DIFFERENTIATION OF MALE HUMAN EMBRYONIC STEM CELLS INTO GERM CELL-LIKE CELLS: A DEVELOPMENTAL MODEL AND AN ASSISTED REPRODUCTIVE TECHNOLOGY

by

FRANKLIN D. WEST

(Under the Direction of Steven L. Stice)

ABSTRACT

Studying human germ cell development has presented many challenges with few relevant models and a lack of primary tissue sources. Embryonic stem cells (ESCs) have recently shown great potential as a viable model producing sperm- and oocyte-like cells that in some cases can even produce live offspring. Additionally, it has been established ESC derived germ-like cells respond appropriately to germ cell signaling, express germ cell markers and undergo meiotic division, further confirming their potential as a developmental model. Beyond basic developmental questions, human germ cells might be used in toxicology, drug screening and cell therapy for infertile couples. However, there are still several major challenges facing the field including heterogeneous populations of differentiated cells, the lack of definitive biomarkers, aberrant developmental timing and potential problems caused by the absence of somatic tissue. The field has major hurdles to overcome, yet ESCs are a key tool to use in unlocking the basic elements of germ cell development that may lead to new fertility treatments. The objectives of these studies were to 1) develop a human ESC differentiation culture system capable of producing homogeneous populations of germ cells, 2) explore the role of mouse embryonic fibroblast and basic fibroblast growth factor in enriching and

differentiating these cells, 3) determine the ability of these cells to be maintained in extended culture and 4) to study human germ cell developmental signaling.

INDEX WORDS: Germ Cell, Differentiation, Human Embryonic Stem Cells, Basic Fibroblast Growth Factor, Bone Morphogenetic Protein 4, KIT Ligand, Cell Therapy, Infertility, Reproduction

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CHAPTER 1

INTRODUCTION

The ability to differentiate embryonic stem cells (ESCs) into germ cells has been established well over 5 years beginning with Hubner et al.'s [1] ground breaking research showing the derivation of oocytes from mouse embryonic stem cells (mESCs). This was quickly followed by the differentiation of sperm cells from mESCs from two other groups [2, 3] and the production of live offspring 3 years later [4]. Despite these successes, there have been very few reports, even from the original groups, noting further progress with only one group producing live offspring, which were short lived. Derivation of germ cells from human embryonic stem cells (hESCs) has proven to be even more challenging with few reports showing limited differentiation with cells only reaching the early meiotic stages [5, 6]. This has left the field with many critical questions concerning ESC derived germ cells including the ability to derive pure germ cell populations, epigenetic reprogramming, advanced differentiation and ultimately if it is possible to efficiently achieve therapeutic goals. Reviews have chronicled the successes in the field [7-9], examined current differentiation strategies, and similarities between hESCs and germ cells [10, 11], which would indicate that it should be fairly simple to derive late germ cells from ESCs. Unfortunately, this has proven not to be the case. In order to improve ESC to germ cell derivation culture systems, it is important to integrate factors found in normal germ cell development.

Several groups have shown significant increases in germ cell numbers using germ cell signaling molecules such as retinoic acid [2] and bone morphogenetic protein family members [3, 6], yet still less than 10% of the cells demonstrated immunoreactivity consistent with germ cells. Other groups have tried to improve differentiation by recreating the germ cell microenvironment using testicular cell condition media [12], testosterone [13] or even by transplanting cells directly into host testis [3, 4]. The results have been encouraging with increases in homogeneity and advances in differentiation, yet the need for further study is apparent with the inability to reliably produce live offspring from mouse germ cell- like (GCL) cells or even production of haploid GCL cells from hESCs.

hESC to germ cell derivation culture systems provide a unique opportunity to study human germ cell development at a basic level, however with more than 10% of couples worldwide considered clinically infertile, the biomedical implications are significant [14]. Fertility problems are caused by a litany of factors including environmental contaminants, health problems and genetic mutations [15-18]. As a relatively easy to obtain and highly proliferative source of human germ cells, ESC derived germ cells could be used in high through put screens for environmental contaminants, health treatments that could adversely affect germ cells, for drug discovery and ultimately as a cell source for cell therapies. The promise of these cells is great, yet the progress has been slow and can only be considered “modest” at best [5, 6].

The studies that comprise this dissertation sought to utilize factors known to be important for in vivo germ cell development, maintaining primary germ cells in culture

and hESCs to develop a unique germ cell differentiation culture system and study human germ cell development with hopes of ultimately producing viable germ cells. These studies were designed to test the following hypotheses: 1) GLCs can be derived from hESCs using and adherent differentiation culture system, 2) mouse embryonic fibroblast (MEF) feeder layers and basic fibroblast growth factor (bFGF) can enhance differentiation creating a more homogeneous population, 3) hESC derived germ cells can undergo meiosis in culture, 4) bone morphogenetic proteins can cause increased germ cell character and enrichment of hESC derived cells, 5) loss of mouse KIT ligand (KITL) in culture can inhibit germ cell differentiation and 6) hESC derived germ cells can be maintained in continual culture thus providing an excellent model system to study germ cell development.

Abbreviations and Nomenclature

ART- assisted reproductive technology

bFGF- basic fibroblast growth factor

BMP- bone morphogenetic protein

dpc- days post coitum

ESCs- embryonic stem cells

GCL- germ cell-like

h- human

KITL- KIT ligand

LIF- leukemia inhibitory factor

m- mouse

MEF- mouse embryonic fibroblast

SMAD- small mothers against decapentaplegic

TGF-β- transforming growth factor beta

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CHAPTER 2

LITERATURE REVIEW:

MALE GERM CELLS DERIVED FROM EMBRYONIC STEM CELLS:

BIOLOGICAL RELEVANCE, CHALLENGES AND FUTURE APPLICATIONS.[1]

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[1] West, F. D., N. L. Boyd, D. W. Machacek, A. Young, and S. L. Stice. To be submitted to *Biology of Reproduction*.

Introduction

Germ cells play a crucial role in biology as the cells that pass on genetic information to future generations of a species. However, the pathway by which mammalian cells turn from an unspecified epiblast cell into functional spermatozoa capable of producing offspring is complex and not fully defined. Differentiation in the mammalian system is different than other model species such as *C. elegans*, *Drosophila* and *Xenopus*, which are predestined by germ plasm, cytoplasm that contain granules of mRNA and proteins essential for germ cell development [1]. Instead mammalian germ cells undergo a dynamic process of specification, migration and differentiation mediated by alternative cell signaling pathways at various stages of development. Embryonic stem cells (ESCs) have long been touted as a tool for studying development with the ability to differentiate in vitro into all 3 germ layers of the body [2-4] and extraembryonic tissue [5]. More recently though, it has also been discovered that ESCs have the ability to differentiate into germ cell-like (GCL) cells in vitro [6-11], which offers a unique window into better understanding the development of germ cells.

In addition to learning the basic mechanisms associated with germ cell development, there is also a large possibility for practical applications for ESC derived male germ cells. Globally,10 to 15% of couples are considered infertile with ~50% due at least in part to the male partner [12]. The cause of male infertility ranges from relatively well understood causes such as sexually transmitted disease, the leading preventable cause of involuntary infertility [13], to Sertoli cell only syndrome, a condition in which all seminiferous tubules show a complete absence of germ cells [14]. Many

individuals who have experienced fertility problems have turned to assisted reproductive technologies (ART) such as artificial insemination and intracytoplasmic sperm injection. However, for people with more severe fertility problems such as Sertoli cell only syndrome, traditional ART techniques are not sufficient. Disorders associated with germ cell development have clearly highlighted a need for a better understanding of extrinsic and intrinsic factors affecting germ cell formation. However, studying human germ cell development has proven to be challenging because of a lack of adult tissue samples and because early germ cell development can only be studied using aborted fetal tissue [15]. One day hESCs could be a source of human germ cells for treating infertility but ethical issues will need to be addressed prior to their use in cell therapies for individuals with severe fertility problems.

In this review, it is my intent to discuss the biological relevance with respect to cell signaling, gene expression and other key developmental processes in the differentiation of ESC into GCL cells. In addition, I will address some of the major challenges associated with studying the differentiation process such as a lack of defining germ cell markers, aberrant developmental timing and the potential problems associated with the absence of somatic tissue. I will also discuss some of the potential biomedical uses and advantages of hESC derived germ cells focusing on male germ cell development with brief references to female germ cell development.

Differentiation of Embryonic Stem Cells into Germ Cells using Embryoid Bodies and Known Germ Cell Signaling Factors

Embryoid Body Germ Cell Differentiation

Differentiation of ESCs into specific lineages has frequently relied on the production of embryoid bodies (EBs) — aggregates of embryonic stem cells that encourage differentiation and are believed to be similar to the early developing embryo [16, 17] — or EB-like structures with a purification step for the specific lineage of interest [18-21]. EBs have also become the main technique employed for differentiation of both mouse [7, 11, 22] and human [6, 23] ESCs into GCL cells with few exceptions [8]. One disadvantage of using EB differentiation is it produces mixed populations of GCL cells at varying stages of development and cells of other lineages. It has consistently been the goal of stem cell biology to create a differentiation culture system capable of producing large homogeneous populations of the desired cell type. This becomes important when transplanting cells for therapy as residual ESCs could easily become tumors and the introduction of foreign cell types into sites such as the testes could have unforeseen repercussions. Mixed populations of germ cells are a result of small microenvironments produced within EBs where cells are affected by varying signaling. Even the addition of exogenous germ cell signaling factors to EBs has resulted in mixed populations [7, 9, 11]. ESCs exposed to signaling factors readily differentiate into germ cells and cells or alternative lineages [9]. A potential way of overcoming this challenge is using a two-dimensional culture system, which eliminates the formation of small microenvironments and cells are equally affected by added germ cell signaling factors. It is also clear that obtaining a more homogeneous germ cell population is dependent upon a better

understanding of germ cell signaling and that studying in vivo systems may hold the key.

Bone Morphogenetic Proteins: In Vivo Specification of Germ Cells and Enhancement of ESC to Germ Cell Differentiation

Bone morphogenetic proteins, members of the transforming growth factor beta (TGF-β) superfamily, are essential for normal germ cell development in vivo [24-27]. In the gastrulating mouse embryo, germ cell specification begins with BMP4 [24] and BMP8b [25] signaling from the extra-embryonic ectoderm and BMP2 [27] signaling from the visceral endoderm to the proximal region of the epiblast (Fig 2.1). BMP signaling molecules act by binding to BMPR-2, which phosphorylates BMPR-1 and activates the small mothers against decapentaplegic (SMAD) signaling pathway with SMAD 1 and 5 being shown to be of specific importance in germ cell formation [28-32]. The activated SMAD signaling proteins enter the nucleus and are believed to activate primordial germ cell (PGC) specifying genes with three prime candidates being *Ifitm3*, *Blimp1* and *Dppa3* [33-35]. Although a direct link between BMP expression and the activation of PGC specifying genes has not been shown BMP4 [24] and BMP8 [25] null mutant mice had few or complete loss of germ cell formation in vivo, and BMP4 heterozygous [24] and BMP2 homozygous [27] mutants had drastically reduced numbers of PGCs. In addition, epiblast explants show significantly reduced ability to form germ cells in the absence of BMP4 signaling, which further highlights the pivotal role of BMPs in germ cell specification. These studies suggest a significant role for BMPs in mouse specification of germ cells, but the role of BMPs in human germ cell development is yet to be determined.

In vitro models using mouse [11] and human [9] ESCs indicate BMPs are important for differentiation into GCL cells. In mESCs, seven days of EB differentiation without additional factors has been found to be sufficient to produce GCL cells [8, 11]. However Toyooka et al. [11] established that differentiation in EB-like aggregates with BMP4 producing trophoblast cells or M15 cells results in GCL cells expressing Ddx4, a marker believed to be expressed exclusively in mouse [36, 37] and human [36] germ cells, within a single day of differentiation. Surprisingly, BMP8b had no detectable effect on the differentiating mESCs [11]. In hESCs, addition of BMP4 to EBs resulted in a dose dependent up regulation in DDX4 gene expression and overall up regulation of synaptomal complex protein 3 (SCP3) [9], a marker expressed briefly in male PGCs once they reach the genital ridge and again during meiosis in the mouse [38, 39]. The addition of BMP7, a BMP family member important for PGC proliferation [40], or BMP8b alone did not make significant increases in DDX4 gene expression relative to treatments without BMPs [9]. However, the addition of BMP4, BMP7 and BMP8b simultaneously resulted in a significant 16 fold increase in DDX4 gene expression, relative to hESCs. The addition of BMP4, BMP7 and BMP8b also increased DDX4 positive cells from 0-3% to 10- 14.5%. These studies support that ESCs can recapitulate what is seen in vivo in the mouse and what is believed to happen in humans with respect to BMP response, which lends further credence to the use of ESCs to study germ cell formation.

Retinoic Acid: Important for Germ Cell Development and Meiotic Activity In Vivo and In Vitro

The role of retinoic acid (RA) in germ cell development has not been fully elucidated, however evidence suggests it is a requirement of normal development in these cells since mice lacking the RA precursor, retinol, exhibit decreased fertility [41, 42]. It is known that retinol is stored mainly in the liver and is bound to retinol binding protein (RBP) for transport to the testis [43]. Mutations in RBP results in developmental arrest of spermatogonial stem cells (SSCs) and loss of further differentiated germ cells [43]. RBP-bound retinol enters the differentiating germ cells and is changed into its functional form, RA (Fig. 2.1) [43], then enters the nucleus and binds to its nuclear receptor—either the retinoic acid receptor (RAR) or retinoid x receptor (RXR) [44]. The RA•receptor dimer is involved in recruiting transcription factors to retinoic acid response elements (RARE), which are associated with genes activated by RA like Stimulated by Retinoic Acid Gene 8 (Stra8), a germ cell protein necessary for meiosis [45, 46].

In the developing mouse embryo, RA has been linked to meiotic activity in germ cells. Inhibiting RA signaling in the female mouse resulted in loss of meiotic activity, while the addition of exogenous RA in culture to male gonads caused increase meiotic activity [41, 46]. Furthermore, Cyp26b1 expression is the in vivo modulator of RA with its expression inhibiting RA meiotic inductive activity. In female mice, Cyp26b1 is initially expressed in the gonad, but is soon inactivated allowing entry into meiosis. However, Cyp26b1 expression continues in the male mouse resulting in a quiescence state.

mESC derived GCL cells also respond to RA. Nayernia et al. [10] used RA to induce the expression of the meiotic marker Stra8 in 60% of cells in differentiation cultures, which were then fluorescence activated cell sorted (FACS) and maintain for two additional months. Two cell lines derived from the sorted cells were exposed to RA for an additional 72 hrs resulting in the expression of the meiotic marker *Sycp3, Acr* and *Dmc1*. Cells exposed to additional RA also showed an increase in the number of haploid products (~30%) relative to differentiated cells that were not exposed to additional RA (~3%). These cells were capable of producing live offspring, representing a landmark in the development of ESC derived GCL cells.

In addition to the important role of RA in meiosis [10], other studies suggest that RA plays a significant role in germ cell proliferation [47]. Geijsen et al. [7] showed that RA added to EB cultures enriched SSEA1 and alkaline phosphatase, two markers classically used to identify germ cells [48-50], positive GCL cells in cultures. In addition, these cells completed meiosis exhibiting a reduction in DNA content. Studies utilizing RA in the differentiation of ESCs to GCL cells has been limited, as has the use of most known germ cell signaling factors in general, with no documented studies in hESCs. However, studies investigating the RA signaling pathway promise to further elucidate its role in germ cell development.

Other Potential Signaling Factors Important for Differentiation of ESCs into GCL Cells

Few factors beyond BMPs and RA have been shown to further enhance ESC to GCL cells differentiation; however based on developmental studies, there are numerous potential candidates (Fig. 2.1). Signaling from KIT ligand (KITL) and its associated receptor tyrosine kinase KIT plays an essential role in germ cell development since

KITL and KIT knockout mice are sterile [51-53]. *Kit* mRNA is first expressed in PGCs at 7.5 days post coitum (dpc) of germ cell development [54]. Prenatal KITL/KIT signaling plays an important role in proliferation [55, 56], suppression of apoptosis [57] and cell adhesion between germ cells and somatic cells [58]. This signaling mechanism is upstream of the MAPK and JAK-STAT pathways [59-62] (Fig. 2.1). KITL/KIT signaling is also important for postnatal germ cell development [63-67] (Fig. 2.1). KITL is expressed by Sertoli cells [68], while KIT is expressed on differentiating type A, intermediate, type B spermatogonia and early preleptotene spermatocytes [63, 69]. Inhibition of KITL/KIT signaling by ACK2, an antibody that acts by irreversibly binding to the receptor, prevents the binding of KITL and activation of the KIT receptor [63, 69]. Loss of KITL/KIT signaling results in the loss of differentiated type A spermatogonia and all downstream derivatives, leading to sterility. Based on its known role in vivo, KITL/KIT signaling could potentially enhance ESC derived PGC proliferation and suppress apoptosis, therefore enriching GCL cells [6-9, 11]. In addition, the percentage of mESC derived GCL cells that complete meiosis is much lower than their in vivo counterparts, 36% and 68% respectively [7]. This may be due to a lack of KITL signaling, limiting further differentiation of ESC derived A spermatogonia into an early spermatocyte or a loss in survivability of pre-meiotic cell types, which occurs in vivo in the absence of KITL/KIT signaling [64].

Glial derived neurotrophic factor (GDNF), a TGF-β family signaling molecule, is essential for self renewal of SSCs [70, 71]. GDNF is produced by Sertoli cells and acts by binding to the germ cell expressed Ret receptor tyrosine kinase and GDNF family receptor-α1 (GFRA1) [72, 73]. This activates a downstream signaling cascade through

the Src family of tyrosine kinases and/or the phosphatidyl-inositol 3' kinase pathway. GDNF knockout in mice is lethal, however heterozygous mice that expressed reduced amounts of GDNF show differentiation of SSC without self renewal, which ultimately led to sterility [71]. Conversely, over expression of GDNF resulted in continual self renewal of the SSC population and a loss of differentiated cell types. These two phenotypes suggest that GDNF plays a crucial role in regulating SSC self renewal and continued proliferation.

Current protocols for differentiating mESCs to germ cells result in a mixed population of meiotic or post-meiotic cells. [7, 10, 11], suggesting that these cells have passed through a SSC stage. Isolation or purification of SSC stage cells without further differentiation into later stage germ cells would be important from a cell therapy perspective. SSCs would be ideal for transplant since these cells, unlike other germ cell stages, have the ability to re-colonize the gonad and return fertility to sterile recipients [74, 75]. Clearly this could be a valuable tool in the assisted reproductive technology arsenal.

Challenges of Differentiating Embryonic Stem Cells into Germ Cells

Embryonic Stem Cells and PGCs Have Similar Characteristics

The many similarities between PGCs and ESC cultures beg the question: What are the phenotypic and functional differences between ESCs and PGCs? ESCs are believed to be similar to the inner cell mass from which they were derived. However there is evidence to support both mouse [76] and human [77] ESCs may be more epiblastic in nature, the stage at which germ cells are first derived [78]. Therefore it is conceivable that epiblastic cells in continual culture could become more GCL in nature,

especially since many of the germ cell specifying and signaling factors are present in ESC culture conditions.

ESCs have traditionally been grown on mouse embryonic fibroblast (MEF) feeder cells. These MEFs express BMP4 [79, 80], a germ cell specifying factor, KITL [79, 81, 82], a germ cell proliferative factor expressed during germ cell migration and in the testis, basic fibroblast growth factor (bFGF) [79], a proliferative factor found in fetal and adult testis [83] , and leukemia inhibitory factor (LIF) [79, 81, 84], a germ cell proliferation factor [85]. The above factors are also important in maintaining pluripotency in mESCs— BMP4 [80], KITL [82], bFGF [86] and LIF [87-90], while bFGF [91] is critical to hESCs' self renewal. In addition to factors already found in ESC maintenance culture, exogenous LIF is commonly added to mESC [89] cultures and bFGF to hESC [91] cultures.

Most ESC lines are, or have been, cultured in fetal bovine/calf serum or knockout serum, which contain a host of undefined factors that could be important for differentiation and maintenance of GCL cells. Knockout serum has also been shown to elicit BMP like activity that can be inhibited by noggin, a BMP antagonist [91]. The presence of germ cell specifying and proliferative factors provides a potential mechanism by which ICM derived ESCs could potentially become germ cells in vitro. Additionally, the necessitation of these factors for the maintenance of ESCs further substantiates the claim that ESCs may truly be PGCs in culture.

A major challenge hindering research on ESC to germ cell differentiation is a lack of known markers specific to germ cells and not expressed in ESCs. Both mouse [7, 11, 92-94] and human [6, 9 , 36] ESCs express the germ cell markers DPPA3, POU5F1,

KIT and the germ cell “specific” marker DDX4. mESCs are also positive for the germ cell markers Ifitm3 [11] and SSEA-1 [95]. Overlap in marker expression among ESC and germ cells is not completely surprising as both cell types are pluripotent. Together, this body of evidence does suggest that ESCs and early PGCs are potentially one and the same or at least closely related.

