Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells

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Historically, our understanding of molecular genetic aspects of human germ cell development has been limited, at least in part due to inaccessibility of early stages of human development to experimentation. However, the derivation of pluripotent stem cells may provide the necessary human genetic system to study germ cell development. In this study, we compared the potential of human induced pluripotent stem cells (iPSCs), derived from adult and fetal somatic cells to form primordial and meiotic germ cells, relative to human embryonic stem cells. We found that ∼5% of human iPSCs differentiated to primordial germ cells (PGCs) following induction with bone morphogenetic proteins. Furthermore, we observed that PGCs expressed green fluorescent protein from a germ cell-specific reporter and were enriched for the expression of endogenous germ cell-specific proteins and mRNAs. In response to the overexpression of intrinsic regulators, we also observed that iPSCs formed meiotic cells with extensive synaptonemal complexes and post-meiotic haploid cells with a similar pattern of ACROSIN staining as observed in human spermatids. These results indicate that human iPSCs derived from reprogramming of adult somatic cells can form germline cells. This system may provide a useful model for molecular genetic studies of human germline formation and pathology and a novel platform for clinical studies and potential therapeutical applications.

INTRODUCTION

Mammalian somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) via the introduction of a small set of transcription factors that encode OCT3/4, SOX2 and KLF4 with or without addition of c-MYC, or an alternate combination of OCT3/4, SOX2, LIN28 and NANOG (1–9). Regardless of the gene combination, however, human iPSC lines bear remarkable similarity to human embryonic stem cells (hESCs) in terms of their morphology, culture and proliferation, gene expression and ability to differentiate to mesoderm, endoderm and ectoderm both in vitro and in vivo in teratoma assays (10,11). A hallmark of pluripotency in vivo and in vitro, however, is the ability to differentiate to the germ cell lineage (12–17). Recently, human iPSCs derived from reprogramming of fetal somatic cells have been shown to differentiate to early-stage primordial germ cells (PGCs), further indicating their similarity in potential to hESCs (4). However, the ability of iPSCs derived from reprogramming of

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RESULTS

BMP-induced differentiation of iPSCs

To examine whether the addition of BMPs can induce germ cell differentiation from iPSCs, as described for hESCs, we differentiated iHUF4 and iP(iMR90) cells for 7 and 14 days with and without the addition of BMP-4, -7 and -8b as previously described for hESCs (15,16). We observed that BMP-induced cultures had an increased expression of the germ cell-specific marker, VASA, as shown by western blotting (Supplementary Material, Fig. S1). We then differentiated all cell lines in parallel, iHUF4, iP(iMR90), H9 and HSF1 for 7 and 14 days via the feeder-free adherent culture with BMP supplementation. We observed that all cell lines maintained a similar hESC-like colony morphology during routine culture and acquired morphologies distinct from undifferentiated cells after 14 days of differentiation (Fig. 1A). Moreover, we observed an increased expression of the germ cell-specific proteins, VASA and DAZL, with differentiation; the level of expression of these proteins was similar at the day 7 and 14 time points between all cell lines (Fig. 1B). Notably, we also detected a low level of VASA and DAZL protein expression in undifferentiated iHUF4 cells, and VASA expression in one sample of undifferentiated iP(iMR90) cells (Fig. 1B), likely indicating the presence of a subpopulation of cells expressing germ cell markers even in the undifferentiated culture. The expression and localization of the VASA protein was also detected by immunostaining for all cell lines, in differentiated cells and also in occasional cells in undifferentiated cultures (data shown for iPSCs, Fig. 1C).

Gene expression analysis

To further assess the differentiation of iPSCs to different lineages, we assayed gene expression after 0, 7 and 14 days of differentiation by quantitative RT–PCR for the pluripotency factors OCT3/4 and NANOG, for the trophectodermal (keratin 7, KRT7), endodermal (GATA-binding protein 6, GATA6), mesodermal (Actin, alpha cardiac muscle 1, ACTC) and ectodermal (neural cell adhesion molecule 1, NCAM1) markers and for the germ cell markers, IFITM1 (PELOTA (PELOTA homolog) and PRDM1A (PR-DoMain containing 1A).

