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Heterogeneity in Human Embryonic Stem Cells May Prevent Endodermal Guided Differentiation

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Abstract

This study aims at exploring the use and peculiarities of stem cell therapy for pulmonary injury and degeneration. The success of stem cell therapy for clinical use needs the validation of several issues. Regenerative Therapy's first and most critical requisite is the *ex vivo* synthesis of desirable cells to replenish those lost by the body in a degenerative disease. The etiology of degeneration differs from disease to disease, likewise, strategies to regenerate lost tissue in its heterogeneousness, should also cater to the regeneration process and to customize the same, the researcher ought to devise strategies to repair or replace or regenerate in the appropriate spatio-temporal format. For example, in the inflammation-degeneration-induced pathophysiological situations of the respiratory epithelium, human embryonic stem cells have been used in a tissue engineering format to induce differentiation and amplification into the desired type of cell, in this case, the non-ciliated squamous epithelial cells. To this end, we were given two human embryonic stem cell (hESC) lines and the main objective of our research work was to get BJNhem19 and BJNhem20 human embryonic stem cell (hESC) lines to differentiate into lung epithelial lineage–specific cells (i.e. alveolar epithelial type I and type II cells and clara cells) which are the key cells to degenerate in most degenerative lung ailments. This in order to generate a potentially unlimited supply of cells of the desired phenotype for use in a novel cell based therapy to repair lung injury. The strategy was to use guided endodermal differentiation by direct administration of one or more growth factors known to be involved in lung development in 2D cell cultures and characterize the cells for the desired markers. According to a tried strategy, the undifferentiated hESC were taken through embryoid body formation and then subjected to induction by defined growth factors in small airways growth medium and bronchiolar endothelial growth medium. Results were not satisfactory and the cells showed poor growth and practically none differentiated appreciably let alone into the desired phenotype. This non-response to induced differentiation is important information as numerous research labs screen various cell lines of embryonic origin with the specific aim to induce differentiation into one or more desired phenotype for functional translation into regenerative therapy. This study therefore fills an existing lacuna in available information regarding behavior of these two hESC available for work to the scientific world in several stem cell banks and shall prevent unnecessary and redundant further screening and save valuable resources.

Keywords: Human embryonic stem cells; Alveolar epithelial cells; Endodermal differentaion; Induction medium

Abbreviations: hESC: Human Embryonic Stem Cells; AEI: Alveolar Epithelial Type I; AEII: Alveolar Epithelial Type II; EB: Embryoid Body; LIF: Leukemia Inhibitory Factor; SCF: Stem Cell Factor; CFU: Colony Forming Assay; CC: Clara Cells; SP: Surfactant Protein; AQP: Aquaporin; ECM: Extracellular Matrix

Introduction

Lung constitutes several types of epithelial and secretory cells residing in distinct anatomical locations. Of these, the alveolar epithelial gas exchange surface consists of two cell types, the type I and type II pneumocytes, also known as alveolar epithelial type I and type II (AEI and AEII) cells, that comprise 95% and 5% respectively of the alveolar lining area [1]. The alveolar fluid balance is regulated by AEI cells [2], structurally these are branched cells with extremely attenuated cytoplasm for gaseous exchange [3]. On the other hand AEII are cuboidal cells, and are located between AEI cells. These cells depict characteristics lamellar bodies and apical microvilli [3]. The main function of AEII cells include the creation and reuptake of pulmonary surfactant [4], regulation of alveolar fluid and synthesis of immunomodulatory proteins (SP-A, SP-D) important for host defense [5]. The columnar Clara cells [6] constitute the majority of the bronchiolar and terminal bronchiolar epithelia. Clara cells actively divided and differentiated to form ciliated cells, secrete glycosaminoglycans that are the major component of the extracellular matrix (ECM), and metabolized airborne toxins by cytochrome P-450 enzyme present in there smooth endoplasmic reticulum [7].

In many life threatening pulmonary diseases, such as acute lung injury, acute respiratory distress syndrome (ARDS), cystic fibrosis and idiopathic pulmonary fibrosis [8-10] endothelial cells along with AEI cells are the primary and initial sites of damage resulting in interstitial edema, increased ECM deposition such as collagen, laminin and fibronectin in the lungs resulting in pulmonary fibrosis and loss of gaseous exchange surface. For lung injury repair, AEII cells and other lung progenitor cells may replace lost AEI cells to re–establish the thin barrier necessary for efficient gaseous – exchange in the alveolar milieu [11].

hESCs recently have been used as a potential source of cells for cell-based therapies in degenerative diseases when loss of function is apparent. Cell based tissue replacement therapy may have the potential to reinstate normal tissue structure and function [12,13]. For successful lung tissue replacement therapy a large number of lung–lineage specific

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cells need to be engineered *in vitro* for transplantation. Wnt/β-Catenin signaling has been identified as a key regulator of stem cell renewal with effect on cell proliferation and differentiation. In this Wnt signaling pathway β-Catenin increases in the nucleus and forms a complex with t–cell factor (TCF) or lymphoid enhancer factor (LEF-1) transcription factor which are differentially modulated by Creb–Binding protein (CBP) and P^{300} co–activators. An increase in β–Catenin or CBP mediated transcription by selectively inhibiting β–Catenin or P^{300} mediated transcription maintains stem cell pluripotency, whereas blockade of β–catenin/CBP signaling facilitates β-Catenin/P^{300} mediated transcription and cellular differentiation [14-16].