Embryonic Germ Cells Lines

Determining which conditions are required to maintain primary germ cells in continual culture is a major challenge, with cells rarely living much longer than seven days [50, 96]. To address this problem, Matsui et al. [81] looked at the effect of KITL, which is expressed by STO cells (an immortalized MEF cell line [97]), on mouse primary germ cells. In addition to showing that KITL had a significant effect in increasing germ cell proliferation, it was also established that LIF derived from the STO feeder cells acted as a mitogen and caused a significant increase in number and survivability of primary mouse germ cells [81]. In 1992, Matsui et al. [98] and Resnick et al. [96] independently discovered the addition of exogenous bFGF to mouse PGCs caused colony formation almost identical to ESCs that could be continuously cultured and are now known as embryonic germ cells (EGCs). These cells form teratomas when injected into nude mice, producing ectoderm, endoderm and mesoderm lineages. EGCs contributed to the germ line when chimeras were formed and thus were transformed into cells resembling the undifferentiated epiblast [96, 98].

Human EGCs were derived from human PGCs using similar conditions as the mouse [99]. mEGCs [98, 100, 101] are positive for the standard mESC markers SSEA-1, Oct4, and alkaline phosphatase, whereas, hEGCs [99, 102] are positive for Oct4,

alkaline phosphatase, SSEA-3, SSEA-4, TRA-160, and TRA-1-81. EBs derived from hEGC give rise to all three embryonic germ layers. The differences between ESCs and EGCs are few including 1) the challenge of maintaining EGCs in continual culture [103, 104] and 2) the lack of expression of SOX2 in EGCs [105], an essential pluripotency factor in ESCs [106]. The lack of SOX2 expression may be one reason why EGCs have a tendency to spontaneously differentiate under serial culture conditions [105]. PGCs in vivo and in vitro (EGCs) closely resemble ESCs by colony morphology, gene and protein expression, response to environmental signaling and differentiation potential. It is evident that further and more comprehensive comparison between ESCs, EGCs and PGCs is needed to discern the differences between these pluripotent cell types.

When Do Embryonic Stem Cells Become Committed to the Germ Cell Lineage?

Redundancy in genetic markers has made it difficult to determine when ESCs have reached a confirmable germ cell state. The author believes that several developmental criteria can be used: 1) re-setting of imprinting gene methylation, 2) meiosis, 3) replacement of histones with protamines, 4) acrosome 5) flagella formation, 6) the ability to fertilize an oocyte and 7) produce live offspring (Fig. 2.2) [7, 10, 11]. None of these criteria have been fully met by hESC derived GCL cells.

DNA methylation plays a critical role in expression of imprinting genes, genes that are variably expressed based on parental origin [107-109]. The addition of a methyl group in differentially methylated regions (DMRs) will silence genes, while the lack of methylation allows for expression. In developing mouse germ cells, imprinting genes undergo demethylation and change from monoallelic to biallelic expression once they reach the genital ridge (10.5 dpc) and remethylation in male germ cells occurs after

14.5 dpc [110-113]. Resetting of imprinting genes (i.e. H19, Igf2r and Snrpn) has been observed in mESC derived GCL cells [7, 10]. GCL cells have also produced haploid cells indicating that they have undergone meiosis. Mouse GCL cells progressed through appropriate DNA synthesis and divisions with diploid, tetraploid and haploid populations [10]. In addition, GCL cells have also been shown to express meiotic markers such as SYCP3, which is involved in the meiotic stage of synapsis [38, 39], and STRA8 [10], an important regulator of meiotic prophase [114, 115]. To date, there are no reports of GCL cells derived from hESCs undergoing complete meiosis, however it is important to note that there has been expression of the meiotic markers SYCP1 and SYCP3 indicating potential meiotic activity (Fig. 2.2) [6, 9]. Presently, only Nayernia et al. [10] have had success with a functional reproductive assay producing 12 offspring out of 65 transferred embryos. Though these offspring were short lived (5 days to 5 months), they were an important proof of principle.

ESC Derived Germ Cells have Aberrant Developmental Timing and Development Relative to In Vivo Counterparts

Normal germ cell development is a highly ordered and structured process which is regulated by spatial and temporal cues. Ddx4 is normally expressed in the mouse at 9.5 dpc [116] and week 7 in humans [36]. Whereas EB in vitro differentiation of ESC to GCL cells resulted in DDX4 expression after three and two days for mESCs [37] and hESCs [9] respectively. Considering that the ICM is collected at ~3.5 dpc in the mouse [117] and day three to five post-fertilization in the human [118], there is a surprising three day difference in Ddx4 expression in mESC derived GCL cells and about a six week difference in DDX4 expression in hESC derived GCL cells relative to in vivo

counterparts. In addition to aberrant germ cell marker expression, entry into meiosis is abnormally early in ESC derived GCL cells. mESCs derived GCL cells enter into meiosis after 20 days of differentiation [7] and male mouse germ cells do not normally enter into meiosis until after 21 days of gestation and 7 days of postnatal development (28 days) [119]). Based on SCP1 and SCP3 expression, hESC derived GCL cells, enter meiosis after 14 days of differentiation [6]. This is drastically different from what is seen in normal male human germ cell development where the complete meiotic process does not normally occur before year 12 to 13 [120]. As previously noted, meiotic entry is intrinsically linked to somatic tissue expression of Cyp26b and RA signaling [41, 46] and therefore the absence of these cell types in differentiation cultures is a likely cause of aberrant developmental timing.

In vivo there is a dynamic cell signaling process that occurs between germ cells and somatic tissue that is critical for normal programming and development. The environment produced by the testis is all but impossible to reproduce in vitro, therefore some groups have partially differentiated ESC derived germ cells and introduced them into the testis [10, 11]. The results were cells that appeared to undergo complete meiosis based on expression of HSC70t [11], a marker expressed in elongated spermatids [121], or visual inspection [10]. Nayernia et al. [10] were able to produce live offspring using their system, yet their GCL cells seemed to remain in a pre-elongated state unlike the cells produced by Toyooka et al. [11] which had flagella-like structures indicative of an elongated spermatid. However, neither of these two studies directly examined the contribution of the somatic tissue in differentiation.

In addition to the previous two studies, one group has attempted to enhance male mESCs differentiation into germ cells by the addition of media that had been exposed to testicular cells for 72 hrs [94]. The idea was that the somatic cells (i.e. Sertoli and leydig cells) found in the testis produce many factors important for maintaining and differentiating these germ cells [122, 123]. Testicular cell conditioned media enhanced male germ cell differentiation, but these male ESC derived GCL cells seemed to form large oocyte-like structures and expressed oocyte specific markers *Fig*α and *Zp3* [94]. The resulting oocyte-like structures from mESC derived germ cells indicates that a better understanding of the role of somatic tissue in differentiation cultures is need. The contribution of somatic cell tissue during sex determination is key in proper germ cell development and the temporal and spatial regulation of these cues have to be taken into account [124-128].

Potential Biomedical Applications of ESC Derived Germ Cells

According to the Centers for Disease Control and Prevention's (CDC) most recent National Survey of Family Growth, 7.1% of American couples are considered clinically infertile, meaning they have unsuccessfully tried to conceive for at least a year. However, infertility is very much a universal problem as 10 to 15% [12] of couples globally are considered infertile with some of the largest populations (~30%) of infertile couples seen in sub-Saharan Africa [129, 130]. It has been shown that ~50% of the time infertility is due, at least in part, to the male component with variability based on socioeconomic, ethnic and regional effects [12, 131]. Male germ cell development can be negatively affected by many factors including environmental contaminants, diseases (e.g. cystic fibrosis), disease treatments (cancer treatments) and genetics

(azoospermia) [132-135]. However, 15% of all cases are labeled as being idiopathic or unexplainable [12, 136, 137]. These facts show a clear need for a system that can be used to study defects in germ cell development.

Current model systems such as *C. elegans, Drosophila, Xenopus* and the mouse are limited with respect to studying human germ cell development due to major species differences ranging from germ cell specification [24, 25, 34, 138, 139] to the expansion and differentiation of SSCs into spermatids [140, 141]. Intuitively, it is best to study human derived tissues, unfortunately, maintenance of human germ cells in culture [103] and obtaining the fetal tissue at the appropriate time to derive these cells is experimentally and ethically challenging [15]. For a model system to be optimal for studying human germ cell development, it should have certain characteristics that 1) are derived from a human source, 2) are relatively easy to culture, 3) maintain genetic stability and 4) are highly proliferative; characteristics all of which hESCs possess.

hESC derived GCL cells have the potential to be used in several ways beyond the basic understanding of germ cell development, including as a drug screening tool. Many drugs have been tested and found to have adverse affects on germ cell development. For example, testicular cancer is the most common type of cancer in men ages 15 to 39 and the treatments are commonly chemo- or radiotherapy [142]. These types of treatments often lead to sterility [142] or even genetic and epigenetic problems, such as aneuploidy [143, 144] and hypomethylation [145], that could lead to birth defects. The list of Food and Drug Administrative (FDA) approved drugs that potentially cause sterility or reduced fertility is expansive including drugs such as sulphasalazine, commonly used to treat inflammatory bowel disease [146], and carbamazepine, used to

treat epilepsy [147, 148]. One of the potential reasons that the negative effects of these drugs were not known initially is most drug screens are not conducted on human germ cells. Present systems cannot recapitulate human sperm motility [147, 148] or acrosome formation [149, 150] — processes that are inhibited by some of these drugs. In addition to screening for adverse drug effects, one could also look for small molecules that positively affect germ cell formation. Pharmacological treatment options for male infertility have been limited, leaving the door wide open for drug discovery for which these ESC derived cells are highly suited.

Germ Cell Therapy

hESC derived GCL cells also have great potential in the field of cell therapy, however there are several technological hurdles that must be overcome. In many cases male fertility issues can be overcome with standard drug treatments or through assisted reproductive technologies (ART) such as artificial insemination (AI) or intracytoplasmic sperm injection (ICSI). However, in some situations where a patient is completely devoid of germ cells (i.e. Sertoli cell only syndrome [151, 152]), ART techniques are rendered useless. It is in these cases that the full potential of ESC derived germ cells can be seen. One could potentially take a somatic cell from an infertile individual and reprogram that cell into an ESC-like state. These patient specific ESCs could then be differentiated into SSCs, the adult stem cell population of the testis that provides a continuous source of germ cells. SSCs could then be transferred to the testis of an infertile patient and lead to germ cell repopulation. In addition to problems associated with differentiating ESCs into germ cells, this strategy faces two other major hurdles: 1) the need for reprogramming and 2) the actual transfer of SSCs into the testis.

Previously, somatic cell nuclear transfer was the only known mechanism for reprogramming of somatic cells, however it has been recently found that the over expression of key genes could cause dedifferentiation of somatic cells into an ESC-like state [153-157]. Generation of induced pluripotent stem (IPS) cells was done first in mouse [153, 155] and then in human [154] fibroblast cells by expressing POU5F1, SOX2, KLF4, and C-MYC. The factors essential for reprogramming are still debated with Yu et al. [156] showing that reprogramming could also be done using NANOG and LIN28, in addition to POU5F1 and SOX2, and eliminating KLF4 and C-MYC. IPS cells seem to be an ESC equivalent expressing genes and surface markers consistent with ESCs, forming all three germ layers and even integrating into the germ line [155, 158]. This technique seems to circumvent most of the ethical and political issues that are associated with somatic cell nuclear transfer, however it presents a new battery of problems. One major concern is that most of these genes are up regulated in various cancer types [159-163] with concerns also being raised by the use of the cancer causing retroviral transduction vehicle [164, 165]. These fears are further validated by the fact that tumors develop in 20% of offspring derived from IPS chimeras [158]. The potential of IPS cells to easily make patient and disease specific stem cells and relieving the scientific community of “serious ethical issues” [166] is inviting, yet the viability of this option still remains to be fully established.

The potential to obtain ESC derived SSCs and restoring fertility to infertile patients is an exciting prospect, yet it has proven to be difficult to even identify in vivo derived cells for transplant. It has been shown that mouse SSCs can be identified and isolated by the presence of THY-1 and α6-INTEGRIN and absence of MHC class I, KIT

and αV-INTEGRIN [70, 167]. Moreover, this has only been done with cells derived from the testis and it is not clear if these markers hold true in a mixed culture system that have cell types representing all germ lineages— as would be the case in an ESC differentiation culture system. Transplant of a mixed population could result in tumors, as a product of remaining pluripotent cells, or placement of foreign (i.e. neurons, cardiomyocytes) cell types into the testis. The transplantation of these cells has not yet been demonstrated in humans, despite the fact that it has been done in several species including mice [74], bovine [168], goats [169] and non-human primates [170]. Species specific problems are always a major concern when considering techniques such as SSC transplants. It is clear that advances in germ cell differentiation cultures and initial human SSC transplant trails, despite the optimistic outlook of this technique, are needed to further advance the therapeutic potential of these GCL cells.

Conclusion

The fact that 10 to 15% of couples worldwide are considered to be clinically infertile is an alarming statistic that supports the need for a robust biologically relevant model [12]. Most traditional model systems (i.e. *Drosophila*, *Xenopus*) have drastic differences in germ cell development, making them not ideal for study [1], and primary human germ cell tissue is extremely limited [15]. Embryonic stem cells are a potential solution to these problems and offer an exciting opportunity to study germ cell development from specification to a mature sperm capable of producing offspring in vitro [6-8, 10, 11]. However, further understanding of germ cell signaling and important developmental processes is essential to enhancing the ESC to germ cell derivation system. The call

for a better understanding is especially true in the lagging hESC field if these cells are ever to be a viable biomedical opportunity.

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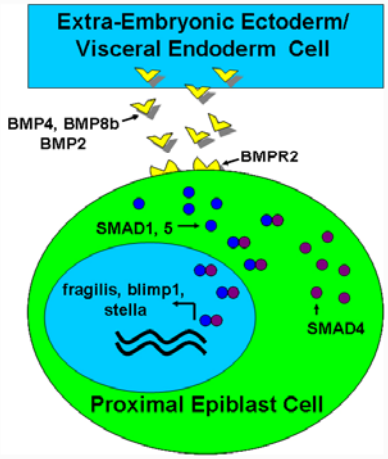
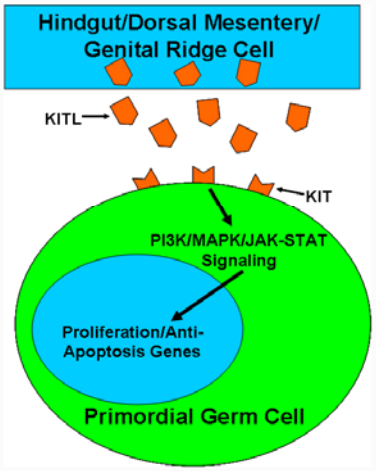
Figure 2.1. Germ Cell Specifying and Developmental Signaling. Prenatal germ cell development begins with BMP4 and 8b specifying signaling from the extra-embryonic ectoderm and BMP2 from the visceral endoderm to the proximal epiblast and activation of BMPRs, SMAD signaling and germ cell specifying genes. KITL/KIT signaling in pre-migratory, migratory and genital ridge germ cells, working through the MAPK and JAK-STAT pathways, induces further proliferation and inhibits apoptosis. Postnatal KITL/KIT signaling also plays a role in proliferation of spermatogonia and inhibiting apoptosis. RA signaling through the RAR or RXR receptor causes activation of meiotic genes such as Str8 to differentiate diploid germ cells into haploid cells.

Prenatal

Specification: BMP Signaling

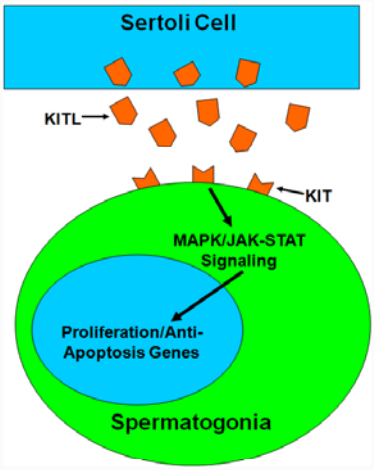


Proliferation: KITL/KIT Signaling



Postnatal

Proliferation: KITL/KIT Signaling



Meiosis: RA Signaling

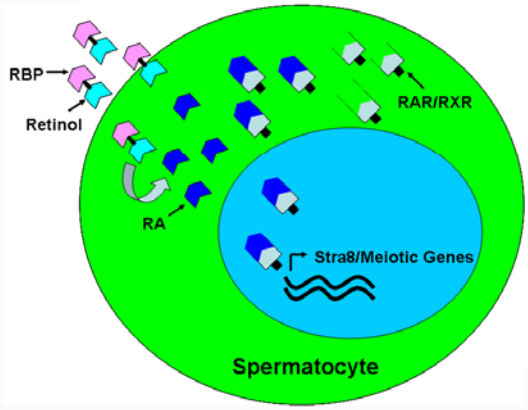
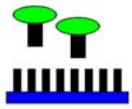
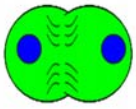
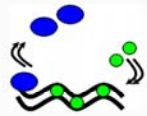
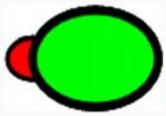
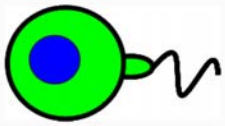
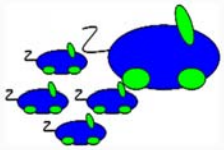


Figure 2.2. Milestone in ESC Derived Germ Cell Development. Certain activities in normal germ cell development are key characteristics and can be used to delineate ESCs from germ cells. mESC derived germ cells have shown the ability to undergo re-setting of imprinting genes [7, 10], meiosis [7, 10, 11, 171], exchange histones for protamines [10, 171], form acrosome [7, 10] and flagella [10, 11] and successfully fertilize an egg [7, 10] and produce offspring [10]. However, hESC derived germ cells have only shown markers indicative of entry into meiosis [6, 9].

Milestones	Mouse ESCs	Human ESCs
 Re-Setting Methylation	Yes	No
 Meiosis	Complete Meiosis	Initial Meiosis
 Histone Replacement with Protamines	Yes	No
 Acrosome Formation	Yes	No
 Flagella Formation	Yes	No
 Fertilization	Yes	No
 Offspring	Yes	No

CHAPTER 3

ENRICHMENT AND DIFFERENTIATION OF HUMAN GERM-LIKE CELLS MEDIATED BY CELLS AND BASIC FIBROBLAST GROWTH FACTOR SIGNALING. [1]

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[1] West, F. D., D. W. Machacek, N.L. Boyd, K. Pandiyan, K. R. Robbins, and S. L. Stice, Accepted by *Stem Cells*. Reprinted here with permission of publisher, 10/01/08.

Abstract

Human embryonic stem cells (hESCs) have recently demonstrated the potential for differentiation into germ-like cells in vitro. This provides a novel model for understanding human germ cell development and human infertility. Mouse embryonic fibroblast (MEF) feeders and basic fibroblast growth factor (bFGF) are two sources of signaling essential for primary culture of germ cells, yet their role has not been examined in the derivation of germ-like cells from hESCs. Here protein and gene expression demonstrate that both MEF feeders and bFGF can significantly enrich germ cell differentiation from hESCs. Under enriched differentiation conditions, flow cytometry analysis proved 69% of cells to be positive for DDX4 and POU5F1 protein expression, consistent with the germ cell lineage. Importantly, removal of bFGF from feeder free cultures resulted in a 50% decrease in POU5F1 and DDX4 positive cells. Quantitative RT-PCR analysis established that bFGF signaling resulted in a up regulation of genes involved in germ cell differentiation with or without feeders, however feeder conditions caused significant up regulation of pre-migratory/migratory (*IFITM3, DAZL, NANOG* and *POU5F1*) and post-migratory genes(*PIWIL2, PUM2*) along with the meiotic markers *SYCP3* and *MLH1*. After further differentiation, >90% of cells expressed the meiotic proteins SYCP3 and MLH1. This is the first demonstration that signaling from MEF feeders and bFGF can induce a highly enriched population of germ-like cells derived from hESCs thus providing a critically needed model for further investigation of human germ cell development and signaling.

Introduction

Infertility is a major problem in the United States with 7.4% of married couples considered to be clinically infertile [1]. To aid these couples, assisted reproductive technologies have been developed, but are ineffective in treating the most severe cases where there is an absence of germ cell production [2]. Human embryonic stem cells (hESCs) offer the means to further understand intrinsic and extrinsic factors involved in early and late germ cell development and survival [3-5]. New developments and discoveries using hESCs should ultimately lead to novel fertility treatments.

Several genes and their products are expressed during germ cell development and are used to follow germ cell differentiation. POU5F1 (also known as Oct4), a transcription factor involved in stem cell pluripotency, is also expressed in primordial germ cells (PGCs) and is highly conserved among species [6]. As PGCs become lineage restricted during germ cell development, POU5F1 becomes exclusively expressed in germ cells and is not expressed in somatic cells [7]. In the mouse, IFITM3 and DPPA3 are specifically expressed in PGCs [8, 9]. Unrestricted germ cells first express IFITM3 during pre-gastrulation followed by DPPA3 expression at the late primitive streak stage of development. Additionally, DDX4 (also known as VASA) is a highly conserved, functionally important germ cell gene that is expressed exclusively in germ cells in numerous species including *Drosophila, Xenopus*, mice and humans [10-13]. DDX4 is an RNA helicase that in *Drosophila* has been shown to be important for germ cell plasm formation, but its role has not yet been fully elucidated in the mammalian system [11]. However, male *Ddx4* homozygous knockout mice are sterile [12] and previous studies have used DDX4 in human ESC to PGC differentiation [3, 14].

