the lid with differentiation media and grown in suspension culture for another 3–5 days. Embryoid bodies .were then collected via pipette, RNA extracted (as above), and qRT-PCR analysis conducted (as above).

***(12) Immunohistochemistry*** (Fig 4) **–** For SSEA-1 labeling, reactions were performed on cells cultured on coverslips in 24 well plates. The primary SSEA-1 antibody (Cat# 480, Santa Cruz Biotechnology, Dallas, TX) was diluted (1:200) in PBS. A secondary anti-mouse IgM conjugated to a green fluorescent molecule (Abcam, Cambridge, MA) was diluted (1:500) and incubated at 4°C, overnight. The cells were then washed 3X in PBS and coverslipped with DAPI solution (VectaShield; Vector Labs). Images were taken using a fluorescent microscope (Olympus Bx61).

For GFP labeling (performed by the Duke University Pathology Lab), chicken or zebrafish embryos, or positive control tissue slides (Mouse GFP positive brain sections), were cut at 5 $\mu$m on a paraffin block and mounted into glass slides. These were dried for at least 30 min at 60°C in an oven. The slides were deparaffinized in three changes of xylene (5 min each), 2 changes of 100% EtOH (3 min each), and 2 changes of 95% EtOH (3 min each). Rehydration was performed in dH2O for 1 min. To block endogenous peroxidase activity, 3% hydrogen peroxide was used for 10 min, followed by a rinse in dH2O to remove antigens. For the primary antibody (Anti-Rabbit GFP Abcam ab290, diluted at 1:100 in PBS [pH = 7.1]), 200 mls of the citrate, pH 6.1, antigen-retrieval buffer from Dako (10X concentrate) were used. The buffer was preheated to 80°C in a Black and Decker vegetable steamer for 20 min. The slides were then cooled to room temperature in running tap

water (about 15 min). Slides were thoroughly rinsed in water and placed in TBST. After antigen retrieval, 10% normal rabbit serum was applied to the slides and incubated for 60 min at room temperature. Afterwards, they were washed with PBS and the excess was drained. After incubation, Vectastain Elite ABC was used, followed by DAB chromagen (Dako), and incubated for 5 min, followed by washing. All slides were counterstained in hematoxylin for 30s. Slides were rinsed in tap water until clear and coverslipped.

