Spontaneous differentiation of germ cells from human embryonic stem cells in vitro

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Little is known of molecular requirements for specification of human germ cells. However, it is likely that they are specified through the action of sequentially expressed genes just as in model organisms. We sought to determine whether human embryonic stem (ES) cell lines, like those of mice, might be capable of forming germ cells in vitro. We compared transcriptional profiles of three pluripotent human ES cells to those of isolated inner cell mass (ICM) cells. We found that ICM cells expressed NANOS1, STELLAR and OCT4, whereas undifferentiated human ES cells expressed these genes along with the germ cell-specific gene, DAZL. Upon ES cell differentiation into embryoid bodies (EBs), we observed a shift in expression from RNA and protein markers of immature germ cells to those indicative of mature germ cells, including expression of VASA, BOL, SCP1, SCP3, GDF9 and TEKT1. Although ability to test the function of these putative VASA positive germ cells is limited, these results demonstrate that differentiation of human ES cells into EBs in vitro results in formation of cells that express markers specific to gonocytes.

INTRODUCTION

Two divergent developmental programs are associated with specification of the germ cell lineage in model organisms. In non-mammalian species of fruitflies, nematodes and frogs, germ cells of both males and females are specified via the inheritance of germ plasm, microscopically distinct oocyte cytoplasm that is particularly rich in RNAs and RNA-binding proteins and segregates with cells destined to be germ cells (1,2). Some of the RNAs and RNA-binding proteins such as Pumilio, Nanos and Dazl are highly conserved between organisms that specify germ cells via germ plasm inheritance, and those that form germ cells independently of germ plasm, such as mammals (2–5).

Fate mapping studies have been used to examine germ cell specification in mammals and have revealed that germ cells are specified in the proximal epiblast in mice (6), in response to signals from the neighboring extraembryonic ectoderm, in particular Bmp4 signaling (7). However, it is notable that the proximal epiblast is not predestined to a germ cell fate since transplantation of the distal epiblast to contact the extraembryonic ectoderm also results in germ cell formation (6). Furthermore, the fate of proximal epiblast cells is ultimately to form both germ cells and extraembryonic mesoderm. Thus, it is likely that the extraembryonic ectoderm provides one of the first signals for germ cell specification in the epiblast, and then, a second as yet uncharacterized signal is required to distinguish extraembryonic mesoderm from germ cells. Germ cells are first recognized following gastrulation, at 7.2 days post coitum (dpc), as an extraembryonic cluster of cells at the base of the allantois that express tissue non-specific alkaline phosphatase (TNAP), Oct4 and stella (8–11). Although epiblast cells migrate through the primitive streak during gastrulation, the physical act of migration does not appear to be necessary for defining germ cell versus somatic cell fates (12–14).

The embryological period equivalent to mouse E5.5–E7.2 in human embryo development occurs shortly after implantation. Thus, the analysis of human germ cell specification in vivo is impracticable due to ethical considerations regarding research during this period. However, recent studies in mice have shown that embryonic stem (ES) cells (derived from the inner cell mass of the blastocyst prior to epiblast formation) are capable of differentiating into female and male germ cells in vitro (15,16). Oocyte differentiation from mouse ES cells was obtained via spontaneous differentiation of adherent cultures, as indicated by analysis of germ cell-specific markers such as

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Vasa, Gdf9 and Scp3, and as corroborated by analysis of morphology and follicular steroidogenic enzyme production (15). Male germ cell differentiation was also demonstrated via differentiation of mouse ES cells into embryoid bodies (EBs) and analysis of germ cell-specific markers (16). Putative PGCs (primordial germ cells) derived from the ES cells were then transplanted into adult testes where they formed cells with morphological characteristics of sperm (16). In addition, while this manuscript was under review, it was further demonstrated that the imprinting status of genes such as Igf2 was appropriate for putative PGCs and that haploid male gametes derived from ES cells were subsequently capable of fertilizing oocytes in vitro and subsequent development to blastocyst stage (17). Taken together, these studies indicated that mouse ES cells are capable of spontaneously forming germ cells in vitro. In contrast, it is not known whether human ES cell lines possess the ability to contribute to the germ cell lineage. Indeed, one might wonder if this ability might be limited given that all human ES cell lines have been derived from embryos donated by infertile couples. Thus, we sought evidence that human ES cells could contribute to germ cell formation in vitro by differentiating three human ES cell lines to EBs and assessing expression of RNA and protein markers diagnostic of germ cell development. Markers examined in studies of mouse ES cell differentiation to the germ cell lineage were included in all experiments (15–17).

