Stem Cell Reports
Article
ISSCR
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# A Method to Identify and Isolate Pluripotent Human Stem Cells and Mouse Epiblast Stem Cells Using Lipid Body-Associated Retinyl Ester Fluorescence

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http://dx.doi.org/10.1016/j.stemcr.2014.05.004


## SUMMARY

We describe the use of a characteristic blue fluorescence to identify and isolate pluripotent human embryonic stem cells and human-induced pluripotent stem cells. The blue fluorescence emission (450–500 nm) is readily observed by fluorescence microscopy and correlates with the expression of pluripotency markers (OCT4, SOX2, and NANOG). It allows easy identification and isolation of undifferentiated human pluripotent stem cells, high-throughput fluorescence sorting and subsequent propagation. The fluorescence appears early during somatic reprogramming. We show that the blue fluorescence arises from the sequestration of retinyl esters in cytoplasmic lipid bodies. The retinoid-sequestering lipid bodies are specific to human and mouse pluripotent stem cells of the primed or epiblast-like state and absent in naive mouse embryonic stem cells. Retinol, present in widely used stem cell culture media, is sequestered as retinyl ester specifically by primed pluripotent cells and also can induce the formation of these lipid bodies.

## INTRODUCTION

Human pluripotent stem cells (HPSCs) are a valuable resource to model disease and early development. Due to differentiation, it is a challenge to retain pluripotency during their culture and expansion. Methods currently used to isolate HPSCs have inherent experimental variability and efficiency, and are (1) mechanical isolation based on morphology (Maherali et al., 2007; Meng et al., 2011) that requires experience, and is laborious and not efficient; (2) quantification of the endogenous expression of stem cell transcription factors (OCT4, SOX2, etc.) (Gerrard et al., 2005; Wernig et al., 2007; Zhang et al., 2011) in live cells, which requires genome modification; (3) fluorescence-activated cell sorting (FACS)-based analysis using cell surface markers (SSEA-4, TRA-1-60, etc.) (Li et al., 2010; Lowry et al., 2008), which requires use of antibody-based staining that is inherently variable; and (4) more recently, a pluripotent stem cell-specific adhesion signature (Singh et al., 2013), which is dependent on the surface properties of cell clusters and thus interrogates the population and not individual cells.

A large number of endogenous fluorophores are present within cells [e.g., NAD(P)H, FADH, cytochromes, etc.] (Stringari et al., 2012) and some studies have used these fluorophores and their fluorescence lifetimes to establish their differentiation (Stringari et al., 2012) and viability status (Buschke et al., 2011). However, these studies failed to establish an association with any unique fluorophore or isolate individual HPSCs. The studies also did not associate the fluorescence with any specific developmental stage or follow it through the process of reprogramming.

In this report, we demonstrate that pluripotent stem cells of the epiblast-like/primed state exhibit a characteristic blue fluorescence in standard media that arises from the sequestration of retinyl esters in cytoplasmic lipid bodies. The fluorescence is easily detected using wide field epifluorescence microscopy. It allows for efficient single cell separation using FACS and propagation. The fluorescence also serves as an early reprogramming marker for induced human pluripotent stem cells (HiPSCs). Finally, we show that whereas mouse embryonic stem cells (ESCs) do not have fluorescent lipid bodies, they are present in pluripotent mouse epiblast-like cells (mEpiSCs) and in the epiblast region of the mouse embryo.

## RESULTS

### Human Pluripotent Stem Cells Have Characteristic Blue Fluorescent Cytoplasmic Lipid Bodies

HPSC cultures on mouse embryonic fibroblast (MEF) feeders in standard media with serum or serum replacement exhibited a blue fluorescence easily observed by epifluorescence microscopy (excitation 325–375 nm, emission 450–500 nm) and readily captured with a cooled charge-coupled device camera (Figure 1A). The blue fluorescence was associated with most cells within colonies with typical human ESC (HuESC) colony morphology, although individual cells had varied levels of fluorescence (Figure 1A). At high magnification, the blue fluorescence was

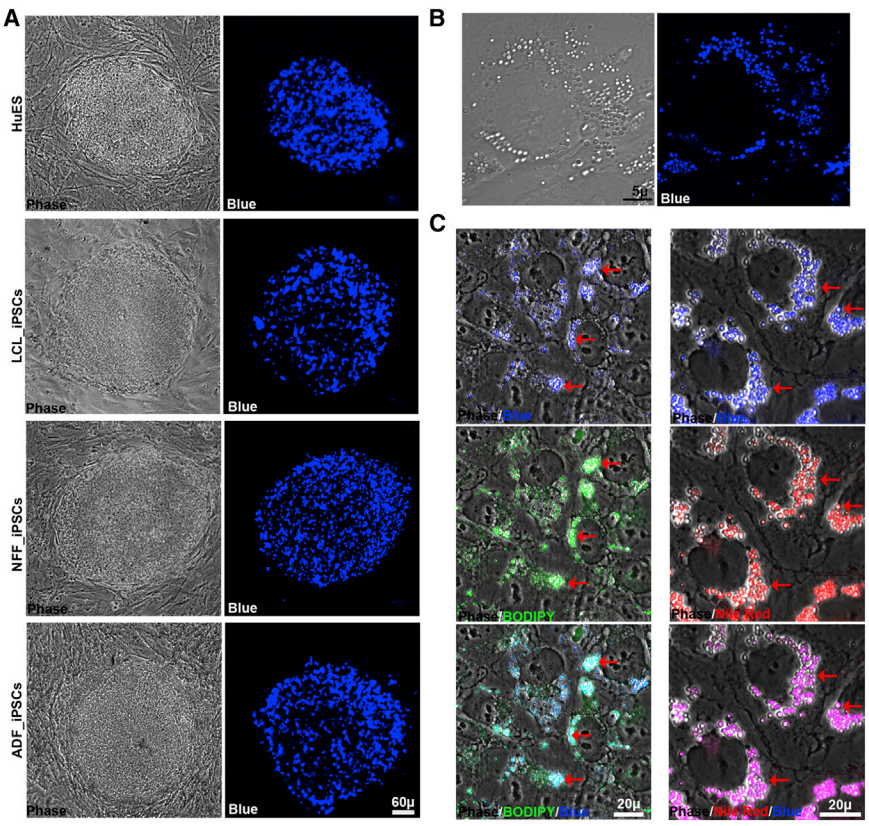



**Figure 1. Human Pluripotent Stem Cells Have Cytoplasmic Lipid Bodies that Exhibit Characteristic Blue Fluorescence**

(A) Blue fluorescence (excitation, 325–375 nm; emission, 460–500 nm) was observed in HPSC (HuESC and HiPS; i.e., NFF_iPS, ADF_iPS, and LCL_iPS) colonies cultured in standard media and culture conditions.

(B) Representative high-magnification confocal image of HuES7 cells showing blue fluorescence confined to spherical bodies.

(C) The fluorescent spherical bodies often show polarized distribution within cells (red arrows, upper row) and stain positive for lipid body-specific markers (BODIPY and Nile red; middle row). The merged images of BODIPY-blue fluorescence and Nile red-blue fluorescence are shown in the lower row.

BF, bright field; NFF, neonatal foreskin fibroblast; ADF, adult dermal fibroblast; LCL, lymphoblastoid cell line. See also Figure S1.

associated with multiple spherical cytoplasmic bodies that were 0.5–1 μm (Figure 1B) and often perinuclear (Figure 1C, red arrows). The fluorescence was retained on fixation with paraformaldehyde and prone to bleaching but recovered in live cells (Figure 1C). The fluorescence is unlikely to be autofluorescence from dying cells because we do not see any autofluorescence at green or red wavelengths (Figure S1C available online). These bodies were stained with lipid body-specific markers BODIPY and Nile red (Figure 1C) and were not associated with other cytoplasmic compartments (Figure S1D). Human neonatal foreskin fibroblasts (NFF), MEFs, mesenchymal stem cells, and HPSC-derived neurons had much lower blue fluorescence (Figures S1A and S1B).

## Blue Fluorescent Lipid Bodies Are Associated with Markers of Human Pluripotent Stem Cells

HPSC colonies often show signs of differentiation in culture apparent as regions of altered morphology. Pluripotency markers such as OCT4, SOX2, and NANOG can be used to determine the differentiation status of HPSCs but these require cells to be fixed and immunostained or engineered to report their expression (Takahashi and Yamanaka, 2006). Cells in HPSC cultures, which stained positive for pluripotency markers, also had highly correlated blue fluorescence (Figure 2A). In colonies, wherever the cells looked differentiated, the fluorescent lipid bodies had either disappeared (Figure 2B) or had decreased (Figure 2C) with a corresponding absence/decrease in the levels of OCT4, SOX2, and NANOG. Mean fluorescence intensity values from cells having both blue fluorescence and pluripotency markers resulted in a tight linear fit: OCT4 ($R^2 = 0.9$), SOX2 ($R^2 = 0.9$), and NANOG ($R^2 = 0.83$; Figure 2A).