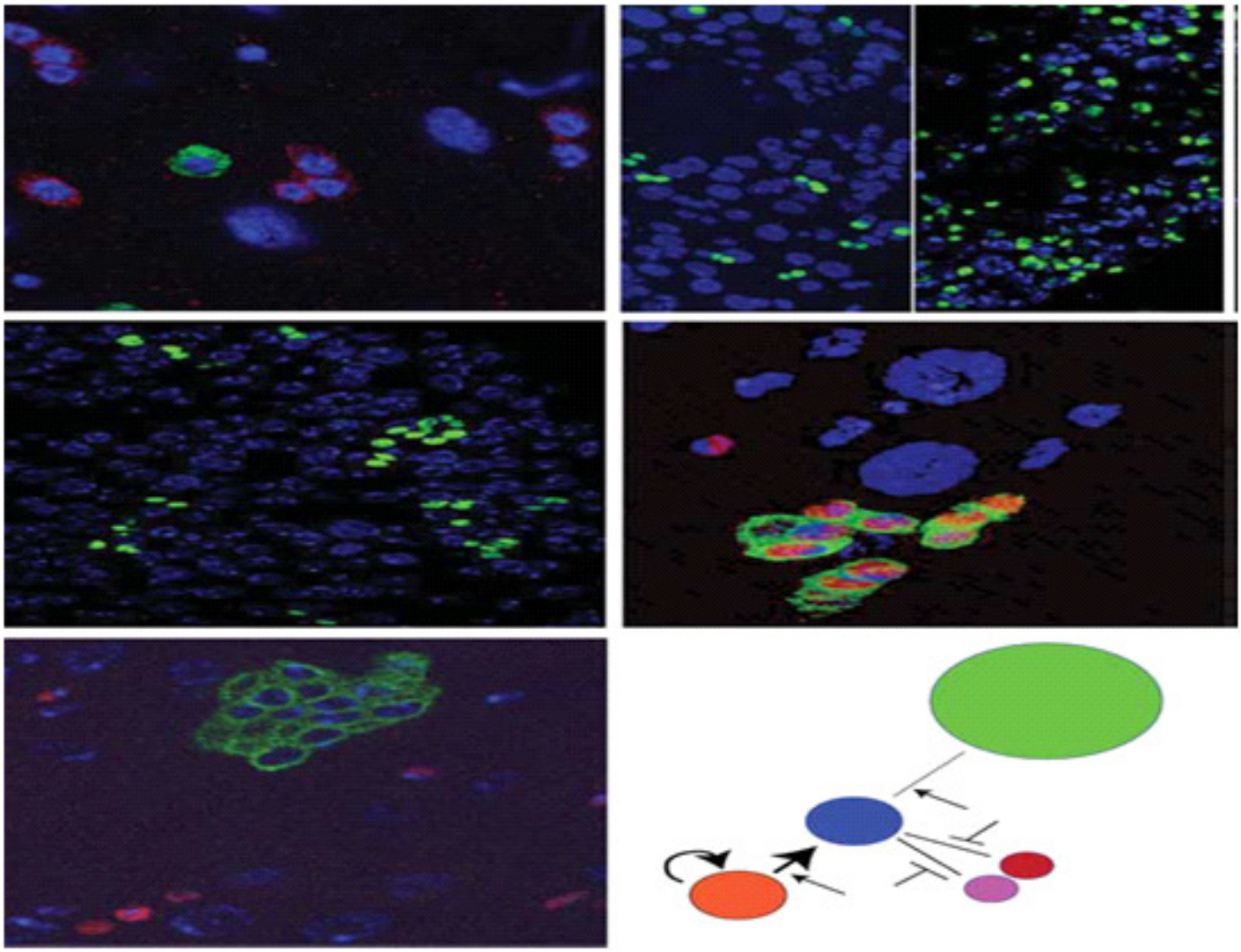

Fig 4 – Immunostaining and Fluorescent Microscopy Using GFP, DAPI as a Marker

***(13) Teratoma formation –*** Chicken and quail iPSC-like cells and control fibroblasts were grown in 6 well plates, detached, and spun down (200g, 5 min). The supernatant was removed, and cells were cleaned and re-spun with PBS (1X, pH: 7.2). The concentration of cells was adjusted to 5 × 106 cells per ml. 5-week-old male SCID mice (N:NIH-bg-nu-xid; Charles River Laboratories, Raleigh, NC) were used for each experiment. Animals were anesthetized with intraperitoneal injections of ketamine–xylazine (50 and 5 $\mu$g/g, respectively) in saline. 100 $\mu$l of

the cell solution was injected into the mouse testes. Afterwards, the mice were let to recover from the anesthesia on a heating pad (Kent Scientific). After 5 weeks, the mice were sacrificed, and the testes were removed to assess