The main aim of our experiment was to differentiate hESCs into lung epithelial lineage–specific cells (AEI, AEII and Clara cells) and develop a cell based strategy to repair lung injury in a mouse model of IPF. Mouse ES and hES H1 cell lines have been previously demonstrated to have differentiated into AEII cells [17-20]. Bleomycin an anti–neoplastic drug has been implicated to cause lung fibrosis as a side effect in patients, and has been used in mouse models to induce fibrosis. Bleomycin acts by damaging DNA and inducing apoptosis of epithelial cells with loss of AEI cells in the alveolus accompanied by EMC protein deposition. It is believed that AEII cells contribute to the repair of injured lungs by an initial limited proliferation followed by differentiation to AEI cells and Clara cells [21].

In order to differentiate hES cells into lung epithelial lineage-specific cells [i.e., alveolar epithelial cells type I (AEI) and II (AEII) cells and Clara cells] and develop a cell-based strategy in order to repair lung injury in a mouse model of IPF. Previous work [22-30] has demonstrated differentiation steps to AEII cells from murine ES cells and the hES H7 cell line.

In this study we tried to differentiate hES BJNhem 19 and BJNhem 20 cells in culture into non–ciliated lung lineage specific cells and characterize them with intra–cellular and surface protein markers and subsequently characterize the cells morphologically [22]. The main idea was to check for down–regulation of pluripotent markers Nanog, SSEA–3 and SSEA–4 and subsequently check for up–regulation of lung lineage specific markers AQP–5 and SP–C. In addition, we were also interested to find out if we could grow EBs from feeder free hESCs. Immunophenotyping was done using FACS and progenitor assessment was assessed by cellular colony formation (CFU).

Materials and Methods

Ethics statement

All animal and animal related works was conducted according to the guidelines prescribed by Faculty Councils for Post Graduate Studies in Science, Technology & Engineering and Agriculture and Veterinary Science (Ref. No. BEHR/1029/2304)

Expansion of hES Cells (H7)

In our published work [22] NIH approved (NIH code WA07) undifferentiated hES cell line 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 irradiated 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 2 bmercaptoethanol (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&DSystems, Minneapolis, MN)]. For cell culture, 6-well 10 cm^2 tissue culture plates, coated with 0.1% gelatin were used, and all cultures were done in a humidified 5% CO_2 incubator at 37°C. The protocol for induction of alveolar epithelial differentiation of hES cells was adapted from established methods. This was our positive control.

Expansion of Human embryonic stem cells (BJNhem19 and BJNhem20)

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 [28-35].

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 2 mM – L Glutamine [36]. 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 10 cm^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 [16-20].

Feeder Free culture of hESCs (BJNhem 19 and BJNhem20)

hESCs were initially cultured in hESC media as aforementioned. Once the cells became confluent these cells were passaged (about once a week) and subsequently cultured in high binding tissue culture plates in conditioned media prepared from MEF [incubation for 24 hours with mitotically inactivated MEFs] [31-35].

MEF were first isolated and cultured in MEF medium. The harvested cells were then treated with Mytomycin-C and ~ 50,000 cells were placed in MEF medium. The MEF media was exchanged with ES medium after 4-6 hours. This ES medium was collected on daily basis (24 hrs as mentioned earlier) and supplemented with 4 ng/ml bFGF before feeding hESCs [37]. LIF (20 ng/ml) as prescribed was not used as it is not the norm and as per WiCell protocol it is unadvised.

Embryoid body formation for H7

On the day of passage, hES cell colonies were inspected, and only

hES cell cultures containing colonies with well-defined boundaries and minimum differentiation were used. Undifferentiated hES cells were treated with 1.2 U/ml dispase (Invitrogen) dissolved in Ca^{2+} and Mg^{2+} -free phosphate-buffered saline (PBS; Mediatech) supplemented with 10% ES cell-qualified fetal bovine serum (FBS; Invitrogen) at 37 uC until the hES cell colonies nearly detached from the plates. Colonies were then washed off the plates, washed twice in ES cell medium without bFGF, and resuspended in EB medium [i.e., KO DMEM, 20% KOSR, 20% non-heat-inactivated fetal calf serum, 1% NEAA, 1 mML glutamine, and 0.1 mM 2b-ME]. Cells were transferred to Corning 6-well ultra-low attachment plates (Corning Inc. Life compared sciences, Lowell, MA) and grown for 4 days in suspension culture in ultra-low attachment plates.

Embryoid Body (EB) Formation (BJNhem19 and BJNhem20)

hESCs were inspected on day 1 of passage and hESC culture colonies with well-defined boundaries and minimum differentiation were utilized. Dispase 1.2 U/ml dissolved in PBS (without magnesium and calcium) supplemented with 10% ES qualified FBS was used for detachment of undifferentiated hESCs at 37°C. This treatment was carried out until all the hESC colonies were detached from the plates. The colonies were then washed off the plates using ES media without bFGF (washing done twice), and the cells were then resuspended in EB Medium (K.O. DMEM, 20% KOSR, 20% non–heat inactivated Fetal Calf serum, 1% NEAA, 1 mM L–Glutamine and 1 mM 2β-ME). Cells were subsequently transferred to ultra-low attachment 6 well plates and were grown for 4 days in suspension culture.