Male mouse germ cells enter a state of mitotic arrest at 13.5 dpc, this quiescent state is not reversed until puberty is reached and the mitotic process is then continued and meioses first takes place [15]. In the mouse, a complex process is orchestrated by a plethora of genes that are activated and inactivated during germ cell development. Synaptonemal complexes formed during homologous chromosome pairing in meiosis involve numerous proteins such as SYCP3 [16, 17]. SYCP3 expression is specific to meiotic germ cells and knockout of this gene results in meiotic disruption and sterility [15, 18]. After the pairing of homologous chromosomes, crossing over occurs, which is controlled by proteins such as MLH1 [19, 20] and is associated with the formation of chiasmata during a crossing over event [19]. MLH1 knockout mice exhibit disrupted meiosis, which results in sterility in both female and male mice [20]. The expression of these genes in human germ cells has been noted, but their study has been limited. Further understanding of these genes and others through in vitro human germ cell models is therefore warranted.

In an attempt to address the need for an in vitro model to better understand these developmental processes, several groups have reported the differentiation of mouse embryonic stem cells (mESCs) and hESCs into germ-like cells. mESCs have produced both male and female germ-like cells with protein profiles consistent with germ cell development [4, 5]. Cells in advanced differentiation cultures have even proven to undergo meiosis and erasure of methylation in imprinting genes, two hallmarks of normal germ cell development [21]. Nayernia et al. successfully showed mESC derived germ cells can produce offspring [22]. Differentiated hESCs have also proven to have protein profiles consistent with normal germ cell development (i.e. DDX4+) and undergo

early stages of meiosis [3, 14]. Unfortunately, both mESC and hESC germ cell differentiation culture systems have produced relatively mixed populations with less than 35% DDX4 positive cells derived from hESCs and co-expression for other pluripotent markers such as POU5F1 was not determined [3, 14]. Traditionally, isolation of stem cell populations based on only a single marker is not as robust as the use of two or more markers.

Previous work has established that mouse embryonic fibroblast feeders and basic fibroblast growth factor (bFGF) are essential for culturing primary germ cells [23-25]; however their role has not been examined in the derivation of germ-like cells from hESCs. Here we show hESC differentiated via an adherent continuous culture on MEF feeders in bFGF supplemented media, resulted in a population of 69% DDX4 and POU5F1 positive cells. To our knowledge, this is the highest proportion of human germ-like cells reported to date derived from either mouse or human ESC. Together, protein and gene expression data indicated MEF feeder cells played a significant role in increasing germ cell enrichment. Supplemental bFGF proved to be of even more importance in deriving and maintaining POU5F1 and DDX4 positive cells under feeder free conditions. Differentiated cultures also showed early meiotic gene and protein expression (e.g., MLH1 and SYCP3). Using this differentiation process, we produced cultures of predominately germ-like cells.

Materials and Methods

hESC Culture Conditions

BGO1 (XY) hESC with normal karyotype were cultured on ICR mouse (Harlan) MEF feeders inactivated by mitomycin C (Sigma). The cells were cultured in 20% KSR

stem cell media, which consists of Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 20% knockout serum replacement (KSR), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin/50 µg/ml streptomycin (Gibco), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 4 ng/ml bFGF (Sigma-Aldrich and R & D Systems). They were maintained in 5% CO_2 and at 37° C. Cells were passaged every 3 days by mechanical dissociation, re-plated on fresh feeders to prevent undirected differentiation with daily media changes as previously described by our laboratory [26].

Enrichment and Differentiation Culture Conditions

Germ-like cells were enriched in an adherent culture system by growing them on feeders or polyornithine (20 µg /ml) and laminen (5 µg/ml) coated (feeder free) plates with or without 4ng/ml bFGF for 3, 10 and 30 days in 20% KSR conditioned media without passaging. Media prepared for feeder free cultures were conditioned by exposing them to MEFs for 24 hours. Cultures were maintained in 5% CO_2 at 37° C and media was replaced every other day. Cells grown for 3 days with bFGF are under identical hESC maintenance conditions and are considered to be hESC controls.

Immunocytochemistry

Cells were passaged onto glass 4 chamber slides (B D Falcon) and fixed with 4% paraformaldehyde for 15 minutes. Antibodies were directed against POU5F1 (Santa Cruz, 1:500), DDX4 (R & D Systems, 1:200), MLH1 (Santa Cruz, 1:200) and SYCP3 (Santa Cruz, 1:200). Primary antibodies were detected using secondary antibodies conjugated to Alexa Flour 488 or 594 (Molecular Probes, 1:1000). Cell observations were made using the Olympus Ix81 with Disc-Spinning Unit and Slide Book Software

(Intelligent Imaging Innovations). The data were quantified using Image-Pro Plus (Media Cybernetics).

Flow Cytometry

Cells were fixed in 57/43% ethanol/PBS for 10 minutes at room temperature. Cells were washed 3 times in PBS and were blocked in 6% donkey serum for 45 min. In feeder free conditions, antibodies were directed against POU5F1 (Santa Cruz, 1:500) and DDX4 (R & D Systems, 1:200). In the presence of feeders, an antibody against Human Nuclei (Chemicon, 1 µl per million cells) was also used to prevent false positives caused by feeders. MEFs were also used as negative controls for POU5F1 and DDX4 expression. Primary antibodies were detected using fluorescently conjugated secondary antibodies Alexa Flour 405, 488 and 647. (Molecular Probes, 1:1000). Cells were sorted and analyzed using a Dakocytomation Cyan (DakoCytomation) and FlowJo Cytometry analysis software (Tree Star).

Reverse Transcription-Polymerase Chain Reaction

RNA was extracted using the Qiashredder and RNeasy kits (Qiagen) according to the manufacturer's instructions. The RNA quality and quantity was verified using an RNA 600 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer. For real time quantitative reverse transcription-polymerase chain reaction *(*RT-PCR) total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer's protocols. Reactions were initially incubated at 25° C for 10 minutes and subsequently at 37° C for 120 minutes. Real-time quantitative RT-PCR (Taqman) assays were chosen for the transcripts to be evaluated from Assays-On-Demand™ (Applied Biosystems Inc.), a pre-validated library of human specific QPCR

assays, and incorporated into 384-well micro-fluidic cards. Two micro liters of the cDNA samples (diluted to 50 µl) along with 50 µl of 2 x PCR master mix were loaded into respective channels on the micro-fluidic cards followed by centrifugation. The card was then sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc.). All failed (undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the ΔΔCT method of quantification (Applied Biosystems Inc.) [27]. Average fold changes from four independent runs were calculated as $2^{-\Delta\Delta CT}$. Significance was determined by running 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons for each gene focusing on temporal, bFGF and feeder effects and their interactions.

Qualitative RT-PCR was performed on RNA using the Qiagen OneStep RT-PCR Kit (Qiagen) following the manufactures instructions. Reactions were initially incubated at 50° C for 30 minutes and then at 95° C for 15 minutes for reverse transcription. The PCR conditions were initiated with denaturing at 95° C for 4 minutes followed by 34 cycles at 94° C for 1 minute, 62° C for 1 minute, 72° C for 1 minute with a final extension at 72° C for 10 minutes. Products were then run 4% agarose gel and examined. Specific primers were used for the amplification of Cyp26b1: Sense 5' TCTTTGAGGGCTTGGATCTG antisense 5' GAATTGGACACCGTGTTGG.

Results

Germ Cell Protein Expression in an Adherent Germ Cell Culture System

DDX4+ POU5F1+ cells were enriched on MEF feeder co-cultures and on polyornithine- and laminin- coated plates for 3, 10 and 30 days without passaging under enrichment conditions. Cells grown under enrichment conditions for 3 days with bFGF are under identical hESC maintenance conditions and are considered to be hESC controls. In most cases DAPI (Fig. 3.1A) nuclear staining of hESCs showed co-localization with the pluripotency marker POU5F1 (Fig. 3.1C) and absence of the germ cell marker DDX4 (Fig. 3.1E, G merge). However, after 10 days of differentiation, the pluripotency marker POU5F1 (Fig. 3.1D) and germ cell marker DDX4 (Fig. 3.1F) showed nuclear co-localization with DAPI (Fig. 3.1B, H merge) with similar results seen at day 30. All treatments showed a sub-population of DDX4+ POU5F1+ cells, which is indicative of early germ cell development (Fig. 3.1). Unexpectedly, we observed DDX4+ POU5F1+ cells in hESC cultures, however other groups have also noted germ-like cells in undifferentiated mouse [28] and human [3] ESCs. DDX4+ POU5F1+ cells were found to be in large clusters, which showed the potential for germ cell signaling events. DDX4 was localized to the nucleolus of DDX4+ POU5F1+ cells (Fig. 3.1F & H, inset). In contrast, no DDX4 or POU5F1 positive staining was observed in co-cultured feeder cells or other hESC derived cells such as human neural progenitor cells (data not shown).

Temporal effect on DDX4+ POU5F1+ expression

Immunocytochemistry showed enhanced germ-like marker expression under enrichment conditions, therefore we used flow cytometry to quantify the enrichment of

DDX4+ POU5F1+ cells. Flow analysis further confirmed that a population of cells was indeed positive for both DDX4 and POU5F1 (Fig. 3.2A). Flow analysis showed that there was a significant ($p<0.05$) temporal effect with an increase in DDX4+ POU5F1+ cell percentage at day 10 (average of treatments 57.62 ±9.20) when compared to the ESC control, day 3 (average of treatments 18.47 ±1.56) and 30 (average of treatments 19.62 ±5.98) with and without feeders and bFGF (Fig. 3.2B). bFGF played a significant role in increasing the percentage of DDX4+ POU5F1+ cells, but only under feeder free conditions (Fig. 3.2B). There was a significant ($p< 0.05$) increase in the percentage of DDX4+ POU5F1+ cells at day 10 (34.63%) and 30 (9.16%) in the presence of bFGF. Yet, bFGF had no significant effect when hESCs were cultured on feeders.

Within day 10 samples, cells without bFGF and feeders showed significantly ($P<0.05$) reduced numbers of DDX4+ POU5F1 cells (Fig. 3.2B). Samples within day 30 were also compared and treatments without MEFs showed reduced numbers of DDX4+ POU5F1+ cells irrespective of the presence of bFGF. This indicates that feeder cell contact plays a role in germ cell enrichment, since cells grown without feeders were grown in MEF conditioned media and would carry feeder derived soluble signaling factors.

MEF Feeders Increased Germ-like Gene Expression

Pre-Migratory and Migratory Stage Gene Expression

Due to bFGF optimizing the performance of feeder free cultures by enriching DDX4+ POU5F1+ cells, germ cell gene expression was only examined in the presence of bFGF. Significant increases in pre-migratory (*IFITM3, DPPA3, POU5F1* and *DAZL*) and migratory (*NANOG* and *DDX4*) germ cell gene expression were observed for both

with and without feeder cultures at day 10 and 30, however cultures grown on feeders consistently showed significantly higher germ cell gene expression than feeder free conditions regardless of day (Fig. 3.3). Cells cultured on feeders had significantly higher *POU5F1, DAZL* and *NANOG* gene expression ($p < 0.05$) at both day 10 and 30, relative to feederless counterparts (Refer to Fig. 3.3 for fold changes). Feeder cell cultured conditions also produced significant ($p < 0.05$) increases for *DDX4* expression at day 10 and *IFITM3* and *DPPA3* expression at day 30 in comparison to without feeder groups (Fig. 3.3). Overall, treatments with feeders showed noted increases in pre-migratory and migratory gene expression relative to the without feeder treatments. In fact, gene expression for *DAZL* was several hundred folds higher when cells were on feeders. In addition, treatments without feeders showed decreased gene expression relative to hESC for *POU5F1, DAZL* and *NANOG* at day 30, while a decrease in germ cell gene expression was never observed in feeder culture conditions.

When comparing enriched feeder cell cultures to undifferentiated hESC, *DAZL* and *NANOG*, genes expressed during pre-migratory and migratory stages were up regulated in day 10 and 30 cultures (Fig. 3.3). Other pre-migratory genes, *POU5F1* and *DDX4*, were also higher at day 10 and *IFITM3* was higher at day 30, relative to hESC control. In contrast, cultures without feeders showed only limited increases. *DAZL* in feeder free cultures was only up regulated at day 10 (Fig. 3.3). All other genes, *IFITM3*, *POU5F1, DPPA3, NANOG* and *DDX4* showed no significant increase relative to hESC controls. This proves that feeder culture conditions cause increased pre-migratory and migratory germ cell gene expression over time, while cultures without feeders show limited temporal increases.

Genital Ridge, Spermatogonia and Meiotic Stage Gene Expression

We then asked the question: Does our culture system up regulate the expression of post-migratory genes of the genital ridge (*PIWIL2*), spermatogonia (*PUM2, DAZ1-4* and *NANOS1*) and meiotic (*MLH1* and *SYCP3*) phases of germ cell development? Therefore, we compared contemporary cultures by quantitative RT-PCR and found that cultures exposed to feeders consistently showed significantly higher germ cell gene expression than feeder free cultures. Feeder cell cultures significantly ($p< 0.05$) increased *PIWIL2, PUM2* and *MLH1* gene expression in day 10 and 30 cultures, relative to their contemporary without feeder cultures (Fig. 3.4). The feeder treatment groups also produced an increase in the *DAZ1-4* cluster (*DAZ1;DAZ2; DAZ3; DAZ4*) at day 10 and *NANOS1* and *SYCP3* at day 30 (Fig. 3.4). Some treatments with feeders produced highly significant changes in post-migratory gene expression, *PIWIL2, SYCP3*, and the *DAZ* family cluster had at least a 10 fold increase in expression ($p<0.05$) relative to feeder free culture. The up regulation of these post-migratory genes is indicative of advanced stages of differentiation when MEF feeder cells are present.

Post-migratory gene expression was increased over time in MEF feeder culture conditions when compared to undifferentiated hESC cultures, but less so in feeder free conditions. In feeder culture conditions expression for *PIWIL2, PUM2*, and *NANOS1* was significantly ($p<0.05$) higher at day 10 and 30 than hESCs (Fig. 3.4). *SYCP3*, a known meiotic marker, was up regulated at day 30 relative to hESCs. Although feeder free cultures generally showed less expression for these genes compared to MEF grown cells, they were up regulated when compared to undifferentiated hESC for the genes *NANOS1* and *PIWIL2* at day 10 and 30 (Fig. 3.4). In contrast to the MEF

included culture, *PUM2* and *SYCP3* showed no up regulation at day 10 or 30 in feeder free conditions. This suggests that feeder conditions generally cause a temporal increase in post-migratory gene expression, where feeder free conditions fail to do so.

Expression of Meiotic Markers in Culture

Increased gene expression of the meiotic genes *MLH1* and *SYCP3* indicate potential entry into meiosis; however this normally does not occur in male germ cell development until puberty. Cyp26b1, a retinoic acid degrading enzyme, regulates sex-specific timing of meiotic entry and inhibits meioses in male mice whereas, male Cyp26b1 knockout mice germ cells enter meiosis in prenatal stages similar to female counterparts [29, 30]. Our RT-PCR results suggested that *CYP26B1* is not expressed in cultures of all treatments (gel not shown). The absence of *CYP26B1* may contribute to the onset meiotic gene expression in cultures of germ-like cells.

To determine wither germ-like cells undergo meiosis in culture, hESCs were differentiated for 10, 16 and 30 days on MEFs in the presence of bFGF and immunostained for MLH1 and SYCP3. Immunostaining showed that > 90% of day 16 cells were positive for MLH1 (Fig. 3.5E-F) and SYCP3 (Fig. 3.5G-H) protein, while no expression of either marker was found in hESCs (Fig. 3.5A-D), day 10 (data not shown) or day 30 cells. In addition, staining was localized to the nucleus, which correlates with their known role in chromosome segregation during meiosis [17, 20, 31] suggestive that germ-like cells have the potential to undergo meiosis in culture.

Discussion

In this study, we used hESC as a model to understand the differentiation process toward the germ cell lineage. After examining multiple treatment variations, we were

able to produce 69% DDX4 and POU5F1 positive germ-like cell population when culturing hESC on MEF feeder cells with bFGF supplemented media. To our knowledge, this is the most uniform population of germ-like cells generated from hESC reported to date. In previous studies, hESCs formed more heterogeneous populations and the best results previously published were no higher than 35% of the population being DDX4 positive [3, 14] in embryoid body differentiation systems in contrast to the adherent culture system described here. Chen et al. [32] used a human adherent germ cell differentiation culture system, however they did not determine in a quantifiable method the number of germ-like cells produced, nor did they examine the role of feeder cells or bFGF supplemented media.

Feeder cells and bFGF have important roles in maintaining both ESC and primordial germ cells [33], but their role in germ cell differentiation has not been investigated. Here the role of bFGF proved to be of even greater importance under feeder free conditions with more than a 100% increase in DDX4+ POU5F1+ cells in feeder free conditions with bFGF at day 10 and day 30, relative to feeder free conditions without bFGF (Fig 3.2). Because the feeder free culture uses MEF conditioned media, this suggests the levels of MEF derived bFGF are not optimal for germ cell differentiation. At the same time, it is unlikely bFGF is the only factor associated with the MEFs affecting our results. In addition to producing bFGF [34], MEF feeders produce KIT ligand (also known as stem cell factor), which is linked to germ cell proliferation and development [35]. These and other factors are logical targets for further understanding of germ cell differentiation in this system. Successful maintenance of pluripotent hESCs is believed to be due to the inhibitory effect of bFGF on the bone

morphogenetic protein (BMP) family pathway [36]. This is the same signaling family proven to be essential for germ cell development in vivo [8, 36, 37] and is present in our system as a part of the knockout serum replacement [36] and thought to be the potential cause of the “undirected” differentiation and enrichment. However, lineage specific induction by BMP family members is concentration dependent [38, 39] and unknown in our extended cultures. Therefore the in vitro conditions in our culture, with the presence of MEFs, may create a microenvironment that limits the differentiation into other cell types [40] and/or promotes germ cell differentiation.

DDX4 has proven to be a robust germ cell marker being one of the few genes determined to be restricted to the germ cell lineage in humans [10]. However, the function and localization of this protein is not fully understood nor has it been well characterized. We showed novel localization of DDX4 protein to the nucleolus, which could indicate a functional role in human germ cell development. The mouse DDX4 homologue, mouse vasa homologue (MVH), has been traditionally noted as being cytoplasmically localized, yet though its function is unclear, it is critical to maintain fertility [12, 41]. DDX4 is an RNA helicase and might be associated with germ cell specific RNA molecules that are localized to the nucleolus. Several other DEAD-box protein family members (i.e. DDX47) have also been found localized to the nucleolus, but they have not been shown to be associated with germ cell development [42]. A better understanding of this germ cell specific protein and its localization is clearly needed and our culture system can provide an important in vitro model for this investigation. Previous studies have shown subpopulations of mouse [28] and human [3] ESCs expressed germ cell markers, which was further confirmed in this study with

the expression of DDX4 in undifferentiated hESCs. The overlapping expression of germ cell markers between germ cells and stem cells may indicate DDX4 has a function in both early germ cells and in maintaining pluripotent cell types.

In this study, changes in the expression of six germ cell pre-migratory and migratory genes and six post-migratory germ cell genes were monitored during the temporal differentiation of hESC in this adherent system. The presence of feeder cells caused dramatic increases in pre-migratory, migratory and post-migratory germ cell gene expression compared to the feederless conditioned media treatment. Once again, the mechanism by which feeder cells cause this increase is not clear, yet important. Due to the fact that cells in the feeder free conditioned media group were less germ-like, we hypothesize that the effect of increased germ cell gene expression is mediated through hESC-MEF cell contact.

Overall, 10 days of differentiation proved to be optimum with noted increases in both germ cell gene and protein markers at this time. The fact that early to late germ cell markers were expressed at this time suggests that day 10 cells were not a homogenous population. Also unexpectedly, the early germ cell specifying genes *IFITM3* only modestly increased overtime, and *DPPA3*, another germ cell specifying gene, showed no increased expression over time. The lack of increased expression of some early germ cell specifying genes may be due to the fact that they may already be expressed at some level in hESC [3]. This has led some to suggest that initial germ cell programming may already be activated in undifferentiated hESCs [3]. If this is the case, then increased expression of early germ cell genes such as *DPPA3* would not be expected, as we observed here. *POU5F1* and *NANOG* were more highly expressed at

day 10 than the starting hESC populations in treatments with feeders, which was unanticipated since these genes are highly expressed in hESCs [43]. However, *POU5F1* expression levels have been linked with specific differentiation pathways. Loss of *POU5F1* has been shown to result in spontaneous differentiation, but up regulation results in differentiation into primitive endoderm [44]. Therefore, *POU5F1* expression may differ depending on the differentiating cell type, and thus differentiating germ cells may require relatively high expression.

Significant gene and protein expression of the meiotic markers SYCP3 and MLH1 were observed under differentiation conditions with feeders. SYCP3 and MLH1 are both early meiotic markers being expressed in prophase, therefore suggesting entry into meioses [16-20, 31]. The increased expression of meiotic genes after only 10 days of differentiation and the presence of protein after 16 days is unexpected given that meiosis and meiosis associated proteins are normally not observed until after puberty. Intuitively, these proteins would not be expected to be present after only 16 days of hESC differentiation in vitro. However this rapid entry into meiosis is consistent with human [3, 14] and mouse [21] ESC derived germ-like cells. Geijsen et al. [21] noted expression of the haploid germ cell marker FE-J1 in mESC derived germ-like cells after 13 days of differentiation, while Clark et al. [3] observed some meiotic activity in hESC derived cells. *SYCP1* gene and cytoplasmic SYCP3 protein were observed, but MLH1 protein was not present after 14 days of hESCs differentiation [3]. Whereas under our conditions, SYCP3 and MLH1 were localized in the nucleus of greater than 90% of the germ-like cells, and both meiotic markers were absent in undifferentiated hESCs. It is encouraging that these proteins were found in the nucleus and potentially in the future,

human haploid cells may be generated from these cultures. We also found an absence of *CYP26B1* expression, a meiotic initiation inhibitor, further suggesting that regulation of male germ cell meiotic events normally observed in vivo may not be mimicked in our cell cultures. In support of our findings, germ cells from *Cyp26b1* knockout males [29, 30] and male germ cells that were ectopically developed, and presumably not exposed to CYP26B1 [45, 46], have been found to directly enter meiosis similar to wild type females. Our study demonstrates that the timing of meiotic activity in germ-like cells is early relative to in vivo counterparts and further inspection for normal meiotic activity is clearly needed. Nonetheless, abbreviated germ cell differentiation may ultimately prove to be an advantage from a therapeutic perspective if germ cell differentiation and maturation can be accelerated.