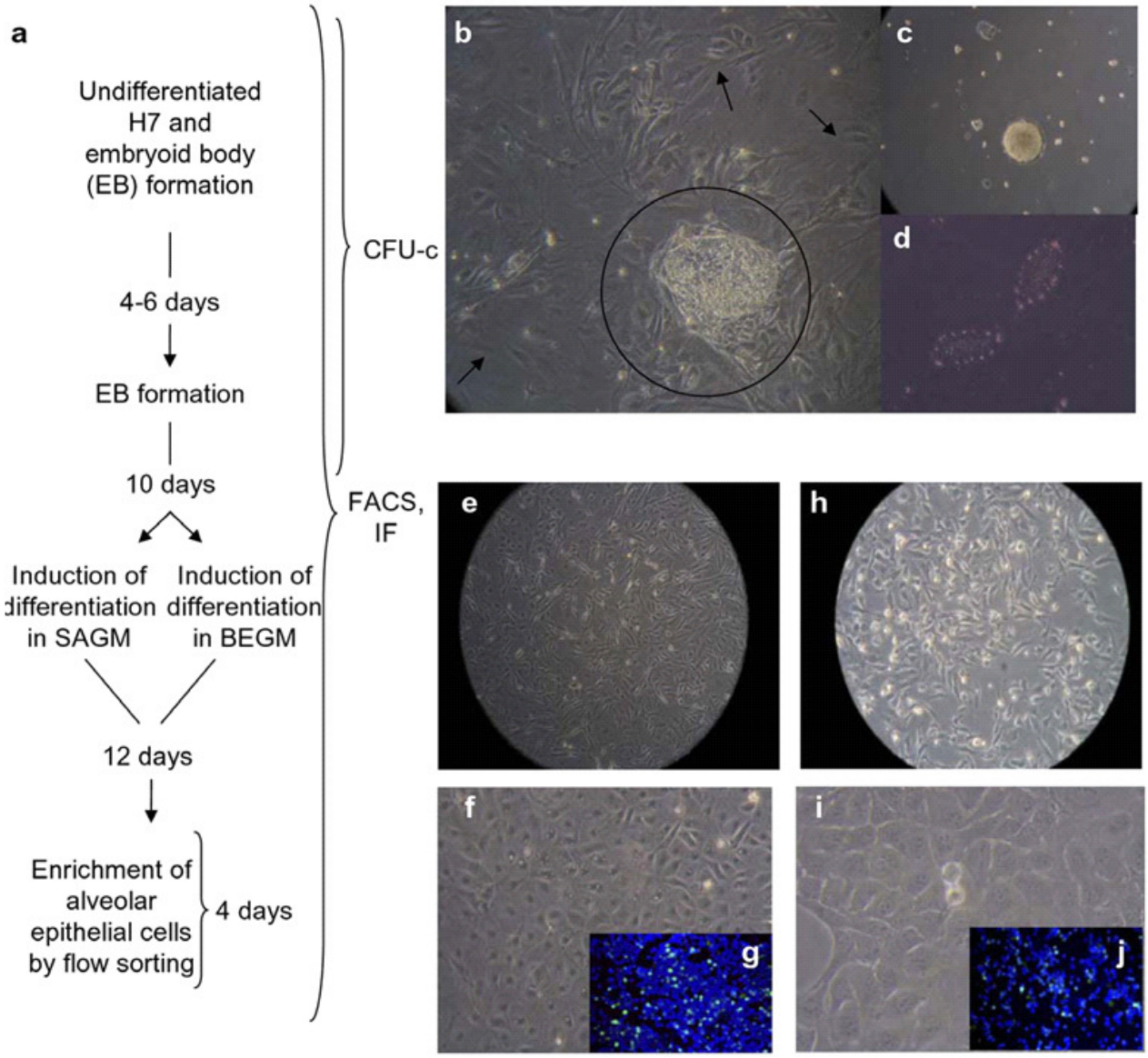


Fig 5 – Differentiation of H7 hES cells to lung epithelial cell-specific lineages. a Outline of formation of EBs from H7 hES cells and differentiation to alveolar epithelial cells in SAGM and BEGM. b Undifferentiated hES cells (within circle) were expanded on c-irradiated MEF feeders for 4–6 days followed by formation of c EBs in suspension culture overnight after aggregation. d day 4 EBs were cultured in ultra-low attachment plates for 10 days and then transferred to gelatin-coated plates and cultured with either e–g SAGM or h–j BEGM for 12 days (g and j are insets of SPC- FITC+ cells). AEII cells were flow sorted as surface SP-C+ cells and enriched in SAGM for an additional 4 days to amplify cell numbers for transplantation. At each stage, cells were fixed in chamber slides for IF microscopy [green probe is FITC-conjugated lineage (epithelial) marker and blue probe is DAPI-stained nuclei of live cells]. f, g show enriched AEII cells and i, j Clara cells

teratoma formation by histology (H&E).

medium" (SAGM) or "bronchiolar epithelial growth medium" (BEGM) for differentiation into lung lineage-

