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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 extra-embryonic 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 testis 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 *Igfr2* 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 reportedly expressed in human ES cells and/or human germ cells. These genes included the *GDF3* (*growth and differentiation factor 3*), *STELLAR* (*STELLA-related*), *NANOG* (the human homolog of mouse *nanog*), *NANOS1*, *DAZL* (*deleted in azoospermia-like*), *VASA*, *cKIT*, *PUM1* (*PUMILIO 1*), *PUM2* (*PUMILIO 2*), *SCP1* (*synatonemal complex protein 1*), *GDF9* (*growth and differentiation factor 9*), and *TEKT1* (*TEKTIN 1*) genes. Of these genes, we found that the *GDF3*, *NANOG* 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*, *PUM1* 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

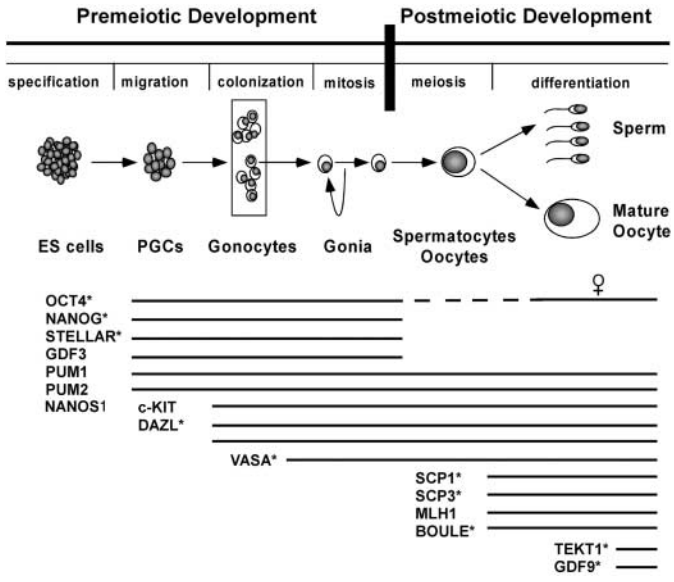

Figure 1. Diagrammatic representation of the different stages of germ cell differentiation in fetal and adult development. Expected expression patterns of genes used to predict each stage of germ cell development are shown by the name of the gene together with the black bars extending to the right of each gene name. All genes shown are enriched in germ cells relative to somatic cells; those that are only expressed in germ cells following gastrulation *in vivo* are indicated by an asterisk. Thus, genes known to be expressed in undifferentiated human ES cells include: OCT4 (73,74); *GDF3*, *Growth and Differentiation Factor 3*; *NANOG*; *STELLAR* (38); *PUM1*, *PUMILIO 1*; *PUM2*, *PUMILIO 2* (3) and *NANOS 1* (4). Genes known to be expressed in PGC development through to later stages of germ cell differentiation include *DAZL*, *Deleted in AZoospermia-Like* (44); *c-kit* (mouse) (75,76); *stella* (mouse) (9) and *Nanos* (mouse and fish) (5,77). Genes known to be expressed from gonocyte formation includes *VASA* (40). Genes expressed during meiosis include: *SCP1*, *Syntaptonemal Complex Protein 1 and 3* (41); *MLH1*, *Mut-L Homolog 1* (78) and *BOULE* (45). Adult oocyte-specific marker: *Gdf9*, *Growth and Differentiation Factor 9* (mouse) (42). Adult spermatid specific marker: *TEKT1*, *Tektin1* (43).

