GENE EXPRESSION AND NEURAL DIFFERENTIATION STUDIES OF EMBRYONIC STEM CELLS

by

JOHN DOUGLAS CALHOUN

(Under the Direction of Steven L. Stice)

ABSTRACT

Few scientific discoveries have generated as much interest and controversy as embryonic stem cells (ESCs). Their discovery has led to a new age in developmental biology and they hold promise as an unlimited supply of material for cell replacement therapy. To date there are no directed differentiation strategies for primate (monkey or human) ESCs that efficiently produce one specific cell type. Further, only recently have efforts been made to characterize the transcriptional profile and mechanism of pluripotency of these cells. For this reason the current study utilizes a technology defined in the mouse to characterize gene expression patterns during early differentiation and create a differentiation protocol for making neural cells from monkey ES cells. By combining microarray analysis and MEDII media, conditioned media from the growth of HepG2 cells, we have characterized gene expression patterns during early differentiation events in hESCs. Treatment of adherent hESCs with 50% MEDII media for 3 days effected differentiation to a cell type with gene expression similar to primitive streak stage cells of the mouse embryo. MEDII treatment up-regulates Cripto, a gene essential for proper anterior-posterior axis and mesoderm formation in mouse embryos. In addition, several genes previously shown to be important for proper development were down-regulated with MEDII

treatment even though the pluripotency markers Oct-4, Nanog, and SSEA-4 were unchanged by the treatment. These include but are not limited to Follistatin, Lefty A, HOXA1, Dapper, and EZH2. Genes down-regulated in this manner are thought to play important roles in pluripotency maintenance of primate ESCs. The current study also utilizes MEDII media to develop an efficient neural differentiation protocol of rhesus monkey ESCs. Embryoid bodies (EBs) formed in the presence of 50% MEDII media exhibited high levels of neural differentiation in comparison to untreated controls. Further, a population of neural progenitors (NPs) was isolated from explants of treated EBs that could be maintained in culture in the undifferentiated state. Mitogen withdrawal led to terminal differentiation into neurons that possessed immunostaining and electrophysiological characteristics of functional neurons. The current study demonstrates MEDII's utility in studying pluripotency and its ability to enrich differentiation to desired lineages.

INDEX WORDS: Embryonic stem cells, neural differentiation, pluripotency, differentiation, microarray, human, primate, mesoderm induction, electrophysiology

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DEDICATION

This dissertation is dedicated to Ashley Renee Moore; Doug, Jane, and Ben Calhoun.

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ABBREVIATIONS

ES ..... embryonic stem

ICM ..... inner cell mass

hESCs ..... human embryonic stem cells

mESCs ..... mouse embryonic stem cells

LIF ..... leukemia inhibitory factor

GSK3 ..... glycogen synthase kinase 3

DA ..... dopaminergic

SHH ..... sonic hedgehog

FGF-8 ..... fibroblast growth factor eight

Sox-2 ..... sry box-2

bFGF ..... basic fibroblast growth factor

NGF ..... nerve growth factor

RA ..... retinoic acid

CHAPTER 1

INTRODUCTION

Few scientific discoveries have generated as much interest and controversy as embryonic stem (ES) cells. Their discovery has led to a new age in developmental biology as well as the ability to alter specific genes in the mouse genome. They hold promise as an unlimited supply of material for cell replacement therapy and model tissues for toxicity testing and drug development. Their short life span has been characterized by interest and debate from the scientific community, the popular press, and world governments. Few people are unaware of the significance of their discovery.

Mammalian Embryogenesis

To fully understand the origin of ES cells, a brief review of early embryogenesis is in order. It has been proven that cells in the early embryo remain totipotent up to the 8-cell stage [1]. The first differentiation event that occurs in the embryo is the development of trophectoderm cells from the blastomeres located on the periphery of the embryo at the 8-16 cell stage of development [2-4]. Once this occurs there is a rearrangement of the embryo to a fluid filled structure called a blastocyst [5, 6]. In the blastocyst, the pluripotent cells are located as a mass at one end of the embryo and are called inner cell mass (ICM) cells. The trophectoderm, which will give rise to extraembryonic membranes, lines the edge of the embryo with the center being a fluid filled cavity [7]. Some of the ICM cells that are adjacent to the cavity then

differentiate into primitive endoderm, which will eventually give rise to extraembryonic tissues as well. The primitive endoderm will eventually separate into two different types based on its location within the embryo. Primitive endoderm that migrates onto the trophectoderm is considered parietal endoderm, while primitive endoderm that remains associated with pluripotent cells is termed visceral endoderm [7]. The cells of the ICM undergo rapid proliferation [8] and apoptosis [9] after implantation to form a population of pluripotent cells with a cavity in the middle. These cells, which are now separated from visceral endoderm by a basement membrane, are termed primitive ectoderm [7]. Primitive ectoderm is a pluripotent cell population that will give rise to the three embryonic germ layers, endoderm, mesoderm, and ectoderm during gastrulation [10-13]. ES cells isolated in the laboratory are inner cell mass cells that have been isolated from blastocyst stage embryos.

Derivation of First Embryonic Stem Cells, Mouse Embryonic Stem Cells

ES cells were first isolated from mouse embryos in 1981 by two research teams working independently of each other [14, 15]. Their discovery arose from work being done with mouse embryonal teratocarcinoma cells. Teratocarcinomas are tumors that arise from germ cells of certain inbred strains of mice that contain derivatives of all embryonic germ layers. Research done with these tumors led to the concept of a stem cell that could give rise to the multiple types of tissue that were routinely observed in teratocarcinomas [16]. It was also discovered that these tumors could be generated if normal mouse embryos were transplanted to an extra-uterine location of a histocompatible host [17]. This knowledge led Martin to hypothesize that teratocarcinoma cells were derivatives of pluripotent cells that would develop normally if they were exposed to growth factors in their normal growth environment. She also suggested that by

supplying the requisite growth factors, stem cells could be isolated directly from embryos themselves. Her hypothesis proved correct and the successful isolation of stem cells directly from pre-implantation embryos was achieved [15]. Initially the term embryonic stem cell was used if cells could meet two criteria. One of these was contribution to the germ line in a chimera, and the other was a limitless life span. Only mouse ES cells have met these two criteria; however, a new set of criteria emerged as ES cells were isolated from other species. The criteria for a pluripotent cell are the derivation from a pluripotent cell population, ability to maintain a normal karyotype, ability to be propagated in an undifferentiated state indefinitely, and the ability to give rise to cell types representative of the three embryonic germ layers. In accord with these criteria, a cell can be considered an embryonic stem cell if it is a pre-implantation embryo derived cell that meets the criteria for a pluripotent cell. However, it should be noted that these criteria do not include contribution to a germline chimera [16]. In dealing with human ES cells an experiment to determine the ability of these cells to contribute to germ line chimeras is considered highly unethical.

In the years to come attempts were made to isolate embryonic stem cells from several vertebrate species. These include medakafish [18], zebrafish [19], chicken [20, 21], rabbit [22, 23], rat [24], Syrian hamster [25], mink [26], pig [27, 28], cattle [29, 30], and sheep [31]. There has been particular interest in deriving stem cells from agricultural species because of the desire to create transgenic livestock.

Human and Primate Embryonic Stem Cells

It was not until 1995 that the successful isolation of embryonic stem cells from a primate species was reported. In that year James Thomson reported the isolation of monkey ES cells.

The cells were isolated from rhesus monkey blastocysts using similar procedures that had been used to isolate mouse stem cells [32]. In 1996 he was able to isolate embryonic stem cells from marmoset monkeys as well [33]. Then in 1998, he reported he had been able to successfully derive embryonic stem cells from human embryos (hESCs) [34]. That same year John Gearhart's laboratory reported the isolation of human stem cells from primordial germ cells [35]. Alan Trounson's laboratory in Australia also reported the isolation of another human embryonic stem cell line [36]. On August 9, 2001 President Bush announced that federal funds would be made available for the study of hESCs provided that they had been isolated prior to the announcement and the embryo from which they were isolated no longer had the ability to develop into a human being. Additional criteria were that the stem cells must have been derived from human embryos created for reproductive processes, the embryo was no longer needed for these processes, informed consent was obtained prior to donation of the embryo, and no financial compensation was made for providing the embryo. In order to facilitate research of cell lines meeting criteria a federal registry was created (http://stemcells.nih.gov/registry/index.asp). The registry currently lists 78 lines as eligible for distribution, but only 15 lines are currently distributed. Even though federal grant money cannot be used for hESC isolations researchers are still free to obtain private funding for stem cell isolation provided that it is clear that federal funding is not used to study these stem cell lines.

While much has been accomplished in the past five years basic laboratory work with hESCs remains difficult. Most culture systems require tedious mechanical passaging of colonies. This requires using pulled Pasteur pipettes to mechanically dissect colonies into smaller pieces. These pieces are plated on fresh feeder layers of mouse embryonic fibroblasts (MEFs) which provide an as yet unspecified anti-differentiation effect. In addition, some level of spontaneous

differentiation occurs in almost all cultures [37]. More recent reports have demonstrated feeder free growth of hESCs on extracellular matrices with conditioned or defined media [38, 39]. However, few laboratories currently culture hESCs with these conditions. Mouse ES cells (mESCs) can be maintained in the undifferentiated state on gelatin alone by the addition of the cytokine leukemia inhibitory factor (LIF). Human LIF does not have the same effect on hESCs. A new report indicates that pluripotency of both mouse and human ESCs can be maintained by inhibiting glycogen synthase kinase-3 (GSK-3), a modulator of Wnt/β-catenin signaling [40]. The report further demonstrates that addition of soluble Wnt3a to hESC culture media was sufficient to maintain pluripotent gene expression. This is the first demonstration of the ability to add soluble anti-differentiation components to hESC cultures to maintain their undifferentiated state.

MEDII Media, a Conditioned Media from Human Hepatocellular Carcinoma (HepG2) Cells

The main goal of this dissertation was to determine if hESCs respond to a biologically active conditioned media from the growth of human hepatocellular carcinoma (HepG2) cell line similarly to mESCs. The conditioned media (MEDII) was previously shown to have a rather unique differentiation effect on mESCs. By treating mESCs with 50% MEDII media supplemented in normal growth media the conversion of these cells to primitive ectoderm was achieved [41]. Consistent with this conversion, mESCs treated with 50% MEDII for three days exhibited down regulation of the pluripotency markers *Rex-1* and *Gbx-2* and up regulation of the primitive ectoderm marker *FGF*-5. These changes in gene expression are displayed by ICM cells undergoing the transition to primitive ectoderm *in* vivo. These cells were termed early

primitive ectoderm-like (EPL) cells. As described previously, primitive ectoderm is a definitive cell population in the progression of differentiation of ES cells to a specific cell type. Subsequent studies with MEDII demonstrated its utility in affecting differentiation outcomes. By making embryoid bodies (EBs), three dimensional aggregates of ES cells grown in suspension culture, from mESCs exposed to 50% MEDII media in the adherent setting the resultant EBs were enriched in mesodermal progenitors in comparison to EBs derived from mESCs [42]. Conversely, if mESCs were aggregated as EBs in the presence of 50% MEDII media these EBs were enriched in neurectoderm in comparison to EBs aggregated without 50% MEDII media [43]. The advantage of using MEDII media lies in its ability to affect differentiation during the initial steps of differentiation from pluripotency. In contrast, many *in vitro* strategies rely on the formation of EBs, in which differentiation to all three germ lineages occurs, and then attempts are made to enrich the complex products of this differentiation towards one lineage. Strategies with MEDII have instead attempted to reduce unwanted germ lineages early in the differentiation process. In the first part of this study, the ability of MEDII to enrich neural differentiation of primate ES cells (pESCs) was evaluated. This study was undertaken to determine the effectiveness of MEDII in generating neural lineages from pESCs. As there is a great deal of similarity between pESCs and hESCs it is highly probable that the results of the study will be transferable to hESCs.

History of Neural Differentiation of Embryonic Stem Cells

A review of neural differentiation strategies is relevant to understanding the techniques employed in the current study. While hESCs and pESCs have been isolated for only for 5 and 8 years respectively, mESCs have been grown in culture for over twenty years as mentioned

previously. The foundation for most *in vitro* differentiation strategies is found in work done with mESCs. The most widely used technique for initiating differentiation of ESCs is the formation of aggregates, or EBs, of cells in suspension culture [44]. Interactions within these aggregates allow the formation of complex cell types [45]. After varying days of growth EBs are then plated as outgrowths in adherent culture. By treating EBs with retinoic acid (RA) at different time points and then plating the EBs on laminin coated dishes, neural differentiation was achieved [46]. Differentiated cells expressed transcripts of neural associated genes and had appropriate electrophysiological properties. Several studies were published with subtle variations in this protocol such as plating on gelatin [47] or tissue culture plastic [48]. All of these studies demonstrated the derivation of neural cells expressing appropriate markers and electrophysiological properties. Other neural cell types such as glia and oligodendrocytes were generated as well.

After several studies were done using these techniques, a new variation on the EB protocol was reported by Dr. Ron Mckay's research group. The Mckay protocol still required the formation of EBs, but instead of using RA treatment, EBs containing cells of all germ layers were plated in selective serum-free media. Non-neural cells, which will not grow in serum-free conditions, do not survive in these conditions and the remaining cells are enriched in nestin positive neural-progenitor cells [49]. Neural progenitors were then efficiently differentiated into functional neurons [50]. The majority of neurons produced by this system were γ-aminobutyric acid-ergic (GABAergic), but a subsequent study demonstrated it was possible to generate dopaminergic (DA) neurons by treating mESC derived neural progenitors with Sonic hedgehog (SHH) and fibroblast growth factor-8 (FGF-8) [51]. These signaling molecules had previously been shown to be important in patterning the ventral midbrain *in vivo* where DA neurons are

naturally produced in the developing nervous system [52]. Perhaps the most elegant study was that of Wichterle et al. in which spinal motor neurons were generated from mESCs in a manner that recapitulates development *in vivo* [53]. Neural progenitors were produced in EBs and then treated with RA as a posteriorizing factor and then with SHH as a ventralizing factor. The importance of these early studies lies in the demonstration that a neural progenitor cell can be generated *in vitro* and further differentiated to a desired cell type by recapitulating *in vivo* signaling events *in vitro*.

A more recent study demonstrates production of DA neurons from ESCs by co-culture with a bone marrow-derived stromal (PA-6) cells [54]. The inducing signal has not been isolated, however the neural induction is suppressed by serum or bone morphogenetic protein-4 (BMP-4). The loss of DA neurons is a hallmark feature of Parkinson's disease, and the ability to produce large numbers of transplantable DA neurons is the goal of many cell replacement programs.

Other strategies take advantage of the concept of the default neural induction model originally described from research with amphibians [55, 56]. By plating mESCs at low density in serum free media supplemented with basic fibroblast growth factor (bFGF) and LIF the direct conversion to a primitive neural stem cell equivalent was achieved [57]. Cells derived in this manner were capable of forming neurospheres, or floating aggregates of nestin positive cells. Neurosphere culture is the most common method for culturing neural stem cells isolated from primary sources [58].

Another slightly more complex technique for generating cell types of a particular lineage has also been developed. The technique, commonly referred to as lineage selection, relies on homologous recombination and the expression of a selection cassette such as an antibiotic

resistance gene. The construct is engineered to be expressed only by a specific promoter that drives the expression of a gene limited to a particular cell type. An example of this strategy initially used in mESCs used an eGFPIRESpac construct driven by the *Sox2* promoter, a gene whose expression was initially defined in neural progenitors [59]. A major drawback to this particular strategy was the discovery that undifferentiated mESCs express *Sox2* mRNA as well. When utilizing the lineage selection approach it is important to be sure the gene whose promoter is being utilized has restricted expression in one particular cell type as all in vitro differentiations exhibit either the persistence of some level of ESCs and/or the derivation of unwanted cell types. While lineage selection is a powerful *in vitro* tool it is unclear whether or not human cells generated in this manner would be candidates for cellular replacement therapy. Regulatory agencies may have concerns about using genetically modified cells for transplantation.

Translation of mESC Neural Differentiation Strategies to Human ES Cells or Primate ES Cells

With the isolation of pESCs and hESCs there was great interest in applying *in vitro* differentiation protocols developed with mESCs to these cells. Initial differentiations of these cells were done *in vivo* and involved the formation of teratocarcinomas, tumors formed by injecting pluripotent cells into SCID mice that contain derivatives of all three germ layers [34]. Initial *in vitro* studies to differentiate hESCs utilized the EB approach [60] and then added RA and nerve growth factor (NGF) to EBs [61]. More recently, specific protocols were used to generate neural cells. In 2001 several published studies reported more direct methods of generating neural progenitors and fully differentiated neurons. Two of the studies used EBs to initially differentiate hESCs and then either used enzymatic [62] or immuno-isolation [63]

techniques to enrich for neural cells. The third study allowed hESCs to become overgrown in adherent culture and then mechanically isolated areas confirmed to contain neural progenitors by immunostaining [64]. Two of the studies demonstrated that the isolated neural progenitors would integrate into the rodent fetal brain after transplantation [62, 64]. These studies illustrated differences between mESCs and hESCs as well. HESCs were not as responsive to RA as mESCs were when using an established mESC neural differentiation protocol. In addition, hESCs driven toward neural lineages readily formed neural tube like structures *in vitro* termed neural rosettes [65]. These structures had not been observed when mESC derived neural progenitors were isolated [66]. These observations demonstrated that while there are many similarities between mESCs and hESCs the two populations do not necessarily differentiate *in vitro* in an identical manner.