Generation of Non-ciliated Pulmonary Epithelial Cells (H7)

Two different culture media were employed to generate nonciliated pulmonary epithelial cells. EBs was transferred to adherent culture in 0.1% gelatin-coated tissue culture plates by limited dispase digestion. One group of EBs was cultured for 12days in small airways growth medium (SAGM) [i.e., Clonetics small airways basal medium (Cambrex Bioscience, Walkersville, MD), bovine pituitary extract 30 mg/ml, insulin 5 mg/ml, hydrocortisone 0.5 mg/ml, gentamycin sulfate-amphotericin B 0.5 mg/ml, bovine serum albumin 0.5 mg/ml, transferrin 10 mg/ml, epinephrine 0.5 mg/ml, and recombinant human epidermal growth factor (rh EGF) 0.5 ng/ml] refreshing media every other day. Retinoic acid 0.1 ng/ml and triiodothyronine (6.5 ng/ml) were excluded from SABM following Ali et al. [17]. From the day12 culture in SAGM, alveolar epithelial cells were flow sorted based on surface expression of SP-C and AQP-5. The .90% SPC+ and AQP-5+ flow-sorted cells were grown in the SAGM medium for 4 more days. A second group consisted of EBs cultured in bronchiolar epithelial growth medium (BEGM) [i.e., Clonetics bronchiolar epithelial basal medium (Cambrex Bioscience), bovine pituitary extract 30 mg/ml, insulin 5 mg/ml, hydrocortisone 0.5 mg/ml, gentamycin sulfate-amphotericin B 0.5 mg/ml, retinoic acid 0.1 ng/ml, transferrin 10 mg/ml, triiodothyronine 6.5 ng/ml, epinephrine 0.5 mg/ml, and rhEGF 0.5 ng and fed similarly every other day with fresh medium.

Expected differentiation/generation of non–ciliated pulmonary epithelial cells (BJNhem 19 and BJNhem20)

In order to generate non–ciliated pulmonary epithelial cells two different culture media were used. EBs after limited Dispase digestion was transferred to adherent culture in 0.1% gelatin coated tissue culture plates. One group of EBs were cultured in small airways growth medium (SAGM) for 12 days. [Small airways basal medium was procured from Promocell, bovine pituitary extract 30 µg/ml, insulin 5 µg/ml, hydrocortisone 0.5 µg/ml, Gentamycin sulfate–Amphotericin B 0.5 µg/ml, bovine serum albumin 0.5 mg/ml, transferrin 10 µg/ml, epinephrine 0.5 µg/ml and recombinant human epidermal growth factor (rhEGF) 0.5 ng/ml] refreshing media every 2 days. Retinoic acid 0.1 ng/ml and triiodothyronine (6.5 ng/ml) were excluded from SABM following Ali et al. Cells were collected from 12 day culture in SAGM, cells were marked with surface expression markers SP-C^+ and AQP-5 for FACS immunophenotyping.

The second group of EBs was cultured in bronchiolar epithelial growth media (BEGM) [Procured from Promocell], bovine pituitary extract 30 µg/ml, insulin 5 µg/ml, hydrocortisone 0.5 µg/ml, Gentamycin sulfate–Amphotericin B 0.5 µg/ml, retinoic acid 0.1 ng/ml, transferring 10 µg/ml, triiodothyronine 6.5ng/ml, epinephrine 0.5 µg/ml, rgEGF 0.5ng/ml. Media was refreshed every 2 days.

Phenotypic Analysis of Cells (BJNhem 19 and BJNhem20)

Immunostaining was performed using specific antibodies. These specific antibodies were either conjugated with secondary antibodies or a secondary antibody had to be added to the primary antibody for staining. Phycoerythrin (PE) labeled Anti – human Nanog (BD Pharmigen, 560873), SSEA–3 (Santa Cruz, 631: SC-21703) and SSEA–4 (Santa Cruz, 813-70: SC – 21704) were used to determine if the hESCs cultured in SAGM and BEGM were still pluripotent or had differentiated into alveolar epithelial (AE) type I or Type II cells. In order to determine if the cells had differentiated in AE type I or II, we checked the cells for the following surface markers namely, Aquaporin 5 (AQP – 5, Santa Cruz Biotechnology, INC., G19: SC–9890) and surfactant associated protein – C (SP-C, Santa Cruz Biotechnology, INC., C – 19: SC-7705). Additionally PerCp-Cy 5.5 Mouse anti – Human CD105 (105) clone: 266, and PE Mouse Anti–Human CD73.clone AD2 (BD Pharmigen).

Based on the manufacturer's protocol, 10^6 cells were taken per sample in 50µl cell suspension in ice cold PBS (1x), 10^5 events were recorded per sort for FACS analysis. For the FACS analysis cells were first stained with conjugated or non-conjugated antibodies and subsequently stained with a secondary antibody when needed. For staining purposes 5-10 µl antibody was added to 10^6 cells in suspension culture and placed on ice for 30 mins. After thorough washing, cells were fixed in 4% formaldehyde in PBS by vortexing and incubated at room temperature for 20 mins followed by permeabilization in either 0.1% Tween–20 or 0.25% Triton X [25].

Cell suspension of 10^6 cells per microfuge tube was prepared per sample and staining was carried out in a single step with a master mix of fluorochrome–conjugated monoclonal antibodies or in some cases where the primary antibody was not available in directly fluorochrome–conjugated form, in two steps of primary unlabelled antibody followed by cross reactive fluorochrome–conjugated specific secondary antibody at 4°C for 30 mins followed by rigorous wash (twice) in ice cold PBS. The antibody stained cell suspension was now analyzed using a BD FACSVerse™ machine and data analyzed using software BD FACSuite version 1.0 or later [25].