Table 1. Real-time PCR in undifferentiated human ES cells

Gene name	HSF-1 ($\times 10^3$)	H9 ($\times 10^3$)	HSF-6 ($\times 10^3$)	*P*-value (significance < 0.01)
NCAM1	7.92 ± 8.8	2.21 ± 1.6	1.86 ± 1.1	0.34
KDR	1.80 ± 1.3	6.48 ± 4.7	4.88 ± 4.2	0.36
AFP	0.437 ± 5.3	1.84 ± 3.1	3.95 ± 0.2	0.39
OCT4	67.2 ± 20	293 ± 119	304 ± 491	0.56
GDF3	1.06 ± 0.1	5.55 ± 1.9[a]	0.288 ± 0.4[b]	0.005*
STELLAR	4.03 ± 5.0	1.26 ± 1.9	0.126 ± 0.1	0.355
NANOG	11.1 ± 2.4	35.5 ± 21	2.76 ± 2.3	0.041
NANOS	1.50 ± 1.6	0.663 ± 0.6	0.681 ± 0.4	0.54
VASA	0	0	0	ND
DAZL	0.083 ± 1.4	0.455 ± 0.7	0.398 ± 0.2	0.83
KIT	1.53 ± 0.1	4.71 ± 7.6	1.08 ± 0.2	0.58
PUM1	11.0 ± 5.0[b]	35.7 ± 6.9[a]	4.82 ± 4.2[c]	0.001*
PUM2	10.1 ± 7.8	67.3 ± 83.7	25.5 ± 28.0	0.42
SYCP1	0	0	0	ND
GDF9	0	0	0	ND
TEKTIN1	0	0	0.0000673 ± 0.000011	0.24

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 *t*-test. Significance ($P \leq 0.01$) between a–b and a–c. ND, 'not done'.

did not observe expression of genes such as *VASA*, *SCP1*, *GDF9*, and *TEKT1* (Fig. 2A) or *BOULE* and *SCP3* (data not shown), which are specific markers of germ cells during their later stages of migration as they enter the gonads and progress through meiosis and gamete morphogenesis (Fig. 2A; Table 1) (40–43)

Since undifferentiated human ES cells expressed a number of markers indicative of putative premeiotic germ cells, we tested whether germ cell specific proteins were also expressed in undifferentiated human ES cells (Fig. 2B). Western blot analysis of human fetal and adult testis tissue was used as a positive control since the transcriptional and translational profiles of these tissues have been well documented (3,4,40,44,45). As expected, the fetal testis expressed PUM2, OCT4, NANOS1, DAZL and VASA proteins, whereas the meiotic proteins, BOULE and SCP3, were confined to adult testis (Fig. 2B). By comparison, undifferentiated human ES cells expressed PUM2, OCT4, NANOS1 and DAZL proteins, but not markers of later germ cell development such as VASA, BOULE or SCP3 (Fig. 2B). These results, along with the transcriptional profiles, indicated that undifferentiated human ES cells express early markers of germ cell development but not later markers of germ cell development such as VASA, at both the mRNA and protein levels.

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). We began 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 certainly not expressed 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 expected random expression of later germ cell markers such as *VASA*, *SCP1*, *SCP3*, *BOULE*, *GDF9* and *TEKT1*. Yet, this was not the case. We observed the