Due to ethical constraints and lack of availability, many researchers have chosen to characterize the *in vitro* differentiation potential of pESCs rather than hESCs. The ability to carry out homologous transplants in a large animal model of cellular replacement therapy makes pESCs even more attractive as models for *in vitro* differentiation studies. However, fewer reports in the literature detailed differentiation strategies for pESCs. The initial differentiation of pESCs to neural lineages was, like hESCs, done *in vivo* by forming teratocarcinomas and analyzing the tumors for specific germ lineages [67]. While pESCs were isolated much earlier than hESCs [32, 33], it was not until 2002 that the directed differentiation of pESCs appeared in the literature [68]. This study utilized the co-culture with PA-6 cell strategy initially defined with mESCs and was capable of generating DA neurons at a frequency of around 35%. The co-culture also induced the differentiation of pigmented epithelia as well. This strategy was useful for generating DA neurons, which are potentially useful for treating Parkinson's disease.

At the time of this study a similar report detailing the differentiation of pESCs to neural cells was published [69]. The first step of their experimental strategy was to create EBs from pESCs. These EBs were plated onto gelatin coated culture dishes and maintained in a serum-free neural cell media for an additional seven days. Neural progenitors were either dispersed with enzymatic treatment or isolated mechanically. These cells were cultured either as floating spheres or as adherent cells in serum-free neural cell media supplemented with the mitogen bFGF. Removal of the bFGF induced terminal differentiation of the neural progenitor cells. Neural progenitors stained positively for the neural progenitor markers nestin and musashi. Terminally differentiated cells stained positively for the pan-neuronal marker MAP2 demonstrating the presence of post-mitotic neurons. Terminally differentiated cells also stained positively for tyrosine hydroxylase, an enzyme necessary for the synthesis of the neural transmitter dopamine, and choline acetyleseterase, an enzyme necessary for the synthesis of choline. This study demonstrated that while pESCs and mESCs have distinct differences, several aspects of the differentiation process to neural lineages are conserved, indicating the ability to translate parts of mESC differentiation protocols to the derivation of neural cell lineages from pESCs.

HepG2 Cell Conditioned Medium (MEDII) Induces or Allows Neural Differentiation

For the current study the ability of MEDII media to influence neural differentiation of pESCs was evaluated. MEDII was shown to be effective in generating neurectoderm from mESCs *in vitro* [43]. MEDII media is the conditioned media from the growth of HepG2 cells. Application of MEDII to mESCs has been shown to affect the differentiation outcome of mESCs. Briefly, the technique involved forming and maintaining EBs in media supplemented

with 50% MEDII (EBM). These aggregates were cultured over several time points, and some aggregates were plated in adherent culture for analysis as well. EBM at days 6-9 were analyzed for *Sox-1* expression, a marker of neural progenitors. In contrast to control EBs, EBM expressed high levels of *Sox-1* as assessed by Northern analysis and *in-situ* hybridization. Morphological analysis of sectioned EBMs revealed dense columnar epithelia consistent with neurectoderm as well. EBM expressed extremely low levels of the endoderm marker alpha-fetoprotein (AFP) and the mesoderm marker Brachyury. Further, terminal differentiation of EBM derived neurectoderm to post-mitotic neurons, neural crest cells, and glia was demonstrated. Gene expression analysis of a panel of markers normally expressed in defined brain regions demonstrated the lack of positional specification imparted to EBM derived neurectoderm. The gene expression studies suggested that EBM neurectoderm was most similar to primitive anterior neurectoderm with characteristics similar to forebrain and midbrain. This contrasts other neural differentiation strategies such as treatment with RA which is known to have a posteriorizing effect during differentiation of mESCs to neural lineages [53]. Therefore, there are several advantages in using MEDII to drive neural differentiation of mESCs including the ability to generate more uniform differentiated populations than standard EB protocols and the generation of non-postionally specified neural precursors that can be terminally differentiated to a variety of cell types. For these reasons, the current study will characterize the influence of MEDII on neural differentiation from pESCs.

Characterization of Undifferentiated hESCS

The derivation of hESCs has ignited considerable interest in these cells as tools for cell replacement therapy and models of early human development. However, the continuous

undifferentiated culture of these cells and the ability to derive a specific cell type are two of the problems faced by hESC researchers. Furthermore, until recently little was known about the transcriptional profile of these cells. Research with mESCs have identified a handful of genes important for pluripotency including Oct-4 [70, 71], Nanog [72, 73], Sox-2 [74], and LIF [75, 76]. More recently, microarray analysis has been used to try to define an expression profile indicative of "stemness" [77, 78]. Both reports used a similar strategy to define "stemness" genes. Three different stem cell populations; hematopoetic stem cells (HSCs), neural stem cells (NSCs), and ESCs, expression profiles were compared to differentiated cells to obtain sets of genes enriched in each type of stem cell. Then, a union list of genes common to all three stem cell types was made. The low degree of correlation between the data sets sparked debate in the stem cell community as to whether or not this strategy was effective in defining genes essential to "stemness". For example, the Ramalho-Santos study created a list of 230 genes enriched in all three stem cell types while the Ivanova study found 283 genes enriched in all three stem cell types. Only six genes, or 1.2%, overlap between the two lists [79]. To further address the issue another study created yet another group of "stemness" genes by using NSCs, ESCs, and retinal progenitor cells (RPCs) [80]. This strategy elucidated a list of 385 genes collectively expressed by all three stem cells. However, when this list was compared with the two previously generated lists only one gene is common to all three lists. If comparisons are made within one cell type only the correlation improves. The authors conclude that as the number of stem cell types used to identify "stemness" genes increases the overlap between the datasets decreases. A closer examination of the studies revealed variables that may contribute to the low overlap of genes. Some of those variables include techniques used to isolate the stem cells, the baseline populations the stem cells were arrayed against, and the data analysis methods used to analyze

the data [81]. Despite the low degree of overlap in "stemness" genes from several studies, global gene expression profiling remains a powerful tool for characterizing pluripotent cell gene expression patterns.

Recent studies reported while the included study was being carried out have utilized microarray technology to characterize gene expression patterns in hESCs. These studies have compared undifferentiated hESCs with differentiated counterparts [82] or hESCs with EC cells and various somatic cell types [83]. By comparing undifferentiated and differentiated cells a set of genes down-regulated during spontaneous differentiation of hESCs was elucidated [82]. While it is not clear what type of differentiation was initiated, in e. g. a default neural induction or a completely random event, it is clear that the strategy was effective in defining genes important for maintaining the pluripotent state of hESCs. The observation that members of the Wnt signaling pathway were enriched in undifferentiated hESCs led to a subsequent study that demonstrated the addition of soluble factors to hESC growth media that mimic the activation of the Wnt signaling pathway could keep hESCs undifferentiated [40]. This certainly validates the power of microarray technology and its application to stem cell biology. A theory that has emerged with the advent of stem cell biology hypothesizes that tumors arise from stem cell populations in the body instead of the de-differentiation of terminally differentiated cells. This theory has emerged from observations of certain similarities between cancer cells and stem cells such as increased nuclear to cytoplasmic cellular ratios, telomerase activity or lengthened telomeres that provide immortality or enhanced lifespan, and activity of signaling pathways essential for embryogenesis and/or development [84, 85]. Along those lines, Sperger et al. compared gene expression profiles of hESCs with hEC cells to determine the similarity of their transcriptional profiles. Genes expressed in both cell types should be indicative of pluripotency

while genes expressed in EC cells alone may give insight into the mechanisms by which tumor cells escape the normal stem cell and differentiation programs. In addition, comparing gene expression profiles of somatic cell types to hESCs will, like comparing hESCs to sponataneously differentiated cells, yield insight into the genetic program of hESCs. A strategy that has not been utilized to date is to compare gene expression patterns of hESCs to closely related derivatives that are still pluripotent yet have begun the beginning phases of differentiation.

HepG2 Conditioned Media (MEDII) Induces Mesodermal Differentiation

The second part of this study sought to characterize the effect of MEDII media on hESCs using microarray analysis, cell cycle analysis, and immunostaining for pluripotency markers. The use of MEDII conditioned media provides a novel and unique strategy for examining gene expression patterns of hESCs and early differentiated derivatives. Further, as studies with mESCs have shown, MEDII may be important for defining an *in vitro* differentiation protocol for deriving mesodermal derivatives from hESCs.

Previous studies with mESCs have demonstrated the ability of MEDII to enrich mesodermal differentiation [42]. Treatment of mESCs with MEDII media effects differentiation of these cells to primitive ectoderm, a transient pluripotent cell population found in mouse embryos immediately prior to gastrulation and formation of the three primary germ layers [41]. As stated above, aggregation of mESCs into EBs in the presence of 50% MEDII media enriches neural differentiation and decreases the levels of differentiation to mesodermal or endodermal lineages [43]. In contrast, if mESCs are exposed to 50% MEDII media for three days (EPL cells) and then aggregated into EBs, the resulting bodies are greatly enriched in nascent mesoderm [42]. Lake et al demonstrate that EBs from MEDII treated cells are morphologically

different than those formed from ES cells. Day four EBs formed from mESCs were composed of a homogenous population of round, smooth, and ordered aggregates, while the morphology of day four EBs formed from MEDII treated mESCs indicated that the cells were more loosely packed and heterogeneous, possibly indicating differentiation had already begun. Further, it was demonstrated by RT-PCR analysis that day three and four EBs formed from MEDII treated mESCs exhibited high levels of expression of the nascent mesoderm markers Brachyury and Goosecoid while day and three ES derived EBs did not. Further, whole mount in situ hybridization revealed large portions of Brachyury expressing cells in most MEDII treated ES cell EBs. From these studies, it was concluded that EBs formed from MEDII treated ES cells exhibited both an earlier appearance and more extensive levels of mesoderm differentiation. Experiments were then done to determine the differentiation capabilities of the mesodermal progenitors. This included demonstrating the expression of Nkx2.5, a transcription factor whose expression is elevated in myocardial cells [86], was detectable earlier and at greater levels by RT-PCR in EBs derived from MEDII treated cells in comparison to EBs made from ES cells. In addition, EBs from both treated and un-treated mESCs were scored at days six through twelve for the appearance of beating heart muscle. Again, EBs from mESCs treated with MEDII displayed beating heart muscle earlier and more thoroughly than EBs from un-treated mESCs. Thirdly, after treatment with the cytokines mIL-3 and hM-CSF EBs from treated mESCs formed macrophages more readily than control counterparts. The final characterization step was to demonstrate that EBs from MEDII treated mESCs fail to form neurons and neurectoderm. Neurons were not detected in EBs from MEDII treated mESCs during days eight through twelve of differentiation while EBs made from mESCs efficiently produced neurons during this differentiation window. However, MEDII treated mESCs could form neurons if treated with RA

instead of aggregated into EBs. These results demonstrated that MEDII treated mESCs were not restricted in differentiation potential but the particular differentiation method was not permissive for neural differentiation.

The studies in this dissertation were performed to asses the similarities of response of pESCs and hESCs to the HepG2 cell conditioned media MEDII. The first study sought to characterize neural differentiaion of pESCs in the presence of MEDII media, while the second study utilized microarray technology to characterize the effect of MEDII media on hESC gene expression profiles. Further, use of MEDII may yield insight into mechanisms of pluripotency maintenance in hESCs as well as provide a novel differentiation strategy for producing mesodermal cell lineages from hESCs.

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CHAPTER 2

DIFFERENTIATION OF RHESUS EMBRYONIC STEM CELLS TO NEURAL PROGENITORS AND NEURONS[1]

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Abstract

Embryonic stem (ES) cells are pluripotent cells capable of differentiating into cell lineages derived from all primary germ layers including neural cells. In this study we describe an efficient method for differentiating rhesus monkey ES cells to neural lineages and the subsequent isolation of an enriched population of Nestin and Musahsi positive neural progenitor (NP) cells. Upon differentiation, these cells exhibit electrophysiological characteristics resembling cultured primary neurons. Embryoid bodies (EBs) were formed in ES growth medium supplemented with 50 % MEDII. After 7 days in suspension culture, EBs were transferred to adherent culture and either differentiated in serum containing medium or expanded in serum free medium. Immunocytochemistry on differentiating cells derived from EBs revealed large networks of MAP-2 and NF200 positive neurons. DAPI staining showed that the center of the MEDII treated EBs was filled with rosettes. NPs isolated from adherent EB cultures expanded in serum free medium were passaged and maintained in an undifferentiated state by culture in serum free N2 with 50 % MEDII and bFGF. Differentiating neurons derived from NPs fired action potentials in response to depolarizing current injection, and expressed functional ionotropic receptors for the neurotransmitters glutamate and gamma-aminobutyric acid (GABA). NPs derived in this way could serve as models for cellular replacement therapy in primate models of neurodegenerative disease, a source of neural cells for toxicity and drug testing, and as a model of the developing primate nervous system.

Key words: embryonic stem cell, human, primate, neural progenitor, electrophysiology, neuron, neural stem cell

Introduction

The isolation of ES cells from monkey and human embryos has generated great interest in using these cells as the basis of cell replacement therapies for degenerative diseases, as well as models of human development. ES cells were first isolated from mouse embryos over 20 years ago [1, 2]. More recently, monkey ES cells were isolated first from the rhesus monkey followed by the isolation of marmoset and cynomologous ES cells [3-6]. Shortly after the isolation of monkey ES cells the same methods were used to isolate human ES cells from *in vitro* fertilized human embryos [7, 8].

Currently, immense interest is focused on finding ways to differentiate ES cells into specific cell types that would be candidates for cell replacement therapies for degenerative diseases. However, the very quality that makes ES cells so appealing, the ability to generate virtually every cell type found in the body, is the Achilles heel of stem cell research. Methods need to be defined that will allow for the production of specific differentiated cell types for cell replacement therapy to become a reality.

Non-human primate and human ES cells have similar markers of pluripotency including the expression of Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase activity. Therefore, we feel that information gained from the study of non-human primate ES cells will be applicable to human ES cells. Rhesus animal models for Parkinson's disease, diabetes, and spinal cord injury have been demonstrated [9-12], and rhesus ES cells can be used to generate cells for allografts into these models thus avoiding potential xeno-transplant rejection as a confounding factor in these studies. Transplants of cells derived from non-human primate ES cells into these models will be used to perfect strategies that can then be applied to

human cell therapies. However, to date, there are no reports of efficient neural differentiation of non-human primate ES cells.

Research done with MEDII, the conditioned medium from the human hepatocellular carcinoma cell line HepG2, indicated that the step wise differentiation of mouse ES cells could be done *in vitro* [13]. It was found that by applying this conditioned medium to adherent cultures of mouse ES cells a uniform conversion of ES cells to a second cell type occurred. Gene expression data confirmed that expression profiles of the two cell types were different with the up-regulation of FGF5 and down-regulation of REX-1, a profile consistent with primitive ectoderm found in the mouse blastocyst [14-17]. In subsequent experiments it was found that the timing and method of exposure to MEDII changed the differentiation outcome. By forming EBs in the presence of 50 % MEDII, neurectoderm was formed at the expense of mesodermal derivatives such as beating heart muscle. Further, the step wise differentiation of this neurectoderm was achieved [18]. Here we report the efficient neural differentiation of rhesus ES cells using the conditioned medium MEDII. Explants of primate ES derived EBs contained large areas of neuralized cells that were MAP-2 and NF200 positive. By sub-culturing the neuralized areas a highly enriched population of neural progenitors has been isolated and characterized. These cells are Nestin, MAP-2, Hu, and Musashi positive. Upon differentiation these cells have a morphology consistent with embryonic neurons including a phase bright cell body with processes. These cells have several electrophysiological properties of neurons including the firing of action potentials and responses to excitatory and inhibitory neurotransmitters.

Materials and Methods

ES cell culture. Primate ES cells were maintained in DMEM/F12 with 15% FBS, 5% Knockout Serum Replacer (KSR, Invitrogen), 2 mM L-Glutamine, 0.1 mM MEM non-essential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, 1,000 units/ml recombinant human leukemia inhibitory factor (hLIF), 0.1 mM βME, and 4 ng/ml bFGF. Cells were grown on mitomycin C inactivated feeder layers and manually passaged with a pulled Pasteur pipette every four days.

Neural induction of primate ES cells. Primate ES were dissociated from adherent culture by incubation with 1 mg/ml collagenase IV (Gibco). When colonies of ES cells could be seen lifting off of the feeder layer they were dislodged with a cell scraper and the suspension of ES cells was spun down, re-suspended in primate ES growth medium (lacking bFGF and hLIF) either with or without 50 % MEDII medium [18]. EBs were grown for seven days in suspension.

EB differentiation. For differentiation, EBs were plated on poly-ornithine and laminin coated 35 mm dishes in DMEM with 10% FCS, L-Glut, and Pen-Strep for 7 days. Previous experiments demonstrated that after 7 days of adherent culture significant differentiation occurred and large areas of neurons could be visualized with phase contrast microscopy. Samples were fixed to avoid loss of neurons in culture.

Sub-culture of neural progenitors. For isolation of NPs, EBs were plated on poly-ornithine and laminin coated 35 mm dishes in DMEM/F12 with 50% MEDII, N2, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-Glutamine, 10 ng/ml bFGF, and 1,000 units/ml hLIF. After proliferation of rosette areas, usually after 7-10 days, these areas were sub-cultured mechanically with a fire drawn Pasteur pipette. Cultures were maintained in DMEM/F12 with

50% MEDII, N2, Pen-Strep, L-Glut, 10 ng/ml bFGF, and 1,000 units/ml hLIF. Further sub-culturing was carried out to enrich the population for NPs.