The combined expression of endogenous and exogenous pluripotency markers [the later derived from viral vectors containing NANOG and OCT3/4 for iP(iMR90) and OCT3/4 for iHUF4] was significantly higher in undifferentiated iPSCs relative to hESCs, but then decreased to the similar levels with differentiation for day 14 (Fig. 2). Upon differentiation, all cell lines expressed markers of endoderm, mesoderm and ectoderm, as well as trophectoderm, indicating a similar differentiation potential between iPSCs and hESCs. Some differences were observed, however; for example, undifferentiated iPSCs had significantly higher expression of ACTC relative to hESCs and the expression of GATA6 was significantly higher in undifferentiated iP(iMR90) relative to other cell lines.

When we examined the mRNA expression of germ cell markers, we observed an increased expression with differentiation for all cell lines with variable but the similar levels of expression between iPSCs and hESCs (Fig. 2). In undifferentiated cells, iP(iMR90) had significantly higher expression of IFITM1 relative to other cell lines and both iPSC lines had significantly higher expression of PELOTA relative to hESCs. However, the expression of PRDM1A was significantly lower in undifferentiated iHUF4 cells relative to other cell lines. We also note that at the RNA level, the expression of VASA is...
observed only at very low and variable levels in all the cell lines as has been observed previously (13–16).

Analysis of VASA:GFP-transduced cells

To compare the efficiency of germ cell differentiation between iPSCs and hESCs, we next transduced each of the pluripotent stem cell lines with a VASA:GFP reporter system and used FACS to determine the percentage of PGCs differentiated, as described previously (15). Lines transduced with the VASA:GFP reporter were designated as follows: vH9, vHSF1, viPS(IMR90) and viHUF4. We observed that the percentage of GFP-positive cells after 7 days of differentiation was very similar between vH9 and vHSF1 cell lines (2.28 and 2.39%, respectively) and comparable with previous reports (Fig. 3A and B). In contrast, we observed that the percentage of GFP-positive cells was more than two times higher in differentiated cultures of viPS(IMR90) and viHUF4 cell lines (4.85 and 5.27%, respectively) relative to hESCs, whereas the baseline percentages of VASA:GFP-positive cells in undifferentiated cultures were similar and very low for all cell lines (vH9 0.64%, vHSF1 0.75%, viPS(IMR90) 0.49%, viHUF4 0.78%; Fig. 3B).

Although western analysis suggested a similar expression of VASA between all cell lines after 7 days of differentiation, the percentage of VASA:GFP-positive cells was higher for iPSCs relative to hESCs suggesting that although the expression level of VASA was similar, the number of germ cells may be different. The western results also indicated a higher expression of VASA for undifferentiated iHUF4 cells; however, no difference was detected in the numbers of VASA:GFP-positive cells by FACS. In general, FACS analysis is likely to be more sensitive than western blotting; however, it is also possible that cells express substantially different amounts of VASA protein at different stages of development, and thus numbers may vary as protein levels remain similar.

Following sorting, VASA:GFP-transduced cells were analyzed by immunostaining for the VASA protein and by quantitative RT–PCR for germ cell markers, pluripotency markers and other lineage markers. We observed that sorted GFP-positive cells were positive for VASA by immunostaining and that no VASA immunopositive cells were detected in the GFP-negative population (Fig. 3C). We also detected very low levels of VASA expression by qPCR in both the VASA-positive and the VASA-negative populations, suggesting a differential expression of mRNA and protein for VASA (data not shown). GFP-positive iPSCs had significantly higher expression of germ cell markers than GFP-negative cells including GCNF (germ cell nuclear factor), IFITM1, STELLAR (STELLA-related) and PELOTA; in viHUF4 iPSCs, we also observed significantly higher expression of the meiotic protein DMC1 (distribution of meiotic control 1) and PRDM1A genes (Fig. 3D). The pluripotency markers OCT3/4 and NANOG were also expressed in germ cells with the expression of OCT4 at significantly higher...
levels in viHUF4 GFP-positive cells; NANOG was expressed in both cell lines, as well, but primarily in the GFP-negative population suggesting that the negative population maintained undifferentiated pluripotent stem cells or cells of other early lineages that still maintained NANOG at low levels. All other lineage markers analyzed were enriched only in the GFP-negative populations.