## Blue Fluorescence Can Be Used to Sort and Enrich for Human Pluripotent Stem Cells

Because individual HuES7 cells exhibit variable levels of blue fluorescence, we examined its relationship to pluripotency and its utility to isolate undifferentiated cells from differentiating cells by FACS. HuES7 cultures resolved into two distinct populations on sorting using blue fluorescence (DAPI channel). The peak fluorescence intensities of the two populations labeled as high blue and low blue differed approximately 100-fold (Figure 3A). The fluorescence intensity of the high-blue population was approximately equal to the fluorescent intensity of the cells stained with 50 ng/ml of Hoechst stain (Figure S2A). A proportion of cells within the high-blue peak was strongly associated with the pluripotent state of the cultures. Largely undifferentiated cultures were characterized by lower peak heights

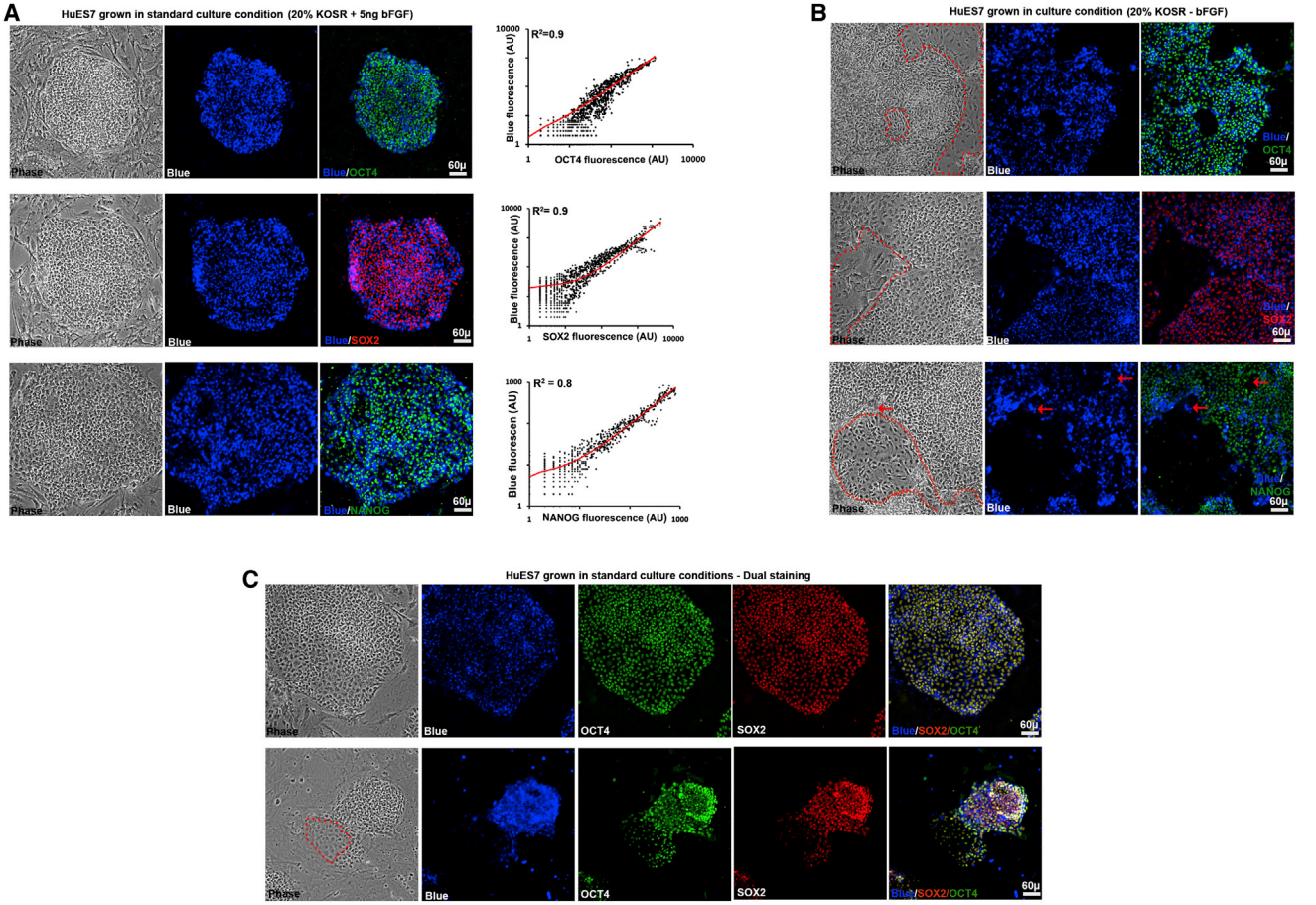


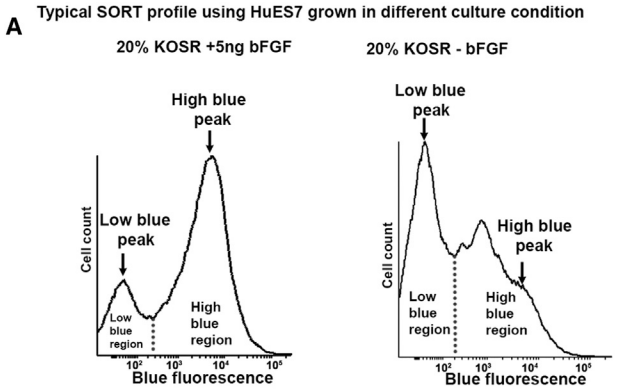

**Figure 2. Blue Fluorescence from Lipid Bodies Is Associated with Pluripotent Stem Cells and Aids in Easy Identification of Differentiated Cells from Pluripotent Stem Cells during Routine Culture**

(A) Cells that express pluripotency markers (OCT4, NANOG, and SOX2) have blue fluorescent lipid bodies. Mean fluorescence intensities of blue fluorescence correlate positively with the fluorescence intensities of pluripotency markers.

(B) HPSCs differentiated by removing bFGF show absence of lipid body-associated blue fluorescence and pluripotency markers. Differentiated regions (marked with red dotted lines) are identified by cellular morphology. The correlation between blue fluorescence and pluripotency markers in differentiating cultures is not evident (red arrow).

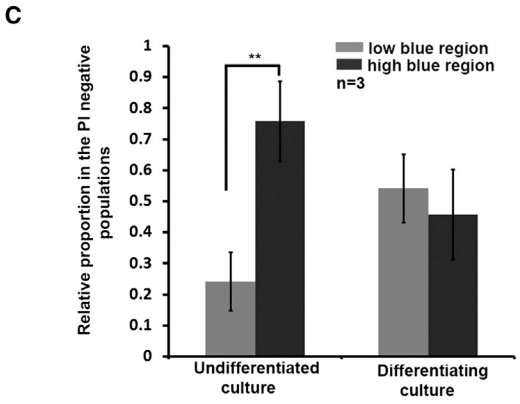
(C) Dual antibody staining (SOX2 and OCT4) of HPSC cultures are in accordance with Figures 2A and 2B above. Fluorescence intensities of equisized ROIs encompassing 10–15 cells across several images were measured to obtain scatter plots.