Immunocytochemistry. Cells were fixed in 4% para-formaldehyde and 4% sucrose for 15 minutes at room temperature. After three 1 minute washes cells were permeabilized by incubation in 50 mM Tris pH 7.6, 250 mM NaCl , 3% normal goat serum, 0.3% Triton X-100, and 1% poly vinyl pyrolidine for 30 minutes. Cells were incubated with primary antibody in permeabilization solution for 1 hour. The following primary antibodies were used: rabbit polyclonal Nestin (1:50 dilution, Chemicon), mouse monoclonal MAP-2 (1:1,000 dilution, Sigma clone HM-2), mouse monoclonal Hu (1:50 dilution, Molecular Probes), and rabbit polyclonal Musashi (1:500 dilution, Chemicon), and mouse monoclonal NF200 (1:400 dilution, Sternberger Monoclonals SMI-32). Cells were then incubated with Alexa 488 and 595 conjugated secondary antibodies (1:1,000 dilutions, Molecular Probes) for 1 hour in permeabilization solution. Nuclei were stained with DAPI (1µg/ml, Roche).

Differentiation of NPs. Eor early differentiation, NPs were incubated in DMEM/F12 (Gibco) with 1X B27 (Gibco), 50 units/ml penicillin, and 50 µg/ml streptomycin. After three days 5-Fluorouracil and Uracil (Sigma) were added at 10 µM each to inhibit overgrowth by mitotic cells. Cells were allowed to differentiate for an additional 5 days before electrophysiology was performed. For more advanced differentiation cells were incubated in Neurobasal (Gibco), 1X B27, 5% FBS, 10 ng/ml hLIF, 10 ng/ml BDNF (R&D Systems). After 24 hours 5-Fluorouracil and Uracil were added at 10 µM each. Cells were differentiated for a total of four weeks and then electrophysiology was performed.

Electrophysiology. Whole-cell recordings were made at room temperature on the stage of an inverted phase-contrast microscope using standard procedures. Briefly, patch electrodes

were filled with a solution containing (in mM): 140 K-gluconate, 5 KCl, 0.2 EGTA, 10 HEPES, 3 MgATP, and 0.3 Na_2GTP (pH 7.2, ~295 mOsm kg^{-1} H_2O). The external solution contained (in mM): 150 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 1.5 $CaCl_2$, 2.5 $MgCl_2$ (pH 7.2, ~310 mOsm kg^{-1} H_2O). Currents were digitized and recorded with a multifunction I/O board and WinWCP software (provided by Dr. J. Dempster, Strathclyde University, Glasgow). Drugs were applied during recordings via a fused silica tube (i.d. 200 μm) connected to multiple reservoirs, the outlet of which was positioned immediately in front of the cell under study.

Results and Discussion

To test the ability of MEDII conditioned medium to promote neural differentiation of rhesus ES cells we formed embryoid bodies (EBs) from the cells with and without 50% MEDII in the medium. EBs formed in the absence of MEDII exhibit a small proportion of rosette structures indicative of neural progenitor cells (NP cells) previously described in differentiating human ES cell cultures [19-21]. However, EBs formed with MEDII exhibited what appeared to be many more rosettes than control EBs (Figure 2.1; A, B). In some EBs treated with MEDII, approximately 90% of the cells in the EB expressed the post-mitotic pan-neuronal marker MAP-2 (Figure 2.1; G, H, and I). Large networks of MAP-2 positive neurons were easily visualized in MEDII treated EBs (Figure 2.1; D, F, and J). High power magnification of MAP-2 staining showed characteristic peri-nuclear staining and small spines on MAP-2 stained neurites (Figure 2.1; K). Differentiated neurons were also positive for non-phosphorylated neurofilament 200 (NF200) (Figure 2.1; L). While some neural differentiation occurs in non MEDII treated EBs, the level of neural differentiation is not high enough to yield enough neurons for subsequent studies. The inclusion of MEDII in the differentiation process increases the amount of neural

differentiation of rhesus ES cells. This suggests that MEDII will promote neural cell fates in differentiating non-human primate and human ES cell lines.

We also noticed that areas of the EBs with intense MAP-2 staining were almost always in close association with the rosettes of NP cells (Figure 2.1; C, D, E, and F). Close examination shows that MAP-2 staining emanates in a spoke-like pattern from the center of these rosettes. More mature neurons encircle the rosette areas and extend neurites toward other MAP-2 positive neurons that arise from other neighboring rosettes. Neurons with a more immature appearance seem to originate from the center of these rosettes. Therefore, we sought to isolate these rosette areas and determine if they express NP markers [19-21]. By using a manual passaging technique we were able to isolate presumptive NP cells and continue their culture independently. By selecting areas that grew in a rosette shape cultures were obtained that can be seen in Figure 2.2; A. By again selecting cells with an NP morphology, [19-23] we could expand these cultures and begin to eliminate cells that were not thought to be NPs (Figure 2.2; B). Occasionally small symmetrical spheres arose after passaging that remained in suspension (Figure 2.2; C). These resembled neurospheres [24-36]. The ability to form neurospheres from ES-derived neural progenitors could be used advantageously to generate more advanced differentiation derivatives in future studies.

To confirm that the passaged rosette cells were indeed neural progenitors we characterized these cultures by immunocytochemistry for the NP markers Nestin and Musashi [37-42]. Clumps of NPs were positive for Nestin (Figure 2.2; D, E, and F) as well as Musashi (Figure 2.2; G). Double staining with the pan-neuronal markers MAP-2 (Figure 2.2; E and F) and Hu (Figure 2.2; G) demonstrated the presence of developing neurons within the colonies of

NP cells. Therefore we feel that the cells isolated from MEDII treated EB explants are indeed NP cells.

NP cells isolated from rhesus ES cell EB explants spontaneously formed neurons with withdrawal of mitogen. To determine the functional capacity of these cells, electrophysiology was performed on neurons derived from the NP cells. Whole-cell intracellular recordings were made from phase-bright cells with at least two visible processes (Figure 2.3; A). In current-clamp mode cells differentiated for 4 weeks maintained a negative resting membrane potential that varied between –23 mV and –59 mV (mean –39 ± 3 mV, *n*=10). When cells were held near –60 mV with steady hyperpolarizing current injection, square depolarizing current commands reliably evoked overshooting action potentials. Cells differentiated for a shorter period of time (3 days) also generated action potentials, but were unable to generate repetitive action potential trains, suggesting these cells expressed a relatively low density of voltage-gated sodium channels (data not shown). In contrast, cells differentiated for 4 weeks could fire action potentials repetitively, consistent with a more mature neuronal phenotype (Figure 2.3; B). In the same cells voltage-clamp experiments were performed to determine if stem cell-derived neurons expressed functional ligand-gated ion channels (Figure 2.3; C). Rapid bath application of glutamate (1 mM; with 100 μM cyclothiazide to block rapid desensitization) evoked robust inward currents (985 ± 416 pA at –60 mV, *n*=5) that reversed polarity at a holding potential near 0 mV. Similarly, rapid bath application of γ-aminobutyric acid (GABA; 100 μM) evoked robust inward currents (654 ± 416 pA at –60 mV, *n*=5; Figure 2.3; C) that reversed polarity near -50 mV and rapidly desensitized. These results suggest that stem cell-derived neurons express functional ionotropic glutamate (AMPA/NMDA) and GABA ($GABA_A$) receptor/ion channels.

In the present study we outline a strategy for the differentiation of rhesus ES cells to neural lineages. Our approach differs from the two previously published reports on differentiation of non-human primate ES cells to neural lineages. The first report described spontaneous differentiation in teratomas [43] and the second used co-culture of the ES cells with the stromal cell line PA6 to induce neural differentiation [44]. In contrast, we have shown that rhesus ES cells can efficiently produce terminally differentiated neurons in a simple cell differentiation culture system without stromal cell co-culture.

To induce neural differentiation of primate ES cells we employed a novel strategy involving the use of the conditioned medium MEDII, which has been shown to drive efficient neural differentiation of mouse ES cells [18]. In that study the formation of neurectoderm was largely homogeneous with 95.7% of the cells scoring positive for NCAM while only 42.13% of cells expressing NCAM in non MEDII treated differentiations. Further, development of neurectoderm *in vitro* followed a time line consistent with developmental progression *in vivo.* Many efforts to produce dopaminergic neurons *in vitro* involve using cocktails of growth factors to obtain the desired phenotype. While some of the strategies developed have been successful, cells may not pass through normal developmental stages that occur *in vivo*. We feel that taking ES cells through a sequential, step-wise differentiation process may produce cells more similar to the naturally occurring cell type. The data presented here suggests that we have achieved the first step of sequential neural differentiation of rhesus ES cells.

In summary, we have used a previously undescribed strategy for the *in vitro* neural differentiation of non-human primate ES cells. MEDII medium was used to generate relatively homogeneous neural differentiation as well as to facilitate the isolation of neural progenitor cells that differentiate to neurons with functional electrophysiological properties. We feel the

electrophysiological data presented is important proof of principle that functional neurons can be derived from rhesus ES cells. The use of primate ES cell derived products in primate models of disease will undoubtedly prove the best model for cell replacement therapy in humans, and we feel the differentiation strategy employed here will be useful in generating a variety of cell types for transplantation into these models. Further experiments will be carried out to determine whether or not the neural differentiation obtained from primate ES cells in this study closely parallels the results obtained with mouse ES cells.

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Figure 2.1: MEDII conditioned medium promotes the neural differentiation of non-human primate ES cells. (A, B) Neural progenitor rosettes in untreated (A) and MEDII treated (B) EBs. Rosettes (arrows pointing to representative rosettes in A and B) were far more common in cells from MEDII treated EBs. (C, D, E, and F) MAP-2 positive neurons were in close association with rosette structures seen in the interior of MEDII treated EBs. Neurites from two rosettes (arrows in C and D) extend processes toward each other (arrowhead in D). Neurons formed rings around rosettes (arrowheads in E and F) while a radial pattern of staining could be visualized in the center of the encircled rosettes. (G, H, and I) Some MEDII treated EBs approached approximately 90% neutralization as characterized by MAP-2 staining. (G) Phase contrast, (H) DAPI, and (I) MAP-2 staining of a MEDII treated EB. (J) Higher magnification view of a network of MAP-2 stained neurons. (K) High power magnification of a MAP-2 positive neuron showing characteristic peri-nuclear staining (arrow) and localization to neurites. (L) NF 200 positive neurons in MEDII treated EB explants. Magnification: A, B, 40X; C, D, G, H, and I, 100X; E, F, J, 200X; K, 600X; and L, 400X.

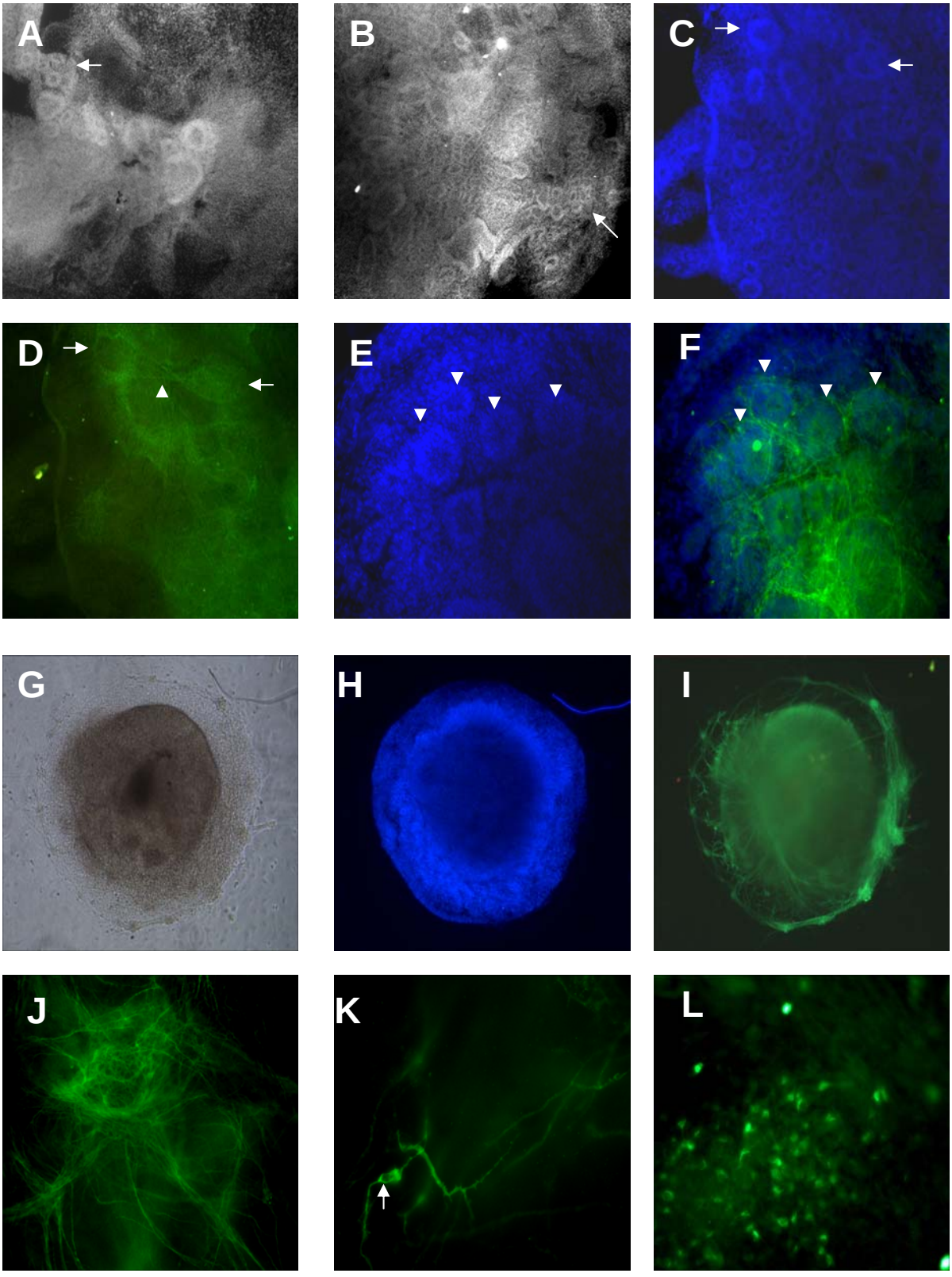
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Figure 2.2: ES derived NP cells enriched from MEDII treated EBs. (A, B) Phase contrast images of NPs after first round of isolation (A) and smaller colony (B) after further sub-culturing by mechanical passaging. (C) Representative sphere that arose spontaneously after passaging. (D) Nestin staining of a colony of putative NP cells and associated neurons. (E) MAP-2 (green), Nestin (red), and DAPI (blue) staining of a colony of NP cells. (F) Hu (green), Nestin (red), and DAPI (blue) staining of a colony of NP cells. (G) High magnification view of NP cells and neurons double stained for Musahsi (red), DAPI (blue), and Hu (green). Magnifications: A, B, C, and D, 100X; E, and F, 200X; and G, 600X.