RESULTS
Many studies in both mice and humans have shown that ES cells have the capacity to proliferate and to differentiate to numerous cell lineages. Using in vitro studies, mouse ES cells have been shown to differentiate into lineages that include hematopoietic, vascular, pancreatic, neural, muscular and germ cells (15–26). Likewise, human ES cells have also been shown to differentiate to several lineages in vitro, including neural, pancreatic, muscular, endothelial, trophoblast and hematopoietic cells (27–36). In all of these studies, a combination of approaches, including analysis of molecular markers specifically expressed in relevant cell types and morphological and/or histochemical approaches, was used to define differentiated cell types. Therefore, in order to identify germ cells in different stages of formation and differentiation during human EB differentiation, we compiled a list of markers that are germ cell-specific (not expressed in somatic lineages), and germ cell-enriched (highly expressed in germ cells with limited expression in somatic cells). From this list, we generated a profile of the expected sequential expression of markers that would define development of the germ cell lineage during ES cell differentiation in vitro (Fig. 1).

Pre-meiotic germ cell markers are expressed in human ES cells
We began our studies by examining gene expression in three lines of undifferentiated ES cells that were either cytogenetically 46;XX (HSF-6 and H9) or 46;XY (HSF-1). As expected from previous reports (37,38), we observed consistently high expression of the human OCT4 gene in all three human ES cell lines (Fig. 1; Table 1). This assured us that our starting population of cells contained pluripotent cells since OCT4 expression is restricted to pluripotent ES cells and germ cells (10.39). Then, we examined expression of additional embryonic stem cell and/or germ cell-specific and germ cell-enriched genes in order to establish baseline profiles of expression of genes reported in human ES cells and/or human germ cells. These genes included the GDF3 (growth and differentiation factor 3), STELLAR (STELLA-related), NANO (the human homolog of mouse nanog), NANOS1, DAZL (deleted in azoospermia-like), VASA, cKIT, PUM1 (PUMILO 1), PUM2 (PUMILO 2), SCP1 (synaptosomal complex protein 1), GDF9 (growth and differentiation factor 9), and TEKT1 (TEKTIN 1) genes. Of these genes, we found that the GDF3, NANO, and STELLAR genes were expressed in undifferentiated ES cells, as previously reported (38). In addition, we also observed expression of the germ cell-specific gene DAZL in all three lines of undifferentiated human ES cells, as well as the germ cell-enriched genes cKIT (a marker of pre-meiotic migrating germ cells), NANOS1, PUMI, and PUM2 (markers of pre-meiotic germ cells of the fetal gonads; Fig. 2A, Table 1). In contrast to these genes, whose expression is diagnostic of pre-meiotic germ cells, we did not observe expression of genes that are known to be restricted in expression to later stages of germ cell differentiation. Thus, we
Table 1. Real-time PCR in undifferentiated human ES cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>HSF-1 (×10^3)</th>
<th>H9 (×10^3)</th>
<th>HSF-6 (×10^3)</th>
<th>P-value (significance &lt; 0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCAM1</td>
<td>7.92± 8.8</td>
<td>2.21± 1.6</td>
<td>1.86± 1.1</td>
<td>0.34</td>
</tr>
<tr>
<td>KDR</td>
<td>1.80±1.3</td>
<td>6.48±4.7</td>
<td>4.88±4.2</td>
<td>0.36</td>
</tr>
<tr>
<td>AFP</td>
<td>0.437±5.3</td>
<td>1.84±3.1</td>
<td>3.95±0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>OCT4</td>
<td>67.2±20</td>
<td>293±119</td>
<td>304±491</td>
<td>0.56</td>
</tr>
<tr>
<td>GDF3</td>
<td>1.06±0.1</td>
<td>5.55±19</td>
<td>0.288±0.4^b</td>
<td>0.005*</td>
</tr>
<tr>
<td>STELLAR</td>
<td>4.03±5.0</td>
<td>1.26±1.9</td>
<td>0.126±0.1</td>
<td>0.355</td>
</tr>
<tr>
<td>NANO1</td>
<td>11.1±2.4</td>
<td>35.5±21</td>
<td>2.76±2.3</td>
<td>0.041</td>
</tr>
<tr>
<td>NANOS</td>
<td>1.50±1.6</td>
<td>0.66±0.6</td>
<td>0.681±0.4</td>
<td>0.54</td>
</tr>
<tr>
<td>VASA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>DAZL</td>
<td>0.083±1.4</td>
<td>0.455±0.7</td>
<td>0.398±0.2</td>
<td>0.83</td>
</tr>
<tr>
<td>KIT</td>
<td>1.53±0.1</td>
<td>4.71±7.6</td>
<td>1.08±0.2</td>
<td>0.58</td>
</tr>
<tr>
<td>PUM1</td>
<td>11.0±5.0^b</td>
<td>35.7±6.9^a</td>
<td>4.82±4.2^a</td>
<td>0.001*</td>
</tr>
<tr>
<td>PUM2</td>
<td>10.1±7.8</td>
<td>67.3±83.7</td>
<td>25.5±28.0</td>
<td>0.42</td>
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<tr>
<td>SYCP1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>GDF9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>TEKTIN1</td>
<td>0</td>
<td>0</td>
<td>0.0000673±0.000011</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Mean normalized expression of genes in HSF-1, H9 and HSF-6 calculated by REST (72). Significance was calculated by ANOVA for each gene followed by two tailed r-test. Significance (P<0.01) between a-b and a-c. ND, 'not done'.