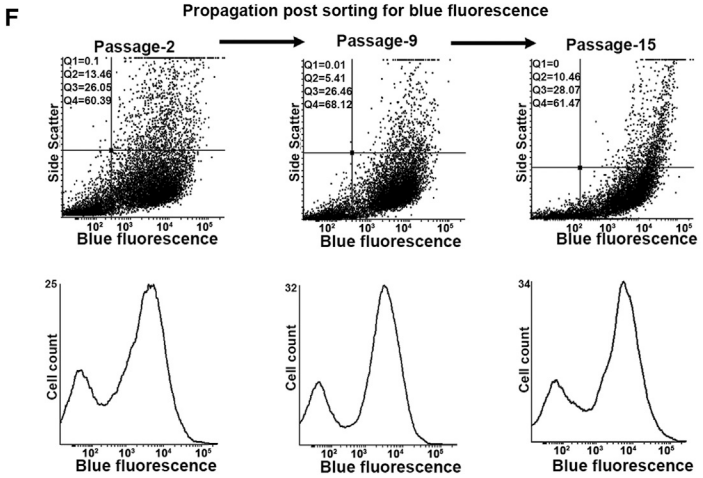
for the low-blue population and higher peak heights for the high-blue population, whereas more differentiated cultures (i.e., cultured without basic fibroblast growth factor [bFGF]) had the reverse profile (Figure 3A). Equal numbers of cells (n = 30,000) from the high-blue region, the low-blue region, and from an unsorted population on mitotically inactivated MEFs in standard media without ROCK inhibitor (ROCKi) were plated. In undifferentiated cultures, the high-blue cell populations always gave rise to larger numbers of colonies with the typical HuES-like morphology compared to the unsorted and low-blue populations (Figure 3B; Figure S2C). In differentiating cultures, sorting for blue fluorescence helped to recover more cells/colonies than unsorted cells, suggesting that sorting for blue fluorescence is beneficial (Figure 3B). To rule out cell death in the low-blue population after sorting, propidium iodide (PI)-negative low-blue cells were plated and imaged after 4 days. The majority of cells acquired a flattened morphology and stained positive for active mitochondria with unfragmented nuclei (Figure S2D), indicating that cells with low-blue fluorescence remained alive and represent the differentiating fraction of cells. The relative proportion of high-blue and low-blue cells in the PI-negative population of cells from multiple undifferentiated and differentiating cultures was determined and was in agreement with the above FACS profiles (Figure 3C). The

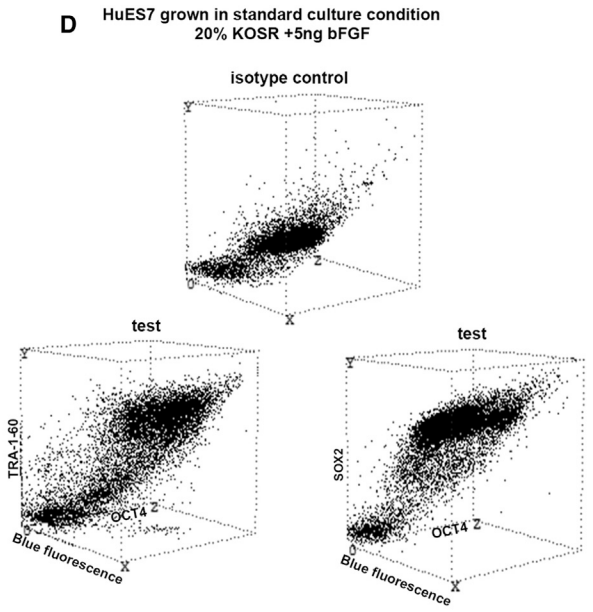


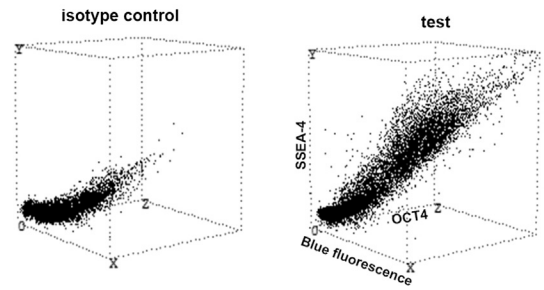

B

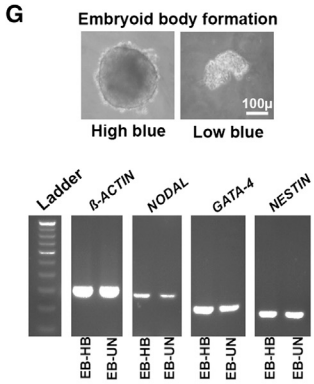
| Populations | No. of colonies/ 30,000 cells plated (undifferentiated culture n=3) mean ± s.d | No. of colonies/ 30,000 cells plated (differentiating culture n=3) mean± s.d |
|---|---|---|
| High Blue Region | 770 ± 42 | 142 ± 11 |
| Low Blue Region | 9 ± 1 | 4 ± 1 |
| Unsorted | 260 ±28 | 52 ± 3 |

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pluripotent nature of the sorted cells was also established with FACS analysis with dual staining of pluripotency markers and blue fluorescence. A high degree of correlation was observed between all the pluripotency markers examined and blue fluorescence (Figure 3D; Figure S2B). In cultures without bFGF, the cells differentiated along with a concomitant decrease in blue fluorescence levels and pluripotency marker levels (Figure 3E; Figure S2B). Repeated sorting/propagation of HuES7 colonies did not alter the blue fluorescence profile, suggesting that sorted cells continue to behave like regular HuES7 cells with some differentiation always observed (represented by cells within the low-blue region; Figure 3F). Furthermore, the high-blue sorted cells were functionally pluripotent whereas cell aggregates of the low-blue population failed to form embryoid bodies (EBs; Figure 3G).

Propagation of HPSC cultures from single cells is reported to be inefficient and dissociation to single cells leads to very low survival (Li et al., 2009). FACS necessitates the dissociation of colonies into single cells prior to sorting. Our results after sorting by blue fluorescence indicate that it is possible to propagate HPSCs after dissociating them into single cells without the use of ROCKi.

In general, HPSC cultures with large percentages of differentiating cells are not known to survive multiple passages. To determine whether such cultures can be rescued, we sorted differentiating HuES cultures and plated the cells with high-blue fluorescence onto MEF feeders. Pluripotent colonies with typical morphology were obtained by day 7 (Figure S2E).

These results indicate that (1) levels of lipid body-associated blue fluorescence correlated positively with pluripotency and self-renewal, and (2) high-speed sorting of single cells for blue fluorescence facilitated the isolation of large numbers of pluripotent stem cells away from differentiated cells. Therefore, sorting by blue fluorescence presents significant advantages over existing protocols of isolating and propagating human pluripotent stem cells.

### Human Somatic Cells Acquire Blue Fluorescent Lipid Bodies Very Early during Reprogramming

Lipid bodies with blue fluorescence are present in both HuESC and HiPSC colonies (Figure 1A) and are far fewer and not fluorescent in human somatic cells (Figure S1A). We monitored the appearance of fluorescent lipid bodies in somatic cells that were being reprogrammed to become pluripotent. Cells from different somatic tissues—i.e., human neonatal fibroblasts and EBV-transformed adult lymphoblastoid cell lines (LCLs)—were reprogrammed using the method of Okita et al. (2011). As early as 7–10 days after transfection, clusters of cells with altered morphology exhibited blue fluorescence while the surrounding MEF layer (used for LCL_iPSC generation) and somatic cells did not (Figures 4A and 4B; Figure S3A). These cell clusters gave rise to typical HiPSC colonies by appearance (Figures 4A and 4B; Figure S3A). All of the blue fluorescent clusters that were examined stained for SSEA-4 (Figure S3A). A total of 30 random cell clusters were monitored from six different experiments and by 20 days, 27 of these clusters became colonies with typical HiPSC morphology (Figure S3B). A total of six colonies (one per experiment) were expanded and all were positive for the pluripotency markers tested (Figure S3C). This establishes that blue fluorescence can be used to monitor reprogramming. We also characterized an NFF_iPSC (NFF-derived iPSCs) and an LCL_iPSC (LCL-derived iPSCs) used in this study for differentiation (Figures S3D and S3E).

Furthermore, the blue fluorescence distribution profiles of the HiPSCs were similar to those of HuES7 cells and were always higher (nearly 100-fold) than their somatic precursors (Figure 4C). The lower level blue fluorescence seen in somatic cells as a single FACS peak could be from NAD(P)H and other intrinsic fluorophores (Andersson et al., 1998; Buschke et al., 2012). These results indicate that the blue fluorescence associated with lipid bodies can serve as a reprogramming marker and aid in the identification of cells being reprogrammed.

**Figure 3. Lipid Body-Associated Blue Fluorescence Can Be Used to Sort and Propagate Pluripotent HPSCs**

(A) Pluripotent stem cells from HPSC cultures when dissociated into single cells and sorted by blue fluorescence resolve into two distinct populations. In an undifferentiated culture, the two populations show a significant difference in their levels of blue fluorescence, distinguishing the undifferentiated cells from their differentiated counterparts. In differentiating cultures, the high blue region broadens, becomes heterogeneous with lower number of cells under the high blue peak.

(B) Colony counts from high blue region, low blue region, and unsorted cells from undifferentiated and differentiating cultures.