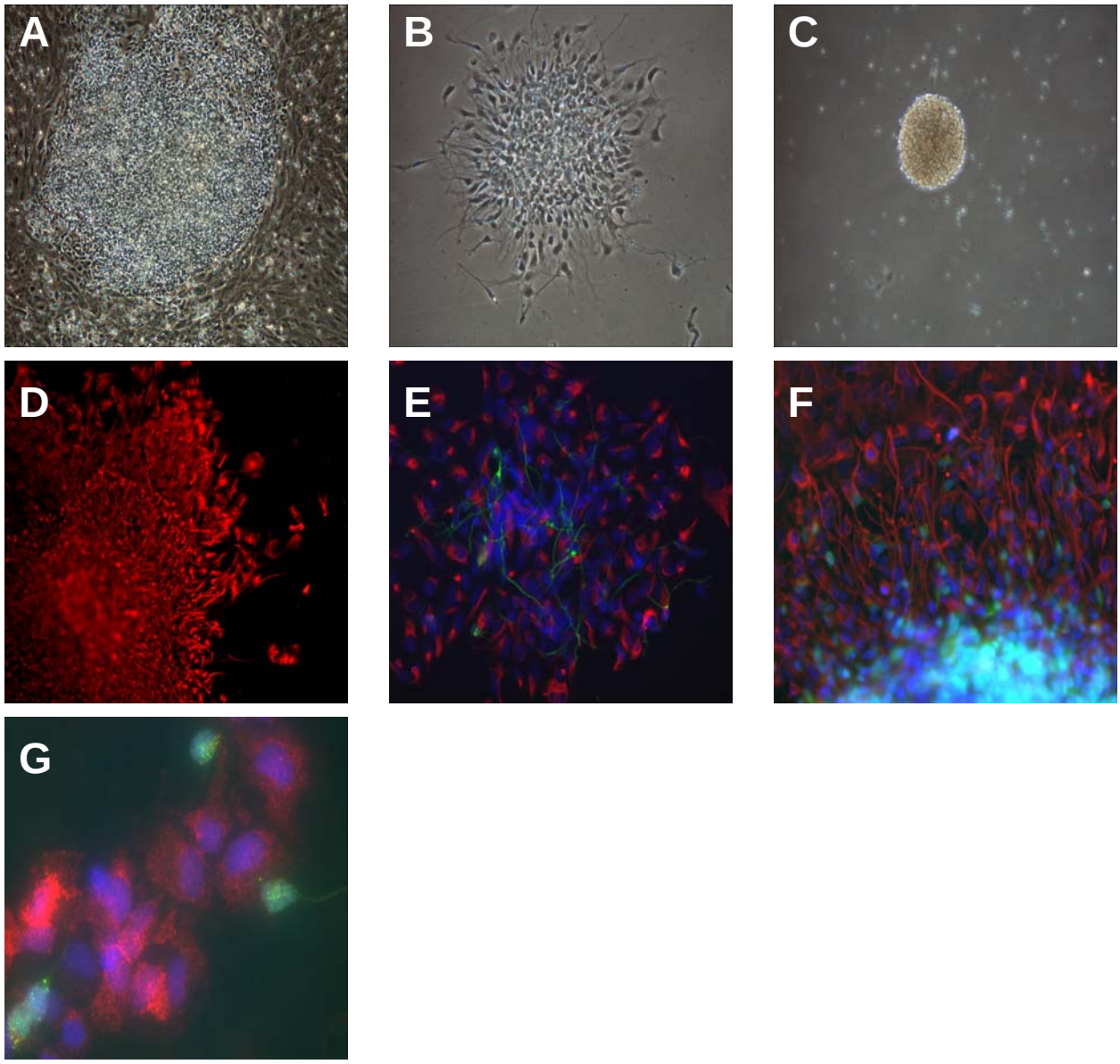
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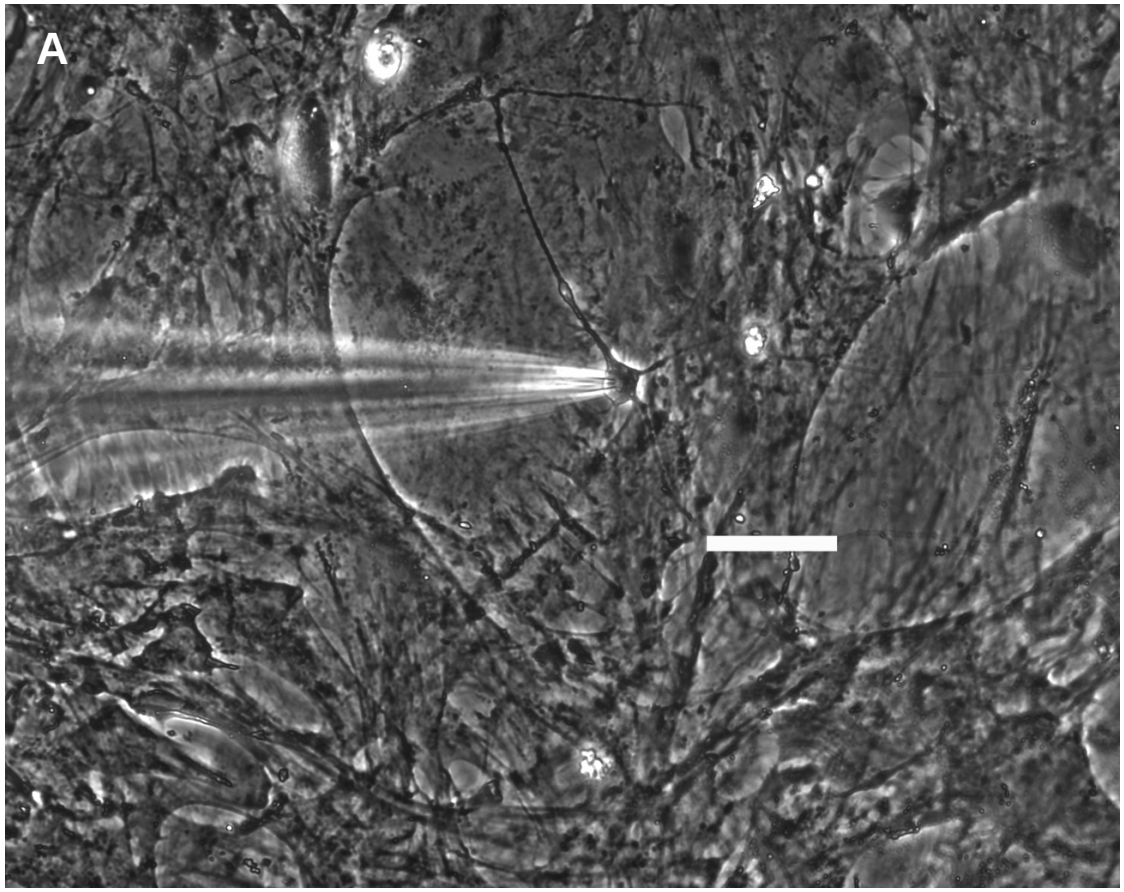
Figure 2.3: Electrophysiological properties of stem cell-derived neurons. (A) Phase contrast image of a stem-cell derived neuron 4 weeks after differentiation attached to a whole-cell recording electrode. (B) current-clamp recording of the voltage response (V) to a square injected current command (I). This cell responded with two overshooting action potentials; 0 mV is indicated by the arrow. (C) Voltage-clamp recording of responses to rapid application (indicated by horizontal bars) of glutamate (100 μM) or γ-aminobutryic acid (GABA; 100μM). Scale bar in (A) is 50 μm.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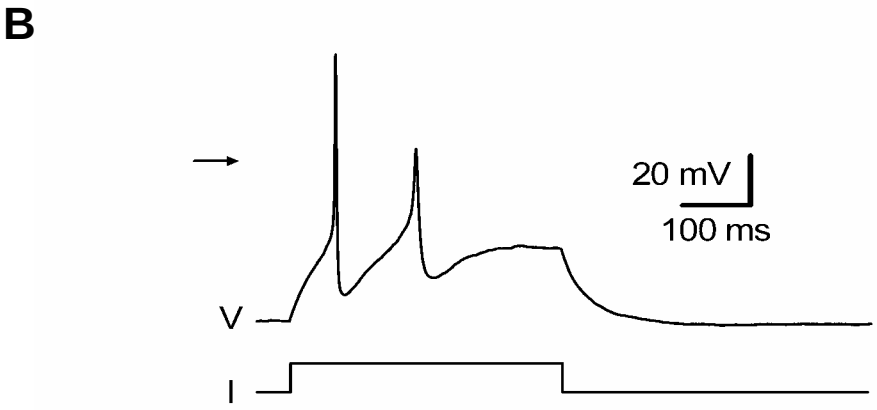
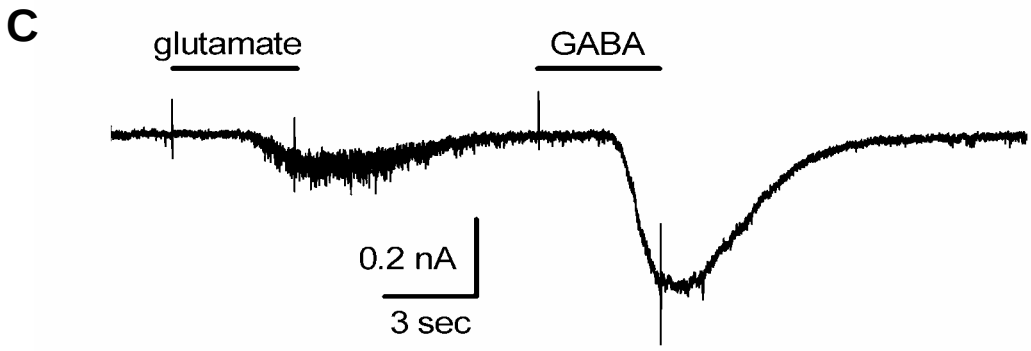

B
20 mV
100 ms
V
I


C
glutamate
GABA
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CHAPTER 3

TRANSCRIPTIONAL PROFILING OF EARLY DIFFERENTIATION EVENTS IN HUMAN EMBRYONIC STEM CELLS[1]

[1]Calhoun, John D., Raj R. Rao, Susanne Warrenfeltz, Romdhane Rekaya, Stephen Dalton, John McDonald, and Steven L. Stice. Submitted to *Developmental Biology*, 1/27/2004.

Abstract

The isolation of human embryonic stem (hES) cells has led to a new age in developmental biology holding promise as an unlimited supply of material for cell replacement therapy. Yet to date there are no directed differentiation strategies for hES cells that efficiently produce one specific cell type. One possible reason may be a lack of understanding of the genes that control signaling events prior to overt differentiation. In this study, we have used HepG2 cell conditioned medium (MEDII), which is known to induce early differentiation in mouse ES cells while retaining pluripotent markers, to query gene expression in hES cells. Treatment of adherent hES cells with 50% MEDII medium for 3 days effected differentiation to a cell type with gene expression similar to primitive streak stage cells of the mouse embryo. MEDII treatment up-regulates TDGF1 (Cripto). Cripto is essential for proper anterior-posterior axis and mesoderm formation in mouse embryos and is a key component of the TGFβ/nodal signaling pathway. Lefty A, an antagonist of Nodal/Cripto signaling normally expressed in the anterior visceral endoderm, is down regulated with MEDII treatment, as is Follistatin, an inhibitor of mesoderm induction via the related activin pathway. In summary the TGFβ/nodal pathway is known to be important for primitive-streak and mesoderm formation and in using MEDII we present a means for generating an in vitro cell population that maintains pluripotent gene expression (Oct-4, Nanog) and SSEA-4 markers while regulating genes in the TGFβ/nodal pathway. Further activation of this pathway may lead to more uniform formation of mesoderm in vitro.

Introduction

Significant research efforts have focused on developing hES cell differentiation strategies (Carpenter, Rosler et al. 2003), but successful *in vitro* differentiation will require a well characterized starting pluripotent hES cell population. Changes in hES cell gene expression, without an alteration in overt pluripotent characteristics or markers in hES cells, may uncover or further define pathways and mechanisms involved in maintenance and then initial loss of pluripotency. We believe a detailed understanding of these mechanisms will be essential for developing hES cells as *in vitro* models for human embryonic development and for harnessing the differentiation capacity that makes hES cells an attractive starting material for cellular replacement therapy.

Previously, a handful of genes expressed in mouse ES (mES) cells, including Oct-4 (Nichols, Zevnik et al. 1998; Niwa, Miyazaki et al. 2000), Nanog (Chambers, Colby et al. 2003; Mitsui, Tokuzawa et al. 2003), Sox-2 (Avilion, Nicolis et al. 2003), and leukemia inhibitory factor (LIF) (Smith, Nichols et al. 1992) have been shown to be essential for pluripotency. Subsequently, microarray analysis has been used to further define an expression profile indicative of "stemness" in mES cells (Ivanova, Dimos et al. 2002; Ramalho-Santos, Yoon et al. 2002). Recent reports have used microarray technology to characterize the transcriptional profile of hES cells. These studies indicate that like mES cells, there is a set of genes expressed in hES cells that are down-regulated upon differentiation (Sato, Sanjuan et al. 2003) or expressed exclusively in hES cells in comparison to somatic, or non-pluripotent, cell types (Sperger, Chen et al. 2003). The power of global gene expression profiling will improve the current understanding of the genetic mechanisms underlying stem cell biology by characterizing

expression patterns of large numbers of genes in pluripotent cell types and differentiated derivatives, thus yielding an expression profile of pluripotency or “stemness” of hES cells.

In the current study, we generated two closely related pluripotent hES cell populations based on an *in vitro* model of early mES cell differentiation and utilized microarray analysis to help identify potential genes involved in early human cell differentiation and/or development events. During early mouse embryonic development, inner cell mass (ICM) cells, the *in vivo* equivalent of ES cells, undergoes differentiation to a second pluripotent cell population termed primitive ectoderm. Primitive ectoderm is characterized by continued expression of pluripotency markers such as Oct-4 and alkaline phosphatase (AP) and increased expression of fibroblast growth factor 5 (FGF5) (Pelton, Sharma et al. 2002). Upon exposure to a HepG2 cell conditioned medium, termed MEDII, mES cells mimicked differentiation to primitive ectoderm *in vitro* (Rathjen, Lake et al. 1999). Further studies demonstrated that MEDII could also enhance differentiation of adherent mES cells toward mesoderm (Lake, Rathjen et al. 2000) or aggregated mES cells to neurectoderm (Rathjen, Haines et al. 2002). Recently we have demonstrated that the effect of MEDII can be translated to primate ES cells. MEDII enhanced the differentiation of aggregated monkey (Calhoun, Lambert et al. 2003) and hES cells (Schulz, Palmarini et al. 2003) towards neural fates. However, these experiments did not investigate MEDII‘s effect on early ES differentiation events, specifically in adherent cultures. In this study, our aim was to better characterize the pluripotent state of adherent hES cells and the effect of MEDII on early developmental differentiation events in hES cells.

Using a combination of different experimental approaches, we have evaluated cellular morphology, cell cycle characteristics, immunostaining, and gene expression of adherent hES cells in response to MEDII. Human ES cells responded to MEDII treatment in a manner similar

to mES cells. Both hES cells and MEDII treated hES cells have a cell cycle profile similar to that of mES and MEDII treated mES cells. Like their murine counterparts, hES cells treated with MEDII expressed pluripotency markers yet exhibit differential gene expression in comparison to hES cells. There were key changes in genes expressed during early embryonic development in these two closely related pluripotent hES cell populations. Cripto, a co-receptor and agonist of activin/nodal/TGFbeta pathway, was up-regulated upon MEDII treatment, suggesting that MEDII may aid in the activation of this signaling pathway. Two antagonists of this signaling pathway, Lefty A and Follistatin were down regulated upon MEDII treatment. GATA-6, Tbx-1, and Ikaros, all markers of developing mesoderm in the mouse embryo, were also up-regulated in response to MEDII treatment. Taken together all of these gene expression changes suggest that treatment with MEDII influences differentiation towards mesoderm in adherent hES cell culture. This is the first study suggesting that MEDII conditioned medium affects components of the TGFβ/nodal pathway in ES cells of any species.

Materials and Methods

Human ES cell culture

BG01 human ES cells (Mitalipova, Calhoun et al. 2003) were maintained on mitomycin C inactivated mouse embryonic feeder (MEF) layers in DMEM/F12, 20% Knockout Serum Replacer, 2 mM L-Glutamine, 0.1 mM MEM non-essential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Gibco/Invitrogen), 1000 U/ml hLIF (Chemicon), 0.1 mM βME (Sigma), and 4 ng/ml bFGF (Sigma). Cells were passaged with 0.05% Trypsin-EDTA (Invitrogen) every three days and re-plated on fresh feeder layers. For microarray analysis,

parallel cultures were plated in either growth media (ES) or growth media supplemented with 50% MEDII media (MEDII) (Figure 3.3).

Generation of serum free MEDII conditioned medium

MEDII medium was prepared as described previously (Rathjen, Lake et al. 1999). Briefly, HepG2 cells were maintained in DMEM/F-12 medium supplemented with 10% FBS, 1X L-Glutamine, and 1X Pen-Strep. HepG2 cells were plated at a density of 16.5 x 10^6 cells in a 175 cm^2 flask and cultured for three days. After three days of culture, serum containing growth medium was aspirated, cells rinsed once with PBS, and fed with 50 ml HepG2 growth media that lacked serum. After three days, media was collected, centrifuged to remove any non-adherent cells, and filter sterilized. Serum free MEDII medium was stored at 4°C until use.

Immunostaining of adherent hES and MEDII treated hES cells

Human ES cells and MEDII treated hES cells were plated on 4 well permanox chamber slides (Nalgene) that had previously been seeded with mitomycin C inactivated MEF feeder layers. Cells were plated at 30,000 cells per chamber and grown in either hES growth medium or hES growth medium supplemented with 50% MEDII conditioned medium. After three days of culture, medium was aspirated, cells rinsed once with PBS, and fixed by incubation with 4% para-formaldehyde 4% sucrose solution for 15 minutes at room temperature, followed by several rinses with PBS.

For Oct-4 staining, cells were permeabilized and blocked by incubation in 1.5T (50 mM Tris pH 7.6, 250 mM NaCl) containing 3% normal goat serum, 0.3% Triton X-100, and 1% poly vinyl pyrolidine for 30 minutes. Cells were then incubated with primary antibody (Santa Cruz Biotechnology, 1:500 dilution) in blocking and permeabilization buffer for 1 hour. After 3 washes in 1.5T with 0.05% Tween 20 (Sigma) cells were incubated with an Alexa 488

conjugated secondary antibody (1:1000 dilutions, Molecular Probes) for 1 hour in blocking and permeabilization buffer. Subsequently, cells were washed 3X in 1.5T with 0.05% Tween 20 and nuclei were stained with DAPI (1µg/ml, Roche).

For SSEA-4 staining, cells were incubated in blocking solution (PBS with 3% normal goat serum) for 45 minutes at room temperature. Cells were then incubated with primary antibody (Chemicon, 1:1,000 dilution) in blocking solution for 30 minutes at room temperature. After 3 washes with PBS, cells were incubated with an Alexa 488 conjugated secondary antibody (Molecular Probes, 1:1000 dilution) in blocking solution for 30 minutes at room temperature. Subsequently, cells were washed 3X in 1.5T with 0.05% Tween 20 and nuclei were stained with DAPI (1µg/ml, Roche). Cells on slides were later visualized on a Nikon TE 2000-E inverted microscope using fluorescence microscopy.

Suspension staining and cell cycle analysis of adherent hES and MEDII treated hES cells

For suspension staining, hES cells were harvested from adherent culture with 0.05% Trypsin-EDTA (Invitrogen), centrifuged at 1,000 rpm at 4°C, and re-suspended in suspension staining buffer consisting of PBS with 0.5 mM EDTA and 3% normal goat serum. After re-suspension in staining buffer, all the subsequent incubation steps involved cells being kept at 4°C. Cells were then incubated in anti-SSEA-4 (Chemicon, 1:200 dilution) at 4°C for 15 minutes. Cell suspensions were washed 2 times in staining buffer, and collected by centrifugation before re-suspension in an Alexa 488 conjugated secondary antibody (Molecular Probes, 1:1000 dilution) in staining buffer. Cells were incubated for 15 minutes at 4°C and washed twice with staining buffer. The cells were later fixed by re-suspension in 70% EtOH and stored at -20°C until they were analyzed on a Becton Dickinson FACSCalibur flow cytometer.