**SC formation by overexpression of DAZL, BOULE and DAZ**

We next tested whether iPSCs possess the ability to initiate and progress through meiosis. For this purpose, we followed previously published protocols to induce meiosis by overexpression of genes of the DAZ gene family and then examined the number and developmental stage of differentiated meiotic cells formed as reported previously (15,23,24). Cells were collected at different time points, up to 14 days after transduction and co-immunostained for SCP3, a component of the SC in meiotic prophase I, and CENtromeric Protein A (CENP-A), a component of the centromere. We observed that the majority of the cells did not have detectable SCP3 staining, indicating that the cells either had not entered meiosis or had already completed meiosis. However, a subset of cells in all the pluripotent stem cell lines had punctate SCP3 staining, a pattern corresponding most closely to the zygotene, pachytene or...
diplotene stages of prophase I (Fig. 4A). The SCP3 staining overlapped with CENP-A and DAPI staining, indicating colocalization to meiotic chromosomes. After overexpression of the DAZ family genes, all lines had cells that were characterized by both punctate and elongated SCP3 staining; we termed cells that were transduced to overexpress DAZ family genes as follows: diPS(IMR90), diHUF4, dH9 and dHSF1. We observed that the diPS(IMR90) cell line had a similar percentage of punctate SCP3 staining relative to both dH9 and dHSF1 cell lines (7.13, 9.36 and 7.88%, respectively) and a similar percentage of elongated staining pattern relative to dHSF1 cells (4.63 and 4.13%, respectively; Fig. 4B). The diHUF4 cells also had a greater percentage of cells with punctate staining (20.86%) compared with all other cell lines, but a
similar percentage of elongated staining relative to dH9 cells (1.23 and 0.75%, respectively).

We further focused on SCP3 staining in undifferentiated cells and cells differentiated with BMPs for up to 14 days, in the absence of overexpression of the members of the DAZ gene family. We were surprised to observe a relatively high percentage of punctate staining for all pluripotent stem cell lines, including undifferentiated HSF1 and iHUF4 cells (19.5 and 23.38%) and remarkably a rare cell with elongated SCP3 staining in both iPSC(IMR90) and iHUF4 undifferentiated cultures (Fig. 4B and C, Supplementary Material, Fig. S2). To examine whether the high percentage of SCP3 staining in these undifferentiated cultures resulted from the presence of cells that had spontaneously differentiated, we manually segregated colonies into those that were morphologically most similar to undifferentiated hESCs and those that were more likely to contain differentiated cells especially along the perimeters. We then observed a substantial decrease in punctate staining patterns with punctate staining reduced to 2.5% of H9 cells, 5% of HSF1 cells, 1.5% of iPSC(IMR90) cells and 3% of iHUF4 cells; moreover, no elongated staining was detected. When we examined whether the differentiation of meiotic cells can be induced with only BMPs, without transduction of DAZ gene family members, we observed that the percentage