Cell Viability (BJNhem 19 and BJNhem20)

Viable cells were measured by trypan blue dye exclusion by light microscopy.

CFU–C assay to determine progenitor cells (BJNhem 19 and BJNhem20)

To check for progenitor cells, CFU–C assay was performed using methylcellulose (Himedia), supplemented with 50 ng of Stem cell factor

(SCF) per ml in IMEM medium. 50,000 cells were plated in 35 mm plates and placed in incubator at 37°C and 5% CO_2 for 10 days. At the end of 10 days colony count was taken under microscope [data not shown] [25].

Results

BJNhem 19 and BJNhem20 hESC fail of to grow on feeder-free cultures

Cells from the hES cell line H7 [22] were differentiated *in vitro* into three lung lineage-specific epithelial cells: AEI cells, AEII cells, and Clara cells as described below. These cells expressed, both intracellularly and on their surface, characteristic marker proteins, detected byfluorescence-activated cell sorting (FACS) and immunofluorescence (IF) microscopy, the mRNA for which were also concomitantly over-expressed as detected by quantitative real-time PCR (qPCR). The protocol for differentiation of pluripotent undifferentiated colonies of BJNhem 19 and 20 hES cells into the lung epithelial cell-specific lineages is shown in Figure 1.

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 [22]. Development of MEF for conditioning of feeder free media in which huESC are to be cultured. MEF from CF-1 (Charles river 12.5 days of gestation from female pregnant mouse was expanded and sub-cultures, less than passage 10 were inactivated by mitomycin-c (10 µg/ml). Inactivated monolayer MEF was conditioned for 24 hours and medium collected and frozen at -20^{0}C for future use (Figure 2A).

The medium contained DMEM, 10% heat-inactivated HI-FBS, and 2mM L-glutamine. Undifferentiated hES cells on mitomycin-c inactivated MEF feeder-conditioned media for 4-6 days followed by

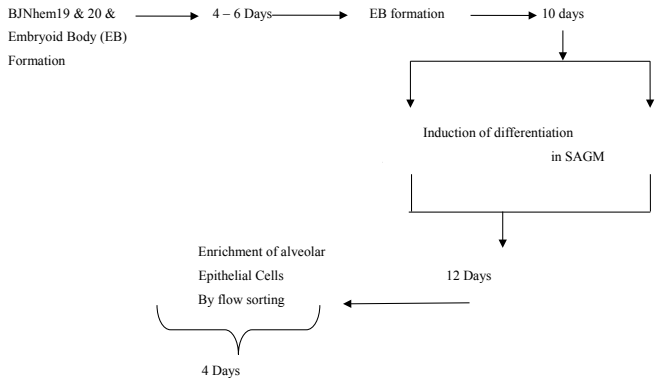

Figure 1: Outline of formation of EBs from Bjnhem20 cells and differentiation to alveolar epithelial cells in SAGM. Undifferentiated.

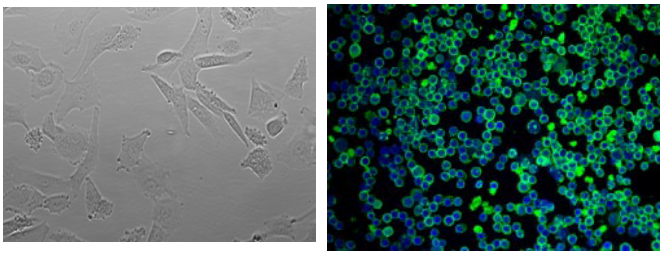

Figure 2A: MEF (sca-1-FITC+).

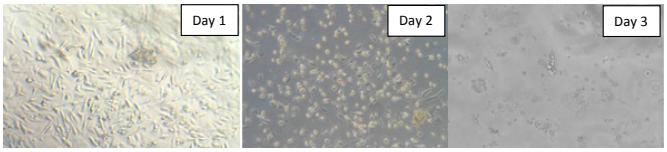

Figure 2B: Feeder-free culture of undifferentiated huESC (BJNhem 19 & 20) in conditioned medium.

formation of EBs. Details of microscopy are as follows: Photographs were taken at 40X magnification using a Leica DMIL microscope (Leica Microsystems GmbH, Germany) and a Zeiss ApoTome (Carl Zeiss Microimaging GmbH, Germany). Photographs were taken with a Zeiss Axiovert 200M microscope and Axiocam MRm and merged using Axiovision 4.6 software (Figure 2B).

Spontaneous down regulation of pluripotent markers in culture with hubaFGF but no LIF

SSEA-3, 4, Oct ¾, nanog spontaneously downregulate in the cells after 72 hours of culture. Retention of pluripotent makers was found till 72 hours of culture following which despite replenishing fresh medium with defined prescribed factors. Transferring cells to mTeSR with matrigel did not improve conditions and it was found that only a fresh culture could revive the cells from mother culture. Sub-cultures were not efficient for propagation (Figure 3).

The percentage of positive cells is shown as mean ± SEM (n=3 independent experiments with flow data collected in triplicate). BD Accuri C6 FACS machine was used to acquire data which was analyzed with Accuri C6 software. Trendline shows a progressive down modulation of pluripotent markers which nevertheless seem to settle to more than 40% at around 5-6 dyas of cultureexcept for Oct ¾.