Summary

Using a novel adherent culture system with MEF feeder cells and bFGF, we have demonstrated successful and reliable production of a population where 69% of cells express germ-like character (DDX4+ POU5F1+) by immunocytochemistry, flow cytometry and quantitative RT-PCR. Enriched cultures showed progressive differentiation with the expression of pre-migratory, migratory, and post-migratory gene expression with some genes being expressed several hundred fold higher than their hESC counterparts. These enriched cultures ultimately demonstrated advanced levels of differentiation with >90% of cells expressing the meiotic markers SYCP3 and MLH1. This robust system clearly has the potential for use in parsing the factors involved in the differentiation of hESCs down the germ cell lineage. Understanding this process could ultimately lead to new and important treatments for infertility.

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Figure 3.1. Expression of DDX4/POU5F1 Protein Is Up Regulated Under Enrichment Conditions. hESCs were cultured on MEF feeders (shown) or polyornithine and laminin coated plates in 20% KSR media with or without bFGF (4ng/ml) for 3, 10 (shown) or 30 days. DAPI **(A)** nuclear staining of hESCs exhibited co-localization with the pluripotency marker POU5F1 **(C)** and absence of the germ cell marker DDX4 **(E)**. A merge of the three images are seen in **(G)**. After 10 days of differentiation, the pluripotency marker POU5F1 **(D)** and germ cell marker DDX4 **(F)** displayed co-localization with DAPI **(B)** with similar results seen at day 30. DDX4 proved to have novel nucleolar localization **(F;** enlarged in the inset**)** and a merge of these images can be seen in **H.** Scale Bars 10 um, insets 5 um.

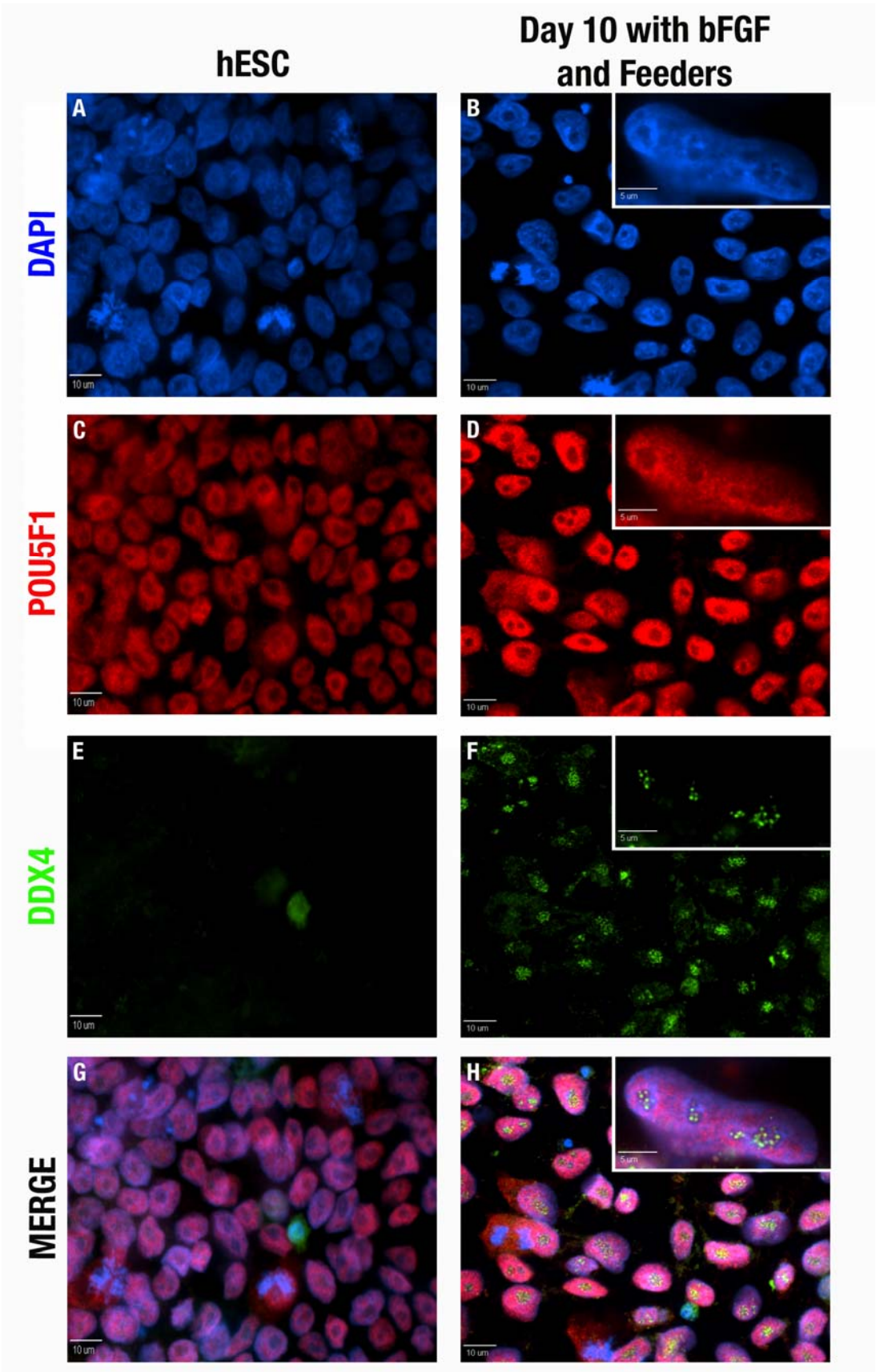
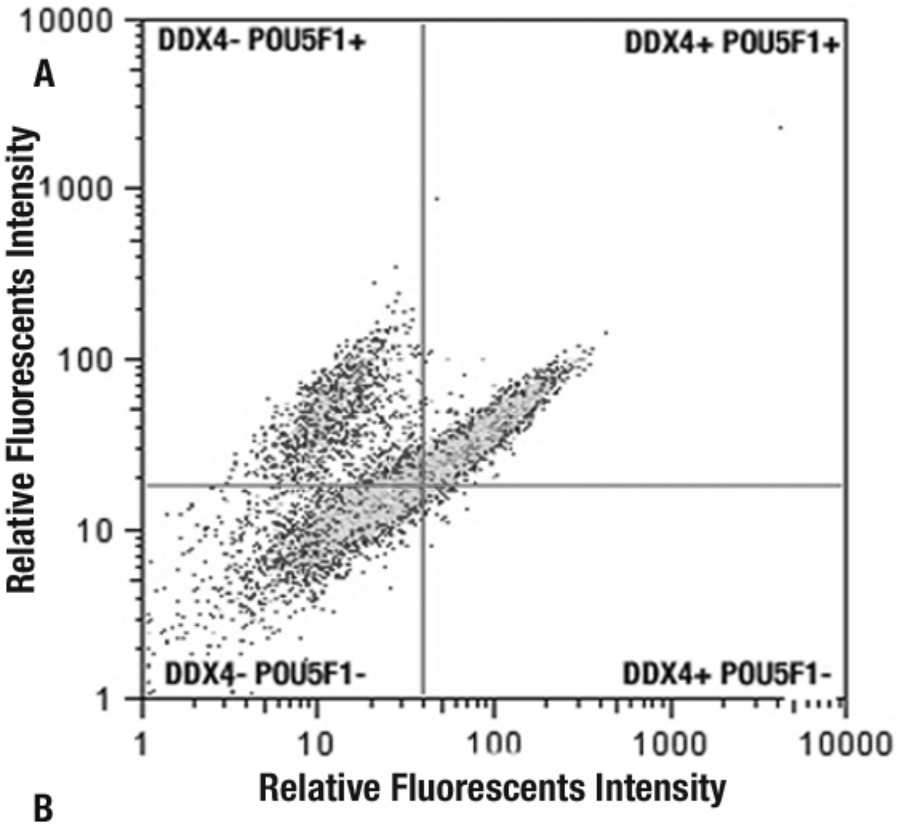

hESC
Day 10 with bFGF and Feeders
DAPI
POU5F1
DDX4
MERGE
A
B
C
D
E
F
G
H
10 um
5 um

Figure 3.2. Enrichment Conditions Result in Increased DDX4+ POU5F1+ Cells with bFGF Playing a Role Under Feeder Free Conditions. Flow cytometry was used to quantify the DDX4+ POU5F1+ cell population (A; Day 10 with MEFs and bFGF shown). Flow analysis showed a significant temporal (******; $p<0.05$; N=4) effect with an increase in DDX4+ POU5F1+ cells at day 10 when compared to the hESC control, day 3, 10 and 30 with and without feeders **(B)**. Under feeder free conditions, bFGF (*****; $p< 0.05$) caused an increase in the percentage of DDX4+ POU5F1+ at day 10 and 30.


A
DDX4- POU5F1+
DDX4+ POU5F1+
DDX4- POU5F1-
DDX4+ POU5F1-
Relative Fluorescents Intensity
10000
1000
100
10
1
1
10
100
1000
10000
Relative Fluorescents Intensity

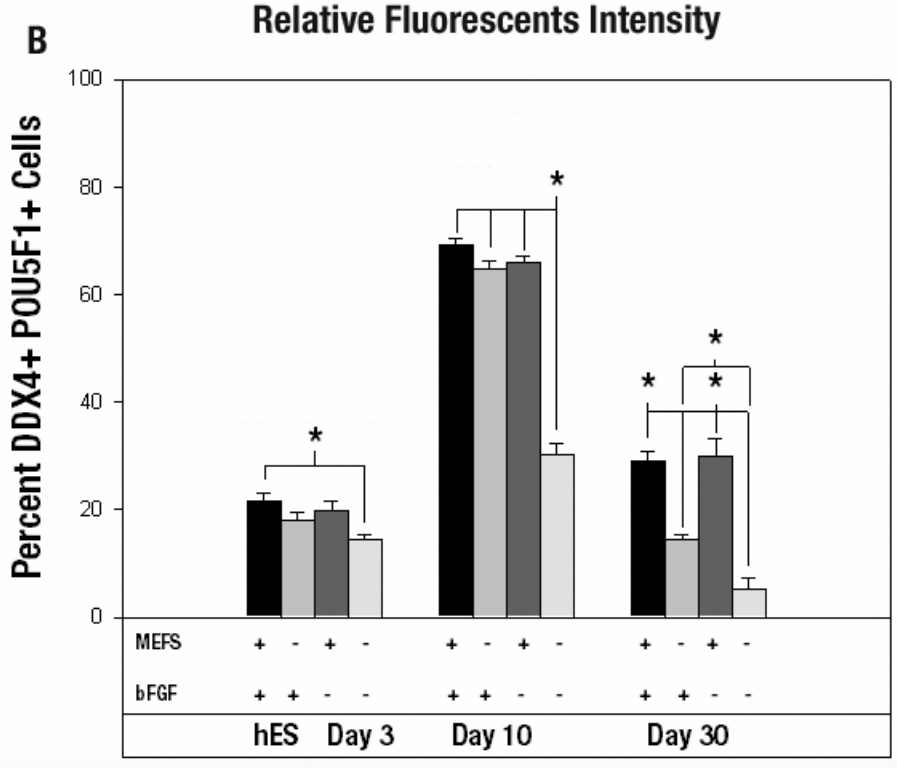

B
Percent DDX4+ POU5F1+ Cells
100
80
60
40
20
0
*
*
*
*
*
MEFS
bFGF
hES
Day 3
Day 10
Day 30

Figure 3.3. Feeder Conditions Resulted in Higher Gene Expression of Pre-Migratory and Migratory Genes. hESCs were grown for 3, 10 and 30 days with and without feeders in the presence of 4 ng/ml of bFGF and were analyzed for gene expression by quantitative RT-PCR. The results proved that cells cultured on feeders expressed significantly (**; $p < 0.05$; N=4) higher POU5F1, DAZL and NANOG at day 10 and 30, DDX4 expression at day 10 and IFITM3 and DPPA3 expression at day 30 relative to feeder free conditions. Temporal effects were significant (*; $p < 0.05$) under feeder culture conditions for DAZL and NANOG gene expression at day 10 and 30 relative to hESCs. POU5F1 and DDX4 were higher at day 10 and IFITM3 was higher at day 30 relative to hESC. Feeder free cultures showed up regulation of DAZL at day 10 and all other genes, IFITM3, POU5F1, DPPA3, NANOG and DDX4 showed no significant increase relative to hESC controls. (Note scale differences.)

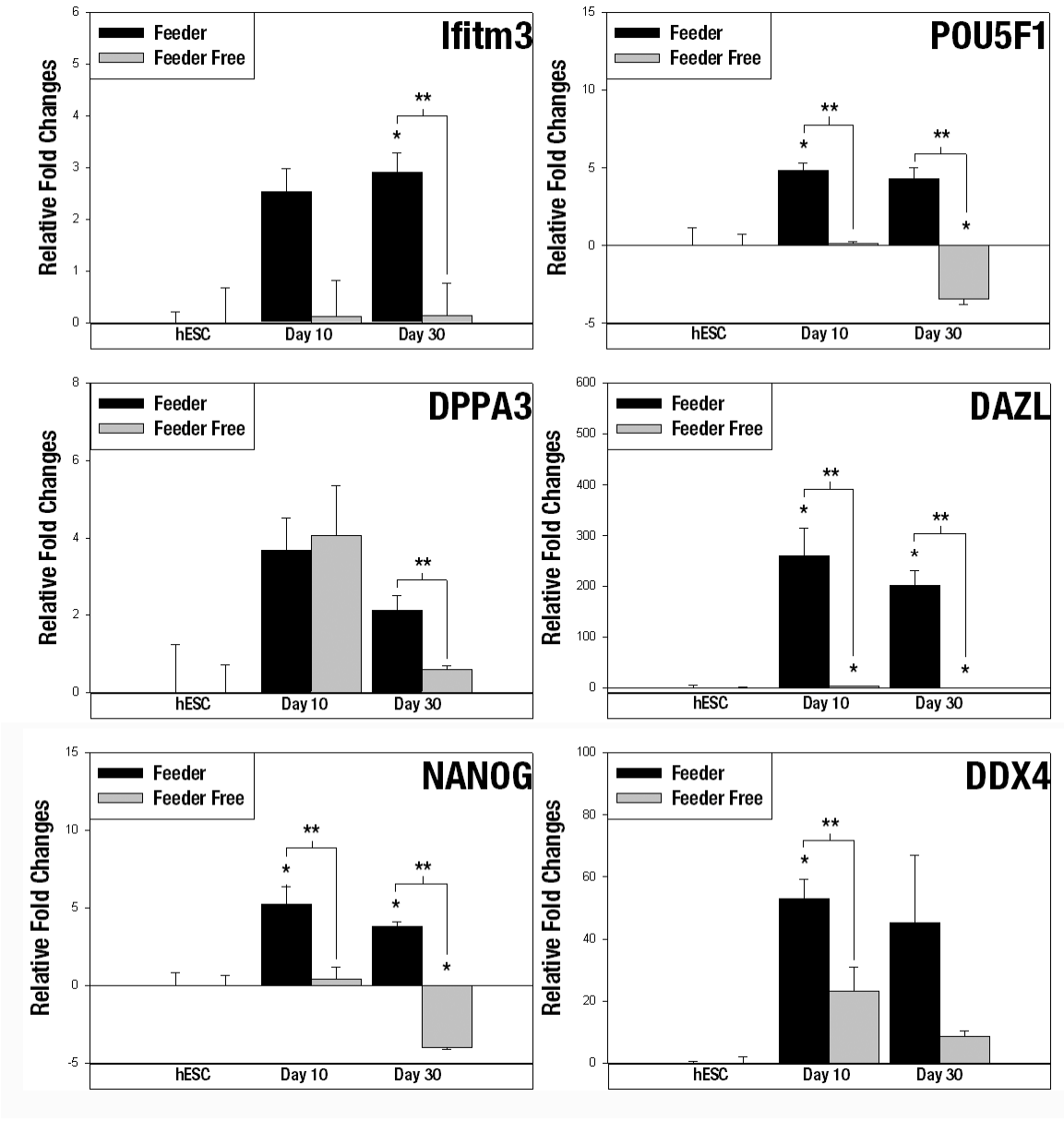

Ifitm3
POU5F1
DPPA3
DAZL
NANOG
DDX4
Feeder
Feeder Free
Relative Fold Changes
hESC
Day 10
Day 30

Figure 3.4. Feeder Conditions Resulted in Higher Gene Expression of Genital Ridge, Spermatogonia and Meiotic Stage Gene Expression. hESCs were grown for 3, 10 and 30 days with and without feeders in the presence of 4 ng/ml of bFGF and were analyzed for gene expression by quantitative RT-PCR. Feeder cell cultures resulted in significantly (**; $p < 0.05$; N=4) increased PIWIL2, PUM2 and MLH1 gene expression for day 10 and 30, the DAZ1-4 cluster at day 10 and NANOS1 and SYCP3 at day 30 relative to feeder free conditions. Temporal effect under feeder culture conditions resulted in significant (*; $p<0.05$) up regulation of PIWIL2, PUM2, and NANOS1 at day 10 and 30 relative to hESCs and SYCP3 expression at day 30. Feeder free cultures were up regulated for the gene NANOS1 and PIWIL2 at day 10 and 30 relative to hESCs, however PUM2, DAZ1-4, SYCP3 and MLH1 showed no up regulation at day 10 or 30. (Note scale differences.)

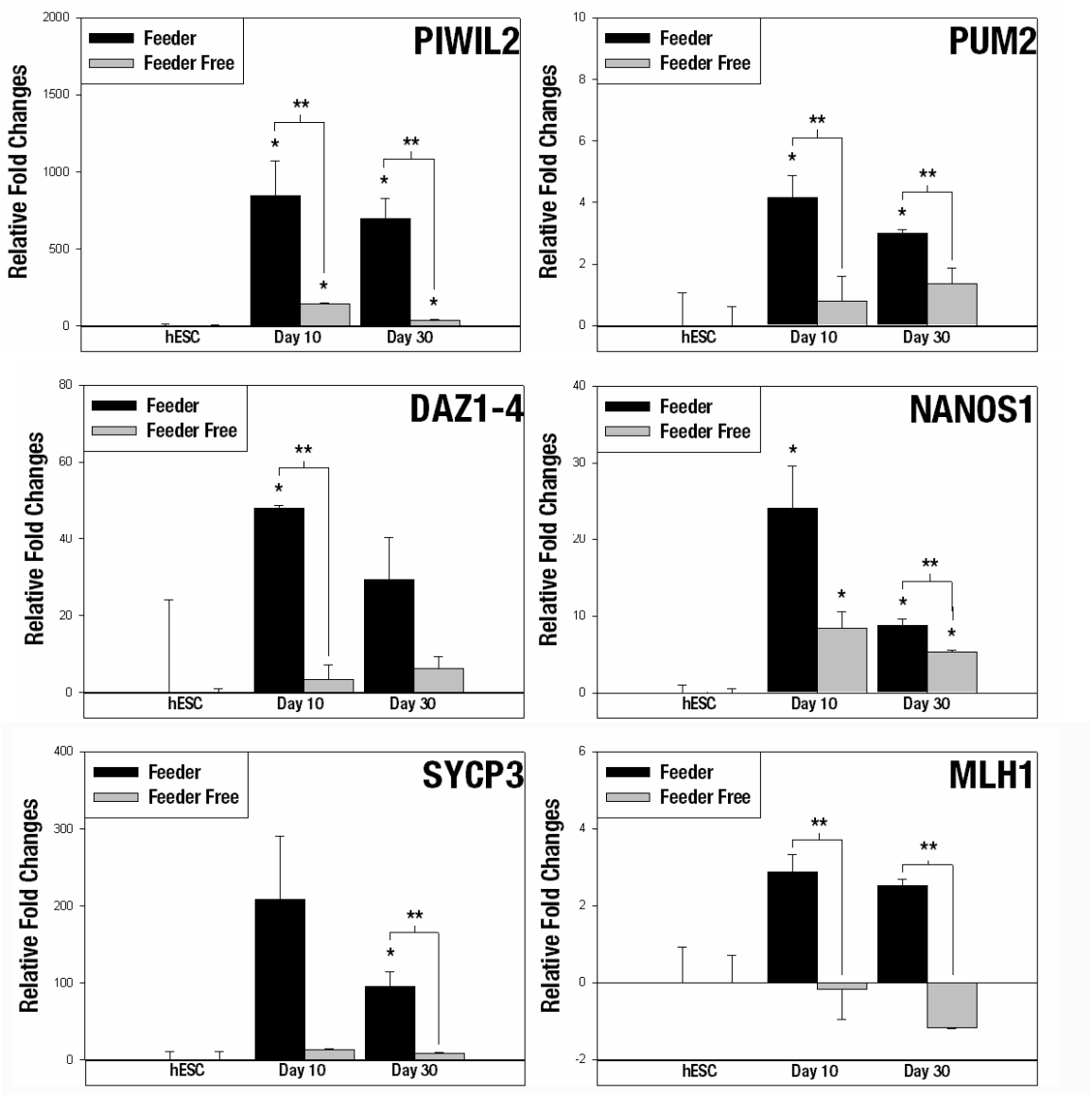
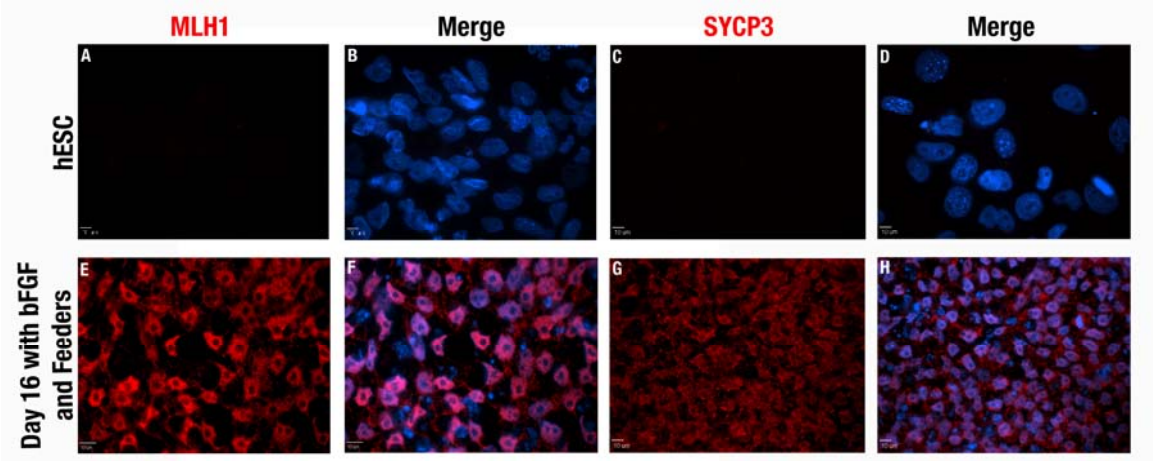

PIWIL2
PUM2
DAZ1-4
NANOS1
SYCP3
MLH1
Feeder
Feeder Free
Relative Fold Changes
hESC
Day 10
Day 30

Figure 3.5. Expression of Meiotic Markers in Differentiated Cultures. hESCs were cultured on MEF feeders in 20% KSR media with bFGF (4ng/ml). Undifferentiated hESCs (control, top row) did not express the meiotic markers MLH1 **(A, B** merge with DAPI**)** or SYCP3 **(C, D** merge with DAPI**)**. After 16 days of differentiation, the meiotic markers MLH1 **(E, F** merge with DAPI**)** and SYCP3 (**G, H** merge with DAPI) displayed co-localization with DAPI. This was not observed at day 10 or day 30. Scale Bars 10 um.