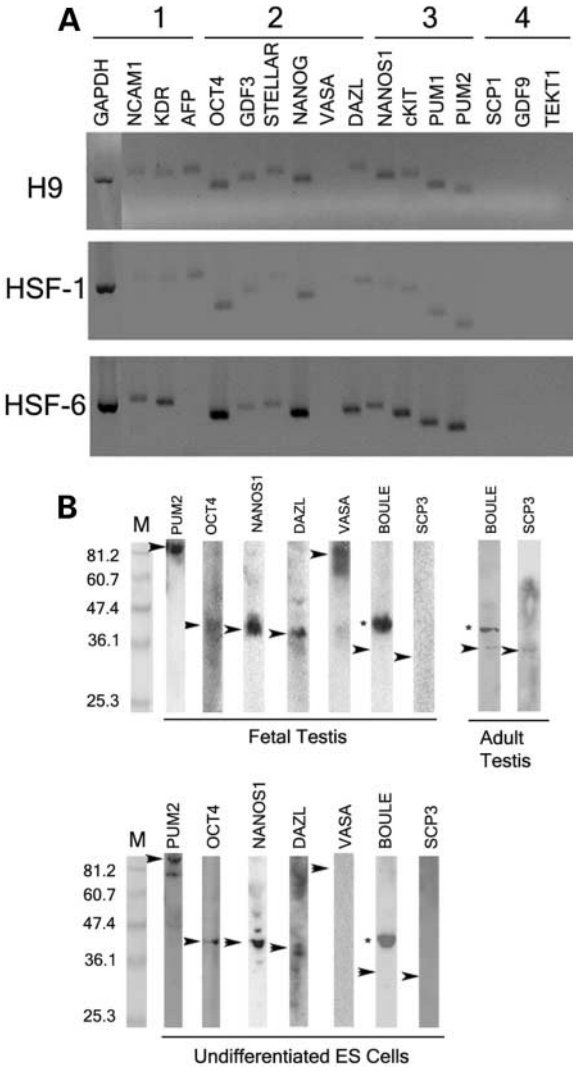

Figure 2. Analysis of expression of RNA and protein markers of the somatic and germ cell lineages in undifferentiated human ES cells. Shown are PCR products separated by gel electrophoresis (**A**). PCR products were generated by quantitative PCR using SYBR green for three different lines of undifferentiated human ES cells, HSF-1, H9 and HSF-6. PCR reactions were performed in duplicate on three different samples from each cell line. Four groups of markers were assayed: (1) somatic lineage markers; (2) ES cell and germ cell specific markers; (3) germ cell enriched markers that have expression in additional somatic cell types; (4) meiotic and post-meiotic germ cell specific expression. Relative expression values were also determined by referencing the C_T value of each test gene to GAPDH (Table 1). Shown is western blot analysis of fetal and adult testis and undifferentiated human ES cells (**B**). Note that the fetal testis contains premeiotic diploid germ cells only; meiosis is not initiated until puberty. As expected, the fetal testis expressed PUM2, OCT4, NANOS1, DAZL and VASA proteins and adult testis also expressed BOULE and SCP3 proteins. Undifferentiated human ES cells have a similar expression profile to fetal testis in that the ES cells expressed the PUM2, OCT4, NANOS1 and DAZL proteins; VASA, BOULE, SCP3 and GDF9 proteins were not detected. Note that, although present, OCT4 expression in the fetal testis is very low, as previously reported (39). The asterisk indicates the non-specific band identified in BOULE pre-immune and immune sera as previously published (45).