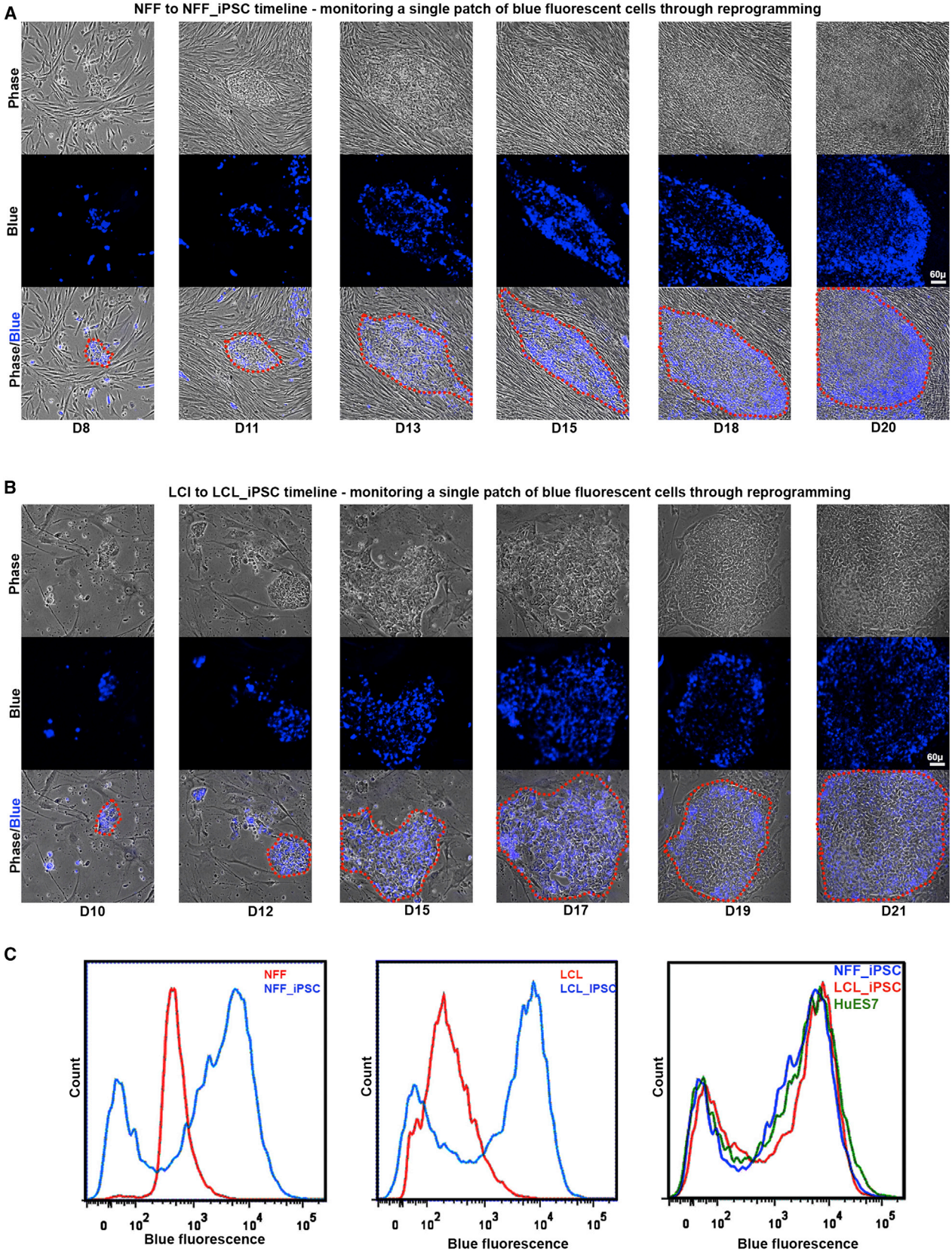
(C) Normalized (viable) cell counts from high blue and low blue regions of undifferentiated and differentiating cultures.

(D and E) FACS analysis of blue fluorescence levels in undifferentiated and differentiating cultures along with dual staining of pluripotency markers.

(F) Repeated sorting/propagation of HPSC colonies (by FACS) did not alter the blue fluorescence profile of HPSC cultures.

(G) Typical embryoid body and cell aggregate generated from high blue and low blue populations from sorted HuES7 cells, respectively. Qualitative RT-PCR analysis of EBs from unsorted and high blue populations of HuES7 to establish functional pluripotency.

Data are from three independent biological replicates; error bars indicate the SD. $^{**}p < 0.01$. EB-HB, EBs from high blue population; EB-UN, EBs from unsorted population. The agarose gel images are obtained from different lanes of a single gel. See also Figure S2.

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### Blue Fluorescent Lipid Bodies Are Absent in Mouse Inner Cell Mass but Present in Mouse Epiblast and in mEpiSC-like Cells

Mouse ESC (mESC) colonies grown in 20% KnockOut Serum Replacement (KOSR) ESC media had very faint blue fluorescence compared to that in HPSC colonies (Figures 5A and 5B). Higher magnification images of BODIPY-stained mESCs showed very few lipid bodies (white arrows) that were almost always at the colony edge, and did not show blue fluorescence (Figure 5C). The faint blue fluorescence observed in mESCs colocalized with the mitochondria-specific dye TMRM ($R^2$ = 0.93). The inset with blue fluorescence (brightness-enhanced) allows better visualization (Figure 5D). This was unlike human pluripotent cells in which the blue fluorescence was localized to the lipid bodies (Figure 5C; Figure S1D). FACS analysis of mESCs showed a single population with low-blue fluorescence whereas HuESCs had a characteristic bimodal distribution (Figure 5E). These results suggest that the blue fluorescence observed in mESCs and HPSCs emanated from different cellular compartments and cells.

Mouse pluripotent stem cells are believed to represent a naive state whereas the human pluripotent stem cells represent a slightly later developmental stage termed as the primed or epiblast-like state (Zhou et al., 2012). To determine whether the blue fluorescent lipid bodies were also present in vivo and specifically mark the primed or epiblast-like cells, we examined mouse embryos at 3.5 days postcoitum (dpc) to evaluate the mouse inner cell mass and at 6.5 dpc, the developmental stage from which mouse epiblast stem cells (mEpiSCs) are derived (Brons et al., 2007; Chenoweth and Tesar, 2010; Najm et al., 2011). Confocal images of 3.5 dpc embryos showed very few BODIPY-stained lipid bodies that were not fluorescent in the blue region and did not localize to the inner cell mass (Figure 5F). The distal epiblast region of the 6.5 dpc embryo had blue fluorescent puncta that were also stained by BODIPY (Figure 5G).

We dissociated and plated 6.5 dpc mouse embryos in EpiSC-specific media (KOSR 15% and FBS 5% with bFGF) (Najm et al., 2011) and propagated the epiblast-like colony outgrowths by mechanical dissociation. The colonies retained fluorescent lipid bodies (Figure 5H). Comparison of mean fluorescence intensities of blue fluorescence with BODIPY fluorescence in both the postimplanted mouse embryo (6.5 dpc) and the mEpiSC-like colonies showed a tight correlation (Figures 5I and 5J; n = 3 independent experiments). These results suggest that blue fluorescent lipid bodies are characteristic of the epiblast-like state and differentiate them from the naive stem cells. To our knowledge, there are no known specific markers for the epiblast stem cells in vivo and the lipid body-associated blue fluorescence may serve as a useful marker in vivo and in vitro.

### Blue Fluorescent Lipid Bodies Are Associated with the Primed State of Pluripotent Stem Cells

We then explored the association of fluorescent blue lipid bodies with stem cells from primed and naive states. When HuES7 cells were cultured in media that promotes their conversion to the naive state (Hanna et al., 2010), it resulted in a significant decrease in blue fluorescence along with a corresponding decrease in the number of lipid bodies in 48 hr (Figure 6A, top and second rows). FACS analysis of HuES7 cells (PI negative) in conversion media confirmed the decrease in blue fluorescence (Figure 6B). The cells in the conversion media had smaller nuclei and were more compact in naive cells (Figure 6A, third row). Levels of SSEA-4, a specific marker of primed cells, showed a significant decrease in the conversion media (Figure 6A, bottom row). Transcript levels of *FGF-5*, a marker for primed cells, also decreased as shown with RT-PCR analysis (Figure 6C).

mES-D3 cells were cultured in media that helps their conversion to the primed state (Takehara et al., 2012). In this media, the mES-D3 cells acquired blue fluorescent lipid bodies, strengthening the association of an epiblast-like state with these organelles (Figure 6D). FACS analysis of the mES-D3 cells (PI negative) also confirmed the appearance of blue fluorescence (Figure 6E). The ES-D3 cells also acquired larger nuclei, similar to primed cells (Figure 6D, bottom row).

