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Maintenance of Feeder Free Anchorage Independent Cultures of ES and iPS Cells by Retinol/Vitamin A

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ABSTRACT

Pluripotent embryonic stem (ES) cells derived from mammalian blastocyst and the adult fibroblast derived induced pluripotent stem (iPS cells) exhibit complete potential to form cells representing all the primary germ layers such as mesoderm, endoderm and ectoderm. These cells are usually co-cultured with mouse embryonic fibroblast feeders to prevent spontaneous differentiation. Feeder free cultures can provide substantial advantage to improve the efficiency and consistency of the culture conditions. In these studies, we demonstrate that a small dietary compound retinol, the alcohol form of vitamin A has capacity to regulate the pluripotency of pluripotent stem cells and maintain highly enriched population of pluripotent ES and iPS cells in feeder free suspension cultures. Retinol maintains long-term cultures of undifferentiated cells via elevated expression of stem cell specific transcription factors Nanog and Oct4. The studies provide evidence that retinol regulates the self-renewal of pluripotent stem cells via the over expression of insulin like growth factor II (IGFII) that engages PI3 kinase signaling pathway via IGF1 receptor tyrosine kinase. The ES cells retain capacity to generate high degree germline competent chimeric animals after microinjection into blastocysts. The studies offer a convenient system for long term maintenance of pluripotent stem cells via the activation of intracellular machinery for self-renewal by a physiologically relevant compound for large-scale production of high quality pluripotent stem cells. J. Cell. Biochem. 113: 3002–3010, 2012.

KEY WORDS: SELF-RENEWAL OF PLURIPOTENT STEM CELLS; FEEDER INDEPENDENT CULTURES; OVER EXPRESSION OF NANOG; RETINOL/VITAMIN A; PI3 KINASE SIGNALING; Akt/PKB SIGNALING

Pluripotent embryonic stem (ES) cells derived from mammalian blastocyst have complete potential to form all types of cells representing the primary germ layers such as endoderm, mesoderm, and ectoderm [Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998]. These cells are believed to be an unlimited source of all types of cells for application in regenerative medicine. ES cells maintain pluripotency via complex interactions of extrinsic signaling by leukemia inhibitory factor (LIF) that activates the Jak/Stat3 pathway [Niwa et al., 1998; Matsuda et al., 1999; Raz et al., 1999], bone morphogenic proteins (BMPs) [Ying et al., 2003; Ogawa et al., 2007], MAPK-ERK [Burdon et al., 1999], and Wnt/βCatenin [Hao et al., 2006; Ogawa et al., 2006] and intrinsic transcription factors such as Nanog, Oct4, and Sox2 [Boiani and Schöler, 2005]. The over expression of Nanog however, is sufficient to prevent the differentiation in mouse and human ES cells [Chambers et al., 2003; Mitsui et al., 2003; Darr et al., 2006].

Recently it has been documented that adult fibroblasts can be reprogrammed into pluripotent stem cells called induced pluripotent stem (iPS) cells by four genes including Oct4, Sox2, Klf4, and c-Myc [Takahashi and Yamanaka, 2006]. The iPS cells have properties identical to the ES cells including differentiation into cells representing endoderm, mesoderm and ectoderm [Yamanaka, 2010]. However, the pluripotent ES and iPS cells tend to differentiate spontaneously in prolonged in vitro cultures.

To prevent spontaneous differentiation, the ES cells and iPS cells are co-cultured with mitotically inactive mouse embryonic fibroblasts (MEFs) [Robertson, 1987] which involves many cumbersome and time consuming steps including preparation of MEFs, inactivation of cells either via mitomycin C or irradiation [Robertson, 1987] and finally the removal of feeder cells, which can slow down the efficiency and limit the large-scale production of undifferentiated stem cells.

In general, the feeder free pluripotent ES and iPS cells are difficult to maintain for extended periods as the cells tend to lose their pluripotency. Undifferentiated mouse ES cells have been maintained in bioreactors for at least 1 month [Zur Nieden et al., 2007] and in

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defined medium with reduced expression of integrins [Hayashi et al., 2007]; in a medium supplemented with different growth factors on serum-free systems [Andäng et al., 2008] or in feeder independent suspension cultures supplemented with polyvinyl alcohols (PVA) [Tsuji et al., 2008].

In these studies we demonstrate that retinol, a small molecule of dietary significance is sufficient to maintain pluripotency of ES cell and iPS cell in long-term culture for more than 20 passages for over 3 months. Earlier we have shown that retinol elevates the expression of Nanog and Oct4 by activating phosphoinositide 3 (PI3) kinase signaling pathway [Chen and Khillan, 2008, 2010]. The phosphoinositide 3 (PI3) kinase signaling is a key signaling pathway in the regulation of cell proliferation, survival, and tumorigenesis [Cantley, 2002] and is critical for the maintenance of mouse and human ES cells. PI3 kinase mediates different pathways from cytokines, including LIF, to the downstream targets via phosphorylation of PtdIns (4, 5)P2 to PtdIns (3, 4, 5)P3 [Armstrong et al., 2006; Watanabe et al., 2006; Huang and Manning, 2009]. In these studies we provide evidence that Retinol maintains the pluripotency of stem cells via upregulation of IGFII, which then activates the PI3 kinase signaling pathway by engaging IGF1 receptor.

We demonstrate that retinol maintains the pluripotency of ES and iPS cells in long-term anchorage independent suspension cultures and maintains high expression of Oct4 and Nanog. The ES cells cultured in anchorage independent cultures retain the capacity to generate high degree chimeric animals with germline transmission. Overall, the studies present a novel mechanism of retinol function in the self-renewal of pluripotent stem cells and a convenient feeder free non-adherent culture system for quantitative production of high quality pluripotent stem cells.

