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Expression Profile of Male Germ Cell-Associated Genes in Mouse Embryonic Stem Cell Cultures Treated with All-Trans Retinoic Acid and Testosterone

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Abstract

Cells that morphologically and functionally resemble male germ cells can be spontaneously derived from ES cells. However, this process is inefficient and unpredictable suggesting that the expression pattern of male germ cell associated genes during spontaneous ES cell differentiation does not mimic the *in vivo* profiles of the genes. Thus, in the present study, the temporal expression profile of genes expressed at different stages of male germ cell development was examined in differentiating ES cells. The effect of all-trans retinoic acid (RA) which is a known inducer of primordial germ cell (PGC) proliferation/survival *in vitro* and testosterone which is required for spermatogenesis *in vivo* on the expression of these genes was also determined. Each of the 12 genes analyzed exhibited one of four temporal expression patterns in untreated differentiating ES cells: progressively decreased (*Dpp3a*, *Sycp3*, *Msy2*), initially low and then increased (*Stra8*, *Sycp1*, *Dazl*, *Act*, *Prm1*), initially decreased and then increased (*Piwil2*, *Tex14*), or relatively unchanged (*Akap3*, *Odf2*). RA-treated cells exhibited increased expression of *Stra8*, *Dazl*, *Act*, and *Prm1* and suppressed expression of *Dpp3a* compared to untreated controls. Furthermore, testosterone increased expression of *Stra8* while the combination of RA and testosterone synergistically increased expression of *Act*. Our findings establish a comprehensive profile of male germ cell gene expression during spontaneous differentiation of murine ES cells and describe the capacity of RA and testosterone to modulate the expression of these genes. Furthermore, these data represent an important first step in designing a plausible directed differentiation protocol for male germ cells.

Keywords

Spermatogenesis; Gene Expression Profiling; Real-Time PCR; Embryoid Bodies

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INTRODUCTION

ES cells, which are derived from the inner cell mass of blastocyst stage embryos (Evans and Kaufman 1981; Thomson et al. 1998), by definition are capable of maintaining pluripotency. They also have the capacity of self-renewal during indefinite propagation *in vitro*, while maintaining a normal karyotype. *In vitro* and *in vivo* assays have shown that ES cells can be induced to recapitulate several aspects of early embryonic development, and can differentiate into cell types from all three germ cell layers dependent upon the culture conditions employed (Keller 2005). Furthermore, the *in vitro* totipotency of mouse ES cells has been demonstrated, with ES cells being able to give rise not only to primordial germ cells (PGCs), but also oocyte-like and sperm-like cells *in vitro* (Dyce et al. 2006; Geijsen et al. 2004; Hubner et al. 2003; Lacham-Kaplan et al. 2006; Toyooka et al. 2003). These gamete-like cells are, in some cases, capable of undergoing meiosis and supporting fertilization, or undergoing parthenogenesis resulting in the formation of blastocyst-like structures containing cells expressing inner cell mass and trophectoderm markers (Dyce et al. 2006; Geijsen et al. 2004; Hubner et al. 2003; Lacham-Kaplan et al. 2006; Toyooka et al. 2003). However, spontaneous differentiation of ES cells into the germ-line is generally low and inefficient with most germ cells undergoing degeneration.