Figure 4. Meiotic progression of iPSCs and hESCs. Meiotic spreads were prepared from undifferentiated cells, cells differentiated with supplementation of BMPs up to 14 days and cells with exogenous overexpression of human DAZ gene family members and then differentiated with BMPs up to 14 days. The meiotic spreads were analyzed by immunostaining against SCP3 (red), CENP-A (green) and DAPI (blue). (A) Meiotic spreads were classified as punctate or elongated SCP3 staining patterns, corresponding to the early leptotene stage (punctate) and the later zygotene, pachytene and diplotene stages (elongated) of meiotic prophase I. Representative images are shown for both groups. (B) Meiotic spreads from dH9, dHSF1, diPS(IMR90) and diHUF4 cell lines were classified as punctate or elongated SCP3 staining patterns and quantified by counting 800 cells from differentiated samples and 200 cells from undifferentiated samples. With overexpression, punctate and elongated SCP3 staining was detected in all cell lines; diPS(IMR90) cell line had both punctate and elongated staining percentages similar to dHSF1, diHUF4 cell line had a similar percentage of elongated staining to dH9 cells, but a high percentage of punctate staining compared with all cell lines. Differentiated cells without overexpression had no elongated staining in any of the cell lines. However, rare cells with elongated staining were detected for both iPSC lines in undifferentiated cell cultures (W). When only morphologically good colonies were manually picked from undifferentiated cultures (G), no elongated staining was seen and the percentage of punctate staining decreased compared with whole culture (W). (C) Representative images are shown for undifferentiated and overexpressed cells. Some cells with punctate staining were observed for the undifferentiated H9 and HSF1 cells, for iPSC(IMR90) and iHUF4 cell lines elongated SCP3 staining was also detected. Elongated staining was observed for all cell lines after overexpression. Scale bar 10 μm (A and C).
of punctate staining varied and either slightly increased compared with undifferentiated whole cultures as in H9 (6.3%), remained similar as in HSFL1 (18%) and IPS(IMR90) (4.8%) or decreased as in iHUF4 (10.4%). Notably; however, there was no elongated staining detected for any of the cell lines with BMP induction alone. Together, these results indicate that although the overall morphology of undifferentiated cultures of pluripotent stem cells (both hESCs and iPSCs) may appear indicative of the undifferentiated state, some cells most likely can transit to a germine fate. Moreover, the addition of BMPs does not substantially increase the number of meiotic cells, whereas exogenous gene expression could promote both initiation and progression from punctate to elongated complex formation. Finally, we observed that both iPSC lines had cells with rare elongated staining in undifferentiated cells, in contrast to hESCs where elongated structures were never observed.

The observed elongated SCP3 staining was similar to previously published staining for hESCs and clinical samples of developing human oocytes (15,25). The nuclei of dH9, dHSFL1 and diPS(IMR90) cells had multiple SCP3-positive strings of different length and thickness; however, the nuclei of diHUF4 cells had a different staining pattern, with fewer and thicker SPC3-positive complexes (Fig. 4C). This, together with the relatively low percentage of elongated staining and high punctate staining in diHUF4 cells, may indicate that these cells might be arrested at early stages of meiotic prophase I.

Haploid cell population analysis

To determine if the overexpression of DAZ gene family members in iPSCs can promote completion of meiosis and generation of haploid cells, FACS was used to isolate haploid cells by DNA content analysis, after transduction and 14 days of differentiation. FACS parameters of haploid cells (1N) were set by using human semen sample procured from an IVF clinic as a control (Fig. 5A). We observed that a small percentage of cells had 1N DNA content in a similar level between all cell lines [diPS(IMR90) 1.6%, diHUF4 1.66%, dH9 1.5% and dHSFL1 1.96%]. A similar percentage of cells (1.34%) was also observed for non-transduced HSFL1 cells in the 1N gating parameter. Although there was no clear peak detected by FACS, the 1N DNA content cells were sorted and further analyzed with the same gating parameter being used for pluripotent stem cells as the control sperm sample.

We examined the DNA content of 1N sorted population by FISH staining using a probe against chromosome 16 (Fig. 5B). Cells with a single copy of chromosome 16 were detected for all cell lines in the sorted putative 1N population [dH9 19%, dHSFL1 21%, diPS(IMR90) 14%, diHUF4 13%; Fig. 5B and C]. Because of the low efficiency of haploid cell generation, it was not possible to design gating capable of retrieving a pure population of 1N cells; instead the 1N population was enriched but also contained 2N cells and debris. For isolated non-transduced HSFL1 cells, no cells with single chromosome 16 were observed, confirming that meiosis was induced by overexpression of DAZ genes in iPSCs as in hESCs (15).