EB formation

The EBs failed to differentiate into AE type I, type II and Clara cells in SAGM medium and BEGM medium respectively. FACs analysis yielded negative for the aforementioned desired cell lines. In addition, both BJNhem19 and BJNhem20 failed to sustain themselves in feeder free cultures.

EBs was formed in suspension culture overnight after aggregation. Day 4 EBs were cultured in ultra-low attachment plates for 10 days and then transferred to gelatin-coated plates and cultured with either SAGM or BEGM. EBs was formed in suspension culture overnight after aggregation. Day 4 EBs were cultured in ultra-low attachment plates for 10 days and then transferred to gelatin-coated plates and cultured with either SAGM or BEGM. Photographs were taken at 40X magnification using a Leica DMIL microscope (Leica Microsystems GmbH, Germany) and a Zeiss ApoTome (Carl Zeiss Microimaging GmbH, Germany). Pfotographs were taken with a Zeiss Axiovert 200M microscope and Axiocam MRm and merged using Axiovision 4.6 software (Figure 4).

Differentiation in induction medium BJNhem 19

Fibroblast like phenotype after 10 days of culture in SAGM (BJNhem20) following the formation of EB-like 3D structures, they were transferred to pro-attachement tissue culture treated plates and subjected to various differentiation induction media. SAGM-induced differentiation yielded a fibroblast-like phenotype where cell growth was rapid. Picture taken by Plympus BX41 (Japan), photo taken by Olympus cool camera (model no. DP20) and image processed by MIcroPublisher 5.0 RTV 5.0 x 10^6 pixels in frame rate 25 fps (Figure 5).

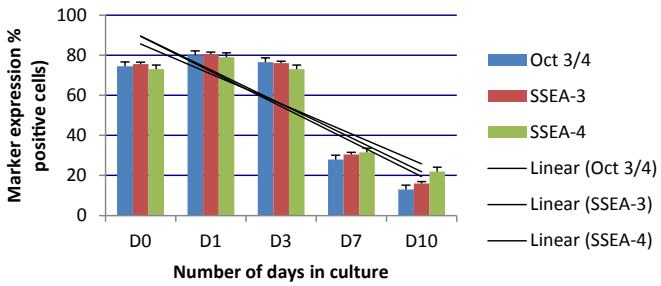

Figure 3: Oct3/4, SSEA-3, SSEA-4 for identification of differentiation stage of the cells in culture.

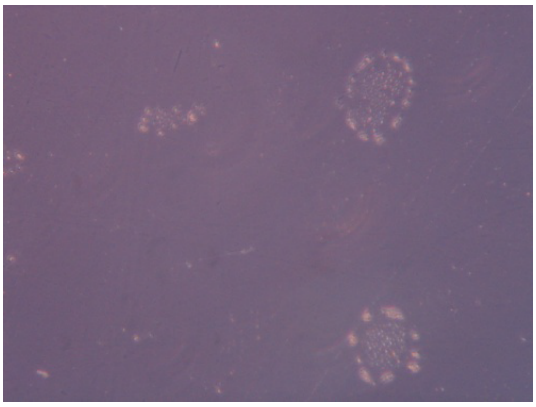

Figure 4: EB formation in ultra-low attachment plates.

Differentiation in induction medium BJNhem 20

Following the formation of EB-like 3D structures, they were transferred to pro- attachement tissue culture treated plates and subjected to various differentiation induction media. SAGM-induced differentiation yielded a fibroblast-like phenotype where cell growth was rapid. Picture taken by Plympus BX41 (Japan), photo taken by Olympus cool camera (model no. DP20) and image processed by MIcroPublisher 5.0 RTV 5.0 × 10^6 pixels in frame rate 25 fps. Fibroblast like phenotype after 10 days of culture in SAGM (BJNhem20). Cuboidal phenotype after 10 days of culture in BEGM (BJNhem20) (Figure 6).

EB were transferred to tissue culture treated plates and grown in induction medium composed of SABM; bovine pituitary extract (BPE) 30 µg/ml, insulin 5 µg/ml, hydrocortisone (HC) 0.5 µg/ml, gentamycin sulfate-amphotericin B 0.5 µg/ml, bovine serum albumin (BSA) 0.5 mg/ml, transferrin 10 µg/ml, epinephrine 0.5 µg/ml, and recombinant human epidermal growth factor (rhEGF) 0.5 ng/ml. Cell count taken using Tali™ Image based cytometer manufactured by Invitrogen, Life Technologies, USA. Photo taken by camera 1.3 megapixels, 4X objective, 4X or 16X digital zoom. SAGM: SABM; bovine pituitary extract (BPE) 30 µg/ml, insulin 5 µg/ml, hydrocortisone (HC) 0.5 µg/ml, gentamycin sulfate-amphotericin B 0.5 µg/ml, bovine serum albumin (BSA) 0.5 mg/ml, transferrin 10 µg/ml, epinephrine 0.5 µg/ml, and recombinant human epidermal growth factor (rhEGF) 0.5 ng/ml (Figure 7).

Additional stimuli for induction

Transient heat shock- exposure of the cells to 67°C for 30 min followed by transfer to 22°C in SAGM results in a mixed phenotype of cells (Figure 8).