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

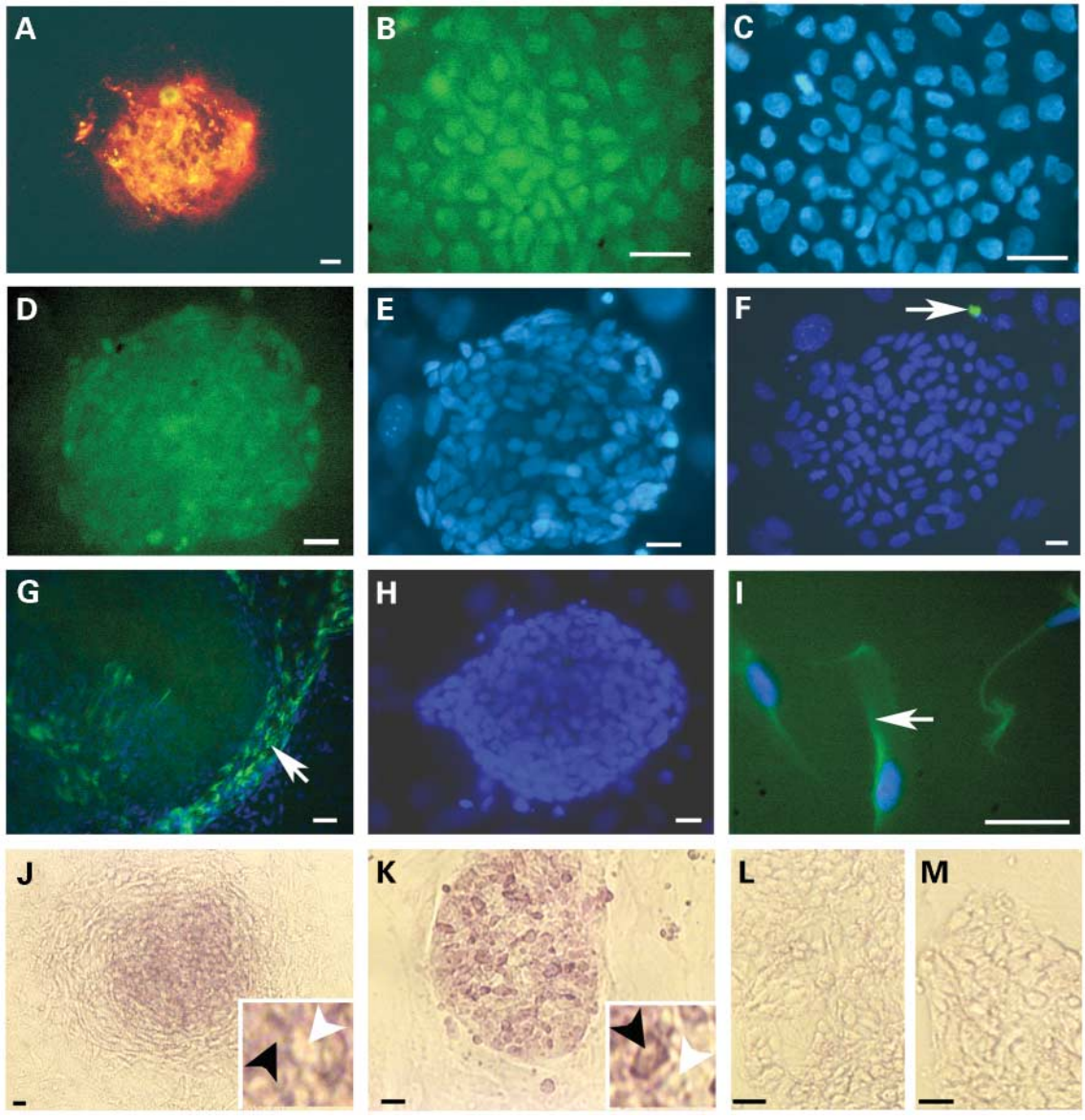

Figure 3. Analysis of cellular and subcellular protein expression in undifferentiated ES cells. Expression was analyzed via immunofluorescence (A–I) and immunohistochemistry (J–M). (**A**) The markers of undifferentiated ES cells, SSEA3 (green) and TRA-1-81 (red), were used to assess status of colony differentiation (orange represents co-localization of SSEA3 and TRA-1-81. Also shown are: STELLAR immunofluorescence (**B**) and DAPI (**C**); DAZL immunofluorescence (**D**) and DAPI (**E**); merged images of immunofluorescence and DAPI for KDR (**F**) white arrow shows positive cell), AFP (**G**; white arrow indicates positive cells), and NCAM1 (**H**). Note that NCAM1 protein is only detected in differentiating ES cells cultured on gelatin (**I**; white arrow indicates positive cytoplasmic expression). Immunohistochemistry of STELLAR (**J**), DAZL (**K**) and VASA (**L**) is also shown. Insets show cells positive for STELLAR (J; black arrowhead) and DAZL (K; black arrowhead); negative for STELLAR (J; white arrowhead) and DAZL (K; white arrowhead). Negative controls included both pre-immune sera (**M**), and processing without the primary antibody (data not shown). Scale bars = 50 μm.

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

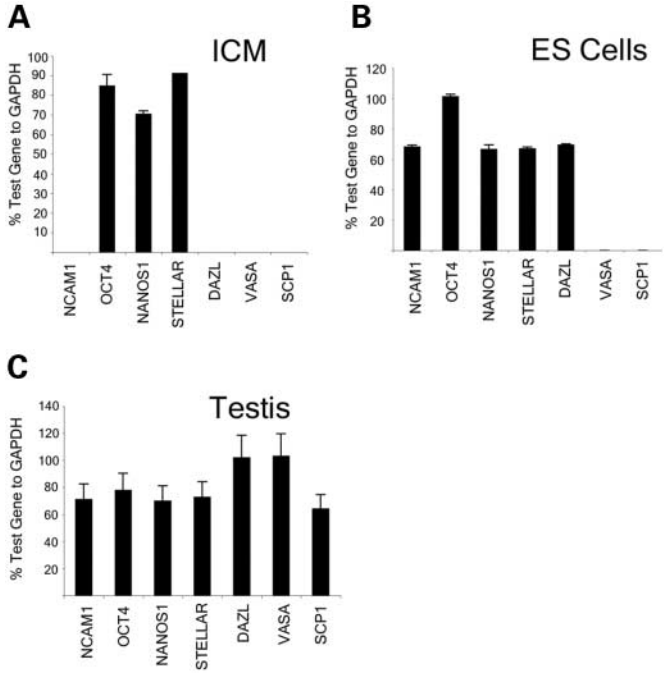

Figure 4. Comparison of expression of a subset of genes in (**A**) human inner cell mass (ICM), (**B**) undifferentiated ES cells, and (**C**) adult testis. Quantitative PCR was used to analyze germ cell specific expression; CT values were expressed as a percentage of GAPDH (GAPDH = 100%) and used to calculate mean normalized expression relative to GAPDH (Table 2). Results are shown as mean and standard deviation of three experiments.