We further analyzed the same populations of 1N cells by ACROSIN staining, a marker of the acrosome in spermatids. We observed distinct perinuclear localization of ACROSIN in the 1N population for all cell lines, including the karyotypically XX lines dH9 and diPS(IMR90) (Fig. 5D). It has been previously shown that human germ cells differentiated in vitro express both male and female genetic programs regardless of their karyotype (13). We observed that 35–72% of cells in the 1N sorted population were positive for ACROSIN (Fig. 5E), which was higher than the percentage of haploid cells in the population, suggesting that ACROSIN may be expressed broadly in meiotic cells before haploidy. This is further indicated by the ACROSIN-positive cells observed in the non-transduced HSFL1 cells, where no haploid cells were detected. However, the percentage of positive cells for the non-transduced HSFL1 cells was lower compared with the transduced dHUF1 cells (31 and 72%, respectively), further indicating the induction of meiosis by overexpression of DAZ genes. Further studies of the ultrastructure of the ACROSIN-positive cells via electron microscopy will aid in diagnosing the functionality of the cells.

DISCUSSION

Human germ cell development is poorly understood with most of our knowledge derived by extrapolation of studies in mice. Nonetheless, although human germ cell development undoubtedly shares similarity to that of the mouse given their similarities in embryology, it is also clear that genetic requirements for human germ cell development are unique. This is illustrated by a number of observations. First, there are several Y chromosome genes, including the DAZ genes, that are absent in mice (26–29). Thus, understanding their role and that of related homologs, such as DAZL and BOL whose functions appear to overlap at least minimally, is difficult (or impossible) on a genetic background that lacks key regulators. Second, similarly in the case of X chromosome, women require two X chromosomes for oocyte development, whereas mice are fertile with just a single X (30–32). Third, it has been observed that reproductive genes and proteins may evolve rapidly (33,34). Indeed, the human genes, STELLAR, GDF3 and NANOG, encode examples of extremely divergent proteins that are expressed in germ cells. Mice and human Stella homologs are just 30% identical at the amino acid level and have distinct differences in expression (14,35). Fourth, it is clear that humans are remarkably fertile compared with other species, with nearly half of the infertility cases linked to faulty germ cell development (36). Finally, humans are remarkably imprecise in carrying out some key aspects of germ cell development that are reportedly the most highly conserved. For example, meiotic chromosome missegregation is rare in most model organisms. In the common yeast, chromosome missegregation occurs in ~1/10 000 cells. In flies, missegregation occurs in 1/1000 to 1/2000 cells and in mice in ≤1/100. In humans, meiotic chromosome missegregation occurs in 5–20% or more of cells depending on sex and age (37). Thus, there are fundamental differences in the genetics, biology and pathology of human germ cell development, compared with model organisms, that merit studies of human germ cell development per se.
Yet, given the timeline of human germ cell development in vivo in the first trimester, it has not been possible to probe gene function on a human genome background.

Recently, the differentiation of human embryonic stem cells (hESCs) has been used to probe gene function in human germ cell development (15). In these studies, human germ cell development was assessed following silencing and overexpressing of genes of the DAZ gene family that encode germ cell-specific cytoplasmic RNA-binding proteins (not transcription factors). Results indicated that human germ cell formation and developmental progression could be modulated by the DAZ gene family with human DAZL shown to function in PGC formation, whereas the Y chromosome homolog, DAZ, and closely related autosomal homolog, BOULE, promoted later stages of meiosis and development of haploid gametes. In spite of these successes, however, further genetic analysis is clearly justified; the use of...
of iPSCs would allow us to take advantage of naturally occurring human genetic variants, including complex deletions and rearrangement, for further analysis.