### The Blue Fluorescence Arises from Retinyl Esters Sequestered in Cytoplasmic Lipid Bodies

Lipid bodies were isolated from HuES7 cells through differential sucrose gradient centrifugation (Bulankina, 2003). They were fluorescent and the fluorescence partitioned into chloroform: methanol ($CHCl_3$:MeOH at 3:1; Figure 7A), suggesting the fluorophore to be hydrophobic. The $CHCl_3$:MeOH extract had absorbance and fluorescence characteristics, i.e., excitation and emission profiles very similar to retinoids (retinol or retinyl esters such as retinyl

**Figure 4. Fluorescent Lipid Bodies Appear Early in the Reprogramming of Somatic Cells**

(A and B) Neonatal foreskin fibroblasts (NFF) and lymphoblastoid cell lines (LCL) show very early appearance of blue fluorescent lipid bodies during reprogramming [D(n), days posttransfection and plating]. The background MEF feeder layer remains nonfluorescent.

(C) The blue fluorescence FACS profiles of human induced pluripotent stem cells HiPSCs (NFF-iPSCs and LCL-iPSCs) are similar and match closely with those of human embryonic stem cells (HuES7).

See also Figure S3.

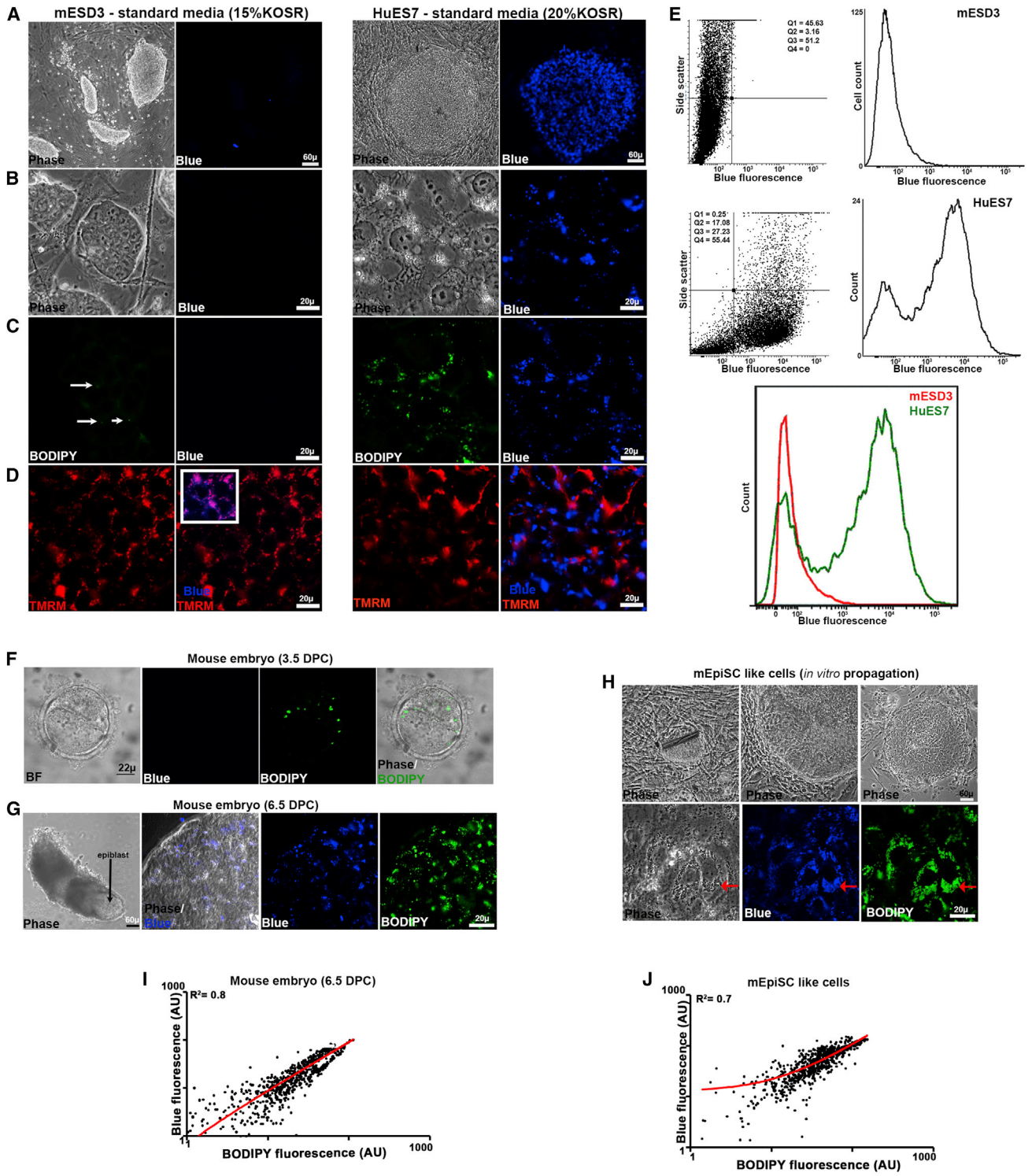


**Figure 5. Blue Fluorescent Lipid Bodies Are Absent in Naive Mouse Pluripotent Stem Cells and Mouse Blastocyst Inner Cell Mass but Present in Cells in Postimplantation Mouse Epiblast Cells and mEpiSC-like Cells**

(A and B) Representative phase contrast and fluorescence images (low and high magnification) of mES-D3 and HuES7 colonies. mES-D3 cells have far lower blue fluorescence compared to HuES7 cells (fluorescence intensity normalized to HuES7 cells).

(C) BODIPY stained and blue fluorescence high-magnification images of mES-D3 and HuES7. mESCs show occasional lipid bodies (white arrows), which are not fluorescent unlike HuESCs.

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palmitate or retinyl oleate) (Morton et al., 1930). The fluorescence spectra of the $CHCl_3$:MeOH extract and retinyl palmitate were identical (Figure 7B). The components of the $CHCl_3$:MeOH extract were resolved with reverse-phase high-performance liquid chromatography (HPLC) and compared with retinoid standards (retinyl palmitate, retinol, and retinyl acetate) by absorbance. The retention time of the primary/main peak of the $CHCl_3$:MeOH extract coincided with that of retinyl palmitate at 18.65 min and was substantially different from retinol and retinyl acetate (6.65 and 7.33 min, respectively; Figure 7C). Because all the fluorescence observed in the $CHCl_3$:MeOH extract (Figure 7B) could be accounted for and was identical to the retinyl palmitate fluorescence, we conclude that the fluorescence emanating from the lipid bodies was largely from retinyl palmitate or a retinoid very similar to it, perhaps retinyl oleate.

Animal cells are unable to synthesize vitamin A. In culture, it is available from serum in the media as retinol, which is taken up by cells and converted to retinyl esters (palmitate or oleate) or oxidized to retinal and retinoic acid (van Berkel, 2009; Guo et al., 2000). We tested for retinol in standard HuES media that contained KOSR, ESC-certified serum, and a commercially available serum-free media ReproFF2 (ReproCell). Samples were extracted with $CHCl_3$:MeOH and resolved with reverse-phase HPLC. All samples contained retinol that matched the retinol standard at 6.65 min (Figure 7D).

We also determined that HPSCs take up retinyl esters from the media, and the blue fluorescence correlated with retinyl ester levels. Cells grown on MEFs in standard media with 20% KOSR, supplemented with retinol or retinyl palmitate at various concentrations (0.5–10 μM), showed a dose-dependent increase in the blue fluorescence of their lipid bodies after 48 hr (Figure 7E).

mES-D3 and R1 cells showed no significant increase in retinoid-associated fluorescence even after 48 hr in mESC media supplemented with 10 μM retinol or retinyl palmitate (Figures S4A and S4B), whereas HuES7 cells had a substantial increase under identical conditions (Figure 7E; Figure S4C). On incubation for 72 hr with retinyl palmitate, mESCs exhibited a slight increase in blue fluorescence. Although retinol caused an increase in blue fluorescence in mESCs, it remained substantially lower than the blue fluorescence observed in HuESCs (Figure S4D).

Essential 8 (E8) media is a recently available, chemically defined serum-free media for HPSC cultures with minimal components and does not contain vitamin A (Chen et al., 2011). HuES7 cells cultured in E8 media showed a rapid decrease in lipid bodies along with its associated fluorescence in 24 hr, and the lipid bodies disappeared in 72 hr (Figure 7F). The addition of retinol (10 μM) to these cultures resulted in the reappearance of fluorescent lipid bodies (Figure 7F). Together with previously described results, this suggests that primed pluripotent stem cells can take up retinoids and that retinol can induce lipid bodies that sequester it as retinyl esters.