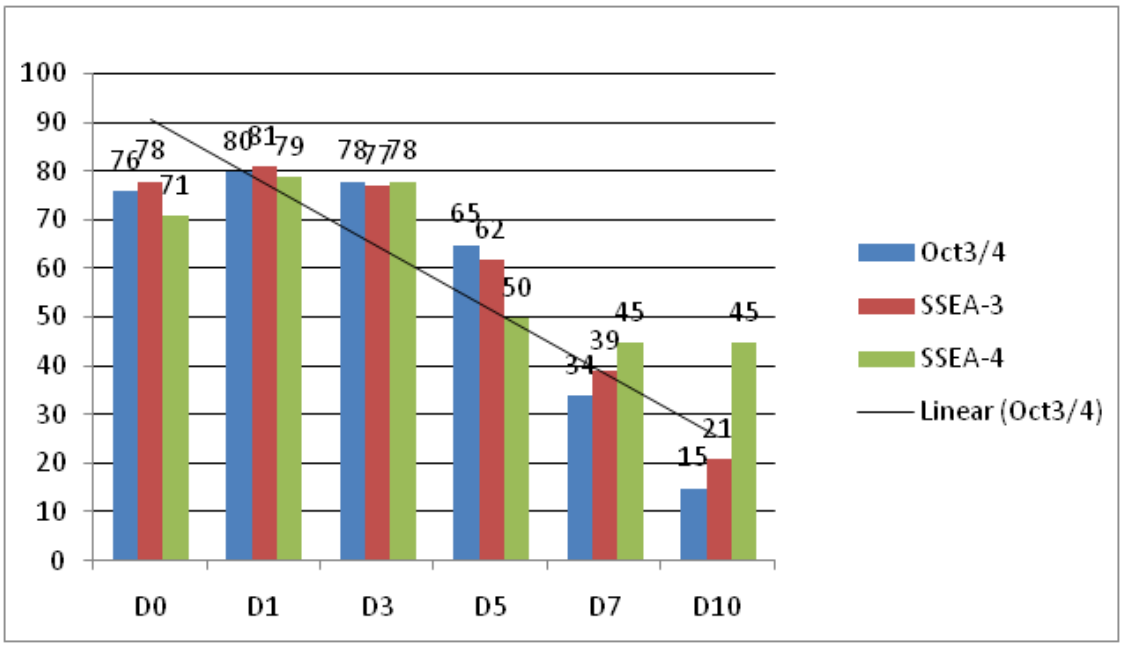


Fig 6 – Percentage of Positive Cells as Mean ± SEM

## Generation of Lung Lineage-specific Airway Cells from Stem Cells – A Case Study with Two Different Sets of Human Cell Lines:

***H7 (Originator WiCells, WI, USA)***

***Expansion, differentiation, and in vivo functional validation of human embryonic stem cells for regenerative therapy using murine lung injury model:***

Murine and human embryonic stem cells have been employed to generate AEI, AEII, and Clara cells[1-6] and also to repair airway injury in fibrosis models (Banerjee *et al*, 2012). As an example as reported by Banerjee *et al*, 2012, human (h) ESC H7 cells were differentiated *in vitro* into lung epithelial lineage-specific cells (i.e., alveolar epithelial cells types I and II cells and Clara cells) as the initial developmental step for a cell-based strategy to repair pulmonary injury in the bleomycin mouse IPF model (Fig 5). Undifferentiated hESCs after culture in embryoid body formation were cultured in either a "small airways growth specific cells. Differentiation of the hESCs was skewed to a predominantly AEII phenotype (i.e., 68% AEII cells, 12% AEI cells, and 4% Clara cells) by culture in SAGM and to a Clara cell phenotype (i.e., 33% Clara cells, 12% AEII, and 2% AE I cells) by culture in SAGM and to a Clara cell phenotype (i.e., 33% Clara cells, 12% AEII, and 2% AE I cells) by culture in BEGM by immunostaining [(aquaporin-5 (AQP-5), caveolin, and ICAM-1 for AEI cells, surfactant proteins C and D (SP-C, SP-D) and aquaporin-1 (AQP-1) for AEII cells, and Clara cell-specific protein-10 (CC-10) for Clara cells) and by electron microscopy. mRNA expression for the AEII marker SP-C increased 15-fold in the hESCs cultured in SAGM whereas expression of the Clara cell marker CC-10 increased 6-fold in the cells cultured in BEGM. It was noted that incubation of the hESCs after differentiation into alveolar and bronchiolar non-ciliated epithelial cells with ICG-001, the small molecule inhibitor Wnt/β-catenin/CBP transcription, changed the cells from