Although western blot analysis provided a general profile of protein expression, it did not reveal expression of proteins in undifferentiated ES cells at the single cell level. Therefore, we used immunohistochemical methods to examine expression of proteins in undifferentiated ES cells (Fig. 3). By confirming that colonies of ES cells stained positively for the cell surface markers SSEA-3 and TRA-1-81, which are known to be expressed only in undifferentiated ES cells (Fig. 3A). We then examined expression of the markers STELLAR, DAZL, VASA, KDR, AFP and NCAM1. We observed that STELLAR protein was localized to the majority of ES cells in most colonies where it was present in both the cytoplasm and nucleus (Fig. 3B and C). Analysis of DAZL protein distribution indicated a distribution similar to that of STELLAR; it was detected in the majority of cells in a given ES cell colony (Fig. 3D and E). In contrast to the expression of the STELLAR and DAZL proteins in the majority of cells in most ES cell colonies, expression of VASA was not detected in any undifferentiated human ES cell colonies (data not shown). Also, in contrast to the expression of STELLAR and DAZL, protein expression of the somatic markers KDR and AFP was only rarely seen and then it was confined to cells at the edges of ES cell colonies (Fig. 3F and G). Notably, we could not detect NCAM1 protein in undifferentiated ES cells (Fig. 3H); this was in spite of the fact that the RNA was clearly detected, as indicated above (Fig. 2). Only with differentiation of ES cells to form EBs, followed by adherent culture for 5 days, was the expression of NCAM1 in the cell cytoplasm of differentiated ES cells observed (Fig. 3I). We confirmed and extended these immunofluorescence results with more sensitive immunohistochemistry. We noted that DAZL and STELLAR were present in the majority of undifferentiated ES cells (Fig. 3J and K), but were not detected in every cell (see insets of Fig. 3J and K). As noted above with immunofluorescence, VASA expression was also undetectable in undifferentiated human ES cells by immunohistochemistry (Fig. 3L).

Expression of germ cell markers in the human inner cell mass

Expression of STELLAR and DAZL mRNA and protein in the majority of human ES cells in undifferentiated colonies was unexpected. In particular, in diverse model organisms that include species of flies, worms, frogs, fish, salamanders, mice and non-human primates, as well as in humans, the expression of DAZ gene family homologs has been shown to be restricted entirely to the germ cell lineage (44,46–63). Thus, the observation that premeiotic germ cell genes, including DAZL, were expressed in undifferentiated human ES cells raised several possibilities: First, the expression of these genes may simply reflect random, unregulated gene expression. However, if that were the case, we might have also detected random expression of other germ cell markers such as VASA, SCP1, SCP3, BOULE, GDF9 and TEKT1. Yet, this was not the case. We observed the...
expression of all the premeiotic germ cell markers that we assayed but none of the meiotic and postmeiotic germ cell markers at both the mRNA and protein levels. Second, it is possible that human ES cells and germ cells share aspects of a common molecular program and therefore express similar genes. We could not discount this possibility based on our data. In fact, expression analysis of genes such as OCT4, NANOG, STELLAR and GDF3 lends further credence to this possibility (38,39). Third, expression of genes such as DAZL may indicate spontaneous differentiation of a subpopulation of ES cells into germ cells. Supporting this possibility was the observation that undifferentiated human ES cells also expressed detectable levels of mRNA markers of the differentiated ectoderm, mesoderm and endoderm somatic lineages [neural cell adhesion molecule 1 (NCAM1), alphafetoprotein (AFP) and tyrosine kinase receptor (KDR), respectively; Fig. 2A; Table 1]. In order to address which of these possibilities was most likely, we compared the expression of several genes in the ICM to that of the undifferentiated human ES cell lines (Fig. 4, A–C). Notably, we found that human ICM cells expressed the gene STELLAR at consistently higher levels than OCT4 (Fig. 4A; Table 2). Furthermore, we also found that ICM cells expressed NANOS1 but not the somatic marker NCAM1, the premeiotic germ cell markers DAZL and later germ cell markers such as VASA or SCP1 (Fig. 4A). Owing to limited availability of human ICMs for analysis, additional markers could not be assayed. Nevertheless, these results illustrated that undifferentiated human ES cells differ in regard to DAZL and NCAM1 transcription compared to their in vivo counterpart ICM cells (Fig. 4A and B). These results also suggest that removal of the ICM from the blastocyst and subsequent culturing on mouse embryonic feeder cells (MEFs) resulted in transcription of DAZL and NCAM1 in ES cells, markers which are normally not expressed in the ICM of blastocysts. Therefore, if we focus on differentiation towards a germ cell-specific pathway, removal of the ICM from blastocysts may result in spontaneous differentiation of cells towards the germ cell lineage based on the expression of the germ cell-specific gene DAZL. However, the next in vivo temporal marker of germ cell differentiation, VASA, is not expressed.