MATERIALS AND METHODS

CULTURES OF ES CELLS AND iPS CELLS

Normal and Green florescent protein (GFP) positive R1 mouse ES cells (A Nagy) and Mouse iPS cells-WP5 (Stemgent Cat. no. 08-0007) were used for the studies. ES cell media contained Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum, 1 mM L-glutamine, 1% non-essential amino acids, 0.1 μM β-mercaptoethanol, 1 mM sodium pyruvate, 1,000 U/ml LIF, 100 units/ml Penicillin and 0.1 mg/ml Streptomycin [Robertson, 1987]. The media for iPS cells contained knockout DMEM with 15% FBS, 1 mM L-glutamine, 1% non-essential amino acids, 530 U/ml LIF, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Fresh cultures of ES cells and iPS cells were started on MEF feeder cells for expansion. The confluent cultures were trypsinized for 5 min with 0.25% trypsin followed by separation of feeder cells by plating on fresh tissue culture plates (BD Falcon Cat. no. 35003) for 1 h. Approximately 0.5×10^6 ES cells were transferred to a non-adhesive 100 mm plate (VWR Cat. no. 25384) in 10 ml ES medium with LIF. All trans-retinol (Sigma–Aldrich prepared as 100 mM solution in 100% ethanol) was added at a concentration of 0.5 μM. Parallel plates without retinol were prepared as untreated controls. Plates were kept in 37°C incubator on a rocker at a speed of 20–25 oscillations/min at a 20–25° tilt. Media was changed every 24–48 h by collecting the cells in 15 ml tube. The tubes were allowed to settle for 15 min. The top 9 ml medium was removed and the bottom 1 ml was transferred to fresh plates with medium supplemented with retinol. The cells were passaged every 5–6 days. The cells were washed in PBS followed by trypsinization in 0.25% trypsin–EDTA at 37°C for 5 min. Colonies were dissociated to single cell by pipetting up and down several times using 5 ml pipette. Fresh media was added and the cells were centrifuged at 1,000 rpm for 4 min. Approximately 0.5×10^6 cells were transferred to fresh plates. The process was repeated every 5–6 days for each passage. The cells at various passages were also stored in liquid nitrogen for future usage. These cells could be thawed and used for fresh suspension cultures without the requirement of feeder cells.

RT PCR ANALYSIS

Total RNA was isolated using STAT 60 solution (TELTEST Friendswood, TX, http://www.isotexdiagnostics.com) following instructions from the manufacturer. The RNA was converted into cDNA using oligo-dT and avian myeloblastosis virus reverse transcriptase using kit purchased from Invitrogen (Carlsbad, CA, http://www.invitrogen.com). The RT PCR was carried out in a total volume of 50 μl using specific primers. The PCR conditions used denaturation at 94°C for 45 s; extension at 72°C for 2 min; and annealing at temperature as specified for each primer pair for 23–30 cycles [Chen and Khillan, 2010]. The products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining using hypoxanthine-guanine phosphoribosyl transferase (HPRT) primers as control. Each assay was performed at least three times.

The primers for RT PCR used were as described [Chen and Khillan, 2010]. The primers for IGFII were, Fwd 5′-CGCTTCAGTTTG-TCTGTTCGG-3′ IGF2 Rev 5′-TGGGTGGTAACACGATCAGG-3′ (source http://jme.endocrinology-journals.org/cgi/reprint/33/1/195).

WESTERN BLOT ANALYSIS

Total protein was extracted with radioimmuno-precipitation assay buffer (Sigma–Aldrich Cat. no. R0278). Anti-GAPDH and anti-Oct4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, http://www.scbt.com), and Nanog antibody was purchased from Chemicon (Temecula, CA, http://www.chemicon.com). Fifty micrograms of protein was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membrane (Bio-Rad, Hercules, CA, http://www.bio-rad.com). The membranes were incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated goat antibody to mouse IgG or rabbit antibody to goat IgG (Santa Cruz Biotechnology), and developed with chemiluminescence reagent (Pierce, Rockford, IL, http://www.piercenet.com). The analysis was repeated at least three times.

ALKALINE PHOSPHATASE ASSAY

The cells were fixed with 4% paraformaldehyde for 2 min at room temperature. Staining for alkaline phosphatase (AP) was performed using a kit from Chemicon following protocols provided by the manufacturer.

IMMUNOFLORESCENCE STAINING

The cells from suspension cultures were plated over cover slips coated with gelatin. The cells were fixed with 4% paraformaldehyde for 20 min at 4°C and permeabilized with 0.5% Triton X for 10 min followed by three washings with PBS. The fixed cells were then treated with cold methanol for 10 min and washed with PBS. The non-specific binding of proteins was blocked by 5% BSA in PBS for 1 h at room temperature. The cells were labeled with goat anti-mouse Sox2 antibody, Oct4 antibody (Santa Cruz Biotechnology) and Nestin antibody (BD Biosciences, San Diego, http://www.bdbiosciences.com) at a concentration of 1:100. Cells were washed for 15 min followed by incubation with secondary antibody at 1:250 and examined by fluorescence microscopy using Olympus Provis microscope.

PREPARATION OF CHIMERIC ANIMALS

ES cells at passage 1 and 5 were microinjected into blastocysts from C57BL6 mice. The blastocysts were transferred into the uterine horns of 2.5 days pseudopregnant CD1 foster mothers to obtain new born pups. All animal procedures were carried out according to the University approved IACUC protocols.