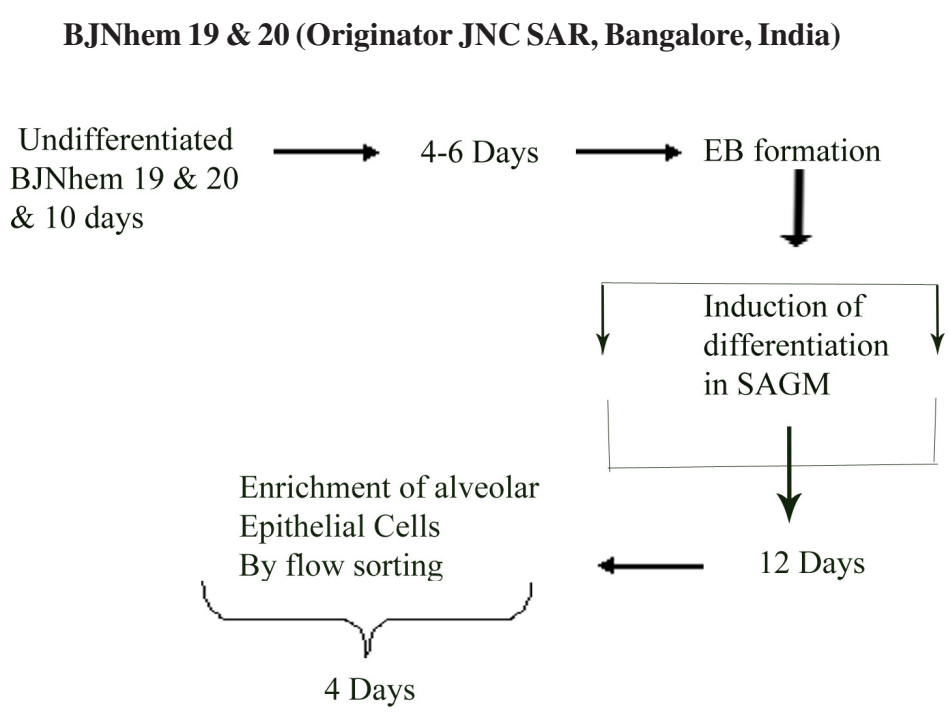


Fig 7 – Outline of formation of EBs from BJNhem20 cells and differentiation to alveolar epithelial cells in SAGM. Differentiation of BJNhem19 and BJNhem20 cells to lung epithelial cell – specific lineage. An outline of formation of EBs from BJNhem19 and BJNhem20 and differentiation to alveolar epithelial cells in SAGM and BEGM.

the AEII phenotype to a predominantly AEI phenotype as demonstrated by flow cytometry and immunocytochemistry[2-5]. Thus inhibition of Wnt/β-catenin positive cells, 12% AEII, and signaling promotes transdifferentiation of type II alveolar epithelial cells to type I cells.

Fig 6 shows intracellular expression of Oct3/4, SSEA-3, SSEA-4 (pluripotent markers) in permeabilized cells were done through flow cytometry for identification of differentiation stage of the cells in culture. The percentage of positive cells is shown as mean ± SEM (n = 3 independent experiments with flow data collected in triplicate). BJNhem19 was grown in defined medium following the standardized recommended protocol for H7 expansion which is briefly described here: Expansion of H7 hES Cells NIH approved (NIH code WA07) undifferentiated hES cell line H7 was obtained from WiCell Research Institute (Madison, WI), and cells from passage 25 to 35 were used. For propagation of the H7 cells in undifferentiated state, the ES cells were initially grown on primary mouse embryonic fibroblast (MEF) feeder cells prepared from timed pregnant CF-1 female mice (day 13.5 of gestation) that had been c-irradiated with 3000 rads for 5 min, and then directly in conditioned medium in which the above cirradiated MEF cells were cultured to ensure purity of human cells and progressively eliminate any mouse feeders from the cultures. The medium contained Dulbecco's Modified Essential Medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), and 2 mM L-glutamine as described previously. The hES cells were cultured in ES medium [i.e., knockout (KO) DMEM supplemented with 20% knock-out serum replacement (KOSR; Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate, 0.1 mM 2bmercaptoethanol (ME) (Sigma-Aldrich Corporation, St Louis, MO), 0.1 mM minimum essential media (MEM), 1% nonessential amino acids (NEAA;

Mediatech, Herndon,VA), 1 mM L-glutamine, and 2 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN)]. For cell culture, 6-well 10 cm2 tissue culture plates, coated with 0.1% gelatin were used, and all cultures were done in a humidified 5% CO2 incubator at 37uC.

***Expansion of human embryonic stem cells:*** Human embryonic stem cell (hESCs) lines BJNhem19 and BJNhem20 were obtained from JNCASR, India which is claimed to be karyotypically normal, sibling human ES cell lines representing the Indian ethnic background. These cells were derived from the inner cell mass (ICM) of grade III poor quality blastocysts that were not suitable for *in vitro* fertility treatment. Both the lines as claimed by JNCASR are pluripotent and have been extensively characterized and cultured continuously for over 250 passages[7].