In the mouse, *in vivo* germ cell differentiation originates on day 6.5 post coitum when primordial germ cells (PGCs) are induced in the proximal epiblast cells of the egg cylinder. A tightly regulated molecular mechanism prevents the PGCs from assuming a somatic fate and they instead undergo an extensive, directed migration toward the future gonads (Kehler et al. 2004; Ohinata et al. 2005; Saitou et al. 2002). PGCs actively proliferate during this migratory phase. Many factors are involved in the proliferation and/or survival of PGCs, including kit ligand, leukemia inhibitory factor (LIF), and tumor necrosis factor α (TNF α) (De Felici and Pesce 1994). Furthermore, retinoic acid (RA), a vitamin A derivative, markedly increases the number of migratory phase PGCs and slows the depletion of gonadal PGCs *in vitro* (Koshimizu et al. 1995; Morriss-Kay and Sokolova 1996). After specification, PGCs must associate with surrounding somatic cells, which provide signals for their subsequent development. In the male gonad, the differentiation of germ cells into spermatozoa occurs in the tubular seminiferous epithelium and is characterized by successive mitotic, meiotic, and post-meiotic phases. Progression through these phases is reliant on crosstalk between the supporting Sertoli and Leydig cells. Specifically, testosterone, secreted by the Leydig cells under the influence of LH and FSH, acts on Sertoli cells to stimulate gene transcription and the secretion of growth factors that promotes germ cell differentiation as reviewed by Griswold (Griswold 1998).

In vivo studies have demonstrated that each phase of spermatogenesis (mitotic, meiotic, and post-meiotic) is characterized by distinct patterns of male germ cell associated gene expression (Di Carlo et al. 2000; Fimia et al. 2001; Gu et al. 1998; Horowitz et al. 2005; Kleene et al. 1984; Lee et al. 2006; Nagano 2007; Oulad-Abdelghani et al. 1996; Rocchietti-March et al. 2000; Wu et al. 2003). Inefficiencies in male germ cell derivation *in vitro* using spontaneous ES cell differentiation protocols likely reflect the inability of the culture to mimic this normal *in vivo* gene expression program. While the expression of male germ cell markers in differentiating ES cells have been previously reported, these studies used mostly non-quantitative methods (Geijsen et al. 2004; Nayernia et al. 2006; Toyooka et al. 2003). Furthermore, simultaneous assessment of cognate protein expression has not been routinely reported. In the present study we have quantitatively examined the temporal expression profile of 12 genes that are expressed during different stages of male germ cell differentiation (Fig. 1) in ES cell-derived embryoid bodies. RA is known to promote PGC proliferation and/or survival *in vitro*, the RA precursor Vitamin A is required for spermatogenesis *in vivo* (Wolgemuth and Chung 2007), and testosterone is essential for spermatogenesis *in vivo* (Griswold 1998). Therefore, we also evaluated the effects of RA and testosterone on male germ

cell associated gene expression in the ES-derived embryoid body cultures. Real-time PCR (QPCR) analysis measured quantitative changes in mRNA abundance of these male germ cell genes while expression of selected proteins was confirmed by Western blot analysis and immunocytochemistry.

MATERIAL AND METHODS

Embryonic stem (ES) cell and embryoid body (EB) cultures

Undifferentiated TL-1 Sv129 mouse XY ES cells were cultured on 0.1% gelatin-coated tissue culture plates (BD Biosciences, San Jose, CA) containing γ -irradiated mouse embryonic fibroblast (MEFs) cells. These murine ES cells have been used by us previously to generate targeted mutations in genes involved in spermatogenesis and knock-out mice, so their capacity to enter the germ line is proven (Zhang et al. 2004; Zhang et al. 2006). To maintain the undifferentiated phenotype, cells were grown in ES cell culture medium (Dulbecco's modified Eagle's medium (HyClone, Logan, UT), 15% heat inactivated fetal bovine serum (HyClone), 1% glutamine (Invitrogen, Carlsbad, CA), 1% nonessential amino acids (Invitrogen), and 1 mM β -mercaptoethanol (Sigma, St Louis, MO)) containing 1,000 U/ml murine leukemia inhibitory factor (LIF) (ESGRO; Gibco, Carlsbad, CA). Confluent cells were dispersed with 0.125% Trypsin-EDTA in Versene (Invitrogen), diluted 2-fold and re-plated on gelatin-coated tissue culture plates containing MEFs and ES cell medium with LIF.