every EB section contained STELLAR-positive cells. Finally, examination of the localization of DAZL protein in EBs revealed that DAZL-positive cells were found in more EBs than either STELLAR- or VASA-positive cells (Fig. 6H–J). However, when adjacent sections were stained for STELLAR and DAZL, most often cells within the same region of the EB stained for both proteins (for example, compare Fig. 6F and I). Furthermore like STELLAR, DAZL-positive cells were more likely to be found evenly dispersed throughout EBs composed of loosely packed cells (Fig. 6I and J). Cell numbers positive for DAZL, STELLAR and VASA were tallied for adjacent sections of the same EB and confirmed visual impressions of immunohistochemistry (Fig. 7). Given these tallies, clearly the fate of all cells that are positive for DAZL and STELLAR on day 0 of differentiation is not to differentiate to VASA-positive cells by days 14 or 21.

Meiosis is a major hallmark of germ cell development; thus, we examined expression of meiotic proteins, namely, the synaptonemal complex protein-3, SCP3 (Fig. 8) and Mut-L Homolog-1, MLH1 (data not shown) in differentiated EBs. *In vivo*, SCP3 expression is first detected as punctate nuclear staining at meiotic leptotene (Fig. 8A). Subsequently, SCP3 localizes along the entire length of the sister chromatids during the pachytene stage of meiosis with MLH1 nodules clearly marking sites of chromosome recombination (Fig. 8B). In undifferentiated human ES cells, only very rarely did we observe SCP3 staining in single isolated cells (Fig. 8D) and MLH1 staining was never observed (data not shown). By day 14 of EB differentiation, we detected cells positive for SCP3 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