The aforementioned ESC lines were grown on primary mouse embryonic feeder cells, for expansion and propagation in an undifferentiated state. The feeder cells were prepared from pregnant Balb C mice (13.5 days of gestation), these cells were then cultured in MEF conditioned media and then treated with Mitomycin – c to stop their differentiation. The MEF was cultured in Dulbecco's Modified Essential Medium (DMEM), 10% fetal bovine serum (FBS) and 2mM – L Glutamine. The hESCs were cultures in ES medium, comprised of Knock out (KO) DMEM supplemented with 20% Knock out serum replacement (KOSR), 1 mM sodium pyruvate, 0.1 mM 2β-Mercaptoethanol (ME), 0.1 mM minimum essential media (MEM), 1% non essential amino acids (NEEA), 1 mM L-glutamine and 2 ng/ml basic fibrobastic growth factor (bFGF). For the purposes of cell culture 6 well 10cm$^2$ tissue culture plates, coated with 0.1% gelatin were used, and all cultures were maintained in a humidified 5% $CO_2$ incubator at 37°C. The protocol for induction of alveolar epithelial differentiation of hESCs was adapted from established methods [2-5].

## All Cells May Not Respond to Tissue Engineering?

A novel strategy was used with spectacular success for differentiating hES cells into endodermal lung lineage-specific cells using human ESCH7 from WiCells, WI, USA[6]. Alteration of the differentiation medium strikingly modified the pathway of differentiation of EBs into different cell types as we found using the H7 ESC. Culture of EBs in a commercially available medium used for maintaining primary culture of mature pulmonary alveolar cells SAGM (excluding tri-iodothyronin and retinoic acid) promoted a predominantly AEII cell phenotype there. In contrast, culture in a commercially available BEGM (with tri-iodothyronin and retinoic acid but without BSA) promoted differentiation to predominantly bronchiolar alveolar cell (i.e., Clara cell) phenotype. Lung lineage-specific cell differentiation was achieved in a relatively shorter span of time (22 days) in contrast to other reported lung lineage culture conditions[8-10]. These culture media, normally used to maintain and grow mature cells, could successfully induce differentiation of pluripotent embryonic stem cells into three types of mature lung lineage-specific non-ciliated cells. Whereas AEI cells and AEII cells are found in the alveolar areas, Clara cells are found in terminal bronchioles. This study demonstrates that from the same clonal population of undifferentiated hES cells, tissue engineering can be used to skew differentiation into one or another type of functionally competent mature cells.

Going on the same hypothesis, we embarked upon inducing directed guided differentiation of two cell lines BJNhem 19 and 20 generated in a JNCSAR lab[11-15]. We used similar coaxing of the ESC as in our work with H7. An important reason for our working with these cells was the originator's claim that the cells could grow in feeder-free conditions. So we tried to condition the media following their published protocol and culture the same. This showed very poor yield which prompted us to shift to using feeders (MEF) for culturing the cells in an undifferentiated state for propagation and use in the subsequent differentiation protocols as was our primary goal.

Despite various tested efforts and additional protocol to induce differentiation through tissue engineering

techniques, lung lineage specific cellular differentiation in both human ESC lines BJNhem 19 and 20 were found to be unsatisfactory. Repeated passages, fresh sub-cultures, feeder-conditioned cultures and on-feeder-cultures, with and without embryoid body formation failed to induce differentiation of these hES in culture into non-ciliated lung lineage-specific cells with intracellular and surface protein markers and morphology characteristic of AEI cells, AEII cells, and Clara cells[11, 16-18].

According to the originator lab's published papers, BJNhem20 cells were seen to divide spontaneously into cardiomyocytes in later passages and their differentiation efficiency was increased with the induction of DMSO[7]. Venu and team observed that only about 5% of early passage EBs showed spontaneous appearance of beating cardiomyocytes but in subsequent later passages this percentage increased to 45.5% ($_p$101), 58.3% ($_p$115), and 62.5% ($_p$135) of beating cardiomyocytes. In order to validate these beating cardiomyocytes these cells were checked for cardiac progenitor marker *Tbx*5 and cardiomyocytes marker α– actinin. Although, only a subset of cultured cells expressed *Tbx*5, all cells analyzed showed α – actinin. These results as per the authors confirm that the BJNhem20 cell line is capable of differentiating into cardiomyocytes. However, it might be noted that the BJNhem19 cell line did not show any spontaneous differentiation in to cardiomyocytes.