Meiotic and post-meiotic germ cell differentiation of human ES cells in vitro

We noted above that undifferentiated ES cells express STELLAR and DAZL in the majority of cells in a given colony. Given that, in vivo, DAZL has only been shown to function in germ cell formation and/or maintenance, we sought to determine whether germ cell differentiation could be achieved with embryoid body (EB) formation in vitro. Under differentiation conditions, genes expressed exclusively or predominantly in pluripotent cell types are expected to decrease whereas markers of the somatic lineages are expected to increase as cells of the somatic lineages differentiate. Indeed, that was the case; the markers, OCT4, STELLAR, NANOG and GDF3 decreased with differentiation, whereas NCAM1, AFP and KDR increased (Fig. 5). Unexpectedly, however, by day 14 of differentiation, expression of both the NANOS1 and DAZL genes decreased dramatically as well. Conversely, there was a sharp increase in expression of the later germ cell lineage markers including the gonocyte marker VASA, the meiotic marker, SCP1, and postmeiotic markers, GDF9 and TEKT1 as EB differentiation progressed (Fig. 5). These results largely parallel those of mice (15–17). However, in contrast to studies in mice, we observed
that human germ cells differentiated in vitro expressed both the male and female genetic programs regardless of whether they were karyotypically XX or XY (Fig. 5). Expression of both GDF9 (an oocyte specific gene) and TEKT1 (a spermatid specific gene) was noted with differentiation of all human ES lines. Since transcriptional profiles indicated that VASA-positive putative germ cells formed with ES cell differentiation, we sought evidence that proteins diagnostic of germ cells might also be expressed in EBs. At days 14 and 21 of culture, EBs were sectioned and stained for the VASA, STELLAR and DAZL proteins via immunohistochemistry. We observed that VASA positive cells were most frequently observed in clusters, at the edges of EBs and throughout small sections of EBs (Fig. 6A and B). We noted that not all cells in a given EB were positive for VASA, a finding consistent with our expectation that somatic cell differentiation was occurring in parallel. We observed that STELLAR-positive cells were more frequent than VASA-positive cells, and were located throughout the EBs, in clusters within EBs, and especially in cells lining the edges of EBs (Fig. 6E–G). As was noted for VASA-positive cells, not...
expression frequently and throughout the EB where the protein was localized predominantly in the cytoplasm (Fig. 8E and F), but also in punctate SCP3-positive nuclear structures (Fig. 8G and H). However, we did not detect MLH1 protein expression even after 22 days of differentiation of EBs.

**DISCUSSION**

**The use of gene expression to assess differentiation of human ES cells**

Human ES cell lines are the in vitro manifestation of cells of the ICM derived from blastocysts. Previous reports have demonstrated that human ES cells are pluripotent and capable of differentiating into a wide variety of somatic cell types during EB formation (64). However, germ cell differentiation from human ES cells has never been reported. In this study, we used a panel of germ cell-specific and germ cell-enriched markers, together with markers of somatic cell lineages, to assess the ability of three lines of undifferentiated human ES cells to form germ cells in vitro. Several lines of evidence suggest that this is a valid approach to assay germ cell development. In particular, this approach has been used successfully to diagnose both female and male germ cell development from ES cells in the mouse (15,16). Indeed, in the case of male germ cells, sorting for differentiated ES cells that expressed VASA and transplantation of these VASA-positive cells into the testis of recipient mice results in the formation of mature sperm in vivo. In contrast, transplantation of unsorted ES cells into the testis of recipient mice resulted in teratoma formation from the ES cells (16). This work provided evidence that VASA-positive ES cells are germ cell precursors in vitro.