For cell cycle analysis, samples that had been suspension stained for SSEA4 and stored at -20°C in 70% EtOH were washed several times to remove residual EtOH and re-suspended in a solution of 50 μg/ml RNase A and 50 μg/ml propidium iodide. Samples were then analyzed by flow cytometry and all data analysis was done using FloJo software.

Microarray analysis of hES and MEDII treated-hES cells

In preparation for microarray analysis, parallel cultures of untreated hES cells and hES cells treated with 50% MEDII were obtained as illustrated in Figure 3.3 A. The treatment was performed over three sequential independent passages and hybridized to six Affymetrix HG-U133A chips to ensure reproducibility of the response to MEDII (Figure 3.3, B). At each three day interval, cultures of untreated and treated cells were harvested and re-suspended in 1 ml Trizol (Invitrogen) and triturated until homogenized. Samples were stored at -80°C until all six samples were prepared. RNA was isolated from the crude homogenate according to the manufacturer's protocols (Trizol, Molecular Research Corporation) using linear polyacrylamide (5μl) to aid in RNA precipitation. Total RNA was further purified with RNAEasy (Qiagen) using the manufacturer's cleanup protocol. RNA quality was assessed by gel electrophoresis and only high quality RNA displaying no degradation by gel electrophoresis, a 2:1 ratio of ribosomal bands, and an A_{260}/A_{280} ratio of 1.9-2.1 was used for biotinylated target preparation.

Biotinylated target cRNA was generated according to the Affymetrix Technical Manual. Briefly, 10μg total RNA was converted to double stranded cDNA using Superscript II (Invitrogen). The cDNA was cleaned by phenol/chloroform extraction and ethanol precipitation. In vitro transcription of the cDNA with the High Yield RNA Transcript Labeling Kit (Enzo) yielded 50-100μg of biotin labeled cRNA target. The cRNA was fragmented in a metal catalyzed acid hydrolysis to a length of 20-200bp (by electrophoresis) and the fragmented cRNA

was hybridized to the Affymetrix HG U133A array for 16 hours at 45°C. Hybridized arrays were washed, stained and scanned according to the Affymetrix technical manual.

For data analysis, .DAT files were converted to .CEL files using Affymetrix MAS 5.0 software. Using the Affy package of Bioconductor (http://www.bioconductor.org) background correction was performed on .CEL files using the background correct function of the RMA algorithm (Irizarry, Bolstad et al. 2003). Perfect match (PM) probe level data was then outputted as a text file. For this study, mismatch (MM) probe data was not used for differential gene expression analysis. PM probe data of the treated and untreated samples was then analyzed using a mixed linear model to determine differential gene expression (Chu, Weir et al. 2002). It is important to note that once data has been processed in the model differences in fluorescence intensity between treated and untreated array data should be due to the treatment alone.

The following mixed linear model was used to analyze the probe level intensities for every gene separately,

$$y_{ijkl} = T_i + P_j + TP_{ij} + t_l + t_l * P_j + T_i * P_j + t_l * T_i + A_k + e_{ijkl}$$

where y_{ijkl} is the transformed $\log_2$ expression intensity generated under treatment (T) i ($i = 1,2$) at the j^{th} ($j = 1,2,...,20$) probe (P) in the array (A) k ($k = 1,2,...,6$) at the passage time (t) l ($l = 1,2,3$) . It is important to note that the number of probes per gene ranged from 11 to 20.

Furthermore, the following assumptions were made about the distribution of the random effects in the model:

$$e_{ijkl} \mid \sigma_e^2 \sim N(0, \sigma_e^2); \qquad A_k \mid \sigma_a^2 \sim N(0, \sigma_a^2)$$

where σ_e^2 and σ_a^2 are the within and between array variances respectively. Further, those two parameters were assumed unknown and were inferred from the data using maximum likelihood based methods.

Data for each of the 22,215 genes was processed on a gene by gene basis simultaneously. Since 22,215 tests were run simultaneously, a Bonferroni adjusted p-value (i. e. 0.05/22,215) was considered to be the measure of stringency to minimize false positive results in the data set. Genes that met the Bonferroni criteria were the primary focus of the study; however this criterion was relaxed to a p-value of 0.001 to increase the data set for a more generalized understanding of the differential expression patterns. Ontologies were processed using the NetAffx (http://www.affymetrix.com) software.

Real time PCR confirmation of differentially expressed genes

Differentially expressed genes to be confirmed by real-time PCR were chosen by three criteria: a p-value well within the Bonferroni cutoff, a high fold change, and the relevance of the gene to developmental processes. Accordingly, Homeobox A1 (HOXA1), Dapper, Follistatin, and Enhancer of Zeste homolog 2 (EZH2) were chosen as genes down-regulated with MEDII treatment (hES cell enriched). Cripto was chosen as an up-regulated gene (MEDII enriched). Oct-4 and Nanog, markers of pluripotency that are not differentially expressed between the two populations were assessed as treatment controls. Sequence information was obtained by interrogating probe information in the NetAffx online analysis software (Affymetrix) and following appropriate links to Ensembl (http://www.ensembl.org) web pages. Sequence information was loaded into Biology Workbench (http://workbench.sdsc.edu) and PRIMER 3 was used to design primers that spanned intron exon boundaries. RNA in excess from isolations for microarray analysis was reverse transcribed using the SuperScript III First-Strand Synthesis

System (Invitrogen) according to manufacturer's protocol. Real Time PCR was done on an Applied Biosystems' ABI PRISM 7700 Sequence Detection System using SYBR Green PCR Core Reagents according to manufacturer's protocols (http://www.appliedbiosystems.com). Data analysis was performed with the REST software package (Pfaffl, Horgan et al. 2002).

Results

MEDII treatment altered adherent hES cell colony morphology

After treatment with MEDII, adherent hES cells were flatter than their non-treated counterparts were and appeared to have less intercellular space (Figure 3.1A, B). However, MEDII treated hES cells continued to grow in colonies and their morphology was distinctly different from that of spontaneously differentiated cells seen routinely in the laboratory (data not shown). We have also observed that MEDII treated hES cells were more resistant to dissociation than untreated hES cell colonies and may have formed tighter connections among cells within the colony.

MEDII treatment did not alter pluripotent marker expression or cell cycle profile in hES cells

To characterize the effect of MEDII on adherent hES cells, immunostaining with known hES cell markers was carried out. BG01 hES cells exhibited stable and uniform expression of SSEA-4 epitope during continuous culture (Figure 3.2). At each stage of passaging during the experiment, parallel cultures of hES cells were plated for assessment of SSEA-4 expression levels. As seen in Figure 3.2, both hES cell and MEDII treated hES cells exhibited a large shift in fluorescence intensity (blue traces, Figure 3.2; A, C) over hES cells or MEDII treated hES cells incubated with secondary antibody only (red traces, Figure 3.2; A, C). A shift was not seen

in the negative control of MEF cells since there was no difference between MEF cells incubated with secondary alone (green trace, Figure 3.2, E) and MEF cells incubated with both primary and secondary antibody (red trace, Figure 3.2, F).

Cell cycle analysis was performed on adherent hES cell and MEDII treated hES cells. The BG01 hES cells used in this study displayed a pluripotent cell cycle profile with a high percentage of cells occupying the S and G2/M phases of the cell cycle. MEDII treatment did not significantly change the cell cycle profile of treated cells (Figure 3.2, D). The treated cells continued to display a pluripotent cell cycle profile with many cells occupying the S and G2/M phases of the cell cycle. In contrast, significantly fewer control MEF cells occupy the S and G2/M phases of the cell cycle (Figure 3.2, F) with the population exhibiting a cell cycle structure consistent with that of proliferating somatic cells in culture.

MEDII causes changes in gene expression but not in pluripotency genes

The global gene expression profiles of untreated and treated adherent hES cells were compared by microarray analysis. It is important to note that the R^2 values, a determination of goodness-of fit for each of the genes ranged between 0.95-0.99, indicating that the mixed linear model adopted fits the data from almost all genes very well (Figure 3.3, C). A plot of difference in gene expression between MEDII treated and untreated hES cells versus the negative log base 2 ($\log_2$) transformed P-value (volcano plot) shows the behavior of all genes analyzed (Figure 3.3, D). A larger number of genes in the hES enriched quadrant of the volcano plot suggest that more genes were down regulated in hES cells in response to MEDII than were up-regulated by MEDII treatment. This is reflected in the statistical summary table (Table 3.1). Focusing on genes that meet the stringent Bonferroni criterion and a fold change greater than 1.5, 73 genes were down-regulated upon MEDII treatment (Figure 3.4) and 20 genes were up-regulated in the MEDII

treated hES cell group (Figure 3.5). It is anticipated that fold change values reported when using a mixed linear model will tend to be lower than global fold change values that are reported in most array experiments (Chu, Weir et al. 2002)**.** This is attributed to a removal of all sources of systematic and random variation contributing to overall fluorescence intensity and the remaining differences in expression being due to the treatment variable alone.

To confirm differential gene expression with MEDII treatment, we analyzed seven genes by real time PCR and their expression was as predicted by microarray analysis (Table 3.2). Oct-4 and Nanog were not differentially expressed (NDE). Cripto was up-regulated upon MEDII treatment with an average fold change of 3.0 over three independent passages. Both HOXA1 and Dapper were down-regulated with an average fold change of 5.5 and 2.80 respectively, whereas Follistatin and EZH2 were down-regulated with an average fold change of 2.54 and 1.169 respectively.

Discussion

Knowledge gained from understanding the signaling events occurring in the embryo will likely lead to improved *in vitro* differentiation strategies for ES cells. We describe here for the first time early positive modulation of genes involved in vertebrate signaling pathway for mesoderm formation (activin/nodal/TGF beta) in hES cells. Understanding initial germ layer induction and formation will be crucial to full realization of hES potential as both a model of development and a source of material for cellular replacement therapy. Accordingly, in this study we have utilized an *in vitro* model of early ICM differentiation to characterize a transitory cell state having two properties, both pluripotent gene expression and expression of genes indicative of primitive streak cells and nascent mesoderm formation. By combining MEDII

medium and microarray technologies we have taken a unique approach to: 1) characterize genes expressed by pluripotent hES cells in comparison to closely associated pluripotent cells from the same parental population, and 2) investigate TGFβ/nodal signally pathway's gene expression in an *in vitro* model of embryo development. We have elucidated genes down regulated in response to MEDII medium at a stage of differentiation before traditional pluripotency markers, such as Oct-4, Nanog, and SSEA-4, are down regulated. In addition, MEDII treated hES cells exhibited an up regulation of several genes in the TGFβ/nodal pathway, consistent with an activation of this pathway. Thus, this study provides a model to test the very early and maybe the initial events in mesoderm induction in hES cells since these are clearly altered cells that still retain the same level of Oct-4 and Nanog expression.

Our initial results suggested that hES cells response to MEDII confirmed what was previously observed in the mES adherent cell cultures. After 3 days in culture, MEDII treated hES cells exhibited an obvious morphology difference in comparison to control hES cells at similar time-points. MEDII treated hES cell colonies were flatter and appeared less refractive than control hES cells. MEDII treated hES cells also appeared to form tighter connections within colonies and no longer exhibited spaces between individual cells in a colony. MEDII treated hES cells were also more resistant to dispersal to single cells during harvesting. This was also previously observed in MEDII treated mES cell colonies that likewise formed a similar flattened colony phenotype (Rathjen, Lake et al. 1999). We also evaluated the cell cycle profile of MEDII treated and control hES cells. Mouse ES cells have a unique cell cycle profile with roughly 50% of cells analyzed residing in S phase and around 20% of cells occupying the G2/M phase of the cell cycle (Jirmanova, Afanassieff et al. 2002; Savatier, Lapillonne et al. 2002; Stead, White et al. 2002). Consistent with these findings, we found that hES cells have a higher

proportion in the S and G2/M phase than in G1 when compared with the profiles of somatic mouse fibroblast cells (Figure 3.2). It was further demonstrated that treatment with retinoic-acid (RA), a known neural differentiation inducing agent for mES cells, changes the cell cycle profile of mES cells to a profile more similar to terminally differentiated cells than epiblast cells (Jirmanova, Afanassieff et al. 2002). However, MEDII treated mES cells retain the unique cell cycle profile which is thought to be indicative of pluripotency (Stead, White et al. 2002). Therefore, treatment of mES cells with terminal differentiation inducing agents drastically changes their cell cycle profile while MEDII treatment does not change the pluripotent cell cycle profile. Similarly, hES and MEDII treated cells exhibit a cell cycle profile with many cells occupying the S and G2/M phases of the cell cycle, like the profile observed in untreated hES cells. Therefore, morphological alteration and cell cycle profiles of MEDII-treated hES and mES cells are similar, suggesting that MEDII does not induce a program of terminal differentiation but instead may at this point induce only early differentiation of hES cells.

Previous studies with MEDII have shown that it causes differentiation to a second pluripotent population that is still Oct-4 positive, recapitulating events that occur *in vivo* in the mouse embryo (Rathjen, Lake et al. 1999). After treatment of hES cells with MEDII medium we observed an altered gene expression for many developmental, cell adhesion and other genes. It is important to emphasize that alteration in gene expression occurred without changes in Oct-4 and Nanog expression in cells that continued to retain their stem cell surface marker SSEA-4. Although it has been demonstrated that MEDII treated mES cells still express Oct-4 (Rathjen, Lake et al. 1999),this is the first report of quantification of Oct-4 and Nanog gene expression using microarray and real time PCR analysis in any ES cell type. Since MEDII treated hES cells have unique features based on the differentially expressed genes, but unaltered in Oct-4 and

Nanog expression and the cell surface marker SSEA-4, one can hypothesize that hES populations are capable of being dynamic in nature without inherent changes in pluripotent markers. Therefore, the use of a handful of pluripotent markers may not be capable of characterizing a dynamic population of hES cells that is undergoing self renewal and/or early differentiation. The TGFβ/nodal pathway is known to be important for primitive-streak and mesoderm formation and the current study using MEDII is the only reported protocol for generating an in vitro cell population having both pluripotent gene expression and increased gene expression in this particular pathway. Cripto is a co-receptor/ligand of nodal that was up regulated upon MED II treatment. Lefty A, an antagonist of Nodal/Cripto signaling normally expressed in the anterior visceral endoderm is down regulated with MEDII treatment (Schier 2003). Additionally, Follistatin is down regulated as well with MEDII treatment. Studies have shown Follistatin may act as an inhibitor of mesoderm induction (Levin 1998; Marchant, Linker et al. 1998). GATA-6 and Tbx-1, genes expressed in the primtive streak, are also up-regulated with MEDII treatment (Chapman, Garvey et al. 1996; Morrisey, Ip et al. 1996). Taken together these results indicate that MEDII affects a gene expression pattern consistent with anterior-posterior axis and primitive streak formation. In previous studies, MEDII treatment of mES cells that then were allowed to form cell aggregates or embryoid bodies had a higher degree of differentiation to mesoderm and further advanced mesoderm derived macrophages and cardiac muscle than non treated mES cells, demonstrating the capability of MEDII to induce mesodermal differentiation (Lake, Rathjen et al. 2000). It is important to note that this previous protocol used embryoid bodies to look at fully formed mesoderm, whereas we utilized an inherently more uniform monolayer approach to observe the initial events that may be directing the hES toward differentiated states such as mesoderm.

In our studies, Cripto (teratocarcinoma derived growth factor 1) was up-regulated in hES colonies after 3 days exposure to MEDII, while down regulated upon differentiation in other ES cell spontaneous differentiation studies, (Sato, Sanjuan et al. 2003). Based on our microarray analysis, Cripto was up-regulated 1.91 fold over the three independent passages in the study with a Bonferroni adjusted p-value of 3.35×10^{-7}. Subsequently, increased expression was verified by real time PCR with an average fold change of 3.01 over the three independent passages. Cripto was initially identified as a gene in undifferentiated mouse and human teratocarcinoma cell lines whose expression decreased with retinoic acid treatment (Ciccodicola, Dono et al. 1989). Cripto was down-regulated when hES are allowed to spontaneously differentiate for 26 days without feeder cells or feeder cell conditioned medium (Sato, Sanjuan et al. 2003). Cripto is a member of the EGF-CFC family of proteins that can act as either a co-receptor or co-ligand of the nodal signaling pathway, by binding to a complex of ActRIB and ActRIIB receptors (Bianco, Wechselberger et al. 2001). Cripto has been shown to be initially expressed uniformly throughout the epiblast with further expression restricted to primitive streak epiblast cells and developing mesoderm (Dono, Scalera et al. 1993; Ding, Yang et al. 1998). Therefore, our results suggest that MEDII may be inducing very early differentiation signaling in the hES cells with Cripto playing a role in this initial differentiation.

Several functional studies have demonstrated the importance of Cripto in early embryonic development. In studies utilizing Cripto homozygous null mutant embryos, it was shown that Cripto was found to be essential for conversion of the proximal-distal axis to an anterior-posterior axis and streak formation. Mutant embryos failed to form a correct node, lacked a primitive streak, and did not form embryonic mesoderm properly (Ding, Yang et al. 1998; Xu, Liguori et al. 1999). Further studies indicated that Cripto null ES cells did not form

beating cardiomyocytes (Xu, Liguori et al. 1998). A more recent study demonstrated that absence of Cripto activity during a specific window of time, led to the conversion of embryoid body derived cells from null ES cells to a neural fate. However, addition of soluble Cripto during the differentiation process restored the ability to differentiate to cardiomyocytes and inhibited neural differentiation (Parisi, D'Andrea et al. 2003). Previous studies have suggested that in the absence of appropriate signals, the default differentiation pathway of pluripotent cells is to generate neural lineages (Tropepe, Hitoshi et al. 2001; Munoz-Sanjuan and Brivanlou 2002). Mouse ES cells differentiated by the embryoid body method have shown increased expression of Cripto in a transient manner (Parisi, D'Andrea et al. 2003). Spontaneously differentiated hES cells showed decreased expression of Cripto (Sato, Sanjuan et al. 2003), but it is useful to note that the hES cells had been cultured in conditions that were not conducive to a mesodermal fate. In addition, the hES cells used in the study were cultured for 26 days as differentiated cells, implying that these cells were probably past the temporal equivalent of the cells in the current study and further past the point of transient Cripto up-regulation. It is also possible that spontaneously differentiated cells follow the default neural pathway while MEDII treatment uniquely recapitulates embryo primitive streak development. Thus, up regulation of Cripto may facilitate derivation of lineages other than the neural one.