RESULTS

RETINOL MAINTAINS SELF-RENEWAL OF ES CELLS IN SUSPENSION CULTURES

Pluripotent ES cells must have two key properties such as self-renewal and capacity to differentiate into all the primary germ layers [Evans and Kaufman, 1981; Martin, 1981]. Earlier we have documented that retinol/vitamin A has capacity to regulate the self-renewal of ES cells by upregulating the expression of Nanog and Oct4 [Chen and Khillan, 2008]. To investigate whether retinol signaling is anchorage dependent, R1 ES cells were cultured as spheroids in suspension culture on non-adherent petri dishes. The cells were agitated constantly on an oscillating platform in the medium supplemented with 0.5 μM retinol.

Most suspension cultures rely on mechanical stirring of the cells. A major drawback of the stirring process is that it encourages cells to form aggregates by cell clumping at least in early stages. To circumvent this clumping, the cells were oscillated by placing petri dishes onto a flat rocking platform. Under these conditions most cells form spheres of 50–100 cells within 48 h and grow via cell proliferation rather than aggregation. The cells were passaged by trypsinization every 5–7 days or as soon as the spheres were approximately 2–3 mm diameter with approximately 300–500 cells.

The cells were cultured for over 20 passages, approximately 3 months by successive trypsinization. Only about $<1\%$ of the cells were lost at each passage most probably due to the damage by trypsinization and the cells formed spheres within 48 h and only a few dead cells were noticed after 48 h. The cells formed spheres with smooth phase bright appearance within 3–5 days (Fig. 1A left panel; passage 16). The trypsinized cells were counted at each passage. As shown in Figure 1B, there was no significant change in the growth of cells at all the passages.

MAINTENANCE OF PLURIPOTENCY OF ES CELLS IN LONG-TERM CULTURES

ES cells maintain pluripotency by extrinsic signaling and intrinsic transcription factors [Boiani and Schöler, 2005]. Undifferentiated ES cells express genes such as Nanog, Oct4, and Sox2, which are the key transcription factors for the maintenance of undifferentiated cells. Our earlier studies have revealed that retinol elevates the expression of Nanog and Oct4 by three- to sixfold [Chen et al., 2007; Chen and Khillan, 2008]. To test whether the anchorage dependence of the ES cell is necessary for the over-expression of these transcription factors, the ES cells at different passages were analyzed by semi-quantitative RT PCR. As shown in Figure 2A (passage 1, 5, and 10), the cells maintained high levels of Nanog and Oct4 mRNA at all the passages compared to control ES cells (lane C). Similar levels of Nanog and Oct4 expression were also observed at later passages such as 16 and 20 (not shown). The untreated cells failed to show the expression of Nanog and Oct4 after passage 1 (data not shown). The cells treated with retinol on the other hand, did not express differentiation specific genes Brachyury, GATA4, and Nestin (Fig. 2A) whereas the non-treated cells exhibited high expression of these genes after first passage (data not shown). The cells were therefore, not maintained further.

The expression of Nanog was further confirmed by Western blot analysis (Fig. 2B) that revealed three- to fourfold elevated expression of Nanog compared to control cells (left lane) supporting our previous results [Chen et al., 2007; Chen and Khillan, 2008]. The elevated expression of these transcription factors was further confirmed by immunostaining analysis. As shown in Figure 2C,D, retinol treated cells at passage 18 expressed high levels of Oct4 and Sox2 compared to control cells. The cells in Figure 2D show slightly uneven edges which is possibly due to uneven settling of the spheres which require approximately 24–48 h to attach to the cover slips. Further, no expression of differentiation specific gene Nestin was observed (not shown) supporting the RT PCR data (Fig. 2A).

ELEVATED EXPRESSION OF NANOG IN RETINOL TREATED CELLS

As shown in Figure 2, retinol upregulated the expression of Nanog. To examine the relative levels of Nanog expression, the retinol treated samples at passage 18 were further analyzed by progressive dilution of protein samples. The samples from cells treated with retinol were diluted with the buffer followed by Western blot analysis (Fig. 3A). The retinol treated cells exhibited three- to fourfold expression of Nanog (Fig. 3A and B) supporting our earlier conclusion on static monolayer cultures [Chen and Khillan, 2008] indicating further that attachment to the surface of petridish is not required for the upregulation of pluripotent stem cell markers.

MAINTENANCE OF STEM CELL MARKERS BY ES CELLS IN THE ABSENCE OF LIF

Usually ES cells spontaneously differentiate within less than 24 h when transferred to medium without LIF and lose expression of ES cell specific markers [Glover et al., 2006]. The retinol treated cells at passage 10 were partially trypsinized for approximately 2 min followed by transfer to the plates coated with gelatin and cultured under differentiation conditions, that is, without retinol and LIF. As