There is a widespread belief that ES cells express many, if not all genes promiscuously. If this were the case, then cell-specific marker analysis would be an inappropriate means to monitor cell differentiation. However, several reports have documented that ES cells express approximately the same percentage of genes as other cell types or perhaps, just slightly more. Approximately 20–30% of genes are expressed in stem cells in contrast to 10–20% in most somatic cells (65–69). These reports would indicate that ES cells do not express all genes in a promiscuous manner. Further, in the current study we also noted that the pattern of gene expression in the germ cell lineage pathway is not random; it begins with expression of known early germ cell-specific genes and proceeds to that of later stage germ cell-specific markers. Finally, in the current study, we connected transcription of germ cell-specific genes to translation. No cell types, other than germ cells, are known to express proteins such as DAZL, VASA, SCP3, GDF9 or TEKT1 in evolutionarily divergent organisms. These observations further support the concept that sequential germ cell gene expression upon differentiation of human ES cells recapitulates legitimate germ cell programs, just as in the case of somatic cell differentiation. Thus, we conclude that undifferentiated human ES cells expressed markers of pre-meiotic germ cells that were not expressed in isolated ICMs. Furthermore, we observed that with ES cell differentiation into EBs, expression of the early gonocyte specific marker VASA was initiated together with expression of SCP3; neither of these genes were...
expressed in undifferentiated ES cells or the ICM. Taken together, our results suggested that gonocyte-like germ cells that expressed VASA and other germ cell specific genes are specified during human ES cell differentiation in vitro.

ES cells express markers of pre-meiotic germ cells
The genetic similarity of human ES cells to the ICM has never been addressed. It is difficult to determine a priori whether it would be likely or not for human ES cells and ICMs to differ significantly in gene expression. Comparisons between cell surface markers present on human ES cells and human ICM cells indicate that these cell types share common antigenic surface profiles and are positive for markers such as SSEA3 and SSEA4 (70). However, differences have also been noted in that ~20% of cells from undifferentiated human ES cell lines, H7 and H14, are positive for SSEA1, a cell surface marker that is not expressed by cells of the ICM (70). Given this heterogeneity in cell surface markers, it may not be surprising that genes like DAZL and NCAM1 are expressed in ES cells even though they are not expressed in the ICM. However, the difference between expression of other markers such as SSEA1 and expression of DAZL in undifferentiated ES cells is that, while the former are expressed in a minority of colonies and

![Gene expression during differentiation of human embryonic stem cells to embryoid bodies. Shown is quantitative PCR using SYBR green. The mean normalized expression of each gene is shown along the y-axis as a function of days of differentiation as shown on the x-axis. Mean normalized expression is relative to GAPDH.](image-url)
cells, DAZL is expressed in the majority of undifferentiated ES cell colonies of three independently derived ES lines and in nearly every cell within a colony. Thus, since DAZL is expressed in almost all undifferentiated ES cells, we hypothesize that the initial program for germ cell specification has been initiated in most undifferentiated human ES cells. To lend credence to these results, we also demonstrated that undifferentiated ES cells expressed other pre-meiotic genes such as PUM2, NANOS1 and cKIT but consistently did not express germ cell markers indicative of later stages of germ cell differentiation such as the meiotic and post-meiotic germ cell-specific genes VASA, SCP1, SCP3, BOULE, GDF9 and TEKT1. As noted in studies in mice, mRNAs of other PGC-specific genes were also expressed in mouse ES cells prior to their differentiation; however protein expression was not assayed (16,17). In fact, it has been suggested that perhaps mouse ES cells resemble the five-day epiblast more closely than cells of the ICM (71). Our results suggest that human ES cells are likely to be distinct from ICM cells and may be more closely related to epiblast cells or embryonic germ cells (which are the in vitro manifestation of PGCs).

**Figure 6.** Immunohistochemistry of day 14 embryoid bodies. Representative immunohistochemistry of day 14 embryoid bodies from HSF-6 and HSF-1 ES cell lines stained for VASA (A, B), STELLAR (E–G) and DAZL (H–J). Negative controls included omission of primary antibody (C) and pre-immune sera (D). Insets magnify regions of the embryoid body, as well as individual cells, that are stained brown by immunolocalization of each protein product (black arrow heads indicate cells stained positive for each protein whereas white arrow heads indicate negative cells). Scale bars = 100 µm.