In this study, we show that human adult and fetal somatic cell-derived iPSC lines can differentiate to PGCs in a similar manner to hESCs, with some differences noted. In addition, we observed that like hESCs, germ cells differentiated from iPSCs entered meiosis, a functional marker of germ cell formation and differentiation, when DAZ family proteins were overexpressed. Furthermore, data indicate that the iPSC lines can differentiate to haploid cells with characteristic staining of ACROSIN for spermatid. With these results, we suggest that iPSCs may provide a useful platform for the study of human germ cell development and infertility defects. Indeed, our data indicate that germ cell differentiation may occur more spontaneously in iPSCs than in hESCs. This may be linked to the process of reprogramming, enhanced expression of pluripotency markers or a preferential differentiation to germline rather than somatic cell derivatives.

Finally, we note that although a major cause of infertility is the production of few or no germ cells, often associated with meiotic defects, today’s treatments for infertility are largely ineffective for those with few or no germ cells (38,39). We envision that the production of germ cells from iPSCs may enable direct screening and assay not only of genetic factors but also for chemicals or small molecules that promote germ cell survival or demise. Ultimately, this may contribute to new strategies for the diagnosis and treatment of infertility, a common health problem that affects 10–15% of reproductive-age couples.

MATERIALS AND METHODS

Cell culture

Human ESCs H9 and HSF1, human fetal-derived iPSC line iPS(IMR90) and human adult-derived iPSC line iHUF4 (lentiviral transfection with OCT3/4, SOX2, KLF4 and c-MYC) were used in the experiments. Human ES cells and iPSC lines were maintained on irradiated MEFs in KoDMEM culture medium for ES cells and DMEM/F12 for iPS cells, supplemented with 20% Knockout serum replacer, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 ng/ml of basic FGF (all Invitrogen) and 0.1 mM 2-mercaptopethanol (Millipore). Cells were passaged every 3–5 days using 1 mg/ml of Collagenase IV (Invitrogen). The feeder-free culture was maintained on matrigel-coated plates (BD Biosciences) with culture medium conditioned on MEFs for 24 h. Cells were differentiated on matrigel-coated plates with differentiation medium; KoDMEM supplemented with 20% fetal bovine serum (Hyclone Laboratories), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptopethanol and 50 ng/ml of BMP-4, -7 and -8b (R&D systems).

Western analysis

Adherent cell cultures were incubated with TrypLE (Invitrogen) for 10 min and collected by scraping. Cells were washed twice with PBS and resuspended with RIPA buffer (Sigma) plus 2 × protease inhibitors (Complete Mini, Roche). Sample tubes were rocked for 20 min and centrifuged for 20 min at 16 000 g at +4°C. Supernatant was measured for protein concentration and 35 µg was denatured at a 1:1 ratio with 2 × Laemmli buffer at 95°C for 5 min, then loaded onto 10% SDS–PAGE gel. The gels were run at 150 V for 70 min (DAZL) or 85 min (VASA) and transferred to a PVDF membrane for 1 h at 100 V in CAPS buffer (10 mM CAPS, 10% methanol, pH 11). Transferred blots were blocked in 5% non-fat milk for 1 h at room temperature. The blot was incubated overnight at +4°C with primary antibody [1:500 for anti-VASA (Abcam or R&D Systems), 1:500 anti-DAZL-1 50] (prepared by the lab), 1:10 000 anti-GAPDH (Abcam), followed by two rinses and 3 × 5 min washes in 0.1% Tween-20 (Sigma-Aldrich) in PBS, pH 7.5. Secondary antibody [1:10 000 anti-rabbit-HRP conjugated (Amersham)] was incubated for 1 h followed by the same washes. ECL+ (Amersham) was used to detect the HRP signal on film.