In association with Cripto, Dapper, a Dishevelled associated antagonist of β-catenin was also down regulated in MEDII hES cells. In a recently published report, characterization of gene expression in β-catenin mutant embryos suggested a link with Cripto expression (Morkel, Huelsken et al. 2003). The authors suggested that β-catenin up-regulates Cripto, in turn increasing Cripto dependent Nodal signaling, through β-catenin, during anterior-posterior axis formation. Ultimately, the increase in Cripto dependent Nodal signaling events activates

mesoderm induction. Previous studies showed that increased levels of Dapper resulted in decreased levels of soluble β-catenin and decreased activation of β-catenin responsive genes(Cheyette, Waxman et al. 2002). Therefore our observed decrease Dapper expression may enhance β-catenin activity in MEDII treated hES cells. We have also verified down-regulation of HOXA1 and EZH2 in addition to the previously mentioned genes involved in primitive streak and mesoderm formation. HOX genes are a group of evolutionary conserved transcription factors arranged in clusters and expressed sequentially in both a spatial and temporal manner (Maconochie, Nonchev et al. 1996; Favier and Dolle 1997). In human teratocarcinoma cells, HOXA1 is the earliest HOX gene expressed after treatment with retinoic acid (Simeone, Acampora et al. 1990; Buettner, Yim et al. 1991), with targeted disruption of the gene shown to be embryonic lethal (Lufkin, Dierich et al. 1991). EZH2 (enhancer of zeste homolog 2) was also confirmed as differentially expressed in our analysis. EZH2 is a polycomb-group gene that maintains chromatin status during differentiation thereby providing a transcriptional memory. Targeted disruption of the gene has been shown to be post-implantation lethal around the time of gastrulation. EZH2 null blastocysts displayed impaired outgrowth thereby inhibiting ES cell isolation (O'Carroll, Erhardt et al. 2001). Another gene of interest down regulated by MEDII treatment was Glypican-4 that has been reported as one of the 25 most significant genes in hES cells (Sperger, Chen et al. 2003). Examination of genes down regulated during the differentiation process before overt loss of pluripotency may thus provide a unique insight into genes operating in the pluripotent state.

In the current study, we have shown that MEDII affects gene expression in hES cells and could be representative of the early transition from pluripotent states to the formation of primitive streak. Our results are in agreement with studies done in the mouse that have shown

MEDII inducing a transitory state characterized by retention of pluripotent gene expression and change in the early embryo developmentally regulated genes (Rathjen, Lake et al. 1999). Further, we have elucidated a set of genes down-regulated upon exposure to MEDII medium that may have a role in the pluripotent state of hES cells or the events leading to an exit in pluripotency. Consistent with this role, we have found changes in expression of several genes implicated in anterior-posterior axis formation, primitive streak formation, and early mesoderm induction. Based on our results it is possible that Cripto dependent TGFβ/nodal signaling plays an early and important role in the initial differentiation from the pluripotent state towards the mesoderm fate. Treatment of hES cells with MEDII medium is the only reported differentiation protocol that shows an increase in Cripto expression. This finding is important because failure to activate Cripto during early differentiation leads to neural fate (Parisi, D'Andrea et al. 2003) and since MEDII can exert this effect in monolayer culture, the resultant product will potentially be more uniform than the product of embryoid differentiation protocols. Thus, continued differentiation with MEDII or isolation of the active mesoderm inducing factors might lead to more uniform mesoderm differentiation and play an important role in generating cell products destined for cellular replacement therapy.

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Figure 3.1. MEDII treatment effects a morphological change in hES cells without loss of pluripotency. Untreated (A, B, E, F, I, J) and MEDII treated hES (C, D, G, H, K, L). Phase contrast images untreated hES cells (A, B). MED II treated hES cells were flatter and more connected (C, D). Both cell types are positive for Oct-4 (E, F, G, H) and SSEA-4 (I, J, K, L). Magnifications: (A, C) 100X, (B, D) 100X, (E-L) 400X.

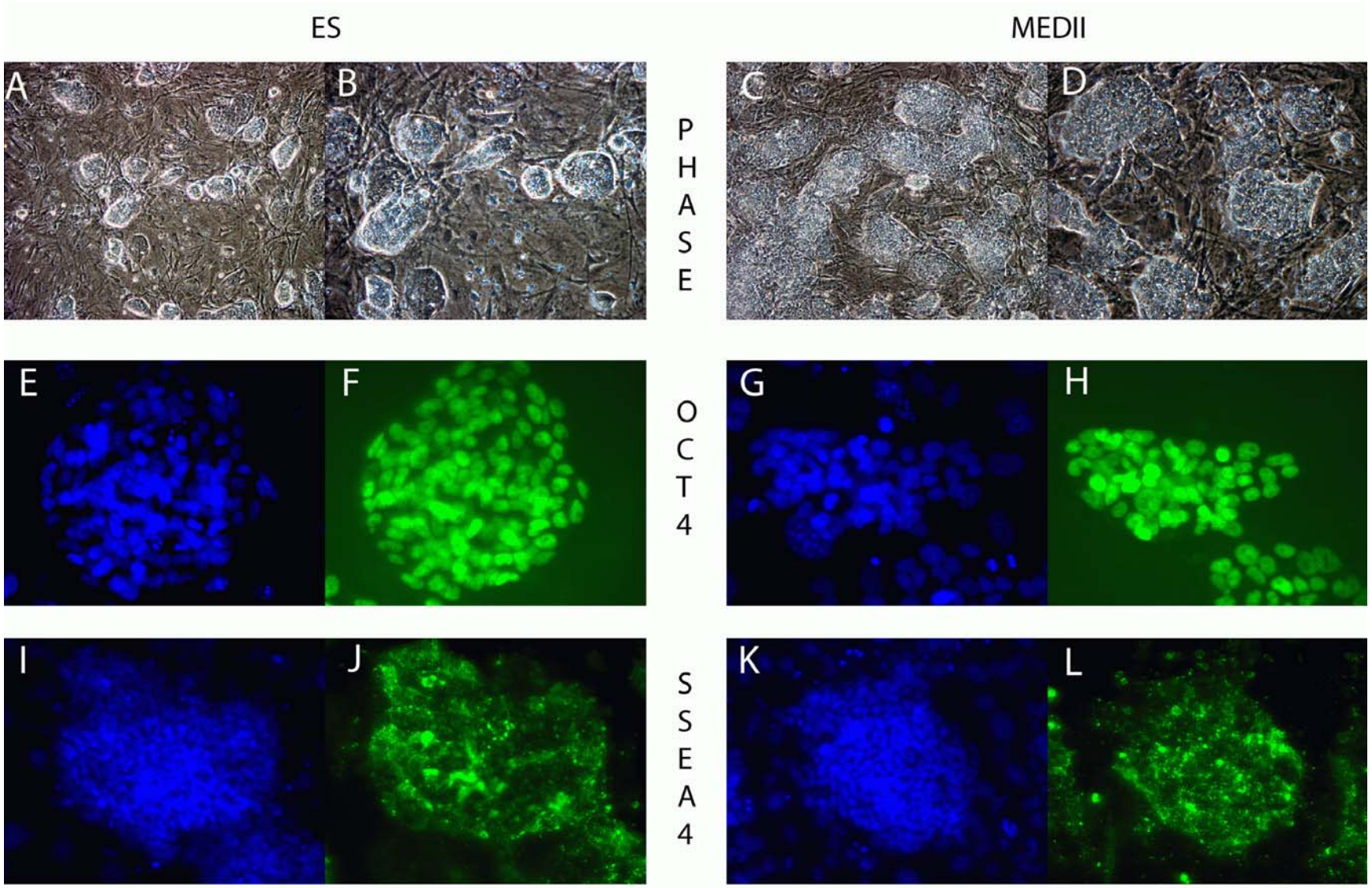
ES
MEDII
A
B
C
D
PHASE
E
F
G
H
OCT4
I
J
K
L
SSEA4

Figure 3.2. Untreated and MEDII treated hES cells have similar cell cycle profiles. Flow cytometry profile of untreated (A) and MEDII treated (B) hES cells stained for SSEA-4 expression. Red traces represent staining done with secondary antibody alone and blue trace represents staining done with both primary and secondary antibody. Cell cycle profiles of untreated (C) and MEDII treated (D) hES cells after gating to remove dead cells, doublets, and non-SSEA-4 positive cells. SSEA-4 staining (E) and cell cycle profile (F) of control mouse embryonic fibroblasts. Green trace represents staining done with secondary antibody alone and red trace represents staining done with both primary and secondary antibody.

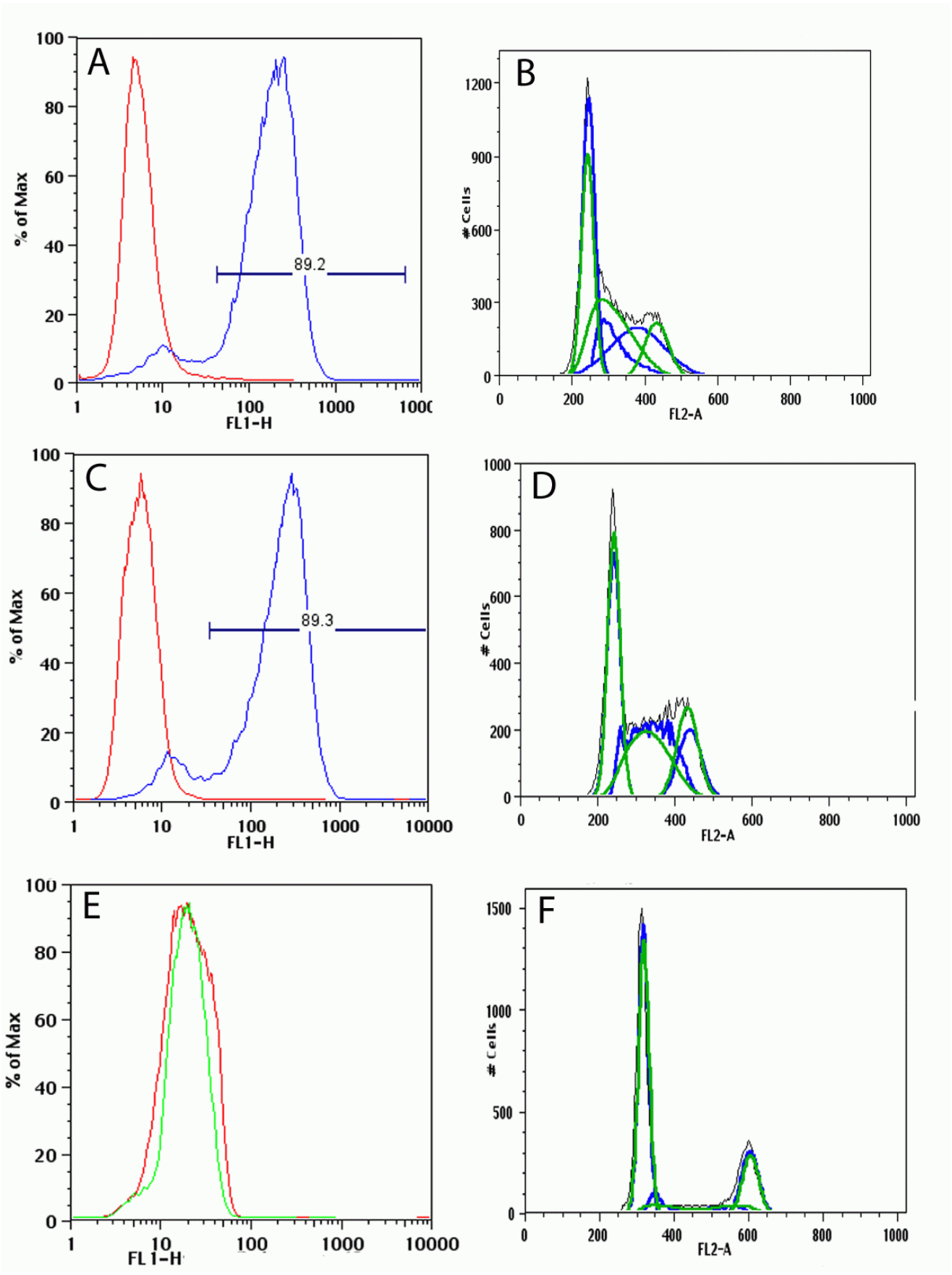
A
% of Max
89.2
FL1-H
B
Cells
FL2-A
C
% of Max
89.3
FL1-H
D
Cells
FL2-A
E
% of Max
FL1-H
F
Cells
FL2-A

Figure 3.3. Microarray analysis of untreated and MEDII treated hES cells. (A) Schematic detailing the experimental strategy employed for the microarray experiments. (B) Scanned images of all six Affymetrix HG U133A chips used in the experiment. (C) R^2 values vs. frequency for each of the 22,215 genes characterized in the experiment after being analyzed by the mixed linear model. (D) Volcano plot of p-value vs. difference in expression value for all 22,215 genes analyzed by microarray between control and treated hES cells.

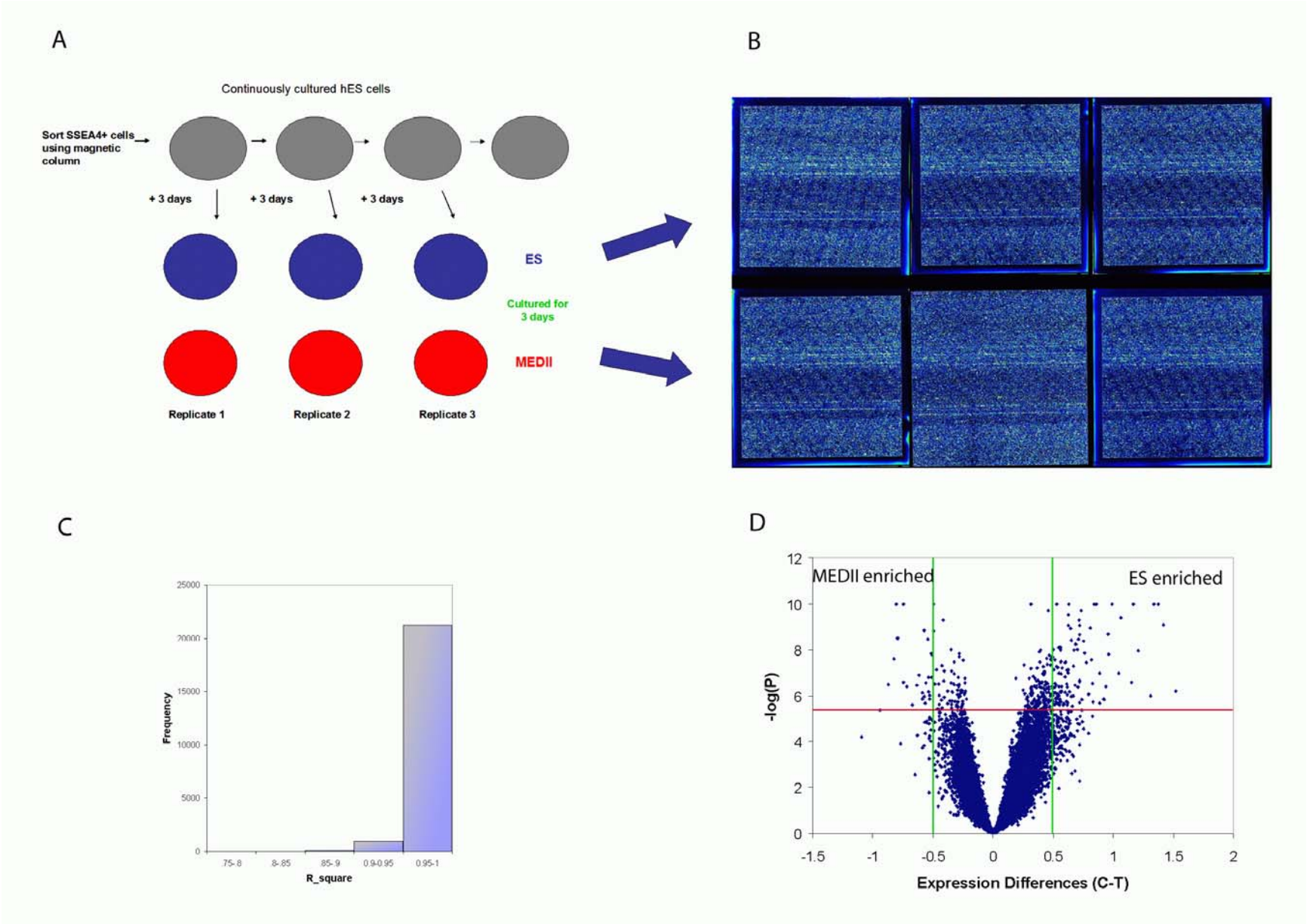
A
Continuously cultured hES cells
Sort SSEA4+ cells using magnetic column
+ 3 days
+ 3 days
+ 3 days
ES
Cultured for 3 days
MEDII
Replicate 1
Replicate 2
Replicate 3
B
C
Frequency
R_square
75-8
8-85
85-9
0.9-0.95
0.95-1
D
MEDII enriched
ES enriched
-log(P)
Expression Differences (C-T)

Figure 3.4. Genes down regulated after MEDII treatment of hES cells. Genes listed were within the Bonferroni adjusted confidence interval and have a fold change of 1.5 fold or greater. Genes were organized according to ontology as defined by Affymetrix's NetAffx online analysis center.