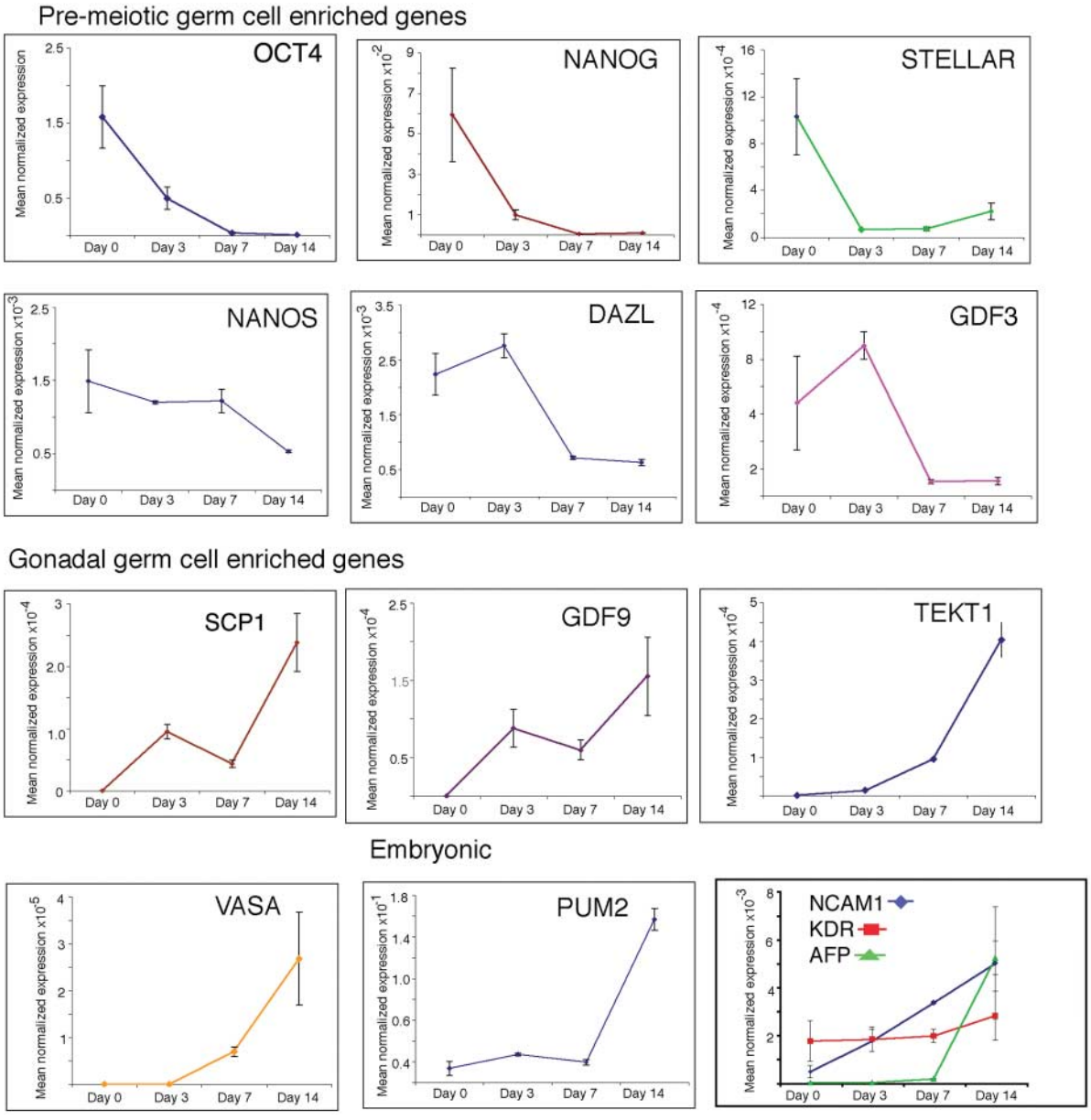

Figure 5. Gene expression during differentiation of human embryonic stem cells to embryoid bodies. Shown is quantitative PCR using SYBR green. The mean normalized expression of each gene is shown along the *y*-axis as a function of days of differentiation as shown on the *x*-axis. Mean normalized expression is relative to *GAPDH*.