MLH1
Merge
SYCP3
Merge
hESC
Day 16 with bFGF and Feeders
A
B
C
D
E
F
G
H

CHAPTER 4

BONE MORPHOGENETIC PROTEIN AND KIT LIGAND SIGNALING ENHANCE HUMAN EMBRYONIC STEM CELL TO GERM CELL-LIKE CELL DIFFERENTIATION.[1]

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[1] West, F. D., M. I. Roche-Rios, S. Abraham, M. A. Bedell, R. R. Rao, M. S. Natrajan, and S. L. Stice. To be submitted to *Biology of Reproduction*, 10/07/08.

Abstract

Signaling mechanisms involved in early human germ cell development are largely unknown and are believed to follow mouse germ cell development; however, there may be species specific differences. Bone morphogenetic protein 4 (BMP4) and KIT ligand (KITL) are necessary in mouse germ cell development and may also play an important role in human germ cell development. Using a hESC to germ cell derivation culture system, we found that endogenous BMP signaling caused germ cell-like (DDX4+ POU5F1+) cell differentiation with the inhibition of this pathway causing a significant decrease in germ cell gene expression and DDX4+ POU5F1+ cells. Moreover, the loss of mouse KITL (mKITL) in enrichment and differentiation cultures resulted in the down regulation of migratory germ cell genes and a 70.5% decrease in DDX4+ POU5F1+ cells, indicating mKITL is involved in human germ cell development. Further we demonstrated, that eliminating KITL feeders but maintaining their secreted extracellular matrix (ECM) sufficiently sustained the increased numbers of DDX4+ POU5F1+ cells in culture, however this resulted in decreased germ cell gene expression. In these studies, we establish that BMP4 and KITL germ cell signaling affects in vitro formation of hESC derived germ cell-like cells and indicates that they may play an important role in normal human germ cell development.

Introduction

Investigation of early human germ cell developmental niche has been hindered, due in part to a lack of biological resources and therefore the majority of mammalian germ cell developmental studies have been conducted in the mouse. Mouse germ cell-like (GCL) cells derived from mouse embryonic stem cells (mESC) undergo meiosis, elongate and even produce live offspring [1-4]. However, murine studies may not always directly translate to advances in human germ cell development, given that similar results using human embryonic stem cells (hESCs) have not been reported. This may reflect species specific differences in germ cell development and is indicative of the challenge in directly translating mESC germ cell results to hESC germ cell development. These differences highlight the need to directly investigate germ cell signaling factors and their relation to early human germ cell developmental events in human cells.

In vivo, several growth factors have proven to be essential in the proper specification and differentiation of developing germ cells in the mouse. These are logical initial signaling factors to investigate in human germ cell development. Two of these factors are bone morphogenetic protein 4 (BMP4) [5], the transforming growth factor beta (TGFβ) superfamily member, and KIT ligand (KITL) [6]. In the gastrulating mouse embryo, germ cell specification begins with BMP4 [5, 7, 8] signaling from the extra-embryonic ectoderm to the proximal region of the epiblast. BMP4 signaling molecules then bind to BMP receptors and activate genes responsible for initial germ cell development. Inhibition of BMP4 signaling has resulted in a partial or complete loss of murine germ cell formation [5, 9-11]. The function of BMP4 in human germ cell

development has not been established. Similar to BMP4, interruption of normal expression of KITL, which is expressed by the somatic tissue along the early germ cell migratory path in both mouse [6] and humans [12], or its receptor KIT also results in a loss of normal migration and proliferation in early mouse germ cells [Runyan, 2006 #299;Matsui, 1990 #552;Chabot, 1988 #296;Geissler, 1988 #297;Mahakali Zama, 2005 #688]. KITL/KIT signaling is also important for postnatal germ cell development [16-18]. Inhibition of KITL/KIT signaling activity results in the loss of differentiated type A spermatogonia and all downstream derivatives, leading to sterility [16, 19]. In addition, KITL/KIT signaling maintains extended mouse primary germ cell cultures [20]. KITL and KIT have both been shown to be present in the human adult testes with abnormal expression being associated with sub-fertility [21-23].

Our previous study showed that mouse embryonic fibroblast cell contact with hESCs was essential for germ cell formation [24]. Further, differentiation of hESCs in feeder conditioned media on polyornithine and laminin coated plates resulted in a significant reduction in germ cell gene expression. One potential source of germ cell signaling is the feeder extracellular matrix (ECM). The ECM plays a significant role in differentiation of early cell types [25-27] including ESCs [28-31] into specific lineages. The role of the ECM in germ cell adhesion and migration has been well studied [32, 33], yet it is not clear whether it is directly involved in germ cell differentiation.

In this study, we use a previously described adherent hESC to germ cell differentiation culture system [24], to determine the effect of BMP4 and KITL on enrichment and differentiation of GCL (DDX4+ POU5F1+) cells. We show that BMP4 enhanced germ cell gene expression and this could be negated with the BMP4

antagonist noggin. Additionally, the differentiation of hESCs on mouse feeder ECM caused a reduction in germ cell gene expression and indicates a dynamic cell signaling process between feeder cells and hESCs. One of the germ cell signaling factors potentially absent in the differentiation of hESCs on ECM was KITL. Using feeders with an *mKitl* deletion, we found that mKITL plays a significant role in enrichment of GCL cells in vitro. These data suggest that hESC derived germ cells provide a robust and much needed system to study human germ cell signaling and development.

Materials and Methods

hESC Culture Conditions

BGO1 (XY) hESCs with normal karyotype were cultured on ICR mouse embryonic fibroblast (MEF; Harlan) feeders inactivated by mitomycin C (Sigma). The cells were cultured in 20% KSR stem cell media, which consists of Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 20% knockout serum replacement (KSR), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin/50 µg/ml streptomycin (Gibco), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 4 ng/ml bFGF (Sigma-Aldrich and R & D Systems). They were maintained in 5% CO_2 and at 37° C. Cells were passaged every 3 days by mechanical dissociation, re-plated on fresh feeders to prevent undirected differentiation with daily media changes as previously described in our laboratory [34].

Enrichment and Differentiation Culture Conditions

As previously described [24], GCL cells were differentiated in an adherent culture system by growing them on ICR MEF feeders (Harlan) in 20% KSR media for 10 days without passaging. Cultures were maintained in 5% CO_2 at 37° C and media was

replaced every other day to stimulate germ cell signaling. In studies using BMP4 and noggin, hESCs were exposed to 10 or 100ng/ml recombinant human BMP4 (R & D Systems) for the first 3 days of differentiation or continually exposed to 100ng/ml of recombinant human noggin (R & D Systems) for 10 days under standard differentiation conditions. Control cells were differentiated under standard conditions in the absence of both BMP4 and noggin. The ECM was prepared as previously described [35]; briefly, mitotically inactivated ICR mouse feeders at a density of 12,000 cells/ cm^2 and maintaining them in culture for four days. Lysis of confluent feeder layers exposed the ECM as a substrate for cell attachment. Feeder cells were then washed with PBS, incubated for 3 minutes in 0.02M NH_4OH and washed three times in PBS. hESCs were seeded on the ECM and differentiated in 20% KSR media as previously described.

To determine the ability of KITL to modulate GCL cell differentiation, hESCs were also differentiated on *Kitl* $^{Sl\text{-}gb}$ feeders from wild type, heterozygous and homozygous mutant mice. The mice were originally obtained from the MRC Radiobiology Unit (Chilton, Didcot, UK) and have been maintained on C3H/HeNCR background for more than 20 generations (M. Bedell, personal communications). The *Kitl*$^{Sl\text{-}gb}$ *mutation was previously shown to be a deletion* resulting in a complete loss of primordial germ cells at 11.5 dpc [Mahakali Zama, 2005 #688]. Briefly, *Kitl* $^{Sl\text{-}gb}$ heterozygous mice were intercrossed and offspring were collected at day 13.5 dpc. The fibroblast cells were isolated from each fetus to prevent cross contamination between homo- and heterozygous individuals. Genotyping was done for each individual by extracting genomic DNA using a 50mM KCl, 10mM Tris-HCl pH 8.3, 2mM MgCl2, 0.1 mg/ml gelatin, 0.45% Nonidet P40, and 0.45% Tween 20 buffer. Samples were then heat

inactivated at 95° C for 10 min. PCR amplification was performed using primers that expand the gb deletion breakpoint. The primers used were: gbA 5'-TGTATCAAAAGGGTCGGGAC- 3', gbB 5'-AGTTCAGTCATAGATTGGAG-3'; gbC 5'-ATTGCTGTACTTGCTGCCTG-3'. Amplification products were analyzed on 7% acrylamide gels.

To assess methylation status of GCL cells during development, cells were passaged onto 4-well chamber slides (BD Falcon), differentiated for 10 days and stained for 5-methylcytidine every day starting at day 4.

Immunocytochemistry

Cells were passaged onto 4-well chamber slides (B D Falcon) and fixed with 4% paraformaldehyde for 15 minutes. Antibodies were directed against POU5F1 (Santa Cruz, 1:500) and DDX4 (R & D Systems, 1:200). Primary antibodies were detected using secondary antibodies conjugated to Alexa Flour 488 or 594 (Molecular Probes, 1:1000). Immunoflurescence imaging was done using the Olympus Ix81 with Disc-Spinning Unit and Slide Book Software (Intelligent Imaging Innovations).

Methylation was assessed by fixing cells with 70% ethanol for 30 minutes. Cells were then treated with 2N HCL and 0.5% triton-X 100 solution for 30 minutes, which was neutralized with a 0.1M Na_2BO_7 solution for 10 minutes. Antibodies were directed against 5-Methylcytidine (Santa Cruz, 1:500) and DDX4 (R & D Systems, 1:200), which were detected using secondary antibodies conjugated to Alexa Flour 488 or 594 (Molecular Probes, 1:1000). Cells were observed using the Olympus Ix81 with Disc-Spinning Unit as mentioned before.

Flow Cytometry

Cells were fixed in 57/43% ethanol/PBS for 10 minutes at room temperature. Cells were washed 3 times in PBS and were blocked in 6% donkey serum for 45 min. Antibodies were directed against POU5F1 (Santa Cruz, 1:250) and DDX4 (R & D Systems, 1:200). Due to the presence of feeders, an antibody against Human Nuclei (Chemicon, 1 µl per million cells) was also used to prevent false positives caused by feeders. MEFs and ENStem human neural progenitors (Millipore) were used as negative controls for POU5F1 and DDX4 expression. Primary antibodies were detected using fluorescently conjugated secondary antibodies Alexa Flour 405, 488 and 647. (Molecular Probes, 1:1000). Cells were analyzed using a Dakocytomation Cyan (DakoCytomation) and FlowJo Cytometry analysis software (Tree Star).

Real-time PCR

RNA was extracted using the Qiashredder and RNeasy kits (Qiagen) according to the manufacturer's instructions. The RNA quality and quantity was verified using an RNA 600 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer. Total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer's protocols. Reactions were initially incubated at 25° C for 10 minutes and subsequently at 37° C for 120 minutes. Quantitative RT-PCR (Taqman) assays were chosen for the transcripts to be evaluated from Assays-On-Demand™ (Applied Biosystems Inc.), a pre-validated library of human specific QPCR assays, and incorporated into 384-well microfluidic cards. Two micro liters of the cDNA samples (diluted to 50 µl) along with 50 µl of 2 x PCR master mix were loaded into respective channels on the microfluidic cards followed by centrifugation. The cards were then sealed and real-time PCR and relative quantification was carried out on the ABI

PRISM 7900 Sequence Detection System (Applied Biosystems Inc.). All failed (undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the ΔΔCT method of quantification (Applied Biosystems Inc.) [36]. Average fold changes from four independent runs were calculated as $2^{-\Delta\Delta CT}$. Significance was determined by running 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons for each gene looking at the effects of BMP4, noggin, KITL and MEF ECM differentiation.

Results

BMP Signaling Causes Increased Germ Cell Gene Expression and Germ Cell-Like Cell Enrichment

We recently demonstrated that an enriched population of DDX4+ POU5F1+ cells can be generated from hESC [24]. DDX4 is a germ cell specific marker in mice [37, 38] and humans [37], while POU5F1 is a marker expressed in pluripotent cell types including germ cells [39-41]. hESCs were differentiated on feeders in 20% KSR media without passaging for 10 days. Media was replenished every other day to encourage germ cell signaling. The number of hESCs (DDX4- POU5F1+ (Fig. 4.1A)) were reduced after differentiation, while a significant number of POU5F1+ cells expressed the germ cell specific marker DDX4+ (Fig. 4.1B and C). DDX4 and POU5F1 expression was specific as co-cultured feeder cells and human neural progenitor cells derived from hESC were consistently negative (data not shown). This level of differentiation was achieved without the addition of exogenous signaling factors, but conditions were still undefined. We examined whether noggin, a BMP antagonist, could inhibit GCL cell

formation in our culture system. hESCs were differentiated under standard conditions in the presence of 100ng/ml of noggin for 10 days (Fig. 4.1D). Under these conditions, up regulation of germ cell gene expression was significantly ($p < 0.05$) inhibited with expression levels resembling an hESC-like state. The specifying gene *IFITM3*, the pre-migratory gene *POU5F1*, the migratory gene *NANOG*, the post-migratory gene *PUM2* and the meiotic gene *MLH1* all remained down regulated with the addition of noggin (Fig. 4.2A). *DPPA3, KIT, DDX4* (Fig. 4.2A), *DAZL, PIWIL2, NANOS1, SYCP1, SYCP3* and *CXCR4* (data not shown) expression was not significantly changed relative to differentiated control (treatment without noggin or BMP4) (Fig. 4.2A). This indicates that BMP activity in these conditions may play a significant role in germ cell differentiation from hESCs.

To explore the ability of exogenous BMP4 in potentiating germ cell differentiation, hESC cultures were cultured as described above except they were exposed to 10 or 100ng/ml of the germ cell specifying signaling molecule BMP4 for 3 days and further differentiated as before for an additional 7 days. *IFITM3* gene expression was significantly increased ($p < 0.05$) in cultures with BMP4 relative to hESCs, however this increase was not significant relative to control treatment without BMP4 (Fig. 4.2A). Conversely, cultures treated with BMP4 exhibited a dose dependent increase ($p < 0.05$) in expression of the specifying gene *DPPA3* and the pre-migratory genes *POU5F1* and *KIT* relative to differentiated control cells (Fig. 4.2A). BMP4 also significantly increased ($p < 0.05$) the expression of the migratory gene *NANOG* (Fig. 4.2A), the post-migratory gene *DAZL* (data not shown), the spermatogonia gene *PUM2* and the meiotic gene *MLH1* relative to hESCs and control (Fig 4.2A). The migratory

gene *DDX4* (Fig. 4.2A) and the post-migratory genes *PIWIL2, NANOS1, SYCP3* (data not shown) showed no significant change with the addition of BMP4, however they were all more highly expressed in the treated cells relative to hESCs. There was no change in expression of the migratory gene *CXCR4* and the meiotic gene *SYCP1* relative to hESCs or control treatment (data not shown). The significant effect of additional BMP4 in 7 genes suggests that BMP4 further enriched GCL character in these cultures.

To further confirm the role of BMP4 in GCL cell enrichment, flow cytometry was conducted to quantify the population of DDX4+ POU5F1+ cells. The addition of noggin significantly ($p < 0.05$) inhibited the enrichment of GCL cells with only 5.9% of cells being DDX4+ POU5F1+, a percentage not significantly ($p < 0.05$) different from hESCs (4.1%) (Fig. 4.2B). Additional exogenous BMP4 did not significantly ($p < 0.05$) increase the percentage of GCL cells, 29.6% of cells being DDX4+ POU5F1+, relative to control treatment (Fig. 4.2B). This suggests that supplemental BMP4 levels in excess of the BMP activity in KSR is not required for the formation of DDX4+ POU5F1+ cells, albeit the addition of BMP4 causes increased germ cell gene expression and potentially enhanced germ cell programming.

Differentiation on Feeder Extracellular Matrix Causes Decreased Germ Cell Gene Expression

Our previous work has shown that differentiation of hESCs into GCL cells requires cell-cell contact with feeders [24]. Differentiation in feeder conditioned media, media exposed to feeders for 24 hrs, on polyornithine and laminin coated plates caused a significant reduction in germ cell gene expression and, in the absence of bFGF, DDX4+ POU5F1+ cells. To determine if feeder ECM promotes GCL cell differentiation,

hESCs were differentiated in 20% KSR media on feeder ECM with every other day media changes and no passaging for 10 days (Fig. 4.1D). Differentiation of hESCs on feeder ECM significantly reduced ($p < 0.05$) pre-migratory genes *IFITM3, DPPA3* and *KIT*, the migratory gene *NANOG*, the post-migratory gene *PUM2* and the meiotic genes *MLH1* and *SYCP3*, relative to control cells differentiated on feeders (Fig. 4.3A). There was no significant change in the pre-migratory genes *POU5F1*, the migratory gene *DDX4*, the post-migratory genes *DAZL, PIWIL2, NANOS1* and the meiotic gene *SYCP1* with respect to hESCs differentiated on feeders (Fig. 4.3A). The down regulation of 7 germ cell genes represents a decrease in germ cell character suggesting that direct communication between the feeders and hESCs is important for the enhancement of germ cell gene expression. Despite the down regulation of germ cell genes in cultures differentiated on feeder ECM, this condition did not significantly affect the number of DDX4+ POU5F1+ GCL cells 34.2% on ECM vs. 30.9% on feeders (Fig. 4.3B). This suggests that GCL cells can be derived on the feeder ECM alone, however differentiation on feeders results in higher germ cell gene expression.

Loss of Mouse KIT Ligand Causes Decreased Germ Cell Gene Expression and DDX4+ POU5F1+ Cells

The effect of KITL, a feeder layer associated factor, was investigated. We used $Kitl^{Sl\text{-}gb}$ mice previously shown to cause a dramatic reduction in PGC numbers in the mouse embryo to create feeders [Mahakali Zama, 2005 #688]. hESCs were differentiated on *mKitl* +/+, +/- and -/- feeders in 20% KSR media without passaging and with media changes every other day for 10 days (Fig. 4.1D). Heterozygous and homozygous null expression of KITL resulted in significant reduction ($p < 0.05$) in the

KITL receptor *KIT*, the migratory genes *CXCR4* and *DDX4*, the post-migratory gene *DAZL* and the meiotic gene *SYCP3* relative to cultures differentiated on *mKITL +/+* feeders (Fig. 4.4A). However, differentiation on *mKITL -/-* feeders had no significant effect on the specifying genes *IFITM3* or *DPPA3,* the pre-migratory gene *POU5F1*, the migratory gene *NANOG* or the post-migratory genes *PIWIL2, PUM2* and *NANOS1* relative to samples differentiated on *mKitl +/+* feeders (data not shown). The percentage of DDX4+ POU5F1+ cells was significantly ($p < 0.05$) decreased when differentiated on m*Kit +/-* (21.5%) and m*Kitl -/-* (10.9%) in a dose dependent manner, relative to hESCs differentiated on *mKitl +/+* feeders (37.0%) (Fig. 4.4B). This suggests that KITL is essential for enrichment of hESC to DDX4+ POU5F1+ cells.