**VASA as a marker of gonocyte formation with ES cell differentiation in vitro**

In the current study, one germ cell specific gene that was not expressed in all three lines of undifferentiated ES cells or in the ICM was VASA. Thus, fortuitously, assaying for VASA expression during ES cell differentiation allows for identification of more mature stages of germ cells in humans, just as in mice (15–17). When we compared expression of VASA with that of DAZL and STELLAR, however, we noted that VASA expression increased by day 3 of EB formation. However, VASA-positive cells constituted only a minority of cells within differentiating EBs. This would suggest that DAZL and STELLAR-positive cells, which constitute the majority of undifferentiated human ES cells in a given colony could not contribute exclusively to VASA-positive germ cells. With differentiation, DAZL and STELLAR positive cells were still identified within EBs, and although we were restricted to analyzing adjacent sections, it appears that the proteins co-localized to the same sub-region of the EB. The precise relationship between initial and later populations of DAZL- and
STELLAR-positive cells, and VASA-positive cells, in differentiating EBs is unclear. However, a potential model is as shown in Figure 9. In addition, it should be noted that the number of VASA positive cells is not consistent between different EBs. The reason for this cannot be addressed at this time. However, we hypothesize that extracellular cues or cell-cell interactions during the earliest stages of ES cell lineage restriction may be important in determining the number of VASA positive cells, upon differentiation. In particular, an extracellular source of BMP4 appears to promote additional VASA positive cells from mouse ES cells in vitro (16).

Comparison with germ cell differentiation from mouse ES cells in vitro

Previously, two independent studies demonstrated that mouse ES cells could form germ cells in vitro (15,16). Both groups took advantage of the ease of genetic manipulation of mouse ES cells and generated undifferentiated ES cell lines that could be sorted for a fluorescent GFP marker under the control of germ cell specific promoters. In one study, putative germ cells were selected for GFP positive cells under the control of the germ cell-specific Oct4 promoter (15). In these studies, expression of mouse Vasa in the putative germ-cell population was noted after 7 days of ES cell differentiation. In a second study, putative germ cells were marked by a GFP reporter under the control of the germ cell-specific mvh (mouse vasa homolog) promoter (16). In this study, expression from the vasa locus was first identified after 3 days of EB differentiation (16). Although both of these studies relied upon reporter constructs to potentially capture germ cells as they differentiated, it was noted that germ cell differentiation occurred spontaneously and at an easily observed frequency. In fact, the reporter constructs were not required to diagnose germ cell development. Here, we report results that correlate closely with those of Toyooka and colleagues in that expression of human VLSA was initiated after 3 days of EB differentiation and VASA-positive cells were most often present in clusters rather than dispersed throughout the EB (16). Moreover, in this study, we found that transcription of later stages of germ cell differentiation including that of the meiotic genes SCP1, SCP3, GDF9 and TEKT1 was only initiated upon differentiation of ES cells to EBs. However, we also observed that the fidelity of meiosis, as judged by SCP3 and MLH1 staining, was severely compromised and we did not observe formation of haploid germ cells in vitro. Like the current study, previous studies also identified Gdf9 and Scp3

Figure 7. Percentage of cells positive for STELLAR, DAZL and VASA in day 14 embryoid bodies. The total number of positive cells was counted in the same embryoid body on adjacent sections for STELLAR, DAZL and VASA. Results are expressed as a percentage of cells positive for the test gene out of the total positive cells counted for each embryoid body.

Figure 8. Meiotic protein expression. Control immunohistochemistry of SCP3 (green), and MLH1 (red) in (A) human leptotene spermatocyte, and (B) human pachytene spermatocytes. (C and D) undifferentiated human ES cells stained with DAPI (C) and SCP3 antisera (D). Cells from EBs at days 14 and 21 were stained for DAPI (E–G) to detect nuclear DNA and SCP3 (F and H) to detect chromosomes in meiosis. MLH expression in ES cells was not be detected by immunofluorescence. Scale bars = 25 μm.
expression in differentiating mouse ES cells (15). However, the formation of sister chromatids and the axial alignment of Scp3 along the length of chromosomes was not demonstrated in these studies nor was any evidence of a haploid genome (15). In contrast, in studies of male germ cell development, putative germ cells were transplanted to the testis and thus may have transited meiosis more faithfully than in vitro studies (16). In addition, more recently, a second group has differentiated male gametes in vitro from embryonic stem cells and found that haploid cells form, albeit at low frequency (17). Taken together, results from in vitro studies in mice and our current studies of human ES cells indicated that germ cell differentiation from ES cells to the stage of VASA expression can be achieved by spontaneous differentiation in vitro. However, it seems likely that completion of meiosis in vitro is inefficient at best.