To promote the formation of embryoid bodies (EBs), approximately 5×10^6 ES cells were transferred to 10 cm bacterial plates (BD Biosciences) containing 10 ml of ES cell culture media without LIF. EBs were maintained in suspension for 5 days with daily medium changes. After 5 days, adherent cultures of EBs were established on gelatin-treated tissue culture plates without MEFs and maintained in ES cell medium without LIF. The adherent cultures of EBs were exposed in triplicate to daily treatments with 2 μ M all-trans retinoic acid (RA) (Sigma), 1 μ M testosterone (Sigma), both RA (2 μ M) and testosterone (1 μ M), or no treatment for 2, 4, 7, or 14 days with the first day of adherent culture considered day 0. Upon termination of the treatment, cells were washed with PBS and total RNA was isolated using TRIzol reagent (Invitrogen).

Reverse transcription and quantitative, real-time PCR

Total RNA (3 μ g) was isolated from the adherent cultures of EBs described above, treated with DNase I (Promega, Madison, WI), and reverse transcribed using random hexamer primers (Roche Diagnostics, Indianapolis IN) and Maloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega) as previously described (Nelson et al. 2001). Equivalent dilutions of the resulting cDNA was subjected to quantitative, real-time PCR (QPCR) amplification for the transcripts listed in Table 1 as previously described (Wood et al. 2003). Briefly, primers for each transcript were designed using the Primer Express 2.01 software (PE Applied Biosystems, Foster City, CA) and tested empirically to assure specific amplification of the target sequence. Each cDNA sample was amplified in triplicate using 2x SYBR Green Master Mix (PE Applied Biosystems). To account for differences in starting material, QPCR was also carried out for each cDNA sample using the Applied Biosystems 18S rRNA 20x primer and probe reagent (PE Applied Biosystems). To define the relative abundance of each transcript in each experimental group, analysis of the resulting QPCR reactions was carried out as previously described (Wood et al. 2003). Briefly, the threshold cycle for the gene-specific and 18S rRNA in each cDNA sample was converted to an arbitrary value using a standard curve generated from serial dilutions of mixed germ cell cDNA. The relative abundance of the target was divided by the relative abundance of 18S rRNA in each sample to generate a normalized abundance for each transcript. The mean and standard error of the mean (SEM) of the normalized transcript abundance was determined for each treatment group at each time point.

The normalized abundance of each transcript was also log transformed to equalize the estimated variances across the experimental groups and subsequently subjected to one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison test to determine statistically significant ($p < 0.05$) differences in mRNA levels between experimental groups.

Western blot analysis

Whole cell protein was extracted from the adherent EB cultures described above and quantified using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Equivalent levels of protein extracts were then fractionated by SDS-PAGE, and transferred to Immobilon PVDF (Millipore, Billerica, MA). Similarly, whole cell protein was extracted from mouse testis (positive control) and mouse heart (negative control), quantified, fractionated by SDS-PAGE and transferred to PVDF. All membranes were blocked with 5% nonfat dry milk and then incubated with primary antibodies (Abcam, Cambridge, MA) against MSY2 (1:1000), DAZL (1: 500), SYCP1 (1:1000), or SYCP3 (1:1000). After washing, goat anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) IgG (Cell Signaling Technologies, Danvers, MA) were used when appropriate at 1:1000, 1:1000, 1:2500, and 1:1000 dilutions respectively. The blots were visualized using enhanced chemiluminescence (Pierce).