## DISCUSSION

HPSCs under culture conditions with serum or serum replacements, such as KOSR, have large numbers of small blue fluorescent lipid bodies. These are strongly associated with pluripotency markers (i.e., OCT4, SOX2, NANOG, SSEA-4, and TRA-1-60) and allow for the identification and isolation of pluripotent stem cells. On sorting, HPSCs resolve into two populations with a near 100-fold difference between their peak blue fluorescence. Only the cells with high-blue fluorescence are pluripotent and give stem cell colonies. The separation of the two populations is easy, efficient, and lends to high-throughput single cell analysis and propagation. Repeated sorting and propagation does not alter the fluorescence profiles and the sorted cells remain as regular HPSCs. The fluorescent lipid bodies also appear early during the reprogramming of somatic cells. This allows for easy detection and isolation of cells being reprogrammed and could help in the identification and interrogation of the early steps in the reprogramming process.

A recent elegant method used fluorescence lifetimes to distinguish lipid body fluorescence from that of NAD(P)

(D) A faint blue fluorescence is observed in mES-D3 cells. When the fluorescence intensity is maximized, it colocalizes with the mitochondria-specific dye, TMRM (inset), in mES-D3 cells.
(E) mESCs exhibit lower and unimodal blue fluorescence distribution whereas HuESCs exhibit a bimodal distribution.
(F) Blue fluorescent lipid bodies are absent in the inner cell mass of the 3.5 dpc mouse embryo.
(G) The epiblast region (from a 6.5 dpc mouse embryo) shows numerous blue fluorescent puncta that stain positive with BODIPY.
(H) Mouse epiblast-like stem cells (mEpiSC) cultured from mouse embryo (6.5 dpc) in mEpiSC media (K15F5) and sequentially passaged (p1, p2, and p3) retain blue fluorescent BODIPY-positive lipid bodies. The lower image shows cells from a p3 culture at high magnification and colocalization of blue fluorescence with BODIPY.
(I and J) Scatter plots of BODIPY mean fluorescence intensities versus mean blue fluorescence intensities in the postimplantation mouse embryo (6.5 dpc) and in mEpiSC-like cells cultured in vitro show high positive correlation. BF, bright field. Fluorescence intensities of equally sized ROIs encompassing 10–15 cells across several images were measured to obtain scatter plots.

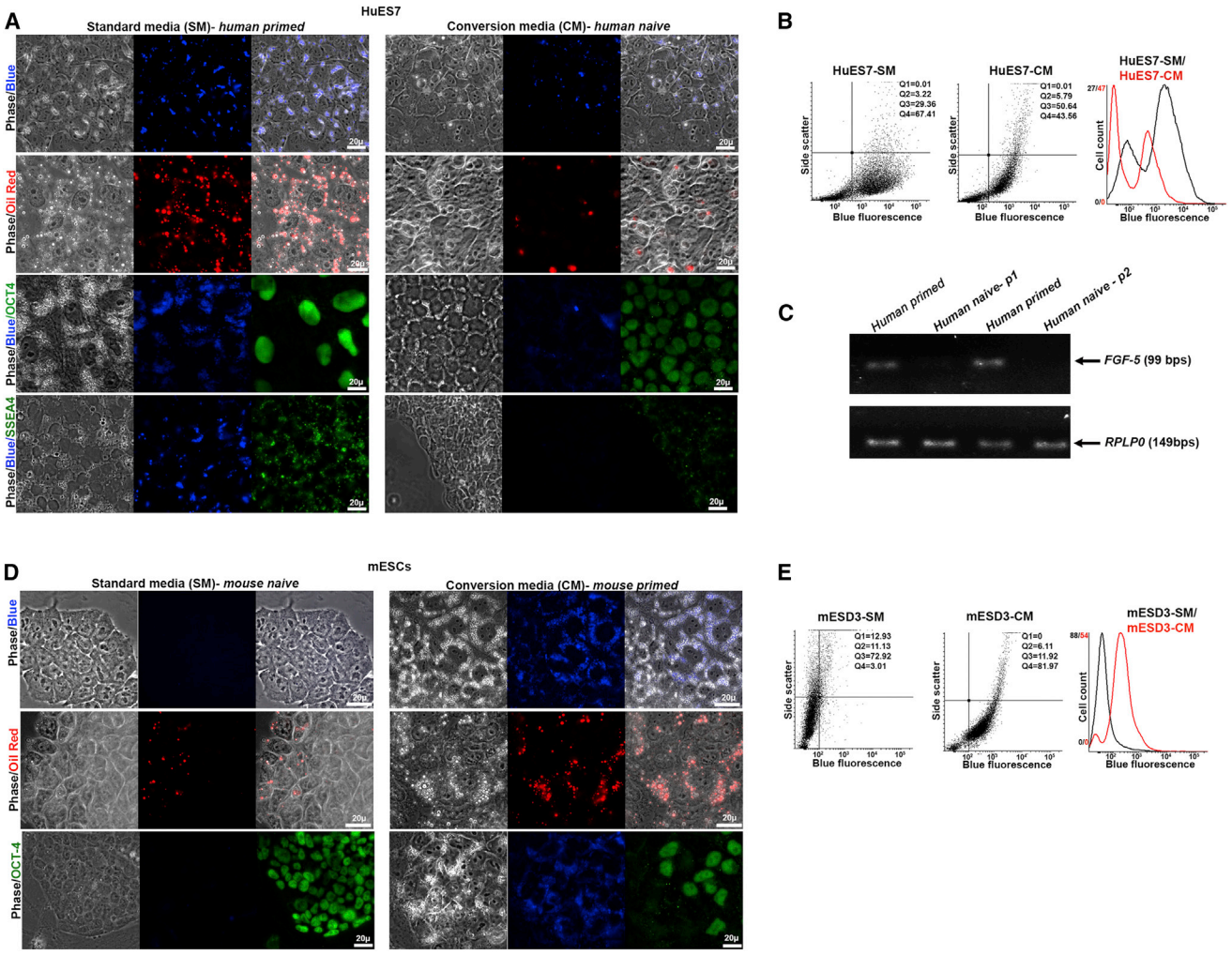



**Figure 6. Blue Fluorescent Lipid Bodies Are Associated with the Primed State of Pluripotent Stem Cells**

(A) Comparison of HuES7 cells grown in standard media (human primed) and in conversion media (human naive) shows decrease in blue fluorescent lipid bodies when grown in conversion media (first and second rows). The nuclear size is altered (third row) and levels of SSEA-4 (a specific marker for primed epiblast cells) decrease (bottom row) when grown in conversion media.

(B) FACS analysis of HuES7 cells grown in conversion media shows a decrease in blue fluorescence levels.

(C) Cells grown in conversion media show a decrease in FGF-5 (specific marker for epiblast cells) transcript levels compared to those in standard media.

(D) mES-D3 cells show an increase in blue fluorescent lipid bodies when grown in conversion media (mouse primed; first and second rows). Cells grown in conversion media also show the expected increase in nuclear size.

(E) FACS analysis of mES-D3 cells grown in conversion media shows an increase in blue fluorescence levels.

H and used their relative ratio to identify pluripotent HuESCs (Stringari et al., 2012). The authors hypothesized that the lipid body fluorescence originated due to ROS-induced lipid peroxide-protein reactions and that stem cells have high ROS levels. We and other groups observe that human pluripotent stem cells have low ROS levels (Haneline, 2008; Pervaiz et al., 2009; Shi et al., 2012; Wang et al., 2013). Our method is based on total blue fluorescence levels determined with fluorescence microscopy or FACS and allows isolation and propagation. We show that the fluorescence emanates from retinyl esters, such as retinyl palmitate in lipid bodies from extracellular retinol that is taken up and esterified. LRAT, which converts retinol to its ester, is expressed in human pluripotent cells. Increasing external retinol or retinyl palmitate causes a dose-dependent increase in the fluorescence of the lipid bodies. The sequestration of retinol as esters in lipid bodies may be for storage and later use as retinoic acid. Retinyl

esters in lipid bodies would resist oxidation to retinoic acid, a powerful differentiation signal for pluripotent stem cells. The storage of retinyl esters is analogous to the sequestration of histones in lipid bodies in *Drosophila* embryos (Li et al., 2012).