Conclusions

In summary, we have documented the transcriptional and translational events of early human germ cell development in vitro during ES cell differentiation. We propose that undifferentiated ES cells are a heterogeneous population of cells that have already initiated a pre-meiotic germ cell program as indicated by expression of the germ cell-specific gene, DAZL. Following differentiation to EBs, we observed the formation of putative germ cells that expressed the germ cell specific markers, VASA, SCP1, SCP3, BOULE, TEKT1 and GDF3. However, we did not observe intact synaptonemal complexes with recombination nodules and suggest that completion of human meiosis in vitro might be promoted, more efficiently and correctly, by additional germ cell factors and/or somatic cell interactions that are native to the in vivo niche.

MATERIALS AND METHODS

Human ES cell culture

Information regarding the human ES cell lines, HSF-6, HSF-1 and H9 (NIH codes UCO6, UC01 and W-9, respectively) can be obtained at http://stemcells.nih.gov/stemcell/. Undifferentiated human ES cell colonies were cultured on irradiated CF1 mouse embryonic fibroblast feeder cells at 5% CO2 in supplemented DMEM medium as previously described (38).

RNA and cDNA production

At days 0, 3, 7, 14 and 22, EBs were collected, centrifuged and resuspended in 600 ml RLT buffer (Qiagen) for RNA extraction. Total RNA was extracted via the RNeasy system (Qiagen) from three independently isolated samples from each of the three different human ES cell lines used in the current analysis. cDNA was generated from 3 µg of total RNA using 250 ng of random hexamers under standard conditions with MIV reverse transcriptase (Promega). PCR was performed with 50 ng of the first strand cDNA reaction. Three inner cell masses (ICMs) were isolated from blastocysts by immunosurgery using antibody against human choriocarcinoma cells and guinea pig complement according to standard procedures. Total RNA from the three independently isolated ICMs was extracted using the PicoPure RNA isolation system (Arcturus) followed by reverse transcription and cDNA production, as above. ICM cell cDNA was concentrated using DNA Clean and Concentrator (ZymoResearch) and resuspended in 15 µl water. cDNA from each ICM was split into eight equal aliquots and used to assay expression of NCAM1, OCT4, NANOS, STELLAR, DAZL, VASA, SCP1 and GAPDH.

Quantitative PCR

Quantitative PCR reactions on human ES cell cDNA were performed in duplicate on each sample in the presence of 4.5 mM MgCl2, 10 mM dATP, dGTP, dCTP and dTTP, 2 µM primers (Table 3), 0.25 U Platinum Taq (Invitrogen), 1 × SYBR green (PerkinElmer), 1 × fluorescent (Biorad) and 2% DMSO. SYBR green PCR amplifications were initiated at 95°C for 5 min followed by 35 cycles of 95°C, 30 s; 60°C, 30 s; and 72°C, 30 s. Quantitative PCR on human ICM cell cDNA was performed using the Assay-on-Demand technology (Applied Biosystems) for NCAM1, OCT4, VASA, DAZL, SCP1 and GAPDH and Assay-by-Design (Applied Biosystems) for NANOS and STELLAR according to manufacturer’s specifications using FAM-490. Results were analyzed using an iCycler IQ™ (Biorad). For SYBR green, equal reaction efficiencies were verified via serial dilution of testis cDNA over a 100-fold range. Mean normalized expression was calculated using REST™ XL software (Relative Expression Software Tool) (72). All experiments included negative controls with no cDNA for each primer pair. Primers were designed to span exons to distinguish cDNA from genomic DNA products.

Western blot analysis

Western blot analysis was performed according to standard procedures (3). Antibody dilutions were rabbit-anti human PUM2 (1/500), rabbit anti-human OCT4 (1/500), rabbit anti-human NANOS (1/500), rabbit anti-human DAZL (149 and 150; 1/500), chicken anti-human VASA (1/500), rabbit anti-human BOULE (1/500) and rabbit anti-human SCP3 (1/5000). OCT4 was purchased (Active Motif).