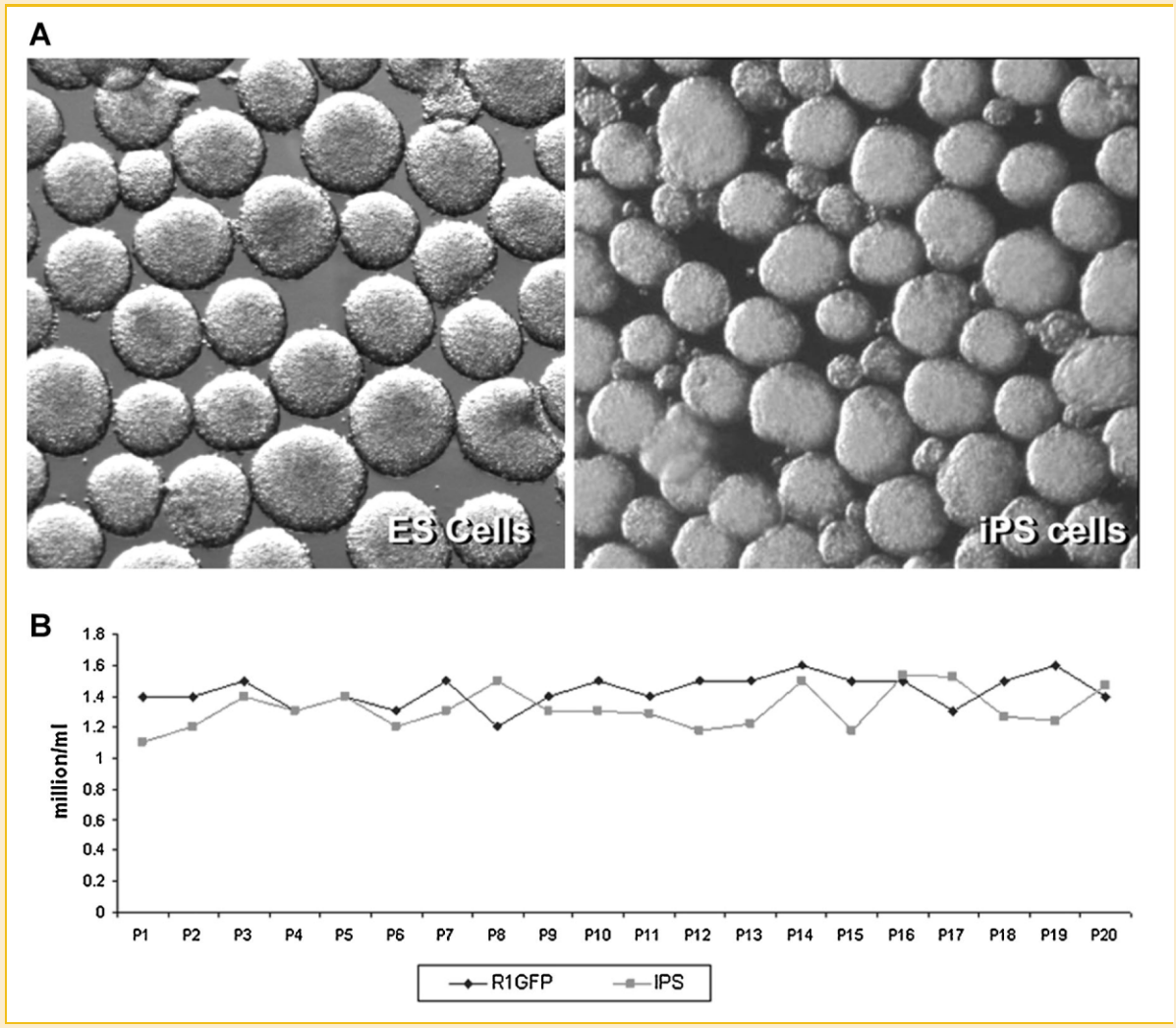

Fig. 1. Retinol maintains self-renewal of ES cells in Feeder independent suspension culture. A: ES cells were cultured in medium supplemented with 0.5 μM retinol and the cells were passaged every 5–6 days by trypsinization. A 5-day culture of R1 ES cells at passage 16 (left panel) and iPS cells passage 10 (right panel). The retinol treated cells formed uniform size phase bright colonies at all the passages. B: The cells at each passage were trypsinized to single cell suspension and the cell count from ES cultures and iPS cultures from 20 passages was plotted. The *Y*-axis shows the total number of cells in millions whereas *X*-axis represents the passage number. No significant difference in cell growth and proliferation was observed over the passages.

shown in Figure 4A, the untreated cells showed morphology of flat fibroblast like cells within 24 h and were totally differentiated at 48 h as observed by negative staining for alkaline phosphatase (AP). The retinol treated cells on the other hand, formed colonies with typical morphology of undifferentiated cells and continued to maintain this morphology even after 48 h. The colonies stained strongly positive for AP indicating the undifferentiated nature of the cells. The cells exhibited flat morphology only after 72 h (compare cells with control cells at 24 h) and were totally differentiated after 96 h (not shown).

The cells cultured at different time points were further analyzed by Western blot analysis. As shown in Figure 4B, retinol treated cells exhibited significant expression of Nanog even after 48 h which was almost comparable to the parental ES cells (lane C). The expression of Nanog however, dropped dramatically at 72 h and disappeared completely at 96 h confirming the data on AP staining. The expression of Nanog in untreated cells on the other hand, was significantly low after 24 h and was undetectable after 48 h confirming a correlation between the expression of Nanog and the pluripotency of ES cells. These data indicate that retinol treated cells maintain pluripotent properties twice longer than the untreated cells in differentiation medium. Therefore, caution must be exercised while performing cell differentiation studies with these cells.

To examine further, the cells from retinol treated cultures at passage 20 were trypsinized and plated over MEF fibroblast feeders. As shown in Figure 4C, that almost 50% of the colonies in control cells exhibited flat morphology of differentiated cells after 3–4 days whereas all the colonies in retinol treated cultures formed undifferentiated AP positive colonies proving