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

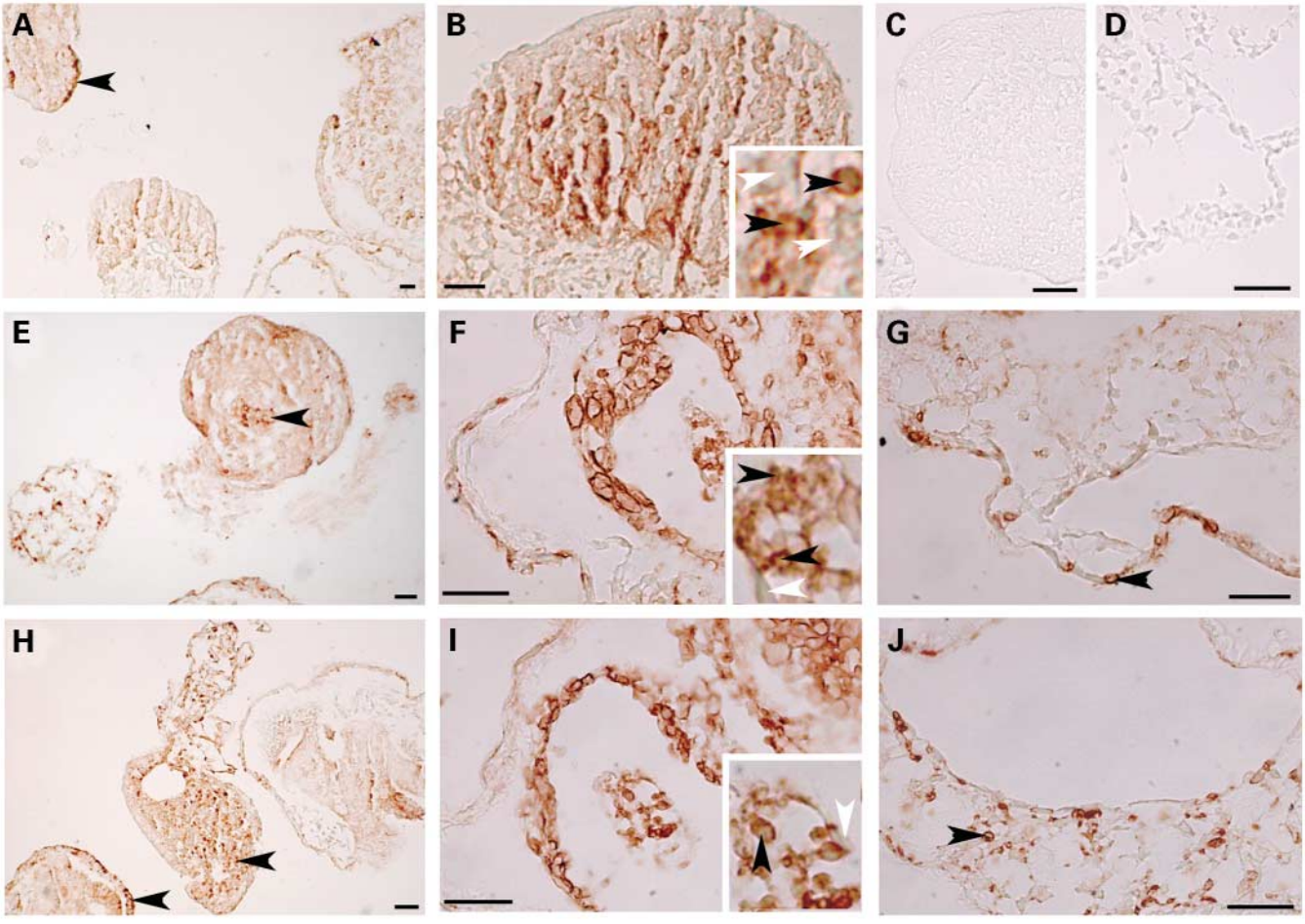

Figure 6. Immunohistochemistry of day 14 embryoid bodies. Representative immunohistochemistry of day 14 embryoid bodies from HSF-6 and HSF-1 ES cell lines stained for VASA (**A**, **B**), STELLAR (**E–G**) and DAZL (**H–J**). Negative controls included omission of primary antibody (**C**) and pre-immune 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.

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).

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

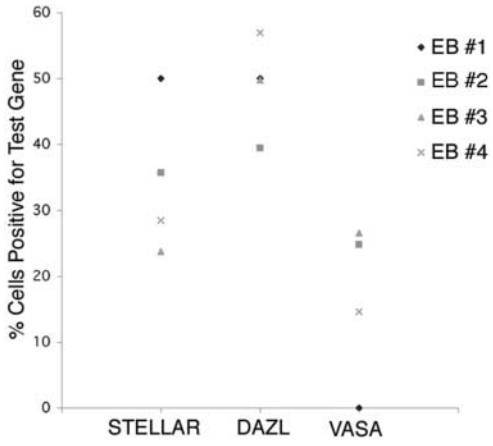

Figure 7. Percentage of cells positive for STELLAR, DAZL and VASA in day 14 embryoid bodies. The total number of positive cells was counted in the same embryoid body on adjacent sections for STELLAR, DAZL and VASA. Results are expressed as a percentage of cells positive for the test gene out of the total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

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 *VASA* was initiated after 3 days of EB differentiation and VASA-positive cells were most often present in clusters rather than dispersed throughout the