Immunofluorescence detection of proteins

For immunofluorescent localization of MSY2 and DAZL under the various culture conditions, adherent EB cultures were established on glass cover slips pretreated with 0.1% gelatin. Similar to the experimental design previously described, the adherent EB cultures were exposed in triplicate to daily treatments of 2 μ M RA (Sigma) 1 μ M testosterone (Sigma), RA (2 μ M) and testosterone (1 μ M), or no treatment for 7 days with the first day of adherent culture considered day 0. After 7 days, cultures were terminated and immunofluorescence was performed as previously described (Evans et al. 1995). Briefly, the cells were fixed in 4% fresh paraformaldehyde, permeabilized with 100% methanol, and blocked with 10% horse serum for 30–60 minutes at 37° C. Cells were subsequently incubated with primary antibodies against MSY2 (1 μ g/ml) or DAZL (1 μ g/ml). Normal rabbit IgG (Jackson Immunosearch, West Grove, PA) or non-immune horse serum were used as negative controls. Cells were then incubated with the anti-rabbit Alexa Fluor 488 (1 μ g/ml) (Molecular Probes, Eugene, OR) secondary antibody. Cover slips were mounted on glass slides with Vectashield and images were recorded using a Leica TCS confocal microscope. All images within a single figure were taken using identical excitation and detection voltages.

RESULTS

Temporal expression profile of germ cell-specific genes in differentiating embryoid bodies

The expression profiles of many of the genes that are associated with male germ cell proliferation and/or differentiation have been reported (Di Carlo et al. 2000; Fimia et al. 2001; Gu et al. 1998; Horowitz et al. 2005; Kleene et al. 1984; Lee et al. 2006; Nagano 2007; Oulad-Abdelghani et al. 1996; Rocchietti-March et al. 2000; Wu et al. 2003). Given the well-defined temporal expression profile of these genes (Fig. 1), they serve as good markers of mitotic, meiotic, and post-meiotic stages of spermatogenesis. To determine the expression profile of these male germ cell markers in differentiating ES cells, adherent murine ES cell cultures, which were established from embryoid bodies (EBs), were maintained in culture for 0, 2, 4, 7, or 14 days. Upon termination of the cultures, RNA was collected and the expression profiles of stella (*Dpp3a*), piwi-like homolog 2 (*Piwi2*), stimulated by RA gene 8 (*Stra8*), testis-expressed gene 14 (*Tex14*), deleted in azoospermia-like (*Dazl*), synaptonemal complex 1 (*Sycp1*), synaptonemal complex 3 (*Sycp3*), mouse-specific Y-box 2 (*Msy2*), outer dense fiber 2 (*Odf2*), activator of CREM in testis (*Act*), protein kinase A associated protein 3 (*Akap3*), protamine 1 (*Prm1*), and protamine 2 (*Prm2*) (Table 1) were examined using quantitative real-

time PCR (QPCR). This panel of genes represents markers from all stages of spermatogenesis (Fig. 1) with several of the genes expressed specifically in the male germ cell (*Piwil2*, *Tex14*, *Stra8*, *Dazl*, *Act*, *Prm1*, and *Prm2*).

The temporal patterns of expression of the germ cell and sperm cell genes revealed by QPCR could be organized into 4 groups: genes whose expression declined progressively (*Dpp3a*, *Sycp3*, *Msy2*); genes whose expression was initially low and then increased (*Stra8*, *Sycp1*, *Dazl*, *Act*, *Prm1*); genes that showed a biphasic pattern of expression with an initial decline and then an increase (*Piwil2*, *Tex14*); and genes that showed relatively little changes (*Akap3*, *Odf2*) (Fig. 2). We could not detect expression of *Prm2* during the time course (data not shown). Using the sequence of gene expression shown in Figure 1 as a guide, the temporal changes in ES cell gene expression were in some cases generally consistent with the anticipated sequence of differentiation of male germ cells (e.g., *Dpp3a*, *Piwil2*, *Dazl*, *Sycp1*, *Act*, *Prm1*), while in other cases the pattern was discordant (e.g. *Stra8*, *Sycp3*, *Msy2*).

Interestingly, when undifferentiated ES cell populations were examined, relatively high levels of *Dpp3a*, *Piwil2*, *Tex14*, *Msy2*, *Sycp3*, *Prm1*, *Odf2* and *Act* mRNA were observed (Fig. 2). However, undifferentiated ES cells did not show expression of *Dazl*, *Stra8*, *Akap3*, *Sycp1* (Fig. 2), and *Prm2* (data not shown). Taken together, these data demonstrated that cultures derived from differentiating EBs express several genes that are markers of male germ cell proliferation and differentiation.