Cell adhesion (2)
Contactin associated protein-like 2, Junction plakoglobin

Embryogenesis and morphogenesis (3)
Glypican 4, Patched, Wnt 5A,

Development (6)
Homeobox A1, Follistatin, Transforming growth factor β1, Lefty A, Lymphoid enhancer-binding factor 1, Dapper homolog 1

Cell growth and maintenance (4)
ALL1-fused gene from chromosome 1q, Papillary renal cell carcinoma (translocation associated), c6.1A, Myotubular myopathy 1

Cell Cycle (2)
MAD2 mitotic deficient-like 1, Nijmegen breakage syndrome 1 (nibrin)

Cellular Structure (2)
Neurofilament 3, Neurofilament light polypeptide

Metabolism (6)
Glutamate decarboxylase 1, Carbonic anhydrase II, Malic enzyme I, Solute carrier family 7, Ornithine decarboxylase 1, Myxoid liposarcoma associated protein 4

Establishment and maintenance of chromatin architecture (1)
Enhancer of zeste homolog 2

Regulation of transcription (3)
Zinc finger protein 84, Aryl hydrocarbon receptor nuclear translocator-like, Cpp/p300 interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2

Signaling (4)
ADP-ribosylation factor-like 7, Ephrin –A4, Chemokine ligand 14, Membrane protein palmitoylated 6

Copper ion binding, transport, homeostasis (3)
Metallothionein 1E, Metallothionein 2A, Metallothionein 1L

Apoptosis (2)
Programmed cell death 2, BCL2 associated athanogene 5

mRNA Splicing (2)
Splicing factor arginine/serine-rich 2, Prp 28 U5 snRNP 100kd protein

Chromosome organization and biogenesis (1)
Histone 1 H2bd

Putative NFKB activating protein (1)
Hypothetical protein 23091

DNA/nucleic acid binding (2)
Lipoma HMGIC fusion partner, Likely ortholog of mouse variant polyadenylation protein CSTF-64

Protein folding/biosynthesis (2)
Ribosomal protein L27, DnaJ (Hsp40) homolog

Proteolysis and peptidolysis (2)
Hypothetical protein FLJ10613, Likely ortholog of mouse heat shock protein 70 kDa

Calcium ion binding activity (1)
Visinin-like 1

Urea Cycle (1)
Argininosuccinate synthetase

Translation initiation (1)
Eukaryotic translation initiation factor 5

DNA Replication (1)
Origin recognition complex subunit like 5

Muscle development (1)
Tropomyosin 2

Transcription (1)
Nuclear phosphoprotein similar to S. cerevisiae PWP1

Sex differentiation (1)
FSH primary response 1

DNA repair (1)
Methyl-CpG binding domain protein 4

Tumor cell survival factor/ATM kinase (1)
KIAA0537 gene product (AMP- related protein kinase 5)

Electron transport (1)
Glutaredoxin 2

Tumor antigen(1)
Paraneoplastic antigen MA1

Respiratory gaseous exchange(1)
Histamine N-methyltransferase

Unknown Function (13)
Jumonji domain conataining 1, Bcl-2-associated transcription factor, Transcription factor B2 (mitochondrial), Hypothetical protein FLJ20516, KIAA0431 protein, Hypothetical protein FLJ12750, KIAA1102 protein, c-Mpl binding protein, SIPL protein, HSKM-B protein, Hypothetical protein MGC29643, Hypothetical protein FLJ21908, Chondroitin 4-sulfotransferase

Figure 3.5. Genes up regulated after MEDII treatment of hES cells. Genes listed were within the Bonferroni adjusted confidence interval and have a fold change of 1.5 fold or greater. Genes were organized according to ontology as defined by Affymetrix's NetAffx online analysis center.

Cell Adhesion (4)
L1 cell adhesion molecule, Ras homolog gene family member E, Cadherin 3 type 1 (P-cadherin, placental), Natural killer cell transcript 4

Immune response (3)
CD74 antigen, Major histocompatibility complex class II DP alpha 1, Major histocompatibility complex class I B

Proteolysis and peptidolysis (1)
Proteasome subunit beta type 8

Signal transduction (1)
Teratocarcinoma derived growth factor 1 (Cripto)

N-linked glycosylation (1)
Transmembrane 4 superfamily member 2

Regulation of transcription (1)
Early growth response 1

Intracellular signaling cascade (1)
Atrophin-1 interacting protein 1

Myosin ATPase activity (1)
Myosin VI

Tumor antigen (1)
Tumor-associated calcium signal transducer 1

VEGF receptor activity (1)
Neuropilin 1

Cell proliferation (1)
Gastrin-releasing peptide receptor

Unknown Function (4)
Hypothetical protein FLJ20171, Hypothetical protein FLJ20273, Hypothetical protein FLJ22693, Similar to zinc finger protein 366

Table 3.1. Summary of statistics for microarray analysis of gene expression.

SigLevel[1]	BONFERRONI	0.001	0.01	0.05
N(up)[2]	**83**	1044	2796	5012
FC(up)[3]	**1.38**	1.24	1.21	1.2
N(Dn)[2]	**203**	1499	2804	4355
FC(Dn)[3]	**1.62**	1.37	1.31	1.28
Fc(All)[3]	**1.55**	1.32	1.26	1.24
%Up[4]	**29**	41	50	54
%Dn[5]	**71**	59	50	46
%genes[6]	**1.3**	11.4	25.1	42.1

(1) Significance Level (2) No. of genes up-or down regulated at indicated significance level (3) Fold changes up or down. Percent of genes that are (4) up or (5) down-regulated (6) Percent of all genes on the microarrays that are differentially expressed

Table 3.2. Real time PCR analysis of untreated and MEDII treated hES cells.

	RNA Isolate[1]	Avg. C_t[2] ES	Avg. C_t MEDII	Std. Dev.[3]		CV[%][4]		Fold Change[5]	P-value[6]
HOXA1	Replicate 1	24.29	26.32	0.02	0.00	0.08	0.01	4.327	0.008
	Replicate 2	24.18	27.40	0.02	0.00	0.06	0.00	9.452	0.001
	Replicate 3	23.94	25.42	0.01	0.03	0.05	0.10	2.655	0.0375
DAPPER	Replicate 1	19.21	20.65	0.00	0.03	0.02	0.14	2.65	0.046
	Replicate 2	20.73	22.51	0.01	0.00	0.02	0.02	3.077	0.0325
	Replicate 3	22.05	23.76	0.03	0.01	0.12	0.04	2.960	0.0155
FOLLIS.	Replicate 1	17.45	19.09	0.01	0.00	0.03	0.01	2.9	0.0685
	Replicate 2	17.16	19.08	0.01	0.00	0.07	0.01	3.19	0.0375
	Replicate 3	15.96	16.62	0.01	0.10	0.06	0.63	1.53	0.0375
CRIPTO	Replicate 1	21.67	19.73	0.03	0.00	0.12	0.02	+3.870	0.044
	Replicate 2	20.61	19.15	0.02	0.00	0.11	0.01	+3.064	0.012
	Replicate 3	22.32	21.25	0.00	0.04	0.00	0.17	+2.097	0.001
EZH2	Replicate 1	20.50	21.29	0.05	0.02	0.23	0.11	1.303	0.088
	Replicate 2	19.36	19.68	0.01	0.00	0.05	0.02	1.104	0.11
	Replicate 3	18.94	19.07	0.01	0.00	0.05	0.01	1.1	0.0455
NANOG	Replicate 1	18.74	19.15	0.00	0.04	0.01	0.22	NDE	N/A
	Replicate 2	21.17	20.53	0.00	0.00	0.01	0.00	+4.643	0.001
	Replicate 3	19.50	19.31	0.01	0.02	0.07	0.10	NDE	N/A
OCT4	Replicate 1	16.39	16.52	0.06	0.34	0.38	2.06	NDE	N/A
	Replicate 2	15.52	16.41	0.01	0.01	0.05	0.04	NDE	N/A
	Replicate 3	15.35	15.21	0.00	0.00	0.03	0.01	NDE	N/A

(1) RNA isolated for microarray analysis was used for real time PCR and replicates are the same as in Figure 3.
(2) C_t represents threshold cycle, the cycle at which fluorescence intensity is considered above background. Standard deviation (3), coefficient of variance (4), fold change (5), and p-value (6) all calculated by REST software. NDE indicates not differentially expressed and N/A indicates not applicable.

CONCLUSION

The goal of this dissertation was to characterize the effect of MEDII media, a conditioned media from HepG2 cells, on monkey embryonic stem cells and human embryonic stem cells. More specifically, the project can be divided into two studies; characterization of the ability of MEDII media to influence neural differentiation of monkey embryonic stem cells, and characterization of the effect of MEDII media on adherently cultured human embryonic stem cells.

As stated previously, the initial studies carried out defining the effect of MEDII media on an embryonic stem cell population were done with mouse embryonic stem cells. During a screen for conditioned media that contained anti-differentiation activity, Rathjen et al discovered that MEDII media had a repeatable and reversible morphology changing effect on mouse embryonic stem cells. The change in morphology was dissimilar to that seen upon spontaneous differentiation. Gene expression studies revealed that mouse embryonic stem cells treated with MEDII media continued to express the pluripotency markers Oct-4 and alkaline phosphatase. However, these cells down regulated a third pluripotency marker Rex-1 and showed increased expression of FGF-5, a marker of primitive ectoderm. Primitive ectoderm is a pluripotent cell population found between blastocyst and gastrulation stage mouse embryos [1]. Further studies demonstrated that altering the method of exposure to MEDII media could enhance either neural or mesodermal differentiation from embryonic stem cells [2, 3].

Study 1: The Ability of MEDII Media to Influence Neural Differentiation of Monkey Embryonic Stem Cells

The first included study's aim was to determine if MEDII media was beneficial in deriving neural cells from monkey embryonic stem cells. In the study we describe an efficient method for differentiating rhesus monkey embryonic stem cells to neural lineages and the subsequent isolation of an enriched population of Nestin and Musashi positive neural progenitor (NP) cells. Neural progenitors are a population of cells capable of undergoing asymmetric division producing another progenitor cell and a terminally differentiated neural cell. Upon differentiation, these cells exhibited electrophysiological characteristics resembling cultured primary neurons. Embryoid bodies were formed in embryonic stem cell growth medium supplemented with 50 % MEDII. After 7 days in suspension culture, embryoid bodies were transferred to adherent culture and either differentiated in serum containing medium or expanded in serum free medium. Immunocytochemistry on differentiating cells derived from embryoid bodies revealed large networks of MAP-2 and NF200 positive neurons. DAPI staining, which fluorescently counterstains nuclei, showed that the center of the MEDII treated embryoid bodies were filled with rosettes. NPs isolated from adherent EB cultures expanded in serum free medium were passaged and maintained in an undifferentiated state by culture in serum free N2 with 50 % MEDII and bFGF. Differentiating neurons derived from NPs fired action potentials in response to depolarizing current injection, and expressed functional ionotropic receptors for the neurotransmitters glutamate and gamma-aminobutyric acid (GABA).

Future directions

Neural progenitors derived in this manner provide a sensible starting material for cellular replacement therapies. Currently, it is not known what specific cell type will emerge as the best

option for cellular replacement therapies. Some feel that an un-specified neural progenitor cell such as that described here would have advantages for replacement therapy. Cells that are not specified could home to injury sites and the local microenvironment could provide differentiation cues to produce the specific cell type needed to repair injured areas. Conversely, it may be that the best candidate cell type for cell replacement therapy will be a terminally differentiated cell type of a specific lineage. In either event, the above described system is flexible and would allow for generation of unspecified progenitors as well as terminally differentiated cell types.

Future experiments could be divided into two categories; transplantation of derived progenitors and differentiated cell types into animal models, and *in vitro* studies designed to better characterize the differentiation process.

The main goal of these types of differentiation studies is the production of material for cell replacement therapies. Studies in humans have demonstrated "proof of principle" that transplanted dopaminergic neurons can help ameliorate symptoms of Parkinson's disease [4]. However, the fetal cells used in this study were not the optimal starting material for reasons pertaining to obvious ethical concerns and limited supply issues. Therefore, generation of large numbers of specific neural cell types would provide unlimited material for cell replacement therapy. These techniques would benefit from experimentation done with large animal models. Models for Parkinson's disease and spinal cord injury have already been worked out in monkeys [5-8]. The goal of these experiments would be to determine if neural progenitors or differentiated cells generated by the protocol described here would overcome disease symptoms. Initial experiments would include transplanting different amounts of neural progenitor cells as a single cell suspension. To start, a similar number of cells to what has been shown to be efficacious in humans [4] would be used, however the protocol may need modification. Several

concentrations of cells would be used and animals would then be assessed for behavior changes (rotational tests for Parkinson's model) and then euthanzied to determine if any integration occurs. Depending on the progress of these experiments, transplants of differentiated cell types could be done as well. Again, the numbers of cells needed for transplantation would need to be determined empirically.

While studies with primates are quite expensive, other studies can be done *in vitro*. Microarray experiments comparing the gene expression profile of undifferentiated monkey embryonic stem cells, neural progenitors, and terminally differentiated cells would provide clues as to what genes are expressed by each cell type. SiRNA technology, which effectively disrupts gene expression, could be performed on hits generated by microarray analysis to functionally assess the importance of specific genes in neural progenitors and terminally differentiated cell types. Knowledge about which genes' activation leads to differentiation may allow researchers to modulate a specific gene or signaling pathway and thereby inhibit or increase differentiation of neural progenitors. Other experiments could include using a panel of neural progenitor markers such as Sox-1, Nestin, and Musashi to carry out time course experiments to determine the optimal embryoid body growth period. Material would also be characterized for expression of pluripotency markers such as Oct-4 and Nanog and lineage markers such as Brachyury for mesoderm and alpha-fetoprotein for endoderm. This would allow for the determination of the optimal time at which there are limited embryonic stem cells, large numbers of neural progenitors, and how much unwanted germ layer contamination is occurring. Studies using MEDII to induce neural differentiation of human embryonic stem cells implicate its potential in the human system as well [9].

Study 2: Characterization of MEDII Effect on Adherent Human Embryonic Stem Cells

The second included study sought to characterize the effect of MEDII media on adherent human embryonic stem cells. The previous reported effect on mouse embryonic stem cells was characterized by a significant change in morphology, down regulation of the pluripotency marker Rex-1, up regulation of the primitive ectoderm marker FGF-5, and continued expression of pluripotency markers Oct-4 and alkaline phosphatase. Rather than limiting the experiment by characterizing the expression pattern of these select genes the current study instead utilized the global gene expression profiling ability of microarray analysis. Earlier attempts to characterize FGF-5 expression in treated and untreated monkey embryonic stem cells and human embryonic stem cells were inconclusive. Further, due to the lack of embryological data on pre- and early post-implantation primate embryos, it is unclear whether or not primate embryos form primitive ectoderm in the same manner that mouse embryos do and if FGF-5 is a suitable marker for that cell type.

Of particular interest was the finding that teratocarcinoma derived growth factor 1 (Cripto) mRNA was up regulated by MEDII treatment. Cripto is a member of the EGF-CFC, family of proteins; which contain an epidermal growth factor (EGF) domain and a Cripto, FRL-1, and Cryptic (CFC) domain, and can act as either a co-receptor or co-ligand of the nodal signaling pathway that binds a complex of ActRIB and ActRIIB receptors [10]. Cripto is initially expressed uniformly throughout the epiblast and then its expression is restricted to primitive streak epiblast cells and developing mesoderm [11, 12]. Therefore our results suggest that MEDII may be inducing very early differentiation signaling in the human embryonic stem cells and Cripto may play a role in this differentiation.