Combinations of extra cellular matrix plus defined medium, such as Matrigel and mTeSR1 were also used (data not presented). Similar results were obtained there also showing a fundamental issue with the cells in growing in an undifferentiated state. Thus one is compelled to conclude that the cells cannot be grown feeder free. Secondly, our attempt to induce respiratory differentiation by culturing cells in serum containing medium followed by SAGM or BEGM was also thwarted. There was no attempt to differentiate the cells via a mesendodermal precursor, then to foregut endoderm and finally to lung, which would be the accepted stepwise protocol in current use. Now, what other protocols would have or not done is a subject of debate.

Given our experience with H7 and BJNhem 19, 20 (Fig 7) what becomes immediately apparent is the suitability of cells / cell lines or responsiveness to induction by tissue engineering

## Various Tissue Engineering Techniques to Induce Differentiation of Pluripotent Cells (Summary Overview):

Controlling differentiation of human induced pluripotent stem cells (hiPSCs) into targeted cell types remain a challenge. Studies show that stem cells respond to microenvironments, made of soluble and matrix-based cues, to regulate their fate and commitment[12].

Biomaterials containing calcium phosphate minerals have been shown to promote osteogenic differentiation of SCs. These materials have been shown to support in vivo bone tissue formation. It has been seen that employing biomineralised poly-(ethylene glycol)-diacrylate-co-acrylyl-6-aminocaproic acid (PEGDA-co-A6ACA) matrices can direct osteogenic differentiation of human bone marrow-derived mesenchymal SCs (hMSC) and human embryonic SCs (hESC). Mineralized matrices containing gelatin methacrylate (GelMA) have been developed to induce differentiation. SCs have been seen to differentiate on these matrices, as well as in 3D macroporous hydrogels, in growth media lacking osteoinductive soluble factors. However, cells on mineralized matrices show spread morphology, while those on non-mineralized matrices are aggregated. This suggests better cell-matrix interactions in mineralized matrices[12].

Pluripotent cells are a potential source of autologous cells for cell and tissue regenerative therapies. A drawback of iPSC application in cell-based therapy is the associated tumourigenesis, especially teratoma formation in the implanted region. Supportive tissue engineering environment and in vivo vascularized chambers have been developed for implantation of human adult SCs and their subsequent differentiation, but without teratoma formation[13].

Natural porcine nucleus pulposus (NP) tissues contain large populations of notochordal cells (NCs). The porcine NP serves as a matrix for differentiation of hiPSCs, which acquired notochordal phenotype. Direct contact between the porcine NP tissue and the hiPSCs is not compulsory for notochordal differentiation, but may produce higher yield. 3D gelatin microspheres (GMs) have been found to be viable matrices for tissue engineering with human adipose-derived SCs (ADSCs). ADSCs and GMs have been assembled into cell-microsphere constructs. ADSCs cultured in monolayer or on GMs can differentiate into adipogenic, osteogenic and hepatic lineages, when maintained in suitable differentiation media.

## Conclusions:

Since their discovery four years ago, induced pluripotent stem cells have captured the imagination of researchers and clinicians seeking to develop patient-specific therapies. Reprogramming adult tissues to embryonic-like states has countless prospective applications to regenerative medicine, drug development, and basic research on stem cells and developmental processes. However, many technical and basic science issues remain before the promise offered by iPSC technology can be realized fully. The ease with which iPSCs can be generated with improved methodology has facilitatedthe development of chemical and siRNA screens as wellas biochemical studies that should further unravel themechanisms of this process. The isolation of iPSCs has also sparked new interest in interconverting mature cell types directly into each other, which has already led to a number of remarkable examples for pancreatic, muscle, and neural cell types. It is likely that many other direct cell switches will be achieved in the near future. It remains to be tested, however, whether transdifferentiation works in the human system as well, and whether lineage-converted cells are functionally equivalent to their in vivo counterparts.

The key steps involved in this process consist of the choice of factors and molecules used, their delivery method, and the choice of target cell type, as well as the parameters of factor expression, culture conditions, methods to identify cells, and the assays used to verify pluripotency. To fully exploit the abundance of new information requires a standardization of certain parameters of the reprogramming process, such as the calculation of reprogramming efficiency and qualification of the pluripotent state.

Finally, the Drosophila IPSCs represents a new model for understanding the fundamental biological mechanisms that control stem cell behaviour. In all of the well-characterized niches, stem cells interact tightly with a non-dividing partner cell that fixes theiranatomical location and has an important role in niche function.