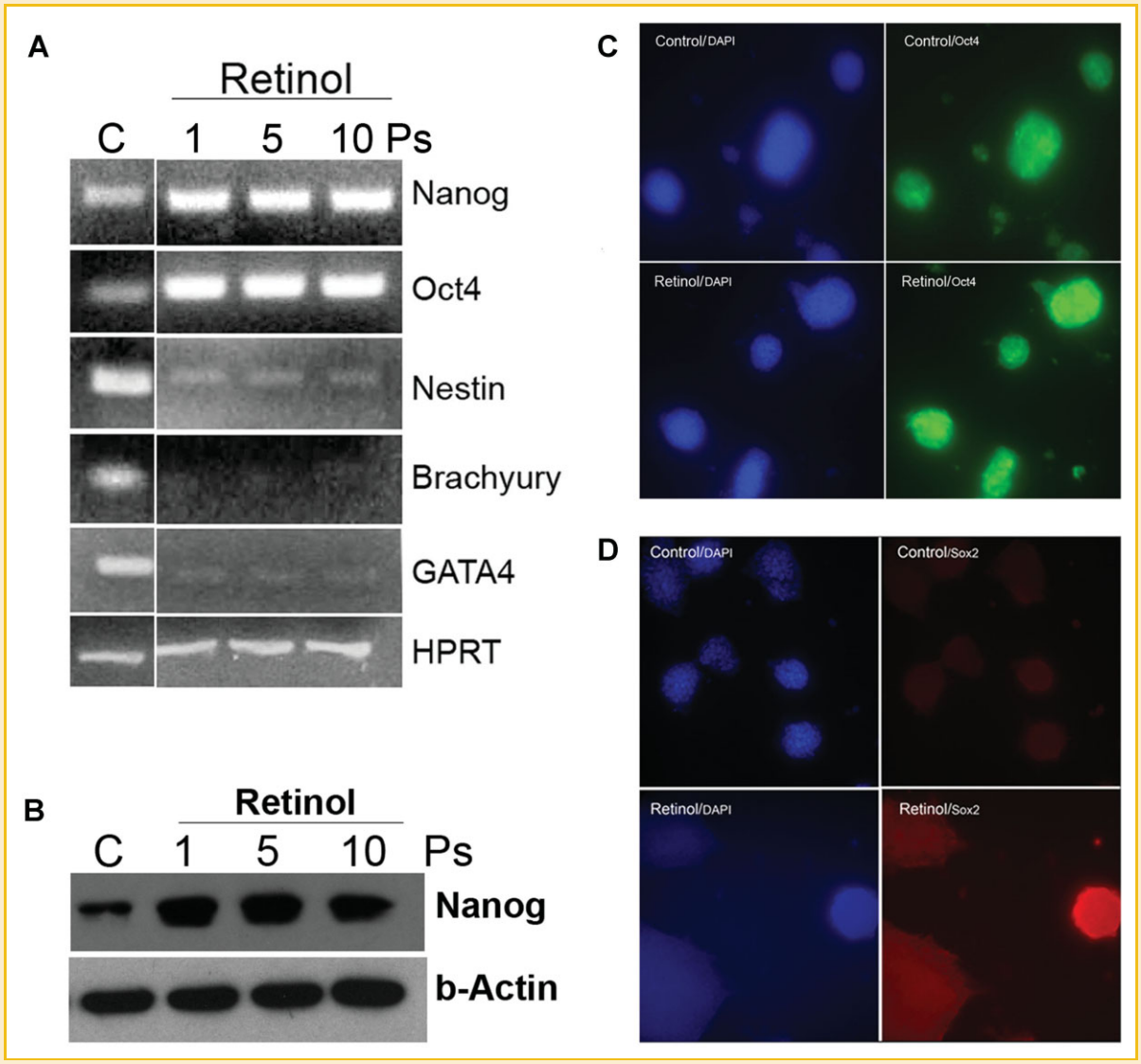

Fig. 2. Retinol treated cells express elevated levels of pluripotent cell specific genes. A: Semi-quantitative RT PCR analysis shows that retinol treated cells exhibit elevated expression of Oct4 and Nanog mRNA at passages 1, 5, and 10 compared to parental cells (C). Retinol treated cells showed almost no expression of differentiation specific genes Nestin, Brachyury and GATA4 at any of the passages. HPRT was used as control for the analysis. B: The analysis of cells by Western blot showed three- to fivefold higher expression of Nanog in retinol treated cells at all the passages. C: Immunostaining analysis of retinol treated cells at passage 18 shows higher expression as noticed by the increased florescence of Oct4 (lower right panel) compared to normal cells. D: Immunostaining analysis of retinol treated cells at passage 18 shows higher expression of Sox2 compared to untreated control cells (upper right panel).

that retinol maintains high percentage of undifferentiated cells in extended cultures.

RETINOL REGULATES ES CELL SELF-RENEWAL VIA UPREGULATION OF IGFII

IGFI and IGFII are the potent ligands for IGF1 receptor. However, only IGFII is expressed in early embryo [DeChiara et al., 1991]. Since ES cells represent the blastocyst stage of the mammalian embryo, we wanted to investigate the effect of retinol on the regulation of IGFII. Total RNA from retinol treated cells was analyzed for the expression of IGFII. As shown in Figure 5A, the ES cells treated with retinol at passage 12 and 16 expressed high levels IGFII as compared to the control cells which showed only barely detectable levels.