HuESC were treated to brief exposure to 67°C for 10 minutes to induce heat shock. The cellular response to heat shock includes the transcriptional up-regulation of genes encoding heat shock proteins (HSPs) as part of the cell's internal repair mechanism [30]. They are also called stress-proteins [31] and respond to heat, cold and oxygen deprivation by activating several cascade pathways [32-35]. Mixed phenotype in SAGM with transient hears shock (BJnhem20).

Characterization of differentiated cell in SAGM

Flow cytometry of surface and intracellular markers of cells differentiated in induction medium (SAGM) for 10 days show very

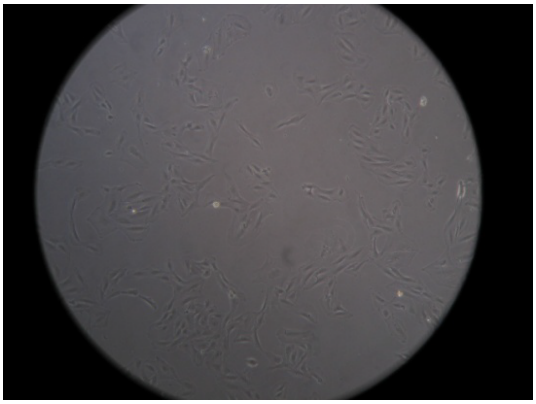

Figure 5: Phenotype of BJNhem 19 after culture in SAGM.

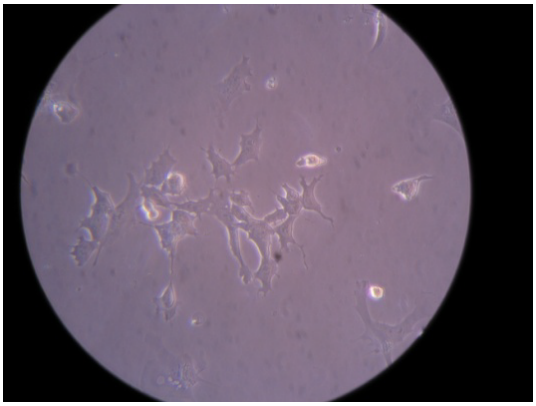

Figure 6: Phenotype of BJNhem 20 after culture in BEGM.

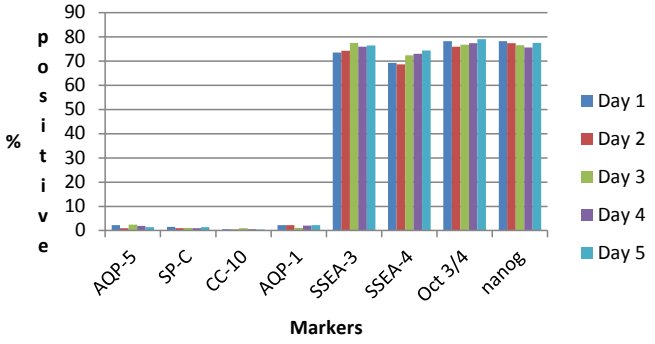

Figure 7: Expression of surface and intra-cellular markers following differentiation in induction medium SAGM in tissue culture treated plates.

little change to the desired phenotype namely lung lineage specific phenotype (there was hardly specific pulmonary ciliated squamous and cuboidal epithelial marker expression). The predominant phenotype obtained was that of fibroblasts, not specific to the lung, and they were some CD45-and some actually CD45+. AEII specific SP-C and AQP-1 and AEI specific AQP-5 expression was <1% and considered negligible.

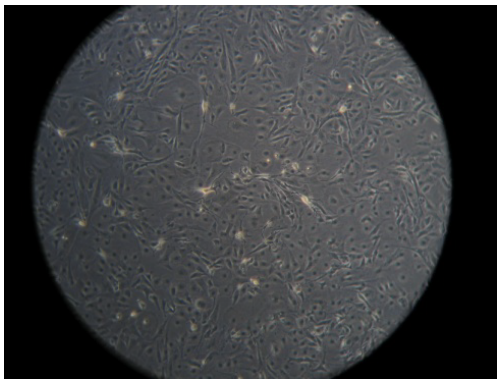

Figure 8: Mixed phenotype in SAGM with transient hear shock (BJnhem20).

Embryoid bodies were transferred into induction medium (SAGM) and cultured in adherent culture for 48 hours and over the next three days (in total 5 days) for optimal growth. Down-modulation of pluripotent specific markers and sequential upregulation of lineage specific (in this case pulomonary lineage) marker expression (intra-cellular protein) was expected. Cells were therefore permeabilized using permeabilization kit (BD biosciences, USA) and stained with specific antibodies against isotype matched control antibodies. With the lineage specific markers irrelevant markes were also used in different cell samples from the same experimental pool. Duplicate samples of cells were stained per experiment and overall experimental output from three independent experiments is presented here. BD Accuri C6 was used to acquire data which was analyzed using BD Accuri C6 software. Cells were stained with specific antibodies (detailed in Materials and Methods section). This figure represents Fibroblast marker expressing cells where the scattergram represents: X-axis CD45-PerCp; Y-axis CD10-FITC (CD45+CD10+ cell population is 31% and is found in the upper right quadrant and CD45-CD10+ cell population is 66% and is found in the lower right LR quadrant) (Figure 9 A-H).