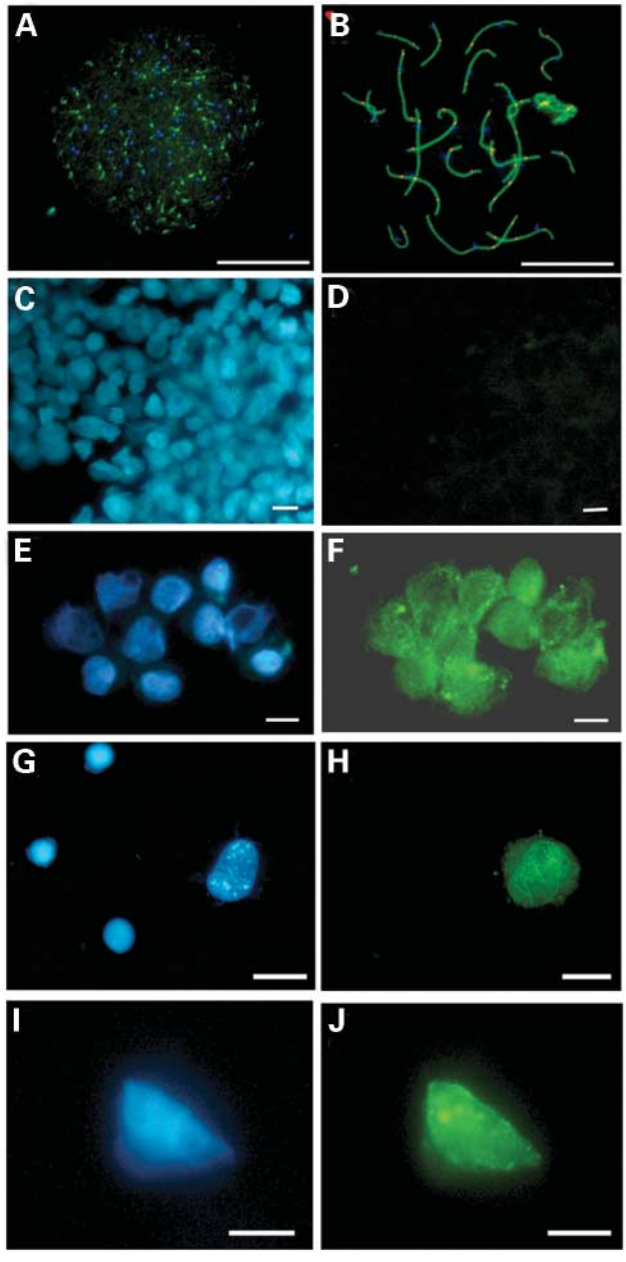

Figure 8. Meiotic protein expression. Control immunohistochemistry of SCP3 (green), and MLH1 (red) in (**A**) human leptotene spermatocyte, and (**B**) human pachytene spermatocytes. (**C** and **D**) undifferentiated human ES cells stained with DAPI (C) and SCP3 antisera (D). Cells from EBs at days 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.

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*

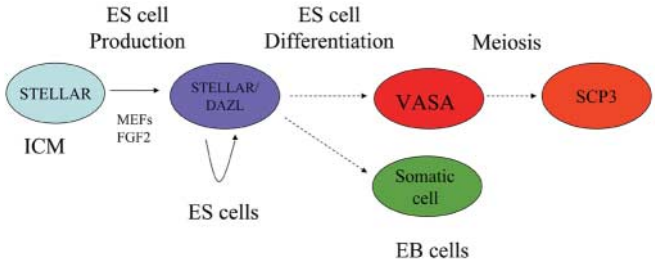

Figure 9. Model of specification of human germ cells based on gene expression in the inner cell mass and ES cell differentiation. See text for further explanation.

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% CO_2 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 MLV 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 $MgCl_2$, 10 mM dATP, dGTP, dCTP and dTTP, 2 µM primers (Table 3), 0.25 U Platinum *Taq* (Invitrogen), 1 × SYBR green (Molecular Probes), 1 × fluorosceine (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^{tm} (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^{TM} 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-mercaptoethanol (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 pippetted 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 H_2O 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 sequence RESVGAAVLREIEDE of human STELLAR (58); chicken anti-human VASA, 1/1000] in PBST before washing twice for 10 min in PBS. Sections were then incubated twice for 10 min in PBS before mounting under a glass coverslip with anti-fade mounting media (Molecular Probes). For avidin biotin immunohistochemistry, following incubation in primary antibodies, sections were incubated for 30 min at room temperature, with either biotin conjugated anti-rabbit (Vector Laboratories) to detect DAZL and STELLAR according to manufacturer's instructions, or biotin conjugated anti-chicken (1/200; Sigma Chemical) to detect VASA. Sections were washed twice for 10 min in PBS followed by incubation for 30 min in ABC reagent (Vector Laboratories). Sections were then washed twice for 10 min in PBS, and incubated in DAB (Vector Laboratories) until the brown color reaction developed. Color reactions were stopped in deionized H_2O before dehydrating sections through ascending concentrations of ethanol to xylene. Sections were permanently mounted under glass coverslips using Vectormount (Vector Laboratories).

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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