IGFII MIMICS THE EFFECT OF RETINOL BY ELEVATING THE EXPRESSION OF NANOG

Since retinol enhanced the expression of IGFII (Fig. 5A), we decided to investigate the effect of IGFII directly. The cells were treated with IGFII followed by Western blot analysis. As shown in Figure 5B (lane marker as IGFII), IGFII alone was sufficient to enhance the expression of Nanog similar to that seen after treatment with retinol (Fig. 5B, lane marked as Retinol). However, the upregulation of Nanog by IGFII was approximately 50% lower than the retinol which indicates that apart from up-regulating the expression of

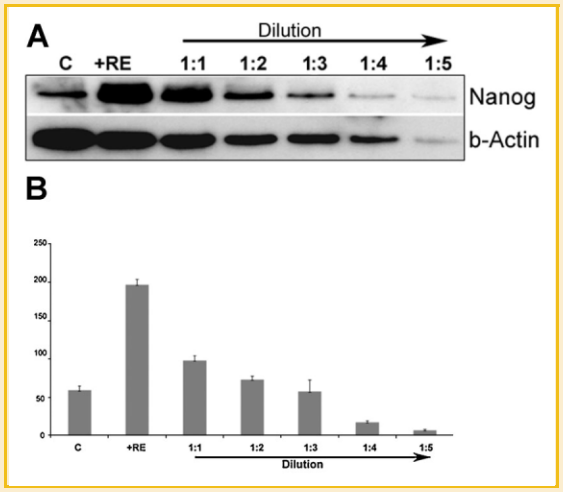

Fig. 3. Enhanced expression of Nanog by retinol. A: The protein samples from ES cells in suspension cultures at passage 18 were progressively diluted 1:1 to 1:5 dilutions. The samples were analyzed by Western blot analysis using Nanog antibodies. The untreated starting ES cells were used as control (C). Retinol elevates the expression of Nanog by three- to fourfold. B: The densitometric analysis of the Western blot samples shows approximately three- to fourfold higher expression of Nanog compared to control sample.

IGFII, retinol may also activate Nanog via yet unknown additional mechanisms.

EFFECT OF IGFII ON ES CELLS IS SENSITIVE TO INHIBITORS OF PI3 KINASE SIGNALING

Earlier we have reported that activation of Nanog by retinol in monolayer cultures is mediated via phosphoinositide 3 (PI3) kinase signaling pathway by engaging insulin like growth factor 1 (IGF1) receptor [Chen and Khillan, 2010]. Since retinol up-regulates the expression of IGFII, we also examined the effect of various inhibitors of PI3 kinase signaling pathway in relation to IGFII. As shown in Figure 5B, the cells treated with PI3 kinase inhibitor LY294002 and IGFII failed to enhance Nanog expression (lanes marked as LY294002 and LY294002 + IGFII) proving that activation of PI3 kinase signaling by retinol is mediated via elevated expression of IGFII. This was further proven by treating cells with picropodophyllin (PPP), a specific inhibitor of IGF1 receptor. IGFII in the presence of PPP failed to show any increase in Nanog (lanes marked as PPP and PPP + IGFII) proving that retinol signaling in ES cells is mediated via IGF1 receptor supporting our previous results [Chen and Khillan, 2010].

Collectively, these data provide strong evidence that retinol regulates the self-renewal of ES cells in non-adherent feeder independent ES cultures by upregulating the expression of IGFII.

RETINOL MAINTAINS PLURIPOTENCY OF iPS CELLS

The iPS cells generated by reprogramming of adult fibroblasts present tremendous potential for regenerative medicine to create patient specific pluripotent cells [Yamanaka, 2010]. Feeder free suspension cultures therefore, can be of significant advantage for large-scale production of high quality iPS cells for regenerative medicine. To investigate the effect of retinol, mouse iPS cells

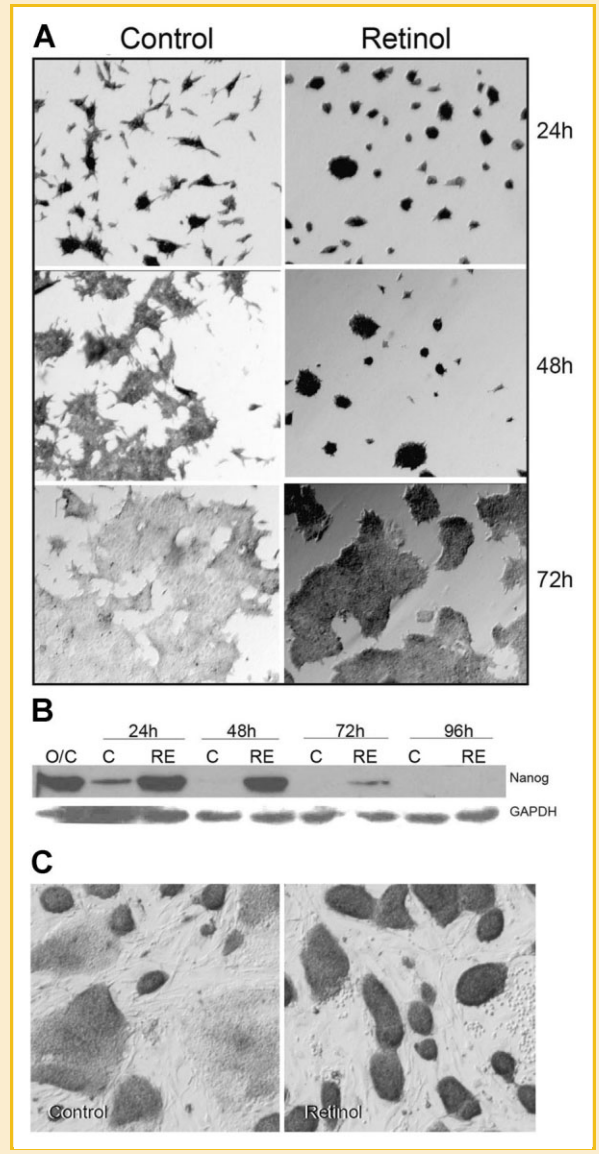

Fig. 4. Retinol prolongs Nanog expression after removal of LIF. A: Retinol treated cells maintained typical morphology of undifferentiated colonies and stained positive for alkaline phosphatase (AP) after 48 h in medium without LIF. The colonies became flat only after 72 h with the loss of AP staining. The control untreated cultures did not form colonies in the medium without LIF and exhibited flat fibroblast like cells within 24 h (40× magnification). B: Western blot analysis of the cells was carried out at different time points after transfer of cells to differentiation medium. As shown, the retinol treated cells maintained expression of Nanog at 24 h and 48 h similar to that seen in the original starting cells (O/C). The level of Nanog decreased dramatically after 72 h. The untreated cells on the other hand, showed significantly lower level of Nanog in the medium without LIF at 24 h (C), which disappeared completely after 48 h. C: The cells from suspension culture at passage 20 were trypsinized and transferred to the plates with feeder cells. Almost 100% of retinol treated cells formed undifferentiated AP positive colonies within 3 days (right panel) whereas in untreated control cells the colonies exhibited morphology of differentiated cells (left panel; 100× magnification).