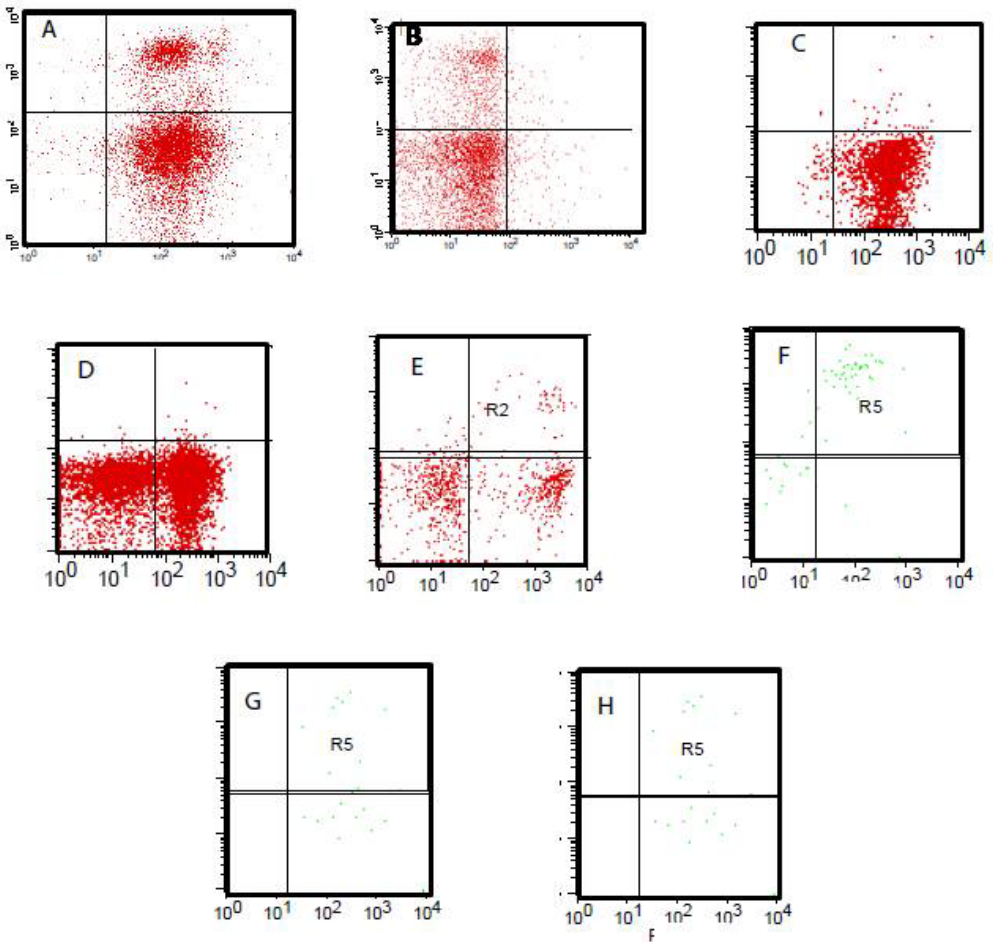

Figure 9: Phenotype assessed by FACS assay following attempts at differentiation in induction medium
A: Fibroblast X-axis CD45-PerCp; Y-axis CD10-FITC (CD45+CD10+31% UR quadrant and CD45-CD10+ 66% LR quadrant) 28% and 51% respectively. B: X-axis SP-C-FITC. Lung lineage specific lineages (SP-C+) <2% (UL quadrant). C: X- axis CD45-PerCp. Hematopoietic lineage (pan-hematopoietic lineage negative). D: CD81+CD47+ (LR 67.5%) Fibroblast specific markers. E: R2 is CD45- gated SP-C+ (UR 1.34%). F: R2 is CD45- gated AQP-1+ (UR 0.67%). G: R5 is CD45- gated AQP-5+ (UR 0.67%). H: R5 is CD45- gated TTF-1+ (UR 0.31%).

Clonogenic potential of hES cells declines with differentiation

CFU-c (colony forming assay counts at day 7 of BJNhem20 in methyl cellulose with SCF-1 in IMDM- undifferentiated ES, EBs and differentiated in SAGM (BJNhem 19). Details of procedure outlined in Materials and Methods section. The presented graphs represent number of colonies formed per). 1 million cells plated in the semi-solid 3D matrix of methyl-cellulose supplemented with IMDM and SCF. CFU-c at day 7 of BJNhem20 in methyl cellulose with SCF-1 in IMDM- undifferentiated ES, EBs and differentiated in SAGM (BJNhem 20). Y-axis represents number of days in culture in varying media and differnetial attachment templates (Figure 10 A-C).

Discussion

This study describes a novel strategy that have been used with spectacular success for differentiating hES cells into endodermal lung lineage-specific cells using human ESCH7 from WiCells, WI, USA [8]. 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 [22-30]. 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.