Effect of retinoic acid and testosterone on the expression of male germ cell markers

Despite expression of genes from all stages of the spermatogenic cycle in the adherent EB cultures, there was no coordinated or sustained expression of any of these genes. Previous studies have demonstrated that retinoic acid and testosterone promote germ cell proliferation *in vitro* and spermatogenesis *in vivo*, respectively (Griswold 1998; Koshimizu et al. 1995; Rohwedel et al. 1999). To determine if these two hormones altered expression of the target genes, adherent EB cultures were established and maintained for 2, 4, 7, or 14 days in the absence or presence of 2 μ M all-trans retinoic acid (RA), 1 μ M testosterone, or both RA (2 μ M) and testosterone (1 μ M). Upon termination of the cultures, RNA was collected and QPCR was carried out to measure the mRNA abundance of *Dpp3a*, *Piwil2*, *Stra8*, *Tex14*, *Dazl*, *Sycp1*, *Sycp3*, *Msy2*, *Odf2*, *Act*, *Akap3*, and *Prm1*.

Although all of the genes examined were expressed on days 2 and 4 of culture, there was no effect of any of the 3 treatments on mRNA abundance at these time points (data not shown). Conversely, when cells were treated with RA for 7 days the expression of *Stra8*, *Dazl*, *Act*, and *Prm1* was significantly increased compared to day 7 untreated control cultures (Fig. 3). In contrast, RA-treated cultures showed a significant decrease in *Dpp3a* mRNA levels at the day 7 time point (Fig. 3). While 7 days of RA treatment had significant effects on gene expression in the EB cultures, testosterone alone did not alter the expression of any gene in our panel compared to the untreated control cultures (Fig. 3). The addition of testosterone to RA did not significantly alter mRNA abundance compared to RA alone for any of the genes tested on day 7 (Fig. 3). When adherent EB cultures were treated for 14 days, RA continued to inhibit *Dpp3a* compared to the day 14 untreated control cultures (Fig. 3). RA significantly increased the mRNA abundance of *Prm1*, but the stimulatory effects on *Stra8* and *Dazl* were not observed on day 14, compared to the day 14 untreated controls (Fig. 3). In fact, *Dazl* expression was significantly reduced in the RA-treated cultures compared to untreated controls. Testosterone significantly increased *Stra8* mRNA levels on day 14 (Fig. 3). When cells were treated with both RA and testosterone, an additive increase in *Act* expression was observed. Collectively, these data suggest a combined role for RA and testosterone in the control of expression of certain genes involved in male germ function.

Protein expression in adherent cultures of differentiated EBs

Seven and 14 days of RA, testosterone, or the combined treatment of RA and testosterone altered mRNA expression of some of the germ cell and sperm cell genes analyzed in our panel. To determine if mRNA expression was associated with protein translation, Western blot analysis was carried out. To assess the effect of hormone treatment on protein expression, adherent EB cultures were treated 2, 4, 7, or 14 days in the absence or presence of RA, testosterone, or both RA and testosterone. Upon termination of treatment, whole cell protein extracts were obtained and equivalent concentrations of each extract was separated by SDS-PAGE and probed for MSY2, DAZL, SYCP1, SYCP3, PRM1, and ACT. Whole cell extracts from mouse heart and testis were used as negative and positive and controls, respectively.