As such, this review has attempted to present a comprehensive comparison of the currently available technologies for iPSC derivation and put forth standards to minimize variability between independent experiments, thus providing a framework to aid in the designing and conducting of future experiments, as well in the evaluation of existing iPSC literature.

## References:


1. Ali NN, Edgar AJ, Samadikuchaksaraei A, Timson CM, Romanska HM, Polak JM, *et al*. Derivation of type II alveolar epithelial cells from murine embryonic stem cells. *Tissue Eng* 2002;**8**:541-50.
2. Banerjee ER, Henderson WR Jr. Defining the molecular role of gp91phox in the immune manifestation of acute allergic asthma using a preclinical murine model. *Clin Mol Allergy* 2012; 10.2. doi: 10.1186/1476-7961-10-2.
3. Banerjee ER, Henderson WR Jr. Characterization of lung stem cell niches in a mouse model of bleomycin-induced fibrosis. 2012;**3**:21. doi: 10.1186/scrt112.
4. Banerjee ER, Henderson WR Jr. Role of T cells in a gp91 phox knockout murine model of acute allergic asthma. *Allergy Asthma Clin Immunol* 2013;**9**:6.doi: 10.1186/1710-1492-9-6.
5. Banerjee ER, Laflamme MA, Papayannopoulou T, Kahn M, Murry CE, Henderson WR Jr. Human embryonic stem cells differentiated to lung lineage-specific cells ameliorate pulmonary fibrosis in a xenograft transplant mouse model. *PLoS One* 2012;**7**:e33165. doi: 10.1371/

journal.pone.0033165. Epub 2012 Mar 28.
6. Ray Banerjee E. Looking for the elusive lung stem cell niche – a perspective. *Translational Respir Med* 2014;**2**:7-31.
7. Inamdar MS, Venu P, Srinivas MS, Rao K, VijayRaghavan K. Derivation and characterization of two sibling human embryonic stem cell lines from discarded grade III embryos. *Stem Cells Dev* 2009;**18**:423-33.
8. Rippon HJ, Ali NN, Polak JM, Bishop AE. Initial observations on the effect of medium composition on the differentiation of murine embryonic stem cells to alveolar type II cells. *Cloning Stem Cells* 2004;**6**:49-56.
9. Samadikuchaksaraei A, Cohen S, Isaac K, Rippon HJ, Polak JM, *et al*. Derivation of distal airway epithelium from human embryonic stem cells. *Tissue Eng* 2006;**12**:867-75.
10. Wang D, Haviland D, Burns A, Zsigmond E, Wetsel R. A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 2007;**104**:4449-54.
11. Kang H1, Shih YR2, Hwang Y2, Wen C3, Rao V2, Seo T4, *et al.* Mineralized gelatin methacrylate-based atrices induce osteogenic differentiation of human induced pluripotentstem cells. *Acta Biomater* 2014; Aug 18.
12. Lim SY, *et al*. In vivo tissue engineering chamber supports human induced pluripotent stem cell survival and rapid differentiation. *Biochem Biophys Res Commun* 2012;**422**:75-9.
13. Liu YI, Rahama MN, Bal BS. Modulating notochordal differentiation of human induced pluripotent stem cells using natural nucleus pulposus tissue matrix; *PLoS One* 2014.
14. Liu YI, Fu S, Rahaman MN, Mao JJ, Bal BS. Native nucleus pulposus tissue matrix promotes notochordal differentiation of human induced pluripotent stem cells with potential for treating intervertebral disc degeneration. *J Biomed Mater Res A* 2014; Jun 2.
15. Kodali AI, Lim TC, Leong DT, Tong YW. Cell-microsphere constructs formed with human adipose-derived stem cells and gelatin microspheres promotes stemness, differentiation, and controlled pro-angiogenic potential. *Macromol Biosci* 2014; Jul 1.
16. Kar S, *et al*. Embryonic stem cells need specific signals to differentiate into tissue specific lineages [Revised manuscript under process in Tissue Science & Engineering, 2014]
17. Hore G, *et al.* Heterogeniety in human ESC may prevent endodermal guided differentiation. [Revised manuscript under process in Stem Cell Research & Therapy, 2014].
18. Sanyal N, *et al*. Human embryonic stem cell lines BJNhem 19 and 20 fail to differentiate into lung lineage specific cells despite induction through guided endodermal differentiation [Manuscript submitted to *Sci Technol*, 2014].