Germ Cell-Like Cells Demonstrated No Change in Genome Wide Methylation

Resetting of the genome methylation pattern in primordial germ cells is an important aspect of normal development [42, 43]. Here we determined methylation in GCL cells relative to hESCs. hESCs were differentiated for 10 days in 20% KSR media without passaging and with media changes every other day (Fig. 4.1D). Cells were collected each day and stained for 5-methylcytidine and DDX4. DDX4+ GCL cells (Fig. 4.5C-D) showed no decrease in 5-methylcytidine expression when compared to DDX4- hESCs (Fig. 4.5A-B).

Discussion

In this study, we demonstrate that signaling factors BMP4 and KITL play a critical role in differentiation of hESCs into cells of a germ cell-like phenotype. Noggin's inhibition of BMP signaling in differentiating cultures decreased germ cell gene expression, and all but eliminated differentiation to DDX4+ POU5F1+ cells. Previous

studies differentiating ESCs into germ cells used undefined culture condition including fetal bovine serum (FBS) or KSR, which may have affected differentiation via BMP activity [2, 44-46] . BMP4 has also been shown to be produced by MEF feeders [47] and may be a major source of germ cell signaling in an adherent culture system. Whereas, feeder cells were not used in embryoid body differentiation studies [45, 48]. Additionally, these studies used differentiation systems where bFGF was removed, which we have previously shown to be important for germ cell enrichment and differentiation [24]. This may explain the significantly higher number of DDX4+ cells seen in our differentiation cultures than in prior differentiation studies using embryoid bodies without bFGF. As anticipated based on the role of BMP4 in specification in vivo [5, 7, 8, 49], adding BMP4 caused an increase in germ cell specifying gene *DPPA3*. Toyooka et al. [4] also showed that BMP4 produced MVH+ cells from mESCs, the mouse homologue of DDX4; however, our cultures already contained DDX4+ cells, making it hard to directly compare these two studies. In addition, our studies showed that BMP4 caused an increase in migratory and post-migratory gene expression, which has not been previously shown and may represent enhanced germ cell programming.

Kee et al. [48] also used exogenous BMP4 in the differentiation of hESCs into GCL cells. They showed that BMP4 induced a 3.4 fold increase in *DDX4* gene expression when compared to differentiated treatments without BMP4. Also, BMP4 caused a significant increase in the DDX4+ population from 3% to 14.5% with the inclusion of additional germ cell enhancing factors BMP7 and BMP8b [48]. We observed a similar fold increase of 3.8 in *DDX4* gene expression when 10ng/ml of BMP4 was added (Fig. 4.2A). This difference was statistically insignificant; however it may be

biologically significant. Further, we did not see a significant increase in the number of DDX4+ POU5F1+ cells in the presence of exogenous BMP4 (Fig. 4.2B). Again, the addition of noggin into our non-supplemented cultures reduced germ cell gene expression and DDX4+ POU5F1+ cells, implying that these cultures may have endogenous BMP activity (Fig. 4.2A). Therefore the addition of exogenous BMP4 beyond that present in KSR may be redundant and may not further enhance GCL cell production.

We report for the first time the role of mouse KITL in the in vitro differentiation of hESCs into germ cells. We found that when mKITL was absent, there was a significant decrease in DDX4+ POU5F1+ cells (Fig. 4.4B). Previously, KITL signaling has been shown to play a pivotal role in promoting proliferation of mouse prenatal germ cells [13, 14] and is believed to do the same in human germ cell development [12, 50]. KITL is also essential in maintaining extended mouse primary germ cell cultures [20, 51-54]. Matsui et al. [51] showed that primary mouse germ cell cultures grown on m*Kitl -/-* feeders had a significant reduction in proliferation and survivability. Even in the absence of feeders, which are known to produce other factors important for primary germ cell culture like leukemia inhibitory factory [51], KITL is able to enhance initial survival of primary germ cells in culture [20]. Here the proliferative response of hESC derived GCL cells to KITL correlates with cultures of primary germ cells and implies that these may model human germ cell development.

KITL/KIT signaling is linked to the migratory phase of early development as KIT is first expressed in mouse primordial germ cells (PGCs) at 7.5 days post coitum (dpc) [55], just proceeding initiation of migration, and KITL is expressed throughout the

migratory pathway and at the homing site [6]. KITL/KIT signaling is believed to play an important role in germ cell development during the migratory phase [13] . This may explain why we found a significant decrease in expression of *CXCR4*, *DDX4*, both are first expressed during migration, in cells on *mKitl -/-* feeders (Fig. 4.4A). KITL/KIT signaling is also essential in postnatal germ cell development and interrupting this signaling leads to a loss of A spermatogonia derivatives, including meiotic cell types [16, 19]. In agreement, we observed a decreased expression in *SYCP3*, a meiotic marker in GCL cells differentiated on *mKitl -/-* (Fig. 4.3). In addition, germ cells just preceding entry into meiosis express KIT and are believed to be responsive to KITL/KIT signaling [16, 19]. Conceivably, *SYCP3* gene expression associated with meiosis may be linked to KITL/KIT signaling, since SYCP3 protein is expressed in the initial stage of the first meiotic division [56, 57]. We did not observe a significant change in the expression of specifying genes *IFITM3* or *DPPA3*, yet we did see a significant decrease in the number of GCL cells. We hypothesize that the absence of KITL does not affect the specification of GCL cells, however it may prevent expansion of these cells. A possible loss in expansion is in agreement with data in the mouse where early mouse germ cells show a loss of proliferation and undergo apoptosis when KITL/KIT signaling is interrupted [13, 14, 55]. Overall, our findings agree with previous in vivo and in vitro germ cell culture data and suggest KITL plays a key role in proliferation and differentiation of these hESC derived GCL cells.

Despite the down regulation of germ cell genes in cultures differentiated on feeder ECM (Fig. 4.3A), the proportion of DDX4+ POU5F1+ cells remained the same as feeder cultured cells (Fig. 4.3B). This result is not totally unexpected since neither

POU5F1 nor DDX4 gene expression was significantly changed in cultures differentiated on ECM. The specifying genes *IFITM3* and *DPPA3* and the post-migratory genes *PUM2, MLH1* and *SYCP3* all showed significant decreased expression relative to cells differentiated on feeder cells. However, these same genes were up regulated relative to hESCs, thus the relatively lower germ cell gene expression in feeder ECM vs the feeder cell groups may represent only a partial failure to recapitulate the germ cell/feeder cell association.

Global demethylation of the primordial germ cell genome is believed to occur in all mammalian species [58, 59]. In our study we did not see a significant decrease in the level of methylation in DDX4+ cells relative to hESCs. Previously, imprinting gene methylation in mESC derived GCL cells was at least partially reset suggesting reprogramming [1, 3]. However, neither of these studies investigated genome wide methylation, only examining 2 or 3 genes. Nayernia et al. [3] went on to demonstrate that GCL cells were able to produce live offspring, yet the offspring had phenotypic abnormalities and were short lived. These abnormalities were ultimately attributed to hypo- and hypermethylation of imprinting genes found in tissue taken from the offspring. In the present study, methylation status of hESC derived GCL cells appears not to be globally reset, however no conclusion can be made about the resetting of individual genes. Epigenetic reprogramming is an important hurdle that must be overcome before hESC derived cells have functional germ cell attributes.

Summary

In summary, this is the first report demonstrating that a loss of KITL in differentiation cultures caused a significant decrease in enrichment of human GCL cells

and germ cell genes that are temporally correlated. BMP4 caused a significant increase in germ cell gene expression and appears to be required for differentiation of hESCs into GCL (DDX4+ POU5F1+) cells. Additionally, other factors and conditions are likely required for proper temporal and spatial germ cell development events to be mimicked in vitro. Building upon these studies, future work will investigate the role of different factors, germ cell niche signaling and comparing germ cell programming of hESC derived germ cells to their in vivo counterparts.

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Figure 4.1. Derivation of Germ Cell-Like Cells from Human Embryonic Stem Cells and Experimental Plan. **A)** POU5F1+ hESCs were differentiated into **B)** DDX4+ (arrowhead) POU5F1+ GCL cells on feeders in 20% KSR media with media changes every other day and without passaging for 10 days. **C)** Flow cytometry indicated a significant DDX4+ population after differentiation. **D)** Differentiation cultures with inhibited BMP activity (noggin), added BMP4, on feeder ECM, *KITL* +/+, +/- or -/ - feeders, and genome methylation were examined with a final end point at 10 days.

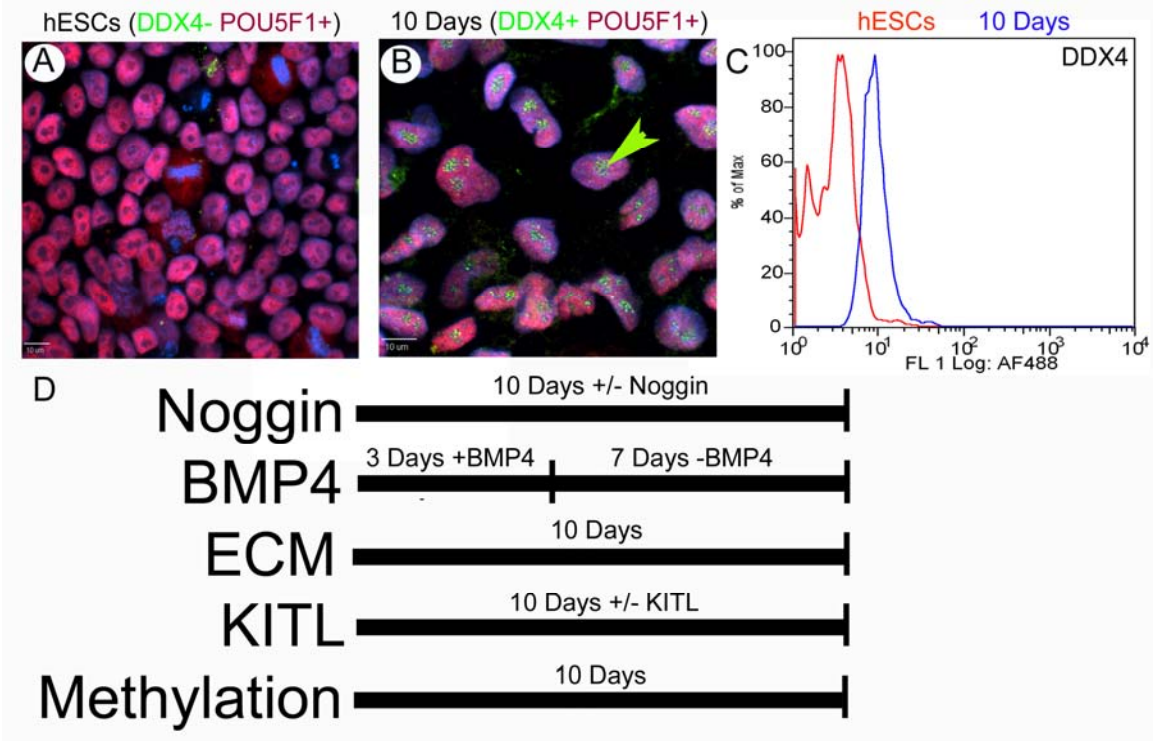

hESCs (DDX4- POU5F1+)
10 Days (DDX4+ POU5F1+)
A
B
C
hESCs
10 Days
DDX4
% of Max
FL 1 Log: AF488
D
Noggin
10 Days +/- Noggin
BMP4
3 Days +BMP4
7 Days -BMP4
ECM
10 Days
KITL
10 Days +/- KITL
Methylation
10 Days

Figure 4.2. BMP Increased Germ Cell Gene Expression and DDX4+ POU5F1+ Cells. (A) hESCs were differentiated in 20% KSR media in the presence of 100ng/ml of noggin, 0ng/ml (control), 10ng/ml or 100ng/ml of BMP4 for 10 days. Noggin significantly inhibited ($p<0.05$) gene expression of *IFITM3, POU5F1, NANOG, PUM2* and *MLH1*, relative to hESCs (control). BMP4 significantly increased ($p<0.05$) *DPPA3, POU5F1*, *KIT, NANOG, PUM2* and *MLH1* expression relative to control. **B)** 100ng/ml of noggin significantly decreased ($p<0.05$) the number of DDX4+ POU5F1+ cells, while 100ng/ml of BMP4 had no effect when compared to control treatment. (*=Statistically Significant from hESCs, # = Statistically Significant from Control (0ng/ml of BMP4 and 0ng/ml noggin)).

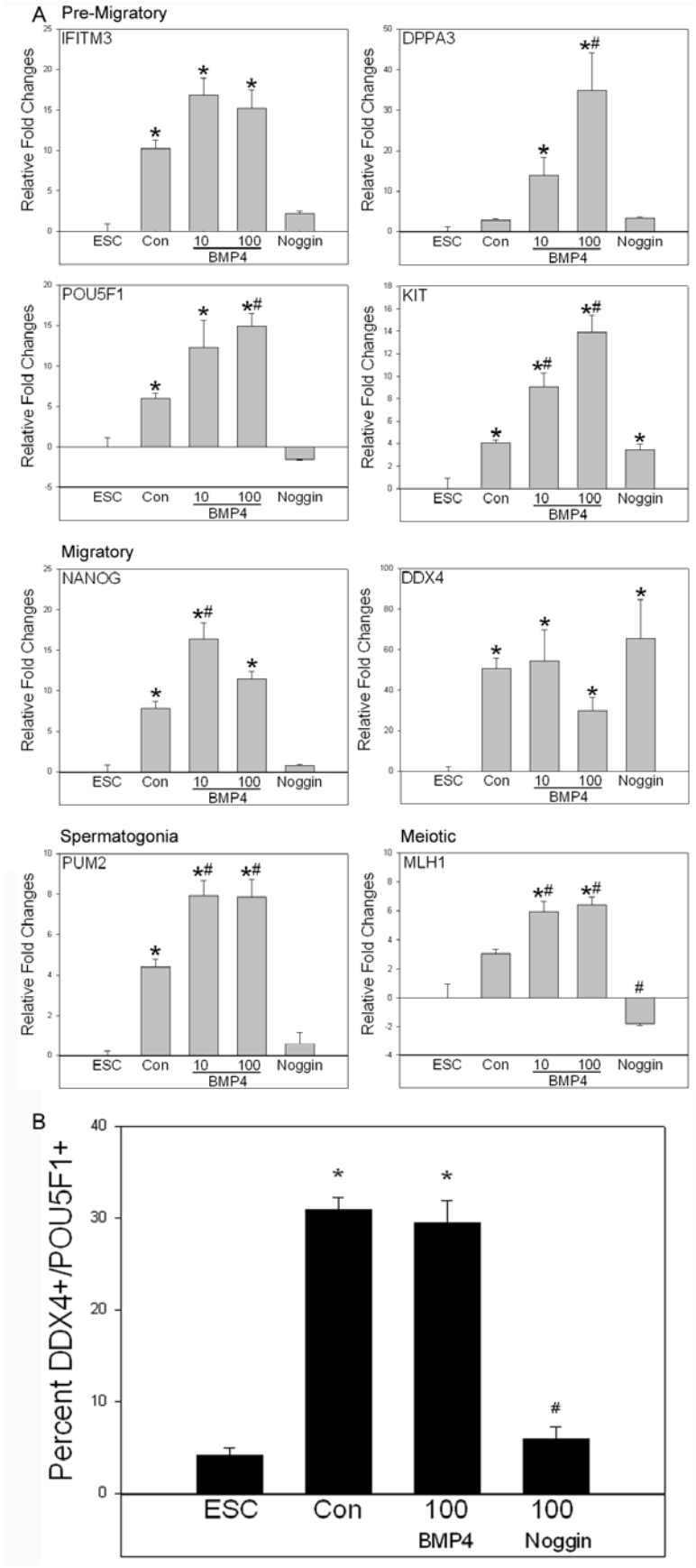

A
Pre-Migratory
IFITM3
DPPA3
POU5F1
KIT
Migratory
NANOG
DDX4
Spermatogonia
PUM2
Meiotic
MLH1
Relative Fold Changes
ESC
Con
10
100
Noggin
BMP4
B
Percent DDX4+/POU5F1+
ESC
Con
100
BMP4
100
Noggin

Figure 4.3. Differentiation on Feeder Extracellular Matrix Decreased Germ Cell Gene Expression. **A)** hESCs were differentiated on feeder ECM and on feeders (control) in 20% KSR media for 10 days. Differentiation of hESCs on ECM significantly decreased ($p<0.05$) expression of germ cell genes *IFITM3, DPPA3, KIT, NANOG, PUM2, MLH1* and *SYCP3* (*=Statistically Significant from Control). **B)** There was no significant change in the percentage of DDX4+ POU5F1+ cells relative to control, but for hESC the percentage of DDX4+ POU5F1+ cells was significantly lower ($p<0.05$) than in both the control and ECM treatment groups (*=Statistically Significant from hESCs, # = Statistically Significant from Control; hESCs and D10 data are the same as Figure 2).

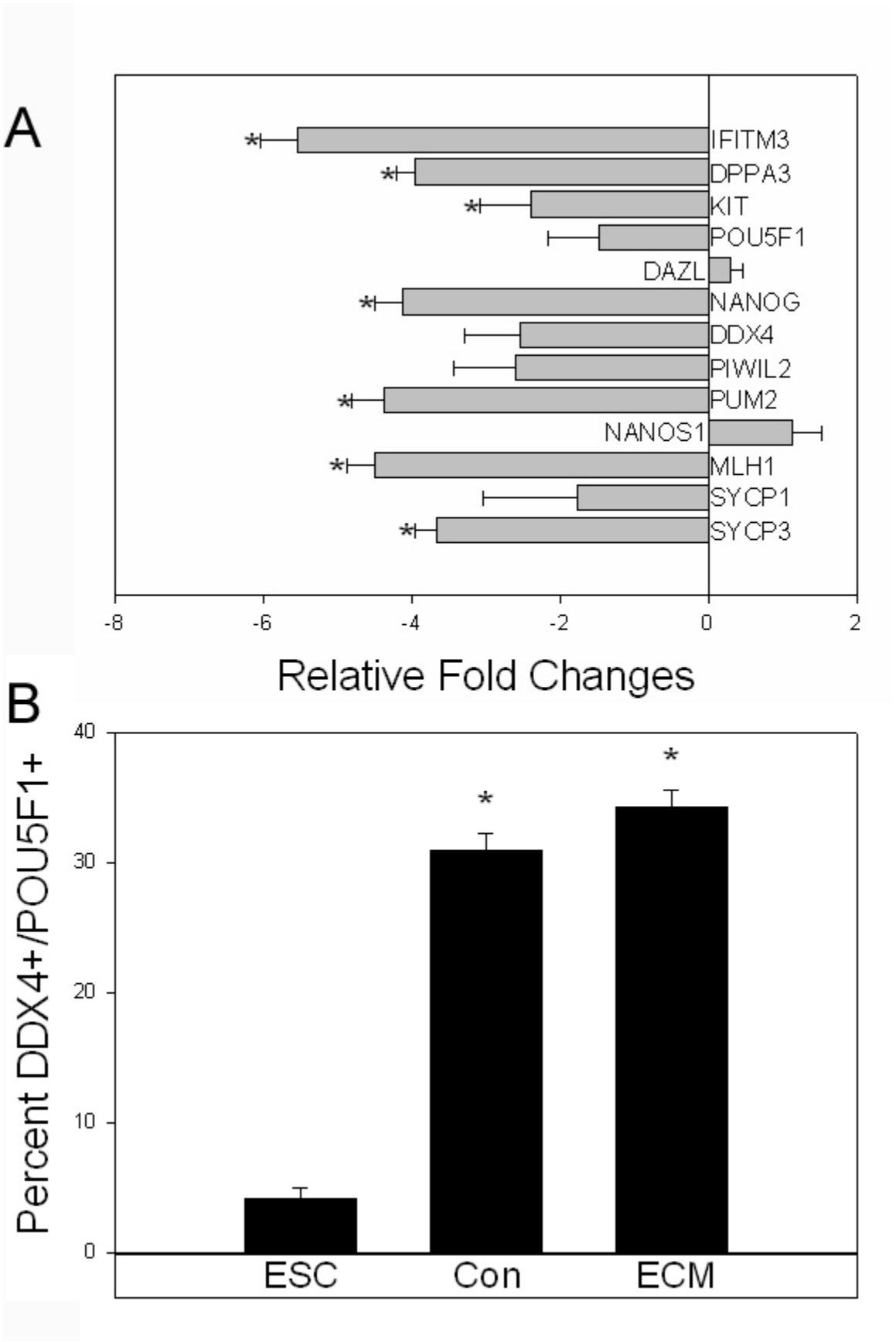

A
IFITM3
DPPA3
KIT
POU5F1
DAZL
NANOG
DDX4
PIWIL2
PUM2
NANOS1
MLH1
SYCP1
SYCP3
-8
-6
-4
-2
0
2
Relative Fold Changes
B
Percent DDX4+/POU5F1+
40
30
20
10
0
ESC
Con
ECM

Figure 4.4. Loss of KITL Expression in Differentiation Cultures Causes Decreased Germ Cell Gene Expression and DDX4+ POU5F1+ Cells. **A)** hESCs were differentiated on *mKitl* +/+, +/- or -/- feeders in 20% KSR media for 10 days. Differentiation of hESCs on *mKitl* -/- feeders significantly decreased ($p<0.05$) *DAZL, KIT, CXCR4, DDX4, MLH1* and *SYCP3* gene expression relative to hESCs differentiated on *mKitl* +/+ feeders. **B)** Differentiation on *mKitl* +/- and -/- feeders significantly decreased ($p<0.05$) DDX4+ POU5F1+ cells relative to hESCs differentiated on *mKitl* +/+ in a dose dependent manner (*=Statistically Significant from hESCs, # = Statistically Significant from *mKITL* +/+ Treatment).