All 6 proteins were detected in testis extracts, which served as a positive control, but not in heart extracts which served as a negative control (Fig. 4A and data not shown). Likewise, MSY2, DAZL, SYCP1, and SYCP3 but not PRM1 and ACT protein were detected in the untreated adherent EB cultures (Fig. 4A and data not shown). Furthermore, the mobility of each protein in the EB cultures was identical to those detected in the testis. This result confirmed that the mRNAs detected by QPCR were, for the most part, translated into proteins in the adherent EB cultures. Interestingly, the expression of DAZL protein in untreated EBs over the time-course was similar to the expression pattern of *Dazl* mRNA over the same time points (compare Fig. 4A and Fig. 2). Conversely, the post-meiotic proteins PRM1 and ACT were not detected in any of the treated or untreated cultures indicating that transcription and translation for some of the genes was uncoupled in the adherent EB cultures. Given that post-transcriptional regulation of protein expression is a prominent feature of spermatogenesis, this observation, and discrepancies apparent from comparing protein expression and mRNA abundance (recognizing that the Western analysis was not quantitative) are not entirely unexpected. These data, do demonstrate that differentiating EBs express markers of early germ cell development, at both the mRNA and protein level.

We confirmed and extended the Western blot results by immunofluorescence detection of MSY2 and DAZL in cultures of adherent EBs which were maintained for 7 days in the absence or presence of RA, testosterone, or both RA and testosterone. We observed that the MSY2 protein was expressed in most of the differentiating colonies of EBs, with high signal levels (Fig. 4B). The effects of the RA, testosterone and the combined treatment were qualitatively consistent with the QPCR results, based on signal intensity. MSY2 was found in the cytoplasm, a typical cellular localization for this protein (Figure 4B) (Gu et al. 1998). DAZL protein localized to the cytoplasm, similar to MSY2.

DISCUSSION

During ES cell differentiation using embryoid body formation, tissue-specific genes and proteins are differentially expressed in a pattern that in some ways mimics mouse embryogenesis *in vivo* (Rohwedel et al. 1999). This model system has been previously used to successfully derive male gamete-like cells (Geijsen et al. 2004; Toyooka et al. 2003; West et al. 2006). While these studies demonstrate the capacity of embryonic stem cells to differentiate into the male germ line, the process has proven inefficient in the number and quality of derived germ cells. Appropriate germ cell proliferation and differentiation *in vivo* is dependent on spatial and temporal regulation of genes within the testis (Fig. 1). This gene expression is regulated by intrinsic, interactive and extrinsic factors (Eddy 2002), and therefore is likely affected by a myriad of differentiation factors, growth factors, signaling molecules and extracellular matrix proteins. Thus, the derivation of a pure population of male gametes and necessary supporting somatic cell components from ES cells will be dependent on culture conditions which elicit a gene expression profile that is optimal for male germ cell differentiation.

At present there has been no quantitative assessment of male germ cell gene expression during spontaneous ES cell differentiation. This information is crucial in order to understand how to modulate culture conditions such that genes are expressed in an appropriate temporal and spatial manner for optimal spermatogenesis. Our QPCR analysis of 12 genes that are expressed at different stages of spermatogenesis (i.e. mitotic, meiotic, and post-meiotic) demonstrates that the mRNAs for these genes are expressed during spontaneous ES cell differentiation and that they exhibit one of four distinct expression profiles (Fig. 2). Interestingly, expression of these germ cell associated genes increased over a relatively short period during ES cell differentiation (i.e. 2–14 days) which is consistent with observations by Geijsen *et al.* (Geijsen *et al.* 2004) and West *et al.* (West *et al.* 2006). Furthermore, the mRNA levels for some of the genes we examined were relatively high in undifferentiated ES cells (Fig. 2). While the differences in the *in vitro* and *in vivo* temporal expression profiles of the germ cell-associated genes may simply reflect differences between the microenvironments of the *in vitro* and *in vivo* cells, these data raise the possibility that there is a group of founder cells already committed to the germ cell lineage within apparently undifferentiated ES cell populations.