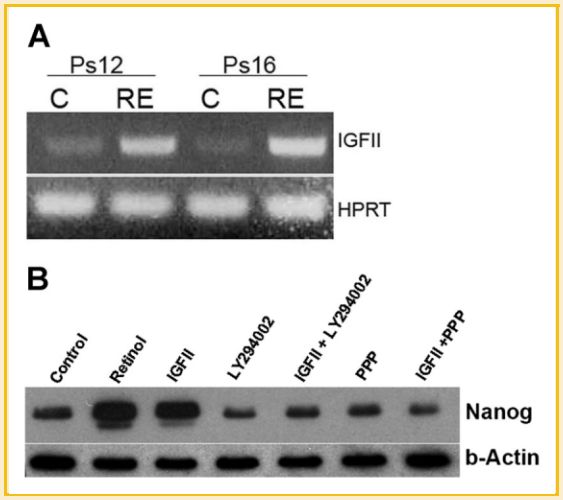

Fig. 5. Retinol signaling is mediated via PI3 kinase pathway. A: Semi-quantitative RT PCR analysis of ES cells at passage 12 and 16 show elevated expression of IGFII after treatment with retinol (RE) as compared to untreated control cells (C). B. ES cells at passage 16 were treated with 0.5 μM retinol; 25 ng IGFII; 10 μM LY294002; IGFII + 10 μM LY294002; 10 μM picropodophyllin (PPP); IGFII + 10 μM PPP for 18 h followed by Western blot analysis for Nanog. IGFII causes the upregulation of Nanog whereas this upregulation is blocked by LY294002 and PPP.

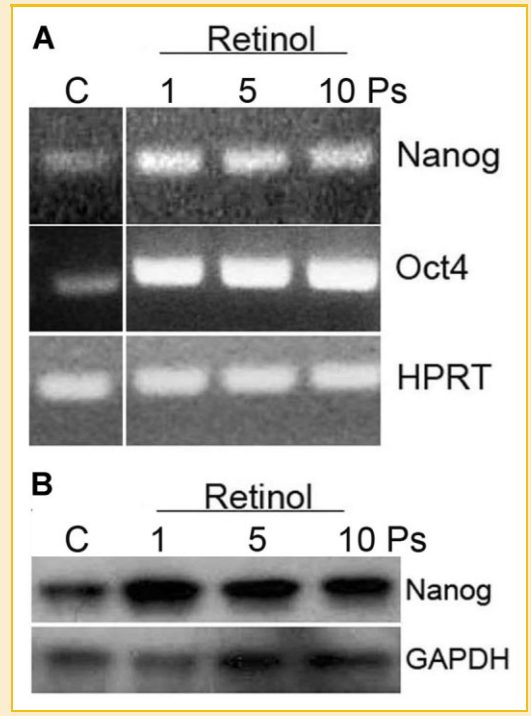

Fig. 6. Long-term culture of iPS cells in feeder independent suspension culture. A: Semi-quantitative RT PCR analysis shows that retinol treated iPS cells exhibit elevated level of Oct4 and Nanog at passages 1, 5, and 10 compared to control parental cells (C). B: Western blot analysis revealed a high expression of Nanog in retinol treated iPS cells at all the passages compared to untreated control cells (C).

obtained from Stemgent, Inc. were cultured in suspension cultures in the medium supplemented with 0.5 μM retinol. Similar to the ES cells, iPS cells also formed spheres at all the passages. Figure 1A right panel shows spheres formed by the iPS cells at passage 10 (Fig. 1A left panel). The undifferentiated iPS cells could be passaged every 5–7 days for over 20 passages without the loss of pluripotency.

The iPS cultures were also analyzed for the expression of Nanog and Oct4. As shown in Figure 6A, RT-PCR analysis of iPS cells at passage 1, 5, and 10 revealed an upregulation of Nanog and Oct4 similar to that observed in ES cells (Fig. 2A). The expression of Nanog was further confirmed by Western blot analysis (Fig. 6B). The iPS cells maintained high expression of Nanog at all the passages as compared to control cells indicating that retinol signaling is conserved between mouse ES cells and iPS cells.

iPS CELLS MAINTAIN UNIFORM GROWTH IN RETINOL MEDIUM

Similar to ES cell cultures, the iPS cells were passaged every 5–7 days by trypsinization and the cells were counted at each passage. As shown in Figure 1B, there was no significant difference in the growth of iPS cells for 20 passages.

RETINOL MAINTAINS GERMLINE POTENTIAL OF ANCHORAGE INDEPENDENT ES CELLS

The gold standard test for the pluripotency is the microinjection of ES cells into blastocyst to create chimeric animals [Brivanlou et al., 2003]. It is therefore, important to confirm that anchorage independence does not affect the potential of ES cells for germ line transmission. ES cells from suspension cultures at passage 1 and passage 5 were microinjected into blastocysts isolated from C57BL6 animals followed by transfer into pseudopregnant females to obtain live born pups. The ES cells at both the passages produced high degree chimeric animals as shown by agouti coat color (Fig. 7 left panel passage 5 cells). Breeding of the chimeric animal with normal females produced progeny that also exhibited agouti coat color proving further the capacity of the cells for germline transmission (Fig. 7B). The progeny of the chimeric mouse could be propagated successfully to establish the line of mice.