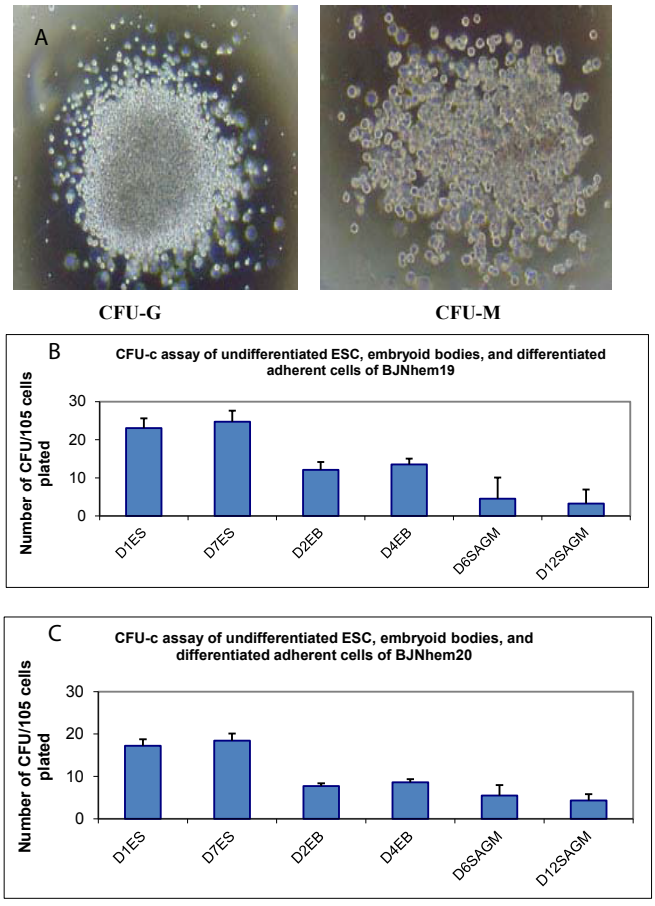

Figure 10: Response to colony formation in presence of SCF-1 (5ng/ml) in methycellulose containing IMDM
A: Photomicrographs of colonies counted at day 7 of culture in defined 3D matrix. BJNhem20 in methyl cellulose with SCF-1 in IMDM- undifferentiated ES, EBs and differentiated in SAGM (BJNhem 19) (Photograph of colonies counted and data presented for Fig.10A, was taken using Bright field phase contrast microscope : model no Olympus BX41 ,camera model no Olympus DP20 at 40X magnification)
B: CFU-c at day 7 of BJNhem19 in methyl cellulose with SCF-1 in IMDM-undifferentiated ES, EBs and differentiated in SAGM (BJNhem 19)
C: CFU-c at day 7 of BJNhem20 in methyl cellulose with SCF-1 in IMDM-undifferentiated ES, EBs and differentiated in SAGM (BJNhem 20)

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 [30-34]. 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.

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 [32]. 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 [31].

In addition to data presented in Figures 1-7, 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. Alternatively, taking the cells by attempting to differentiate the cells via a meso-endodermal precursor, then to foregut endoderm and finally to lung, do not seem a viable way to generate tissues for regenerative therapy as shortening steps and curtailing time is our principal aim.

Our observations show:

1. Neither huESC (BJNhem 19 nor 20) could grow independently of feeder as claimed in the literature from the originator lab.
2. Inactivated mouse embryonic fibroblast feeders can support their growth but after 10 passages, a pronounced downturn as far as pluripotent marker expression in undifferentiated condition is concerned, is found. This may not be considered a good sign for a so-called immortal cell line.
3. Embryoid bodies looked typical following culture in high attachment plates in EB media on day 3 of culture.
4. Spontaneous cardiac differentiation was not tested but upon induction under influence of various differentiation media with defined components, insignificant number of cells showed any endodermal lineage specific differentiation, at all, and a predominantly fibroblast like lineage was detected.
5. These differentiated cells showed pronounced fibrin-like protein synthesis and deposition upon in vitro culture on tissue culture treated high attachment plates.

This brings up the important question of the suitability of certain cells versus others in the work for induced differentiation into a certain cell type of a certain germinal lineage. The cells showed fibroblast like phenotypeupon inuced guided differentiation with defined growth factors. The cells showed a high level of pluripotent markers despite being cultured in a pointedly differentiation inducing medium. The cells showed low CFU-c or clonogenic potential and the cells show resistance to differentiation into the desired cell phenotype although other ESC lines such as H1, H7, KCL002-WT4, Shef-6 and others do [36-55].

To form a large diffusible interface capable of conducting respiratory gases to and from the circulation, the lung must undergo extensive cell proliferation, branching morphogenesis, and alveolar saccule formation, to generate sufficient surface area. In addition, the cells must differentiate into at least 40 distinct lung cell lineages. Specific transcriptional factors, peptide growth factor receptor-mediated signalling pathways, extracellular matrix components, and integrin-signalling pathways interact to direct lung morphogenesis and lung cell lineage differentiation. Some cell lines may be better adapted or in more precise terms, possess a predisposition to responding to becoming some germinal layer-derived cells versus others and therefore while undertaking tissue engineering exercises with follow-up functional assays on the functional attributes of an engineered cell, these factors need be documented. Stem cell banks may participate in the formation and collation of a comprehensive database enumerating these attributes and systems biology analysis of their epigenetic tendencies may reveal patterns and predictive value of such signals that may save valuable resources when one tried to engineer ESC into specific cell types. As literature suggests, human ESC line discrepancy in differentiation to endoderm lineage is not surprising [56]. Lastly heterogeneity in human ESC available to the research community has often been explored extensively making the work of tissue engineers all the more time consuming and sometimes futile. There is an urgent need for detailed information in the form of a database so that while selecting ESC for tissue engineering purposes for studies in regenerative medicine and developmental biology information is available upfront to enable informed choice of a pluripotent cell line being preferred over another for induction into desired functional cells.

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