This study also demonstrates that the treatment of ES cell-derived embryoid bodies with RA significantly modifies the expression of genes associated with the male germ cell lineage (Fig. 3). Several groups have demonstrated that RA induces differentiation of ES cells into neural, adipose, skeletal muscle, cardiac muscle and vascular smooth muscle cells in a time- and concentration-dependent manner (Dani *et al.* 1997; Drab *et al.* 1997; Strubing *et al.* 1995; Wobus *et al.* 1994). In the present study, RA also regulated gene expression in a time- and concentration-dependent manner. Specifically, optimal changes in gene expression were induced when cells were treated with 2 μ M RA with lower concentrations having no effect on gene expression and higher concentrations almost abolishing expression of the genes we examined (data not shown). Likewise, RA treatment produced a time-dependent change in gene expression with 7 days inducing increased expression of *Stra8*, *Dazl*, *Act*, and *Prm1* while 14 days of treatment only showing increased mRNA levels of *Prm1*. In complementary studies, Geijsen *et al.*, found that treatment of ES cells or embryoid bodies with RA preserves cells positive for SSEA1 and alkaline phosphatase in differentiating ES cells (Geijsen *et al.* 2004). Collectively, these data suggest that RA makes an important contribution to ES cell differentiation into the male germ cell lineage.

Interestingly, the effects of testosterone on gene expression were also time dependent. Specifically, there was no testosterone treatment effect in the 7d cultures. However, after 14 days of culture, *Stra8* mRNA levels were increased (Fig. 3). Furthermore, treatment of cells for 14 days with both RA and testosterone induced a dramatic increase in the expression of *Act*. In the male gonad, the differentiation of germ cells into spermatozoa occurs in the tubular seminiferous epithelium and depends on the supporting Sertoli and Leydig cells. That is, testosterone is secreted by Leydig cells which stimulate Sertoli cell expression and secretion of growth factors (Griswold 1998). The growth factors in turn promote germ cell differentiation. Thus, the presence of testosterone during ES cell differentiation may support gene expression in the absence of differentiated Leydig and Sertoli cells.

CONCLUSIONS

In summary, our observations demonstrate the capacity of murine ES cells to express genes characteristic of male germ cells *in vitro*. We have also shown that RA and testosterone influence this process. Thus, these data represent an important first step in designing a plausible directed differentiation protocol for male germ cells such that further investigation may yield culture conditions that increase the level of male germ cell gene expression in a pattern consistent with the developmental program of spermatogenesis.

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Figure 1. Schematic representation of temporal gene expression during spermatogenesis
Male germ cells from the mitotic, meiotic, and post-meiotic stages of spermatogenesis are pictured including A⁰ spermatogonia (A⁰ SG), A and B spermatogonia (A/B SG), preleptotene spermatocytes (PL SC), leptotene and zygotene spermatocytes (L/Z SC), early pachytene spermatocytes (EP SC), late pachytene spermatocytes (LP SC), secondary spermatocytes (2' SC), round spermatids (RS), elongating spermatids (ES), and sperm (S). The temporal expression profiles of *Dpp3a*, *Piwil2*, *Stra8*, *Tex14*, *Dazl*, *Sycp1*, *Sycp3*, *Msy2*, *Odf2*, *Act*, *Akap3*, *Prm1*, and *Prm2* during the normal spermatogenic cycle (Di Carlo et al. 2000; Fimia et al. 2001; Gu et al. 1998; Horowitz et al. 2005; Kleene et al. 1984; Lee et al. 2006; Nagano 2007; Oulad-Abdelghani et al. 1996; Rocchietti-March et al. 2000; Wu et al. 2003) are indicated by the black bars.

Figure 2. Temporal expression profile of male germ cell genes in untreated adherent EB cultures QPCR was carried out using cDNA from adherent EB cultures maintained for 0 (D0), 2 (D2), 4 (D4), 7 (D7), or 14 (D14) days to measure the relative mRNA abundance of *Dpp3a*, *Piwil2*, *Stra8*, *Tex14*, *Dazl*, *Sycp1*, *Sycp3*, *Msy2*, *Odf2*, *Act*, *Akap3*, and *Prm1*. 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 18S rRNA. Bars represent standard errors. The individual genes are arranged according to the temporal pattern of expression shown in Figure 1.