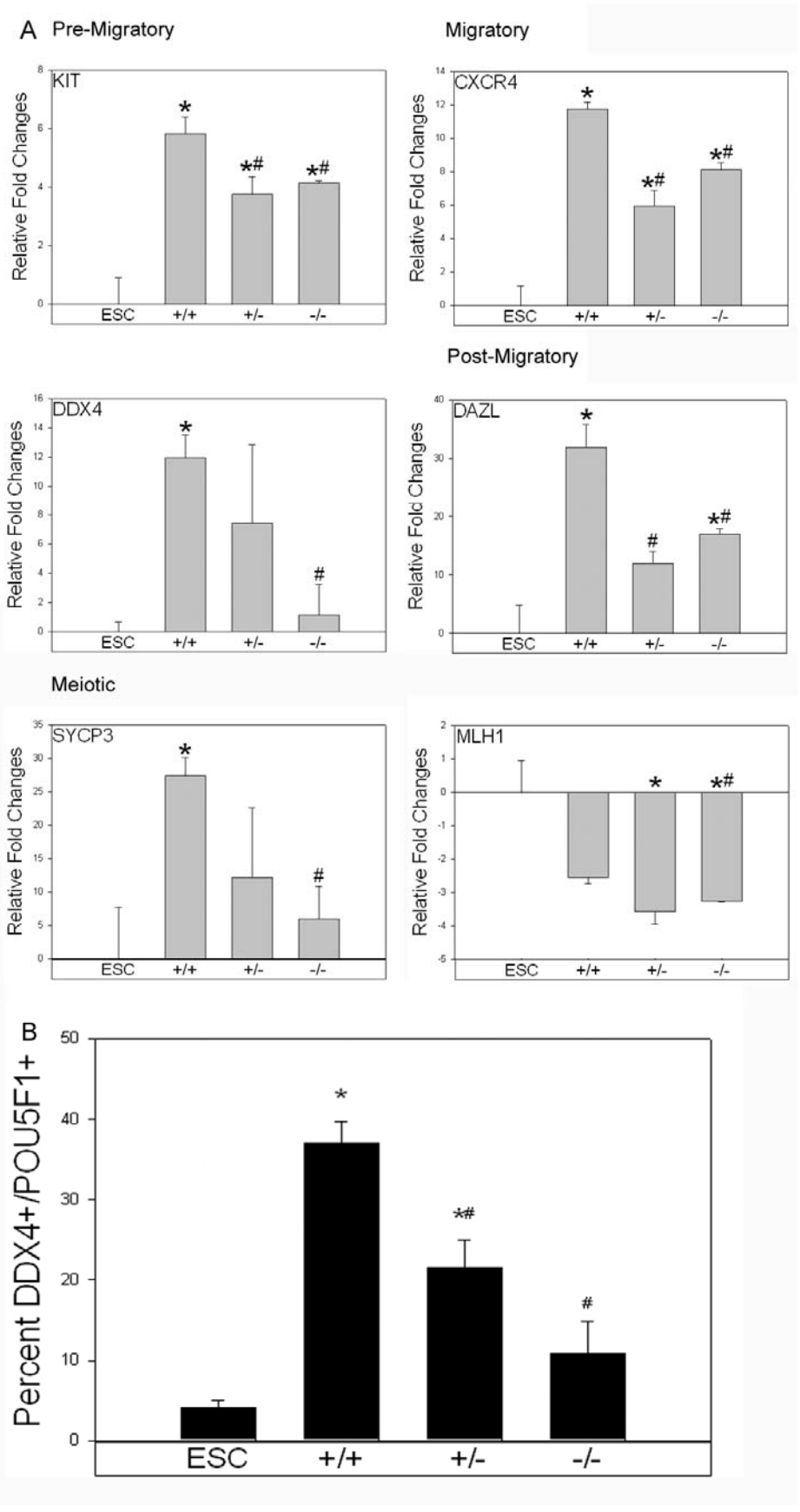
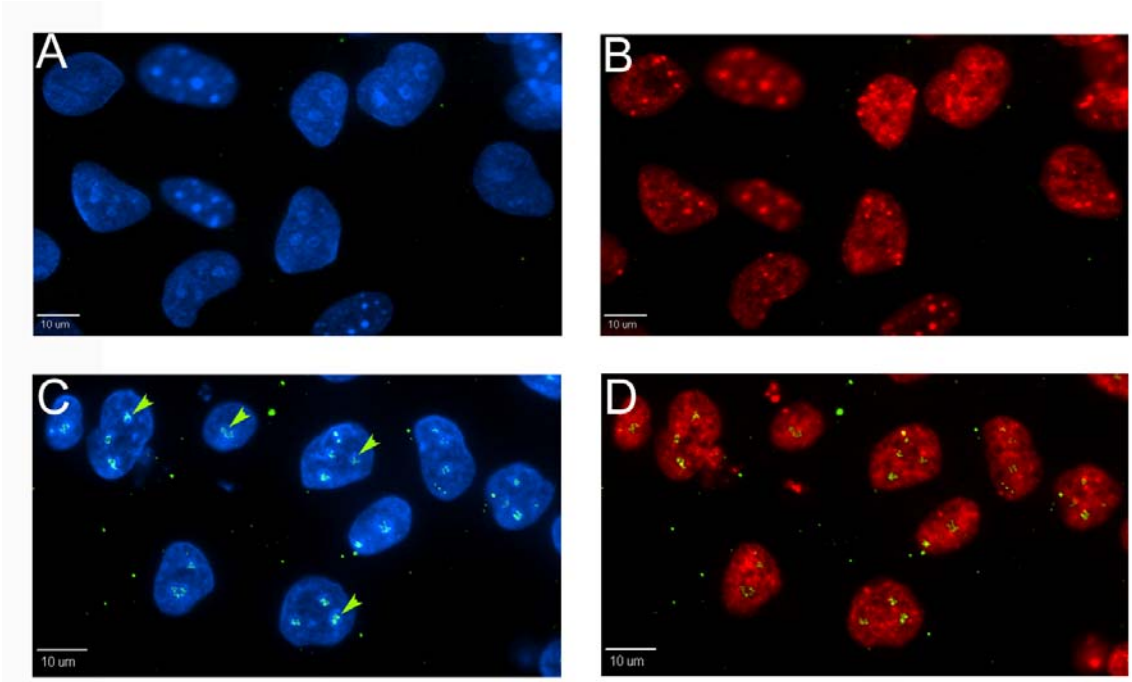

A
Pre-Migratory
Migratory
KIT
CXCR4
Relative Fold Changes
ESC
+/+
+/-
-/-
Post-Migratory
DDX4
DAZL
Meiotic
SYCP3
MLH1
B
Percent DDX4+/POU5F1+

Figure 4.5. Germ Cell-Like Cells Showed No Change in Genome Wide Methylation.

hESCs were differentiated on feeders in 20% KSR media for 10 days. Samples were stained for DDX4 (green, arrowhead), 5-methylcytidine (red) and DAPI (blue). **A, B)** Undifferentiated DDX4- hESCs and **C, D)** differentiated DDX4+ cells were both positive for 5-methylcytidine. Scale Bars 10 um.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CHAPTER 5

CONTINUAL CULTURE OF GERM CELL-LIKE CELLS

Introduction

Germ cells are an intense topic of study because they are the only cell type of the body capable of giving rise to all other cell types, pass on genetic information to future generations and reprogram somatic cells into a pluripotent. In addition to the basic science behind germ cells, more practical applications such as the inability of millions of couples to produce offspring have spurred further interest. However, the mechanisms behind germ cell development and their unique properties have remained undisclosed.

To further elucidate the mechanisms specifically behind mammalian germ cell development, several groups have created in vitro culture systems capable of supporting extended culture of germ cells [2, 3]. These cells now known as embryonic germ cells (EGCs) have been derived from numerous species including humans [4, 5]. The successful derivation of human EGCs (hEGCs) would seemingly lead to an excellent system to study germ cell development, yet their propensity to differentiate has made them prohibitive to work with and has led to few advances [4, 6, 7]. This has left the field searching for new opportunities to study human germ cell development.

In 2003, Hubner et al. [8] demonstrated for the first time that germ cells could be derived from mouse embryonic stem cells (mESCs). These cells expressed the germ cell specific marker MVH, were capable of producing the reproductive hormone estradiol and large follicle-like structures. Follicle-like cells ultimately appeared to

undergo spontaneous activation and parthenogenesis producing blastocyst-like structures. Male germ cells have also been produced from mESCs with sperm-like cells appearing to undergo re-setting of imprinting genes, capable of elongation and producing offspring [9-11]. Human embryonic stem cells (hESCs) have demonstrated the ability to differentiate into germ cell-like (GCL) cells expressing the germ cell markers DDX4 (also known as VASA), POU5F1 and early meiotic activity with the expression of SYCP3 and MLH1[12-14], which both play an essential role in the crossover event [15-19]. These culture systems have shown the ability of ESCs to differentiate into GCL cells. Yet it is not clear whether these cells can be maintained in continual culture or if they will spontaneously differentiate into other cell types as their in vivo counterparts have done, thus potentially limiting their usefulness [4, 6, 7].

The objective of this study was to determine if hESC derived GCL cells can be maintained in continual culture without further differentiation or dedifferentiation back to hESC as was previously observed in germ cell cultures. Further developing a previously described culture system [13], we demonstrate that hESC derived GCL cells can maintain up-regulation of 12 germ cell genes for over 20 passages. In addition, continual culture of GCL (DDX4+ POU5F1+) cells results in further enrichment of intently differentiated populations where ultimately 60% of cells were DDX4+ POU5F1+. These results suggest that unlike primary germ cells, hESC derived GCL cells can easily be maintained in continual culture and may serve as a superior model for human germ cell development.

Materials and Methods

hESC Culture Conditions

BGO1 (XY) hESCs with normal karyotype were cultured on ICR mouse (Harlan) MEF feeders inactivated by mitomycin C (Sigma-Aldrich). The cells were cultured in 20% KSR stem cell media, which consists of Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 20% knockout serum replacement (KSR), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin/50 µg/ml streptomycin (Gibco), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 4 ng/ml bFGF (Sigma-Aldrich and R & D Systems). They were maintained in 5% CO_2 and at 37° C. Cells were passaged every 3 days by mechanical dissociation, re-plated on fresh feeders to prevent undirected differentiation with daily media changes as previously described by our laboratory [20].

Continual Culture Conditions

GCL cells were differentiated in an adherent culture system by growing them on feeders with 4ng/ml bFGF for 10 days in 20% KSR media without passaging as previously described [13]. After differentiation, cells were trypsin passaged every 4 days for 20 passages with flow cytometry and RNA analysis done at passages 5, 10 and 20 with hESC and day 10 controls to determine percentage of DDX4+ POU5F1+ cells and germ cell gene expression levels. Cultures were maintained in 5% CO_2 at 37° C and media was changed every other day.

Flow Cytometry

Cells were fixed in 57/43% ethanol/PBS for 10 minutes at room temperature. Cells were washed 3 times in PBS and were blocked in 6% donkey serum for 45 min.

Antibodies were directed against POU5F1 (Santa Cruz, 1:500), DDX4 (R & D Systems, 1:200) and Human Nuclei (Chemicon, 1 µl per million cells) to prevent false positives caused by feeders. MEFs and ENStem human neural progenitors (Millipore) were used as negative controls for POU5F1 and DDX4 expression. Primary antibodies were detected using fluorescently conjugated secondary antibodies Alexa Flour 405, 488 and 647. (Molecular Probes, 1:1000). Cells were analyzed using a Dakocytomation Cyan (DakoCytomation) and FlowJo Cytometry analysis software (Tree Star). Significance was determined by running 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons for each time point. Comparisons between treatments where the p-value < 0.05 were considered significantly different.

Quantitative Reverse Transcription-Polymerase Chain Reaction

RNA was extracted using the Qiashredder and RNeasy kits (Qiagen) according to the manufacturer's instructions. The RNA quality and quantity were verified using an RNA 600 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer. For real-time quantitative reverse transcription-polymerase chain reaction *(*RT-PCR), total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer's protocols. Reactions were initially incubated at 25° C for 10 minutes and subsequently at 37° C for 120 minutes. Real-time quantitative RT-PCR (Taqman) assays were chosen for the transcripts to be evaluated from Assays-On-Demand™ (Applied Biosystems Inc.), a pre-validated library of human specific QPCR assays, and incorporated into 384-well microfluidic cards. Two micro liters of the cDNA samples (diluted to 50 µl) along with 50 µl of 2 x PCR master mix were loaded into respective channels on the microfluidic cards followed by centrifugation. The cards were

then sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc.). All failed (undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the ΔΔCT method of quantification (Applied Biosystems Inc.) [21]. Average fold changes from four independent runs were calculated as $2^{-\Delta\Delta CT}$. Significance was determined by running 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons for each gene. Comparisons between treatments where the p-value < 0.05 were considered significantly different.

Results

Germ Cell-Like Cells Can Be Maintained in Continual Culture

hESCs were differentiated for 10 days in 20% KSR media on feeders. Differentiated cells were serially passaged for 20 passages with flow cytometry analysis performed at 5, 10 and 20 passages with hESC and day 10 controls to determine the percentage of DDX4+ POU5F1+ GCL cells. After 10 days of differentiation, flow cytometry indicated that 18.7% of cells were DDX4+ POU5F1+, a significant ($p< 0.05$) increase relative to the 4.0% found in hESCs (Fig 5.1). Passaging of enriched cells resulted in a significant ($p< 0.05$) increase in DDX4+ POU5F1+ relative to hESCs and day 10 controls with 58.0% of cells being DDX4+ POU5F1+ at passage 5 (Fig. 5.1). This increase was maintained at passages 10 and 20 with 58.7% and 60.7% of cells being DDX4+ POU5F1+ respectively. Previously, we demonstrated that DDX4+ POU5F1+ cells could not be maintained for 30 days, a point that temporally corresponds with serial passage 5, under differentiation conditions without passaging.

Our results once again support this with <1.0% of the population being DDX4+ POU5F1+ at day 30, which is a significant ($p < 0.05$) decrease relative to day 30 (P5) continual culture cells (58.0%), hESCs (4.0%) and day 10 controls(18.7% ; Fig. 5.1). These results suggest that DDX4+ POU5F1+ GCL cells can be maintained in continual culture for at least 20 passages and that passaging is essential to maintain a germ like phenotype.

Continually Cultured Germ Cell-Like Cells Maintain Up Regulated Germ Cell Gene Expression

To confirm the ability of GCL cells to be continually cultured, germ cell gene expression levels were determined in serially passaged DDX4+ POU5F1+ enriched cultures. Continually passaged cultures demonstrated significant ($p < 0.05$) up-regulation of the pre-migratory and migratory genes *IFTIM3, POU5F1, NANOG* and *DDX4* after 10 days of differentiation relative to hESCs (Refer to Fig. 5.2 for fold changes). This up-regulation was maintained for 5, 10 and 20 passages for genes *IFITM3, NANOG, POU5F1*, and *DDX4*. The pre-migratory genes *DPPA3* and *KIT* did not show up-regulation after initial differentiation, however these genes showed significant ($p < 0.05$) increases in gene expression after 5 passages relative to hESCs (Fig. 5.2). This increase in gene expression was maintained for 10 and 20 passages.

Markers of post-migratory and meiotic stages of germ cell development also exhibited considerable increases after differentiation, which were maintained after continual passaging. The post-migratory genes *DAZL, PUM2* and *NANOS1* and the meiotic genes *MLH1, SYCP1* and *SYCP3* demonstrated significant ($p < 0.05$) up-regulation relative to hESCs after 10 days of differentiation (Fig. 5.3). *SYCP3* gene

expression was maintained at passages 5, 10 and 20, however *DAZL, PUM2, NANOS1, MLH1* and *SYCP1* all showed further increases ($p < 0.05$), relative to day 10 controls, after 20 serial passages. These results indicate that serially passaging enriched DDX4+ POU5F1+ cultures can maintain or even enhance increased levels of early and late germ cell gene expression.

Previously, we observed significant down-regulation of germ cell genes after 30 days under differentiation conditions without passaging [13]. In this study, day 30 (passage 5) continually passaged cultures possessed up-regulation of all genes at significantly ($p < 0.05$) higher levels than day 30 cultures that were not passaged (Fig. 5.2, Fig. 5.3). This further supports that continual passaging after differentiation is essential to maintain enriched cultures of GCL cells.

Discussion

The inability to continually culture germ cells has been a considerable hurdle in studying germ cell development, especially in humans. An in vitro model would provide a system that is easily manipulated for in-depth analysis of germ cell signaling and developmental processes. In this study, we demonstrated that enriched cultures of hESC derived GCL cells could undergo 20 passages and maintain, or further enhance, a high level of enrichment (> 58%) and germ cell gene expression. We believe this is the first time that ESC derived GCL cells have shown the ability to maintain germ cell character after serial passaging and propagation. This system offers great potential and flexibility to further study the little known factors important for germ cell development.

Under continual culture conditions, enrichment of DDX4+ POU5F1+ cells was not only maintained but was further increased from 18.7% to 60.7% of cells. Further

increases were also noted in gene expression of the pre-migratory genes *DPPA3* and *KIT*, the post-migratory genes *DAZL, PUM2* and *NANOS1* and the meiotic genes MLH1 and *SYCP1* after serial passaging. In most cases, these increases were incremental with passage 20 cells showing significantly higher expression than initially differentiated day 10 cells. These increases suggest that continually passaged cultures are not static and that further differentiation is potentially occurring. However, this would not be surprising as continual culture conditions are similar to differentiation conditions with the exception of being passaged. It appears that continually passaging these cells is essential to maintaining a germ cell phenotype. Cells that were not passaged showed a loss of the DDX4+ POU5F1+ populations at day 30 and a decrease in most germ cell gene expression relative to cultures that were continually passaged for 30 days (passage 5). Primary germ cells have the ability to turn into other cell types under differentiation conditions. Therefore germ cells derived from hESCs that are maintained under differentiation conditions may potentially change from GCL cells to cells of other lineages. However, this hypothesis still remains to be tested.

Previous groups working with mouse [3, 22] and human [5] primary germ cells have been able to maintain cells in extended culture using systems similar to the one described here. Since these initial reports, there have been numerous studies documenting the inability to maintain primary germ cells in culture [4, 6, 7]. However, the two key factors found to be essential in the continual culture of these cells are KIT ligand (KITL) and basic fibroblast growth factor (bFGF) [2, 3, 22]. Both of these factors are in our continual culture system and we have previously demonstrated that they are important in enrichment and differentiation of GCL cells [13, 23].

This report demonstrates for the first time that ESC derived GCL (DDX4+ POU5F1+) cells can be maintained in continual culture for at least 20 passages, while sustaining increased early and late germ cell gene expression. In addition, these cultures also demonstrated significant increases in GCL cell enrichment and late germ cell gene expression after serial passaging. The ability to maintain hESC derived GCL cells in continual culture suggests that these cells may have a significant advantage over primary germ cells as a tool to study germ cell development. It is the hope that culture systems such as these may ultimately provide a means to better understand the basic principles of germ cell development and in time, treatments for reduced fertility and sterility.

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Figure 5.1. hESC Derived Germ Cell-Like Cells Can Be Maintained in Continual Culture for 20 Passages. hESCs were differentiated for 10 days in 20% KSR media on feeders and passaged 20 times with flow cytometry performed on hESCs, day 10 and 30 and passages 5, 10 and 20. After 10 days of differentiation, 18.7% of cells were DDX4+ POU5F1+, a significant ($p < 0.05$) increase relative to the 4.0% found in hESCs. Passaging of enriched cells resulted in a significant ($p < 0.05$) increase in DDX4+ POU5F1+ relative to hESCs and day 10 controls with 58.0% of cells being DDX4+ POU5F1+ at passage 5, 58.7% at passage 10 and 60.7% at passage 20 (* indicates significantly different from hESCs; # indicates significantly different from day 10). Cultures that were allowed to differentiate for 30 days without passaging (Non-Passaged (NP)) showed a significant ($p < 0.05$) decrease in DDX4+ POU5F1+ relative to day 30 (passage 5 (P5)) continual passaged cells (+ indicates significantly different from non-passaged cells).