Hypotonic immunohistochemistry for meiotic markers

Human ES cells at day 0 were digested for 15 min at 37°C with 1 mg/ml collagenase type IV/1 mg/ml dispase (Gibco BRL) in
knockout DMEM high glucose (Gibco BRL) containing 20% knockout serum replacer (Gibco BRL), 1 mM glutamine (Gibco BRL), non-essential amino acids (Gibco BRL), 0.1 mM β-mercaptopethanol (Sigma Chemical) and 4 ng/μl FGF2 (R&D Systems). Colonies were gently dislodged, centrifuged at 1000 rpm, 5 min, and then resuspended in freshly prepared hypoextraction buffer pH 8.2 (30 mM Tris pH 8.2/50 mM sucrose/17 mM citric acid/5 mM EDTA/0.5 mM DTT/0.5 mM Pefabloc; Gibco BRL). EBs and ES cell colonies were then lightly teased apart before further incubation in hypoextraction buffer for 30 min. ES cell colonies and EBs were then collected into 100 mM Sucrose before teasing apart using 20 gage needles to form a single cell suspension. The single cell suspensions were pipetted onto glass slides, previously treated with 1% paraformaldehyde in PBS pH 9.2 containing 0.25% Tween 20. The slides were then in 0.04% photoflo (KODAK) in distilled H2O followed by incubation for 30 min in Antibody Dilution Buffer (ADB) containing 10% Normal Donkey Serum (Jackson ImmunoResearch Laboratories)/3% BSA (Sigma Chemical)/0.5% Tween 20 (Sigma Chemical). Slides were incubated overnight at 37°C with rabbit anti-human SCP3 (a gift from Christa Heyting, Wageningen University) and mouse anti-rat MLH (Ongogene) diluted in ADB at 1/500 and 1/25, respectively. Slides were washed for 10 min in ADB followed by 24 h in ADB at 4°C. Slides were incubated with secondary antibodies (rhodamine-conjugated anti-mouse for MLH; FITC-conjugated anti-rabbit for SCP3; 1/100 dilution), 45 min at 37°C. Slides were washed four times in PBS and sealed under a coverslip with anti-fade mounting media (Molecular Probes).

Immunohistochemistry on paraffin-embedded tissue sections

Human testis specimens were fixed by immersion in Bouin’s fixative (VWR Scientific); EBs were fixed in 4% paraformaldehyde in PBS (pH 7.4), 1 h, processed to paraffin, and cut at 5 μm serial sections onto slides. Slides were blocked by incubation in PBS/0.1% BSA/0.3% Tween 20 (PBST)/10% normal goat serum as appropriate (Vector Laboratories) for 30 min. Slides were incubated with primary antibody overnight at 4°C (rabbit anti-human DAZL; 1/100; rabbit anti-human STELLAR: 1/1000 [antibodies made against peptide C14 with rabbit anti-human SCP3 (a gift from Christa Heyting, Wageningen University)] and mouse anti-rat MLH (Ongogene) diluted in ADB at 1/500 and 1/25, respectively. Slides were washed as above, incubated for 30 min in ADB followed by 10 min. Colonies were washed as above, incubated for 30 min with 4% normal donkey serum (Jackson ImmunoResearch Laboratories), and incubated with primary antibodies diluted in PBS [1/100, DAZL; 1/100, STELLAR; 1/1000, VASA; 1/30 SSEA1; 1/30 SSEA3; 1/30 SSEA4; 1/30 TRA-1-81; 1/30 TRA-1-60 (all from Chemicon International Temecula, CA, USA)]; 1/50 NCAM1; 1/50 KDR (Assay Designs Inc., Ann Arbor, MI, USA) and neat AFP (Becton Coulter) for immunofluorescence. For Avidin–biotin immunohistochemistry antibodies were diluted (1/500, DAZL; 1/1000, STELLAR; 1/1000 VASA in PBS). All primary antibodies were incubated for 1 h at room temperature. Colonies were then washed twice in PBS and incubated with secondary antibodies as described above. Colonies were viewed using a Leica microscope fitted for immunofluorescence after mounting with anti-fade mounting media or PBS (DAB).

Whole mount immunohistochemistry

Undifferentiated human ES cells were grown on feeder layers for at least 4 days before removing the media and fixing in 4% paraformaldehyde in PBS (pH 7.4) for 15 min. Colonies were then washed twice for 10 min in TBS + 0.05% Tween 20 (TBST), followed by incubation in 0.1% Triton X in PBS for 10 min. Colonies were washed as above, incubated for 30 min with 4% normal donkey serum (Jackson ImmunoResearch Laboratories), and incubated with primary antibodies diluted in PBS [1/100, DAZL; 1/100, STELLAR; 1/1000, VASA; 1/30 SSEA1; 1/30 SSEA3; 1/30 SSEA4; 1/30 TRA-1-81; 1/30 TRA-1-60 (all from Chemicon International Temecula, CA, USA)]; 1/50 NCAM1; 1/50 KDR (Assay Designs Inc., Ann Arbor, MI, USA) and neat AFP (Becton Coulter) for immunofluorescence. For Avidin–biotin immunohistochemistry antibodies were diluted (1/500, DAZL; 1/1000, STELLAR; 1/1000 VASA in PBS). All primary antibodies were incubated for 1 h at room temperature. Colonies were then washed twice in PBS and incubated with secondary antibodies as described above. Colonies were viewed using a Leica microscope fitted for immunofluorescence after mounting with anti-fade mounting media or PBS (DAB).

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