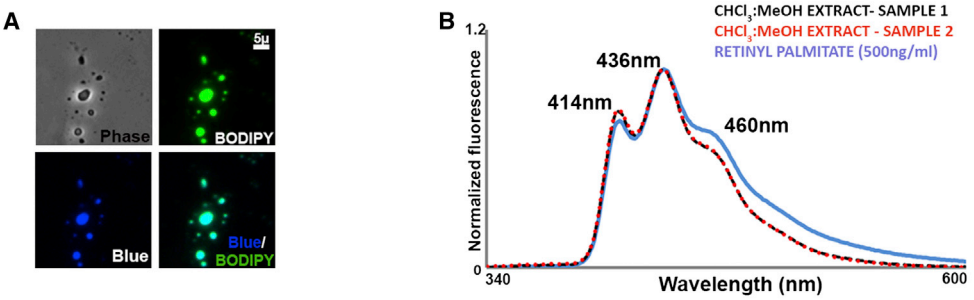
Retinoids (vitamin A) are important for a number of physiological and developmental processes including reproduction. Retinoic acid, one of the retinoids, plays an essential role in early development and maintenance of specific tissue types. Khillan and colleagues demonstrated that retinol can be used to maintain mESCs in a pluripotent state in feeder-free cultures (Chen et al., 2007). Retinol increases the expression of NANOG and promotes self-renewal by activating PI3K/AKT and the IGF-1/IRS-1 pathway (Chen and Khillan, 2010; Chen et al., 2007). Wang et al. (2011) showed that retinoic acid receptor gamma, liver receptor homolog 1, and retinoic acid analogs promote faster reprogramming of mouse and human somatic cells. Vaajasaari (2009) and Rajala et al. (2011) have also reported that retinol increases the expression of OCT4 and NANOG in HuESCs. These results show that retinol and some of its nonoxidized derivatives can have a significant role in the self-renewal of pluripotent stem cells.

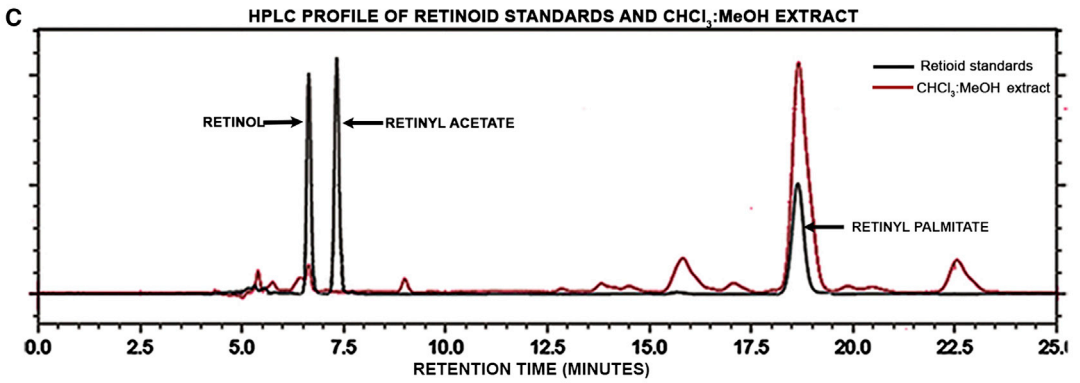
mESCs in media with serum or serum-replacements have very few lipid bodies and low levels of blue fluorescence. mESCs do not take up retinol or retinyl palmitate at levels seen in primed cells, although the retinol typically present at ~200 nM or less is actively sequestered by HPSCs. Prolonged incubation of mESCs with retinyl palmitate did not increase blue fluorescence, whereas retinol caused a slight increase. Examination of available transcriptomes of mouse and HuESCs indicate significant differences in transcripts related to retinol uptake and metabolism. *RBP*, *STRA6*, *LRAT* that encode proteins that bind, transport, and convert retinol to its ester, are expressed many fold more in human pluripotent cells than in mESCs (http://amazonia.transcriptome.eu, U133A [Enver et al., 2005]; NCBI GEO accession number GSM87830, MoES.C57B). *STRA6* is a G protein-coupled receptor and suggested to induce lipogenesis when activated by retinol (Muenzner et al., 2013). This may explain the presence of fluorescent lipid bodies only in epiblast-like or primed pluripotent human cells, and why HuESCs in E8 media lack lipid bodies and when supplemented with retinol acquire them. *STRA6* mutations in humans also have varied and strong phenotypes (Chassaing et al., 2009). Retinol could play a significant role in pluripotency and it may be useful to have retinol/retinyl esters in the media.

We considered the possibility that lipid bodies are only present in primed or epiblast-like cells. Examination of preimplantation (3.5 dpc) and postimplantation (6.5 dpc) mouse embryos showed the presence of blue fluorescent lipid bodies only in the latter. Retinol/retinoic acid are involved at this developmental stage (Huang et al., 2001). Mouse epiblast-like stem cells derived from postimplantation embryos also led to colonies similar to HuESC colonies, with fluorescent lipid bodies. In media that shift human pluripotent stem cells to the naive state or mESCs to the primed state, the cells show the appropriate shift in blue fluorescent lipid bodies. These results suggest lipid bodies that sequester retinoids to mark the epiblast-like state provide opportunities to explore their functional roles and can distinguish naive from primed pluripotent stem cells. Lipid bodies are also associated with specific metabolic states, and may mark a primed metabolic state because changing the media dramatically alters their levels.

The role of lipid bodies, in cellular physiology and development, is becoming increasingly evident. It may have a role to play in primed pluripotent cells. In most somatic cells, lipid bodies, usually present in small numbers, are heterogeneous in composition within and between cell types (Ducharme and Bickel, 2008; Li et al., 2012; Walther and Farese, 2012). They consist of mostly neutral lipids associated with specific proteins that vary between cell types. Cells with lipid bodies that sequester retinyl esters are uncommon. Retinal pigmented epithelium and hepatic stellate cells are two examples. Lipid bodies are also sites of lipogenesis, lipid storage, and synthesis of lipid signaling molecules, such as arachadonic acid, which has been implicated in the maintenance of pluripotency. Recent studies have implicated retinol to activate the *JAK/STAT* pathway and to regulate lipid accumulation via *SOCS3* and *PPARγ* (Berry and Noy, 2012; Berry et al., 2012). Electron micrographs of primate pluripotent stem cells portray structures that could be lipid bodies but were assumed to be lysosomes or glycogen granules (Johkura et al., 2004). Lipid bodies, although reported in primate pluripotent stem cells, were not associated with epiblast stem cells (Cibelli et al., 2002; Stringari et al., 2012). Oleate, a lipid, has also been related to pluripotency (Ben-David et al., 2013). E8 media does not contain any lipid or retinoids and HPSCs in E8 remain pluripotent, so retinoids are not essential for pluripotency. The lipids necessary for cell growth are synthesized endogenously. HPSCs grown in E8 with retinol acquire lipid bodies in 24–48 hr, indicating their ability to acquire these bodies. It is currently not clear what advantages retinol provides in the propagation of pluripotent stem cells other than the increased expression of NANOG and OCT4 in HuESCs (Rajala et al., 2011).

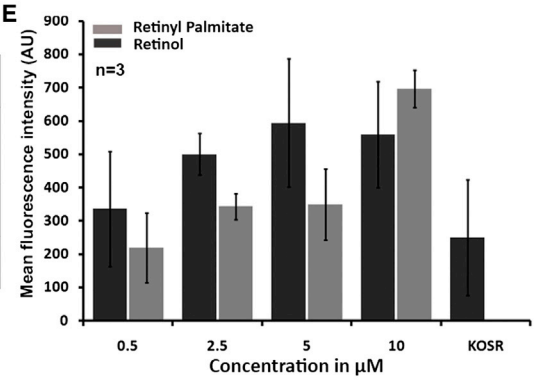
In conclusion, we have identified retinyl ester-containing lipid bodies to be associated with primed or epiblast-like pluripotent stem cells. The fluorescent lipid bodies can be easily used for routine identification, isolation,

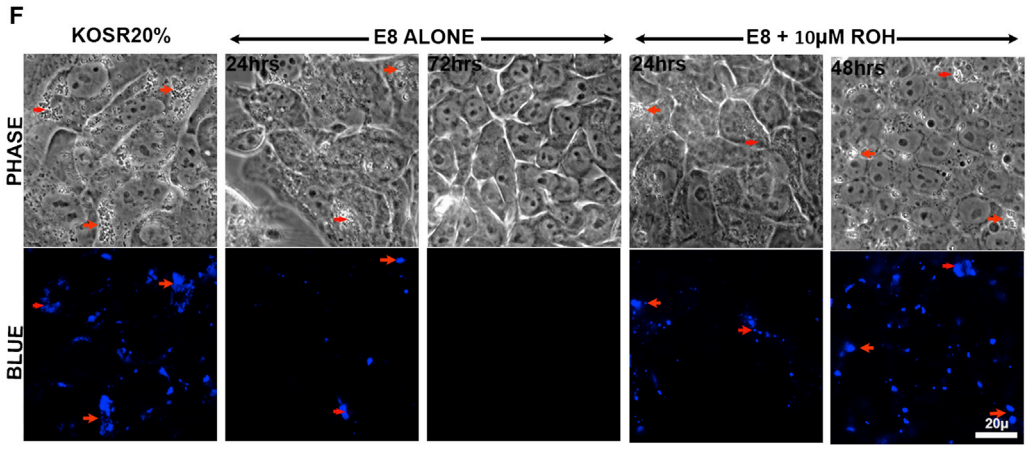



D

| | Standard retention time | FBS | FF2 | KOSR |
|---|---|---|---|---|
| Retinyl palmitate | 18.65min | no peak | no peak | no peak |
| Retinol | 6.65min | 6.65min | 6.647min | 6.647min |
| Retinyl acetate | 7.33min | no peak | no peak | no peak |

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and propagation of primed pluripotent stem cells. They are early markers of reprogramming cells in standard media and possibly in media such as E8, in the presence of retinol or its esters. In addition, the processes that underlie this striking difference between naive and epiblast-like stem cells should help us understand the mechanisms involved in pluripotency and pluripotent states.