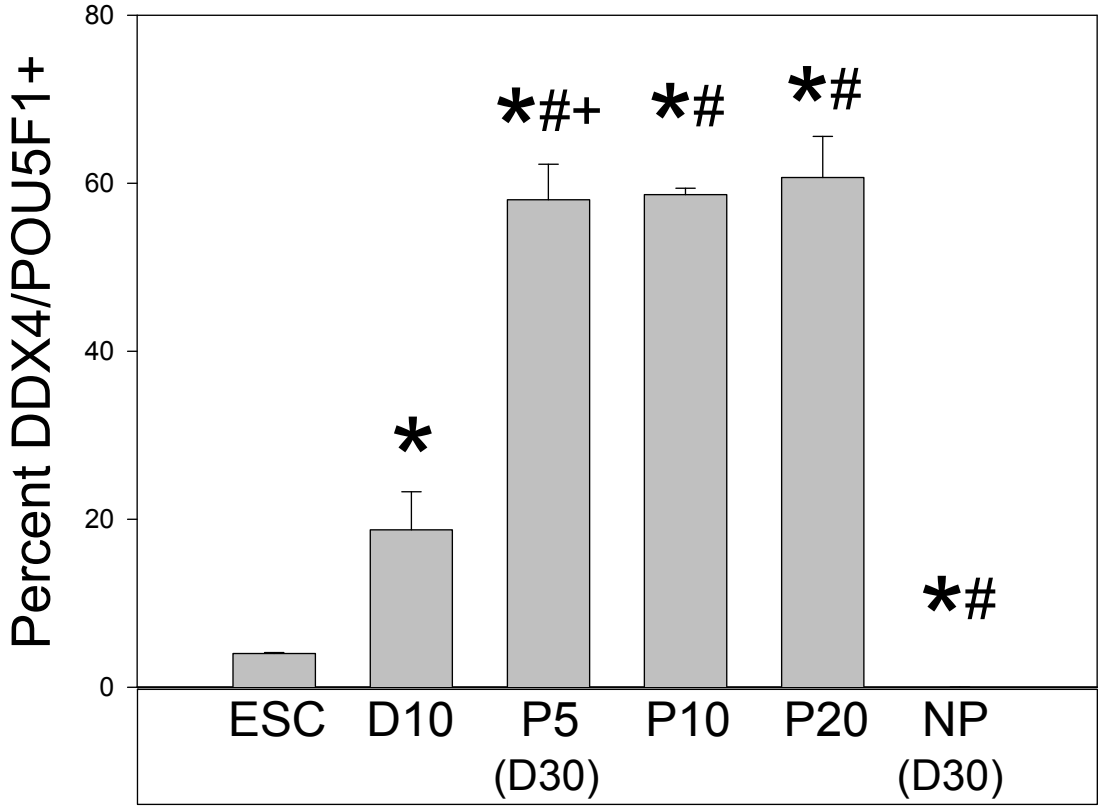

Percent DDX4/POU5F1+
80
60
40
20
0
*
*#+
*#
*#
*#
ESC
D10
P5
(D30)
P10
P20
NP
(D30)

Figure 5.2. Continually Cultured Germ Cell-Like Cells Maintain Up Regulated Pre-Migratory and Migratory Germ Cell Gene Expression. *IFTIM3, POU5F1, NANOG* and *DDX4* demonstrated significant ($p < 0.05$) up-regulation after 10 days of differentiation relative to hESCs. *IFITM3, NANOG, POU5F1,* and *DDX4* maintained up-regulation for 20 passages. *DPPA3* and *KIT* did not show up-regulation after initial differentiation, however these genes showed significant ($p < 0.05$) increases after 5 passages relative to hESCs. This increase in gene expression was maintained for 20 passages (* indicates significantly different from hESCs; # indicates significantly different from day 10). Day 30 (passage 5(P5) continually passaged cultures possessed up-regulation of all genes at a significantly ($p < 0.05$) higher levels than day 30 cultures that were not passaged (+ indicates significantly different from non-passaged cells).

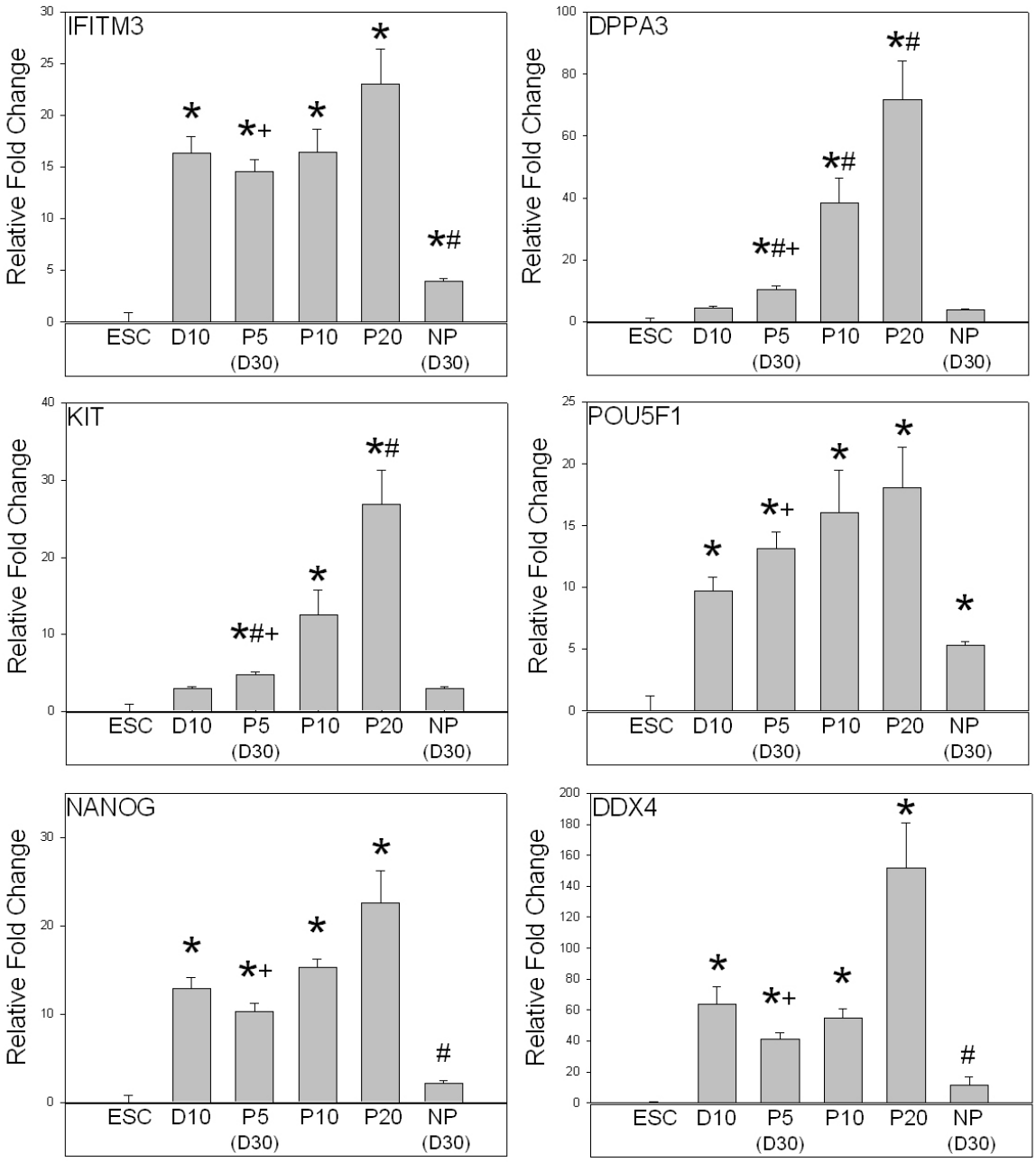
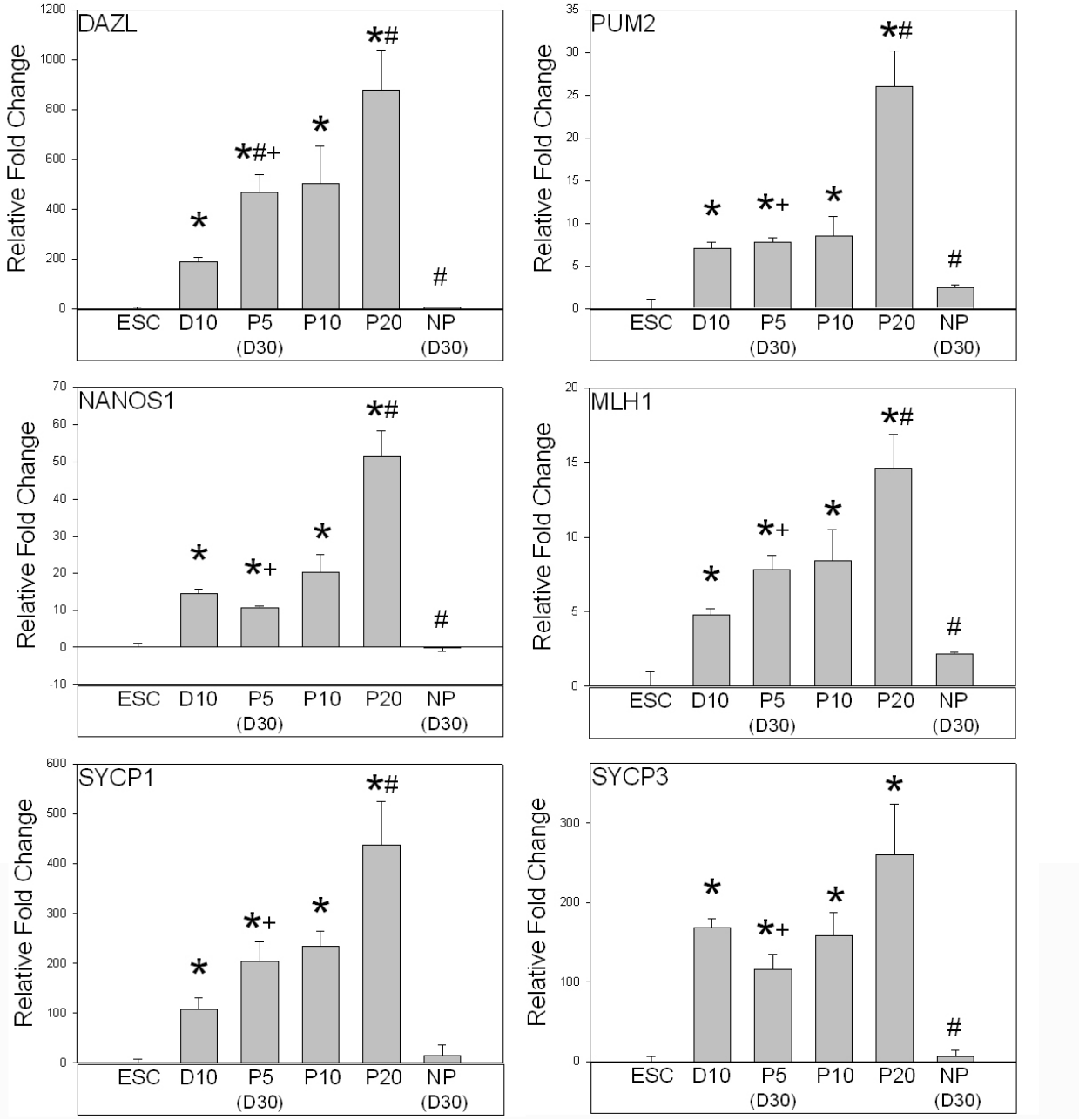

IFITM3
DPPA3
KIT
POU5F1
NANOG
DDX4
Relative Fold Change
ESC
D10
P5
(D30)
P10
P20
NP
(D30)

Figure 5.3. Continually Cultured Germ Cell-Like Cells Maintain Up Regulated Post-Migratory and Meiotic Germ Cell Gene Expression. Post-migratory genes *DAZL, PUM2* and *NANOS1* and the meiotic genes *MLH1, SYCP1* and *SYCP3* demonstrated significant ($p < 0.05$) up-regulation relative to hESCs after 10 days of differentiation. *SYCP3* gene expression was maintained at passages 5, 10 and 20, however *DAZL, PUM2, NANOS1, MLH1* and *SYCP1* all showed further increases ($p < 0.05$), relative to day 10 controls, after 20 serial passages (* indicates significantly different from hESCs; # indicates significantly different from day 10). Day 30 (passage 5 (P5)) continually passaged cultures possessed up-regulation of all genes at significantly ($p < 0.05$) higher levels than day 30 cultures that were not passaged (+ indicates significantly different from non-passaged cells).


DAZL
PUM2
NANOS1
MLH1
SYCP1
SYCP3
Relative Fold Change
ESC
D10
P5 (D30)
P10
P20
NP (D30)

CHAPTER 6

CONCLUSION

Germ cells are arguably one of the most fascinating cell types in the body passing on essential genetic information to future generations of a species. In order to do this, mammalian primordial germ cells (PGCs) undergo an extensive developmental progression beginning with the specification of undetermined epiblast cells and ending with spermatozoa and oocytes. The mechanisms by which these processes are orchestrated are highly regulated temporally and spatially control, where signaling at one stage of development causes expansion and at another stage causes differentiation. The interest in germ cell development also extends beyond basic science with more than 10% of couples worldwide being considered clinically infertile. In addition, the ability to produce a limitless source of oocytes for cloning and the opportunity to study the mechanisms by which these cells reprogram somatic cells into a pluripotent state has also piqued interest. To further explore the unique properties of germ cells and their basic development, emphasis has been placed on the development of various model systems.

There are currently numerous model systems, yet many of these systems (i.e. *Drosophila* and X*enopus*) have significant limitations with respect to studying mammalian germ cell development due to key differences. This challenge becomes even more significant when studying human germ cell development where availability of primary tissue samples is limited. To circumvent some of these issues, several groups have attempted to develop primary

germ cell culture systems [1-3], however these systems have proven to be inadequate [4-6]. More recently, embryonic stem cells (ESCs) have shown the ability to turn into germ cell-like (GCL) cells that are able to undergo advanced differentiation including meiosis and elongation [7-9]. However, ESC derived GCL cells come with a host of other issues indicating a significant need for a better culture system to study germ cell development.

In these studies, we developed a novel adherent germ cell differentiation culture system utilizing MEF feeders and bFGF where an unprecedented 69% of human embryonic stem cells (hESCs) were differentiated into GCL (DDX4+ POU5F1+) cells as assessed by immunocytochemistry, flow cytometry and quantitative RT-PCR. Enriched cultures showed significant increases in more than 12 different germ cell genes representing early and late germ cell development. In addition, we demonstrated that these GCL cells could be maintained in continual culture for more than 20 passages, while sustaining high levels of homogeneity and up regulated germ cell gene expression. Advanced differentiation of these enriched cultures ultimately led to populations where >90% of cells expressed the meiotic markers MLH1 and SYCP3 at the protein level. This had not been previously demonstrated in hESC derived GCL cells.

In addition, our results suggested that these cultures could potentially be used as a model system for human germ cell development. Studies in the mouse have shown that bone morphogenetic protein (BMP) family members and KIT ligand (KITL) signaling are important for germ cell formation and development, however the roles of these signaling molecules are not clear in humans. Our studies represent the first report that a

loss of mouse KITL in differentiation cultures causes a significant decrease (70.5%) in enrichment of human GCL cells and germ cell gene expression. We also showed that the addition of exogenous BMP4 caused a significant increase in germ cell gene expression and inhibition of endogenous BMP activity caused a loss in germ cell gene expression and enrichment. This suggests that BMP activity is important for differentiation of hESCs into GCL cells. This robust system clearly has the potential for use in parsing the factors involved in the differentiation of hESCs down the germ cell lineage. Understanding this process could ultimately lead to a better understanding of germ cell development and new and important treatments for infertility.

Future Studies

Despite the significant successes reported in these studies, there are still many issues that remain to be addressed and improvements that could be made to the current system. One concern is the use of undefined culture systems relying on feeder cells and knockout serum replacement, which produce or contain unknown and variable concentrations of germ cell signaling factors. Initial studies indicated high numbers (21%) of GCL (DDX4+ POU5F1+) cells in starting hESCs populations, which under 10 days of enrichment and differentiation conditions yielded a more homogeneous population of 69% DDX4+ POU5F1+ cells. However, later studies showed a significantly deceased starting DDX4+ POU5F1+ population of ~4% with results ranging from 29% to 37% after 10 days of differentiation. This variability between studies is potentially due at least in part to the use of undefined conditions. This highlights a clear need for the development of a defined culture system that can only be derived through a better understanding of germ cell maintenance factors.

One significant improvement to the current system would be the use of a reporter system to determine when cells have reached a germ cell state. For example, if we had used a DDX4 promoter driven GFP reporter system, we would have been able to isolate and purify GCL cells by fluorescence activated cell sorting (FACS). This would allow us to do gene expression analysis on hESCs and DDX4- and DDX4+ cells in differentiated cultures, providing a cleaner gene expression profile. DDX4+ cells could have also been removed from hESC starting populations. This would have eliminated the question of whether the increase in DDX4+ POU5F1+ was due to enrichment or differentiation. In addition, a DDX4 reporter system would have allowed us to continually culture the same cells and easily assess germ cell differentiation at additional time points. The development of similar reporter systems would have also helped in determining the initial expression of meiotic markers such as SYCP3. Systems such as these could have significantly strengthened our findings.

These studies also do not take into account the significant contribution of somatic tissue in germ cell development. In vivo, somatic cells play key roles in specification of germ cells, sex determination, maintaining the spermatogonial stem cell niche and meiosis, processes that cannot be completely recapitulated in culture. Several groups working with mESC derived GCL cells have attempted to reproduce these microenvironments by factors such as testosterone [10] and through testicular cell conditioned media [11]. However, all of these strategies have met with limited success and the only current system that has produced offspring relies on cell transplants into the testis [12]. In order to test the ability of our cells to develop into normal spermatozoa, it may be possible to do cross species transplants. This process faces

the hurdle of species specific differences, however other groups have shown interesting results [13, 14]. More recently, xenotransplants where somatic tissue and germ cells are placed into ectopic sites have significantly increased success rates of cross species transplants [15, 16]. These techniques maybe valuable in attempts to get hESC derived cells to surpass the early meiotic stage.

In addition, these current studies look at the broad effects of different culture conditions on gene and protein expression and germ cell development as a whole. However, it would be useful to take a more in-depth approach and study the specific effects of a germ cell signaling molecule on gene and protein expression at defined stages of germ cell development. For example, it has been shown that retinoic acid (RA) plays an important role in the meiotic stage of germ cell development; activating genes with RA response elements such as stimulated by retinoic acid gene 8 (STRA8) [17, 18]. However with over 200 genes estimated to be involved in meiosis, the exact role of RA, its targets and their downstream effects are not well defined. Using a system such as the one described here, one could add RA and determine if it binds to its receptors, translocates to the nucleus and activates the appropriate genes, producing the proteins of interest. The targets of these activated proteins could then be determined. There are numerous advantages to using a cell culture system to ask questions utilizing these highly proliferative and easily manipulated cells.

These cells have proven to be easy to manipulate in some respects, however it has not been proven whether these cells will be easy to genetically manipulate. If hESC derived germ cells are able to be genetically manipulated, this would be a significant advantage over their hESC predecessors, which are not and often undergo silencing of

introduced genes. The inability to stably transduce hESCs has made it hard to introduce genes to enhance differentiation, to silence genes using siRNAs or even introduce simple reporter systems that could be used to track cells inserted into in vivo models. GCL cells have shown the ability to enter early meiosis, therefore it is conceivable that one could introduce other meiotic genes to further enhance meiotic activity or add factors that can aid in epigenetic reprogramming of these cells. This also opens the door for gene therapies that could reverse the genetic defects that initially caused sterility or reduced fertility in some patients.

In the future, GCL cells also present a great opportunity as a cell therapy. These cells could be differentiated into spermatogonial stem cells (SSCs) and be reintroduced into patients who lack germ cells, such as patients who have been treated for cancer or exposed to toxic substances. However the ability of existing culture systems to derive spermatogonia is not apparent. Currently, we have cells that appear to enter early meiosis, which would normally indicate that cells have passed through the spermatogonia stage of male germ cell development. It is not clear if this is true for these male ESC derived cells as they appear to enter directly into meiosis, which is indicative of female germ cell development. If these cells do indeed produce spermatogonia, it is potentially possible to determine if there are a spermatogonial-like cells based on surface markers [19, 20]. However, determining if SSCs exist in differentiated germ cell cultures based on cell surface markers also presents unique challenges. For example, THY1+ MHCI- KIT- cells may clearly be SSCs when collecting cells from the testis, which limits the number of potential cell types. However, differentiated cultures of ESCs can potentially give rise to any cell type in the body

including other cells that may have similar cell surface profiles. There is currently only one definitive test to determine if hESC derived SSCs are fully functional and that is by transplanting these cells into a sterile testis and allowing for repopulation of the gonad. These cells should then be proven to be capable of fertilize an oocyte and producing offspring. Being one of the final cell therapy goals, there are many issues that still remain to be addressed before this stage of study is truly viable.

The present studies showed little change in the methylation status of GCL cells, yet there is still great potential to use these cells to study signaling that is important for epigenetic modifications. In vivo derived germ cells have shown the ability to undergo methylation erasure during early germ cell development. However, the exact mechanisms that control active demethylation are unclear. ESC derived germ cells are potentially primed to undergo demethylation as their in vivo counterparts are and may represent an easier cell type to study demethylation and cell reprogramming. In addition, if these cells can be directed down the female germ cell lineage, GCL cells represent an infinite source of oocytes, the only cell type known to naturally reprogram adult cell types. This could increase the speed at which reprogramming studies can be done.

The possibilities of hESC derived GCL cells are seemingly endless with potential from basic science to drug screens to cell therapies. In these studies, we developed a robust culture system that can reliably produce high numbers of GCL cells from human embryonic stem cells. It is hoped that the results found here and in future studies will help advance the field and produce real world clinical applications.

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