DISCUSSION

Pluripotent ES cells are believed to be an unlimited source of all types of cells for regenerative medicine [Donovan and Gearhart, 2001]. Recent developments in reprogramming of adult fibroblasts into iPS cells, has demonstrated a similar potential for application of these cells in regenerative medicine [Yamanaka, 2010]. A major limitation however is that the pluripotent cells lose their pluripotency in prolonged cultures. In general, human and mouse ES cells and iPS cells are maintained over MEFs derived feeders [Robertson, 1987; Thomson et al., 1998] that involves several cumbersome and time consuming steps that limits the quantitative production of undifferentiated cells. In addition, variability of different batches of MEFs can also influence the growth of stem cells. Particularly, the co-culture of human ES cells with mouse feeder cells can render these cells unfit for clinical applications. Therefore, the conditions that maintain high quality

Fig. 7. Retinol treated cells form high degree chimeric animals with germline potential. Microinjection of retinol treated R1 ES cells (agouti background) at passage 5 into C57BL6 embryos produced chimeras with high contribution of ES cells (left panel). Mating of the chimeric animal with normal C57BL6 females produced progeny with agouti coat color proving germline competence of the cells (right panel).

undifferentiated cells can be of tremendous advantage for downstream applications.

Suspension cultures of ES cell are routinely performed to generate embryoid bodies to induce differentiation [Höpfl et al., 2004]. However, long-term cultures of pluripotent stem cells in suspension cultures are difficult to maintain. Short-term cultures of mouse ES cells have been tested in medium with polyvinyl alcohol (PVA) [Tsuji et al., 2008], specific growth factors [Ying et al., 2003; Andäng et al., 2008], integrin suppresses agents [Hayashi et al., 2007] as well as in bioreactors [Zur Nieden et al., 2007]. It is not been tested whether anchorage dependence is important for maintaining these cells in long-term cultures. Our earlier studies have documented that retinol can maintain pluripotency of ES cells in long-term cultures [Chen and Khillan, 2008]. The current studies we describe long-term cultures of pluripotent stem cells in suspension cultures. We demonstrate that a physiologically relevant component retinol/vitamin A is sufficient to maintain the long term feeder free suspension cultures of ES and iPS cells without the requirement of additional growth factors. Since retinol is a physiological compound, it is expected to have minimal toxic effects on the cells.

The ES cells in suspension cultures exhibit elevated expression of Oct4 and Nanog in response to retinol (Fig. 2A,B) which is accompanied by the suppression of differentiation specific genes which indicates that attachment of cells to surface of petri dish is not the prerequisite for the pluripotency of ES cells. Moreover, the growth rate of the ES cells and iPS remained unaffected for at least 20 passages.

Interestingly, the ES cells maintained the expression of Nanog and Oct4 even after the removal of LIF from the medium (Fig. 4). The maintenance of stem cell properties for extended periods without the LIF could be attributed to the higher levels of Nanog, which supports the earlier reports that over-expression of Nanog prevents the differentiation of ES cells [Chambers et al., 2003; Darr et al., 2006].

The conditions for culturing cells in suspension were modified to encourage the formation of spheres via cell proliferation and to prevent cell aggregation. It is possible that the initial spheres may have been formed by aggregation, but the subsequent growth appeared to result predominantly from multiplication of cells as there was no evidence of multiple aggregates. Almost all the retinol treated cells formed phase bright colonies of undifferentiated cells (Fig. 4C).

The suspension cultures of ES cells offer a convenient alternate for long-term maintenance of pluripotent cells. The conditions described here involve simple steps and inexpensive ingredients. The cells can be repeatedly frozen and thawed similar to normal static cultures. Single cell suspensions can be achieved by simple steps of trypsinization. In addition, the suspension cultures allow the convenience of collecting cells at anytime for analyses without disturbing the whole culture. It is also noteworthy that retinol/vitamin A alone is sufficient to maintain highly enriched population of pluripotent stem cells. Although the starting cells expressed a detectable level of differentiation specific genes Brachyury, GATA4, and Nestin, their expression disappeared after retinol treatment (Fig. 2A) suggesting the possibility of elimination of cells via terminal differentiation [Chen and Khillan, 2010].

The maintenance of high quality undifferentiated cells by retinol can be attributed to the elevated expression of Nanog [Chambers et al., 2003; Mitsui et al., 2003]. It is important to note that recent reports by Zhang et al. [2011] have also shown that retinol maintains the self-renewal of mouse germ cells. Our earlier studies have revealed that retinol regulates the self-renewal of stem cells by activating PI3 kinase signaling pathway by engaging IGF1 receptor [Chen and Khillan, 2010]. The studies here now demonstrate that activation is caused by upregulating the expression of IGFII growth factor (Fig. 5B) thus extending the mechanisms further upstream. The genomic analysis of the upstream promoter region of IGFII will reveal the mechanism by which retinol regulated this gene.

Although the IGFII mimicked the effect of retinol on Nanog activation, it is also possible that retinol regulates some additional mechanisms as well. It is therefore, interesting to note that a key role of IGFII/IGF1 receptor axis in the pluripotency of human ES cells has also been documented recently [Bendall et al., 2007]. A similar effect of retinol in human pluripotent cells will have significant

implications for culturing feeder independent human ES and iPS cells for clinical applications.

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