## EXPERIMENTAL PROCEDURES

### FACS and Colony Evaluation

Flow cytometry and sorting were performed using 2–2.5 million cells/ml. The colonies were enzymatically dissociated with ESGRO Complete Accutase (EMD Millipore; SF006) and resuspended in KO-DMEM (Life Technologies; 10829-018), 100 μM 2-mercaptoethanol (Life Technologies; 21985-023), 10 nM nonessential amino acids (Life Technologies; 11140-050), 100 mM L-glutamine (Life Technologies; 35050061), 0.5% fetal bovine serum (Life Technologies; 10270), and 5 ng/ml recombinant human bFGF (Peprotech; 00-18B) and sorted using a BD FACSAria II special order system (BD Biosciences) in the presence of 1 μg/ml PI (Sigma-Aldrich; P4864). Only live cells (PI negative) were analyzed and separated using a 375 nm near-UV laser and a Trigon detector with a 450/50 nm bandpass filter (DAPI channel) to detect the blue fluorescence signal. The PMT voltage was set at 350 V, and 30,000 live cells from the high-blue, low-blue, and unsorted populations treated in the same way as the sorted populations were plated on MEF feeder layers conditioned with HuESC media. On day 5, colonies were counted and morphologies of the colonies evaluated in three independent experiments. Data analysis was performed using Cyflogic 1.2.1, (http://www.cyflogic.com) and FlowJo (http://www.flowjo.com). Use of cells from animal tissue and use of pluripotent stem cells and LCLs were been approved by the Institutional Animal Ethics Committee, Institutional Biosafety Committee, and by the Institutional Committee for Stem Cell Research and Therapy.

### Isolation and Characterization of Lipid Bodies from HuES7 Stem Cells

Lipid bodes were isolated using a modified density gradient ultracentrifugation method (Bulankina, 2003). Three hundred colonies were scraped and resuspended in 2 ml of 2 M sucrose solution in 10 mM Tris-HCl, and 1 mM EDTA buffer pH 7.4. The cell suspension was vortexed four times for 30 s separated by 2 min on ice and then passed four times through a 26G needle. Two milliliters each of 0.27 M and 0.135 M sucrose in Tris-EDTA buffer was layered sequentially onto the cell suspension in clear ultracentrifuge tubes (Beckman, part no. 344060) and centrifuged at 150,000 × *g* in a Beckman SW 40 Ti rotor for 1 hr and allowed to decelerate without braking. The topmost layer containing the lipid bodies was collected and stored at −20°C until it was used.

### Staining of Lipid Bodies in Mouse Embryos at 3.5 dpc and 6.5 dpc and Generation of Mouse EpiSC-like Cells

The preimplantation (3.5 dpc) and postimplantation (6.5 dpc) embryos were isolated from CF1 mice using the method of Shea and Geijsen (2007). The embryos were fixed with 4% paraformaldehyde, stained with BODIPY 493/503 (Life Technologies; D-3922) and imaged for green and blue fluorescence. Mouse EpiSC-like cells were derived from 6.5 dpc embryos using a previously described protocol (Najm et al., 2011). Flattened-out colonies were observed by 24–48 hr. The colonies were mechanically dissected into small pieces and propagated once every 3–4 days. The colony morphology and presence of lipid body-associated blue fluorescence were observed and evaluated at each passage.

### In Vitro Conversion of Pluripotent Stem Cells between Naive and Primed or Epiblast-like States

To convert mESCs to EpiSC state, i.e., naive to primed state, mES-R1 and D3 cells were grown in HuESC medium with 10 ng/ml of human Activin (Peprotech; 20-14) and 10 ng/ml bFGF on dishes coated with 0.1% gelatin (300 Bloom; Sigma-Aldrich; G3500) in 1× PBS. The medium was replaced daily. The cells were closely monitored for change in morphology and for the appearance of lipid bodies along with the associated fluorescence characteristics. For conversion of HuES7 cells to the naive mESC-like state, primed state cells were grown on mitotically inactivated MEF in media with the following composition: 48 ml DMEM/F12 (Invitrogen; 11320), 48 ml Neurobasal (Invitrogen; 21103), 1 ml N2 supplement (Invitrogen; 17502048), 2 ml B27 supplement

**Figure 7. The Blue Fluorescence in Lipid Bodies Arises from Retinoids Obtained from Sera or Serum Replacement Components**

(A) Lipid bodies isolated from HuES7 cells using sucrose gradient centrifugation retain blue fluorescence and stain positive for BODIPY.

(B) Chloroform: methanol extracts of fluorescent lipid bodies and retinyl palmitate have identical fluorescence spectra. The fluorescence traces of individual samples were normalized to their maximum emission values.

(C) C18 reverse-phase HPLC of the $CHCl_3$:MeOH extract and retinyl standards shows the major peak of the extract and retinyl palmitate to have identical retention times.

(D) Reverse-phase HPLC analysis of $CHCl_3$:MeOH extracts of FBS and serum-free commercially available ESC-grade media shows presence of retinol.

(E) Lipid bodies in HuES7 cells cultured in standard HuESC media supplemented with retinol and retinyl palmitate show dose-dependent increase in blue fluorescence.

(F) HuES7 cells cultured in chemically defined E8 media (lacks retinol) show a steep decrease in lipid bodies (red arrows) and blue fluorescence with time and regain blue fluorescent lipid bodies in 48 hr when E8 is supplemented with retinol.

Data are from three independent biological replicates; error bars indicate the SD. See also Figure S4.

(Invitrogen; 17504044), 2 μg recombinant human LIF (Peprotech; 300-05), 1 mM glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, penicillin-streptomycin, 5 mg/ml BSA (Sigma; A9647), and small molecule inhibitors at the final concentrations mentioned here: PD0325901 (1 μM; Tocris Bioscience; 4192), CHIR99021 (3 μM; Tocris Bioscience; 4423), and forskolin (10 μM; Tocris Bioscience; 1099) modified from an earlier protocol (Hanna et al., 2010). The medium was changed daily and colony morphology, disappearance of lipid bodies, and change in fluorescence were monitored. The HuES7 cells and the primed to naive converting cells were propagated up to three passages by trypsinization, and at each passage, cells were harvested for RNA isolation.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.05.004.

## AUTHOR CONTRIBUTIONS

T.M., O.M., R.M., and P.B.M. planned and did experiments, analyzed results, and wrote and had final approval of the manuscript. M.M.P. conceived and designed the study, analyzed results, and wrote and had final approval of the manuscript.

## ACKNOWLEDGMENTS

This work was supported by funds to M.M.P. from the NCBS (TIFR) and DBT (India; BT/01/IYBA/2009) to O.M. as an independent investigator. T.M. and M.P.B. were supported by CSIR fellowship no. 09/860(0118)/2011-EMR-I and no. 09/860(0094)2009-EMR-I, respectively. R.M. and O.M. were supported by BT/01/IYBA/2009. We thank Jayant Udgaonkar for valuable comments on the manuscript, Mrinalini Puranik (IISER, Pune) and Sudha Rajamani (IISER, Pune) for useful input, Suchitha Soman for help with confocal microscopy, H. Krishnamurthy and CIFF (NCBS) for FACS and confocal microscopy, C-CAMP (DBT) for initial HPLC analysis, Dr. G.H. Mohan and the NCBS Animal Facility, Joginder Singh, and A.T. Sabareesan for help with spectroscopy, Doug Melton (Harvard University) for the HuES7 line, Imtiaz Zafar for initiating HuESC cultures in the laboratory, and members of the laboratory and Vishal Bhardwaj for discussions. Plasmids for subcellular compartment analysis were kind gifts from Jennifer Lippincott-Schwarz, NIH Bethesda, USA and Suresh Subramani, UC San Diego, USA. We also thank Geriatric Clinic, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore for LCL lines and Shagufta Parveen from the School of Regenerative Medicine, Bangalore for providing mesenchymal stem cells. A patent application for the method has been filed by the authors' institution.

Received: September 25, 2013
Revised: May 1, 2014
Accepted: May 7, 2014
Published: June 12, 2014

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