Figure 3. Expression profile of male germ cell genes in adherent EB cultures treated with RA, testosterone, or both RA and testosterone

QPCR was carried out using cDNA from adherent EB cultures maintained for 7 (white bars), or 14 (black bars) days in the absence (-) or presence of RA, testosterone (T), or both RA and testosterone (RA+T). The relative abundance of each gene in each experimental group was normalized using 18S rRNA. The data represent QPCR results from 1–4 independent experiments, normalized to day 7 untreated control samples. The data was subjected to one-way ANOVA and Tukey-Kramer's multiple comparison tests. Statistically significant differences ($p < 0.05$) in normalized mRNA abundance between experimental groups are

indicated by different letters (a,b,c,d). The individual genes are arranged according to the temporal pattern of expression shown in Figure 1.

Figure 4. Western blot analysis and immunofluorescence localization of germ cell gene expression
 A. Western blot analysis of MSY2, DAZL, SYCP1, and SYCP3 in adherent EB cultures. Whole cell extracts from adherent EB cultures maintained for 2 (d2), 4 (d4), 7 (d7) or 14 (d14) days in the absence (–) or presence of RA, testosterone (T), or both RA and testosterone (RA+T) were analyzed for MSY2, DAZL, SYCP1, and SYCP3 protein expression. Whole cell extracts from mouse heart and testis were analyzed on the same blot and represent negative and positive control samples, respectively. Representative blots are shown. B. Immunofluorescence localization of MSY2 and DAZL in adherent EB cultures. Adherent cultures of EBs were maintained for 7 days in the absence (–) or presence of RA, testosterone (T), or RA and testosterone (RA+T). Upon termination, the cultures were processed for immunofluorescence using anti-MSY2 and anti-DAZL antibodies or non-immune serum (neg. control). MSY2 and DAZL were visualized using confocal microscopy ($\times 250$ magnification). White bars in each image represent 10 μ m in length.

TABLE 1

Quantitative RT-PCR primer sequences

Gene	Accession #	Forward Sequence (5'>3')	Reverse Sequence (5'>3')
<i>Dpp3a</i>	AY082485	TGTCGGTGCTGAAAGACCCTAT	TTTCCTTCGAGCCTTTTTTGTC
<i>Piwi2</i>	NM_021308	TGACCTGTGCATCCCCCTTCT	TCCCCACAAGCTTCATATCCA
<i>Tex14</i>	NM_031386	GCGTATCGCAGTCGGCA	CCATGTGCAGCACTGGGA
<i>Stra8</i>	NM_009292	GTTTCCTGCGTGTTCCACAAG	CACCCGAGGCTCAAGCTTC
<i>Sycp1</i>	NM_011516	CGCTACAACCACATGCTTCG	GGAACGCTGCTTAGATCTCCTC
<i>Sycp3</i>	NM_011517	ATGCTTCGAGGGTGTGGG	TTCCACCAGGCACCATCTTT
<i>Dazl</i>	NM_010021	AATGTTCAGTTCATGATGCTGCTC	TGTATGCTTCGGTCCACAGACT
<i>Msy2</i>	NM_016875	CACCAAGGAGGATGTCTTTGTTC	CCAACACTCCGCAGAACTTC
<i>Odf2</i>	NM_013615	CTGCCTTGTTAAGGTGTTGATGTC	TCATGGCCTTGAAGGATACCA
<i>Act</i>	AK006414	GTGTGCAGCCTGCACCAA	ACTGGCGGTCTTGAAAGCA
<i>Akap3</i>	NM_009650	ACGCCACTTTGACTTTGTAACCA	AAGACACCAATAAGGCTCATTCTG
<i>Prm1</i>	NM_013637	AGGTGTAAAAAATACTAGATGCACAGAATAG	TTCAAGATGTGGCGAGATGCT
<i>Prm2</i>	NM_008933	GAATAGTCACCTGCCCAAGCA	GCAGCTCAGGGCTCAGACA