Immunostaining of fixed cells

Cells were fixed with 4% paraformaldehyde (USB corporation) for 15 min and washed 3 × 5 min with PBS (Invitrogen). Cells were then permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 30 min and blocked with 4% chicken serum (Sigma-Aldrich) in PBS for 30 min. Rabbit polyclonal anti-VASA primary antibody (Abcam) was used with 1:200 dilution and incubated overnight at +4°C. Cells were washed 3 × 5 min with 0.1% Tween-20 in PBS. Chicken anti-rabbit Alexa 488 secondary antibody (Invitrogen) was used with a dilution of 1:300 and incubated for 1 h at room temperature. Cells were washed again as before and 1 ng of DAPI (Sigma Aldrich) was added to the cells.

VASA:GFP transduction and flow cytometry

Undifferentiated cells were transduced on matrigel with a lentiviral vector carrying GFP gene under the VASA promoter (15) for 6 h at +37°C. Conditioned medium was added 2:1 and cells were further incubated overnight. Virus supernatant was washed off four times with PBS, and the selection was started the following day with geneticin (Invitrogen) for 7 days. Cells were passaged to MEFs and then moved back to matrigel followed by differentiation with BMPs. Cell cultures were incubated with 1 mg/ml of Collagenese IV for 10 min and with TrypLE (Invitrogen) for 10 min and collected by scraping. Cells were washed three times by medium exchange and centrifugation. Single cell suspension was obtained filtering cell solution through a 40-µm cell strainer (BD).

Gene expression analysis

Total RNA was prepared as described in the RNeasy Mini Kit (Qiagen) with on-column DNase I digestion. About 1 µg of total RNA from each sample was used for random primed reverse transcription, which was carried out as described in the product protocol (SuperScript™ III First-Strand Synthesis System for RT-PCR, Invitrogen), and 1.25 µl of the cDNA was preamplified using 96 different 0.2× Taqman assays (Applied Biosystems, Carlsbad, CA, USA) as primers in a reaction; 5 µl 2× buffer (from CellsDirect™ One-Step qRT-PCR...
DNA content staining for FACS analysis

Cells were collected as described above and fixed with 70% ethanol for 1 h at room temperature. Cells were then incubated in a staining solution [0.1% Triton X-100, 0.2 mg/ml of RNase A and 0.02 mg/ml of propidium iodide (Invitrogen)] for 15 min at +37°C. The cell suspension was used for FACS.

Fluorescent in situ hybridization

Sorted cell populations were collected by cytopsin at 180g for 3 min. Slides were fixed with Carnoy’s fixative (1:3 acetic acid:methanol) for 5 min and air-dried. The slides were then dehydrated in ice-cold 70, 80 and 100% ethanol, 2 min each and air-dried. FISH probe against chromosome 16 (Vysis) was denatured on slides at 85°C and hybridized at 37°C overnight. Slides were then washed with 2× SSC and 0.1% SDS in 2× SSC at 50°C, 5 min each. Prolong Gold anti-fade with DAPI was applied to slides.

ACROSIN staining

Sorted cell populations from DNA content analysis were cytopsin as described above. The slides were fixed with 4% paraformaldehyde for 15 min and then incubated with 1% Triton X-100 for 15 min, followed by washes with 0.1% Tween-20. Slides were blocked with 4% goat serum for 1 h at room temperature and stained with antibody against ACROSIN (Santa Cruz) diluted 1:50 with 1% goat serum at 4°C overnight. Slides were washed and goat anti-rabbit Alexa 488 (Invitrogen) secondary antibody was applied at 1:1000 for 1 h at room temperature, followed by washes and mounting as described above.

Statistical analysis

The statistical analysis for gene expression results for BMP-induced differentiation was performed with one-way ANOVA, followed by the Bonferroni post-test. All four cell lines were compared within each time group; undifferentiated and 7 and 14 days differentiated cells for each gene separately. Significance was accepted at $P < 0.05$. The statistical analysis for the gene expression of GFP-positive and GFP-negative population was performed with an unpaired t-test. Significance was accepted at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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