Several functional studies have shown illustrated the importance of Cripto in early mouse embryonic development. Targeted disruption of the gene has been done by two independent groups. These studies assessed Cripto homozygous null mutant embryos and discovered that Cripto is essential for conversion of the proximal-distal axis to an anterior-posterior axis and streak formation. Mutant embryos fail to form a correct node, lack a primitive streak, and do not form embryonic mesoderm properly [12, 13]. Further, a study of Cripto null ES cells demonstrated that these cells could not form beating cardiomyocytes [14]. A more recent study demonstrated that without Cripto activity during a specific window of time, EB derived cells from null ES cells were directly converted to a neural fate. However, addition of soluble Cripto during the differentiation process could restore the ability to differentiate to cardiomyocytes and inhibit neural differentiation [15]. Previous studies have suggested that in the absence of appropriate signals the default differentiation pathway of pluripotent cells is to generate neural lineages [16, 17]. Mouse embryonic stem cells differentiated by the EB method show increased expression of Cripto in a transient manner [15]. However, spontaneously differentiated human embryonic stem cells show decreased expression of Cripto [18] but these cells had been cultured in conditions which may not produce mesodermal fates. These human embryonic stem cells were cultured twenty six days as differentiated cells and therefore it is likely that these cells had differentiated beyond the temporal equivalent to cell types produced in the included study. It is thought that the current study using MEDII media is the only reported protocol for generating a cellular population with pluripotent gene expression and increased Cripto expression. This protocol has advantages over embryoid body methods in that the treatment creates a uniform monolayer of differentiated cells. This leads to the hypothesis that MEDII treatment will be important in generating uniform populations of non-neural cell types from human embryonic

stem cells *in vitro*. An interesting new study that used microarray analysis used to characterize gene expression in β-catenin mutant embryos in comparison to wild type embryos also demonstrated differential Cripto expression between the two embryo types. Two stages of embryo development were used, E6.0 pre-streak embryos and E6.5 early-streak stage embryos. Interestingly, Cripto was the most down regulated gene when comparing mutant and normal embryos at E6.0. In addition, Cripto was the most down regulated gene at E6.5 as well. Nanog, a pluripotency marker, and Brachyury, FGF8, and Eomes were as well deregulated in E6.5 β-catenin mutant embryos [19]. In addition, if an active form of β-catenin is expressed in null embryos Cripto expression is increased. The authors also demonstrate that β-catenin regulates Cripto expression. Based on comparisons of β-catenin mutant embryo gene expression with Cripto and Wnt 3 mutant embryos, the authors suggest a two step model of β-catenin dependent embryo development. In the first step β-catenin up-regulates Cripto during anterior-posterior axis orientation. This leads to increased Cripto expression which induces Cripto dependent Nodal signaling. This signal in turns leads to step two, mesoderm formation, which is controlled by β-catenin activation by Wnt3 and subsequent activation of nascent mesoderm genes such as Brachyury. Interestingly, HepG2 cells, the cells from which MEDII is generated, are characterized by a mutation in β-catenin. These cells express a truncated form of the protein that is 75 kDa instead of the normal 92 kDa protein found in normal cells and tissues. This truncation is thought to remove the GSK-3β, a signaling pathway member that phosphorylates β-catenin leading to its degradation, phosphorylation site and binding sites for other proteins [20, 21]. It is unclear what the active components of MEDII media are, but it is indeed interesting that the cells that produce the media are characterized by an activating mutation of the very protein recently

proposed to be responsible for anterior-posterior axis formation and subsequent mesoderm formation in mouse embryo.

Our data also indicate changes in expression of other genes indicative of primitive streak like cells. Lefty A, an antagonist of Nodal/Cripto signaling normally expressed in the anterior visceral endoderm, is down-regulated with MEDII treatment [22]. Follistatin is down-regulated as well with MEDII treatment. Studies have shown Follistatin may act as an inhibitor of mesoderm induction [23, 24]. Two genes expressed in the primitive streak, GATA-6 and Tbx-1, are up-regulated with MEDII treatment [25, 26]. Taken together these results indicate that MEDII potentially induces a gene expression pattern consistent with anterior-posterior axis and primitive streak formation.

To summarize the current findings a new model of MEDII's effect on hESCs is proposed (Figure 4.1). As mentioned above, two known inhibitors of mesoderm induction, Follistatin and Lefty, are down regulated with MEDII treatment. In addition, Cripto which is required for functional Nodal signaling is up regulated. As the diagram indicates, cells would then be capable of receiving an activating signal of the heterodimeric complex of Activin type II and ALK4 receptors. Activation of these receptors is known to activate the Smad transcription factors which in turn activate transcription of mesoderm inducing genes. The current study suggests this would explain the ability of MEDII to influence neural differentiation of ES cells.

In addition, Figure 4.2 is a schematic detailing components of the Wnt signaling pathway. The current model stipulates a two state model. In the absence of Wnt signal, β-catenin is phosphorylated and degraded. In the presence of Wnt signal, β-catenin is translocated to the nucleus and activates transcription of developmentally associated genes. Wnt3 has been shown to be essential for the formation of mesoderm in the mouse embryo. Further, as stated above

Wnt3 can maintain the pluripotent state of hESCs grown on Matrigel rather than feeder layers. As the discussion above indicates, β-catenin can increase levels of Cripto. Increased Cripto in turn causes increased Nodal signaling which causes mouse embryos to undergo axis formation, which occurs immediately prior to mesoderm induction. Due to the knowledge that the transforming mutation of HepG2 cells, the cells which generate MEDII, is an activating mutation of β-catenin, this leads to the new hypothesis that activated β-catenin in HepG2 cells causes HepG2 cells to secrete signaling molecules that cause ES cells to undergo changes in gene expression similar to ICM cells being converted to primitive streak stage cells.

Future directions

The data generated by microarray analysis of MEDII treated human embryonic stem cells and the discussion above leads to the new hypothesis that MEDII induces changes in human embryonic stem cells consistent with those seen during mouse embryo axis formation and early mesoderm induction. Further, it leads to the hypothesis that MEDII contains a soluble active factor upstream of Cripto regulation during development. The most likely factors would be Nodal and/or Activin based on their known roles in patterning the mouse epiblast. Studies are already underway using MEDII with Zebrafish embryos to determine what effect the conditioned media may have on influencing their development. For these experiments, serum free MEDII was generated as well as pure poly A^{++} RNA from the HepG2 cells themselves. RNA will be injected into Zebrafish embryos which will be allowed to develop to the twenty-four hour period and then fixed for morphology assessment and possible in-situ hybridization studies. If possible, embryos will be bathed in 50% MEDII media as an alternative way of exposing the embryos to the conditioned media. This approach may not be feasible due to embryo toxicity issues. In keeping with the hypothesis that Nodal and/or Activin may be the active factor in MEDII,

primers have been designed and will be used by RT-PCR to determine if transcripts are present for Nodal and/or Activin.

These experiments make the assumption that up regulation of Cripto is indeed the important and necessary response for the effect MEDII treatment has on human embryonic stem cells. One way to determine if this is the case would be to design a siRNA construct that would knock down mRNA levels of Cripto, thereby effectively preventing its function. An experiment could then be performed which contained four experimental groups; untreated human embryonic stem cells, 50% MEDII treated human embryonic stem cells, untreated siCripto human embryonic stem cells, and 50% MEDII treated siCripto human embryonic stem cells. One would examine the phenotype of the 50% MEDII treated siCripto human embryonic stem cells to determine if the siRNA construct inhibiting Cripto expression blocked the morphology and gene expression changes seen in 50% MEDII treated human embryonic stem cells.

Further experiments are needed to further characterize MEDII's effect on human embryonic stem cells. By using a panel of genes that could be monitored by either RT-PCR or Real Time PCR time course experiments could be carried out to determine the optimal treatment window. Genes to monitor would include Oct-4, Nanog, Cripto, Eomesodermin, Wnt3a, Nodal, Activin, BMP-4, and Brachyury. As mentioned previously, Oct-4 and Nanog are markers of pluripotency and monitoring their expression will tell whether or not there is loss of pluripotency. Cripto and Eomesodermin are markers of the developing primitive streak [27] and axis formation of mouse embryos. Wnt3a is known to induce epiblast cells to form mesodermal rather than neurectodermal lineages after gastrulation. Addition of BMP-4 to mouse embryonic stem cells directs their differentiation to mesodermal lineages. Brachyury, a target of Wnt3a is not only a marker of nascent mesoderm, but is essential for mesoderm formation as well [28]. By

extending the treatment groups of the experiment to two, four, and six days, and monitoring the above mentioned genes, an optimal treatment time to initiate early differentiation and possibly mesoderm induction could be determined.

As stated earlier, studies with mouse embryonic stem cells demonstrated that embryoid bodies made from adherently cultured cells exposed to 50% MEDII media were comprised largely of nascent mesoderm [3]. After optimizing the time period of treatment of human embryonic stem cells with 50% MEDII media, embryoid bodies should be made from these cells. These embryoid bodies could be assayed for mesoderm development by several methods including in-situ hybridizations or RT-PCR analysis for Brachyury expression. As a guide, time points similar to those used for the mouse embryonic stem cell studies could be used initially, and as results were generated the experimental protocol would be refined. As a further demonstration of the ability to form mesodermal derivatives, the embryoid bodies would be plated adherently and scored for beating cardiomyocyte formation, a common assay for mesodermal differentiation from embryonic stem cells. Timing would be crucial to this assay as well, but multiple time points could be assayed after plating. While there have been demonstrations of the ability of human embryonic stem cells to form beating heart muscle, it is unclear whether or not human embryos undergoing mesoderm induction exhibit expression of Brachyury. This could be a potential pitfall for the experimental protocol. By having a gene expression and functional assay meaningful data should be obtained.

Ultimately, it would be beneficial to obtain mesodermal cells from adherent cultures without having to make embryoid bodies, which introduces some level of unwanted cell types. In mouse embryos, Wnt3a signaling induces mesodermal differentiation [29]. Therefore, soluble Wnt3a may induce mesodermal differentiation of human embryonic stem cells. However, a

recent study demonstrated that human embryonic stem cells grown without a feeder layer could be maintained in the pluripotent state by Wnt3a [30]. A possible line of experiments would involve first treating human embryonic stem cells with 50% MEDII and then adding Wnt3a. This experiment would build on the hypothesis that MEDII treated human embryonic stem cells are equivalent to cells in the mouse embryo immediately prior to primitive streak formation and are primed to receive a mesodermal induction signal. Cells would be assayed for expression of the nascent mesoderm marker Brachyury to determine if the treatment pushes cells toward mesodermal lineages.

The suggested experiments are examples of ways in which the ability of MEDII media to induce mesodermal differentiation of human embryonic stem cells may be assessed. Mesodermal cells would be useful for deriving cells beneficial to heart attack patients as well as those in need of endothelial cells. While much effort has been put into deriving neural cell lineages from human embryonic stem cells, fewer studies have been carried out characterizing the mesodermal differentiation capacity of these cells. These studies will be important as models of development as well as for the generation of cell replacement therapy material.

In summary, the current studies utilize MEDII media, a technology developed with mouse embryonic stem cells, to define an *in vitro* differentiation strategy for the efficient production of neural lineages from monkey embryonic stem cells, including the first demonstration of functionality of derived neurons. In addition, by taking advantage of MEDII's ability to influence differentiation of embryonic stem cells to stable intermediates of differentiation still retaining pluripotency, the study provides a snapshot of gene expression changes occurring during early differentiation events in pluripotent cells. Not only will this be an important model for developmental events occurring in pre-implantation embryos, but data

suggests the protocol may be an efficient way to overcome the default neural differentiation of human embryonic stem cells and yield cells of mesodermal and endodermal lineages.

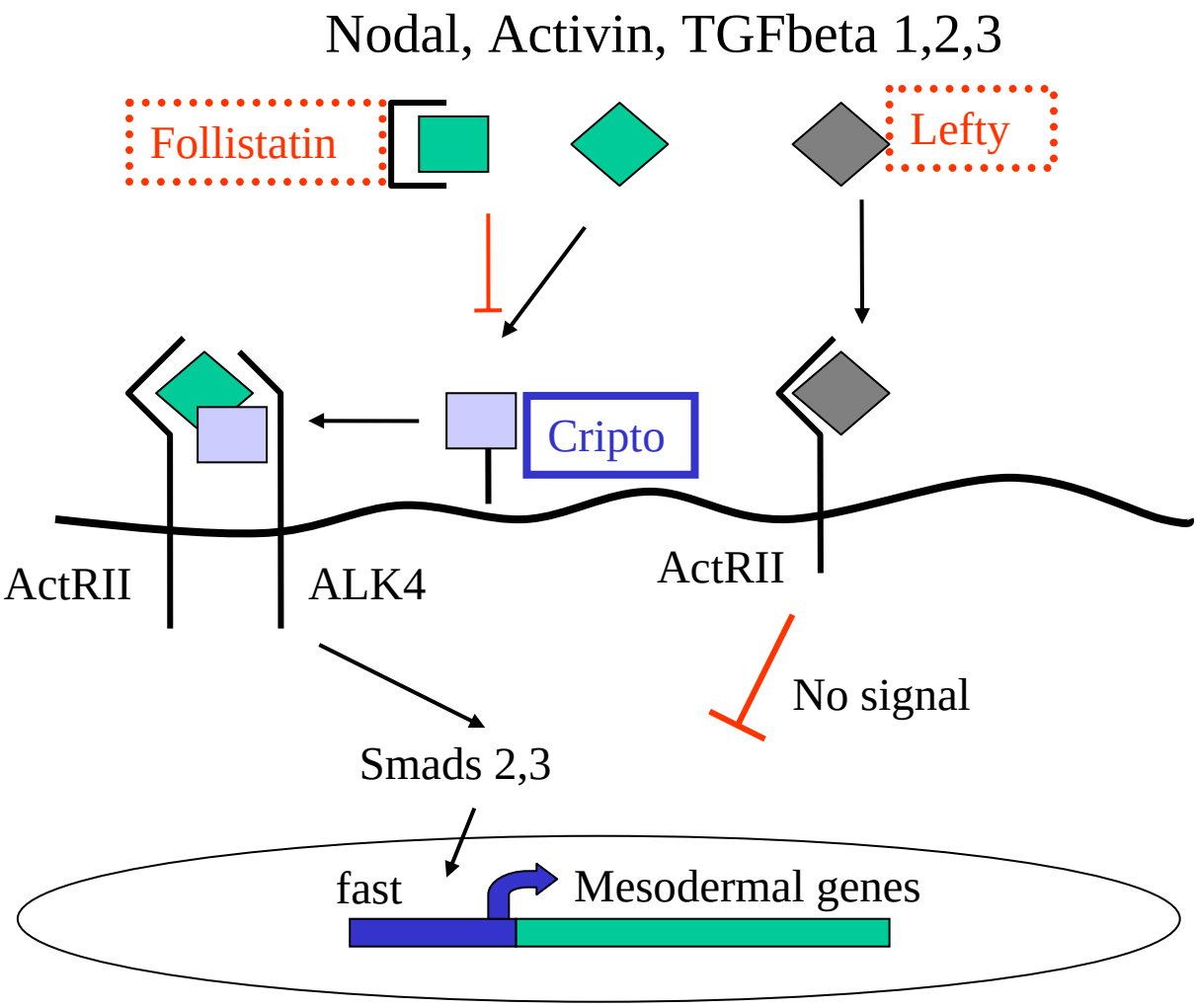

Figure 4.1. Proposed model of MEDII action. Genes in red, Follistatin and Lefty, are down regulated after MEDII treatment while Cripto, in blue, is up regulated after MEDII treatment.

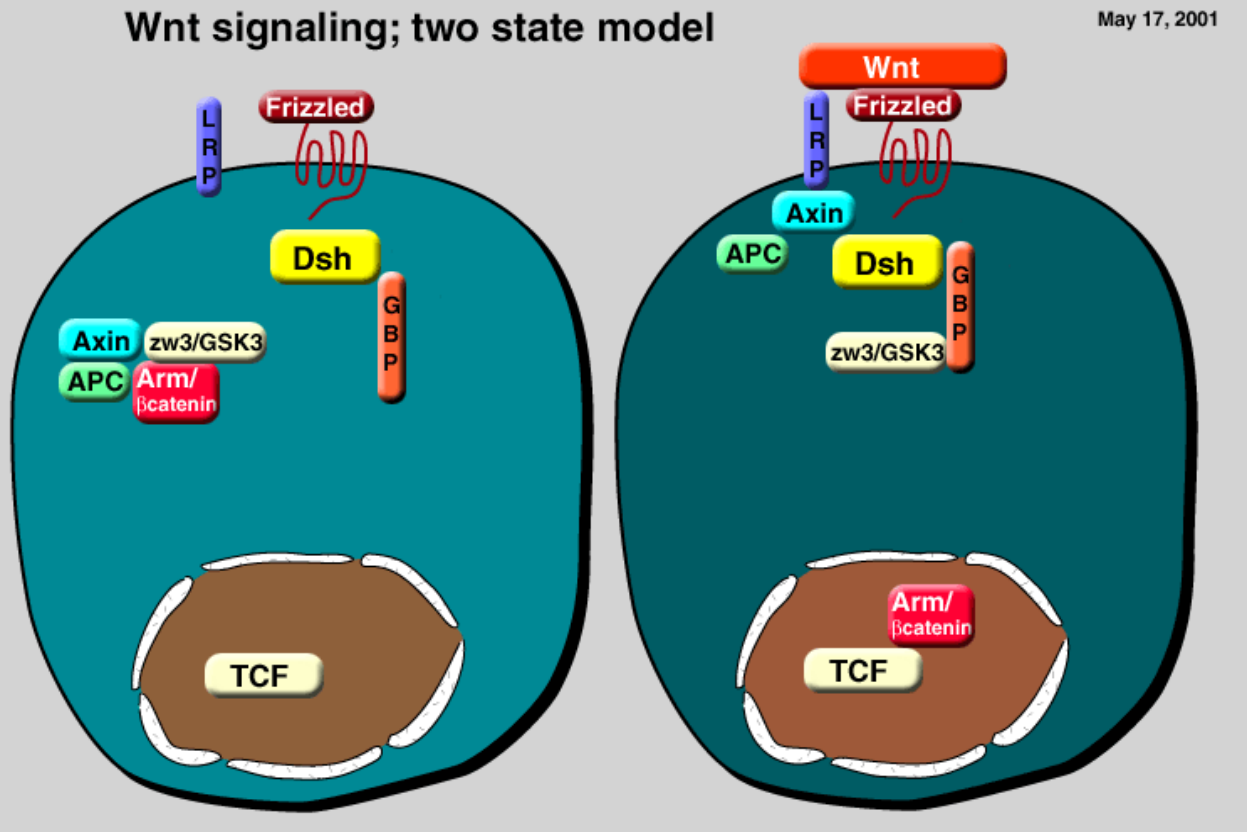

Figure 4.2. Two state model of Wnt/β-catenin signaling. In the absence of Wnt signal (left panel) β-catenin is phosphorylated by GSK-3 and degraded via the proteasome. In the presence of signal (right panel) β-catenin is not phosphorylated and enters the nucleus and interacts with TCF transcription factors. http://www.stanford.edu/~rnusse/pathways/celldouble.html

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