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REST/NRSF regulates genetic stability and cell fate in human embryonic stem cells

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REST/NRSF regulates genetic stability and cell fate in human embryonic stem cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

By

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2013

ABSTRACT OF THE DISSERTATION

REST/NRSF regulates genetic stability and cell fate in human embryonic stem cells

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Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2013

Professor April Dawn Pyle, chair

REST (RE1 silencing transcription factor), also known as NRSF (neuron-restrictive silencer factor), is well-known as a transcriptional repressor of neural genes in non-neural tissues. Dysregulation of REST activity is thought to play a role in diverse diseases including cardiac hypertrophy, Down Syndrome, Huntington's disease and cancer. Previous studies examining the pluripotency transcriptional network in mouse as well as human embryonic stem cells (hESCs) have revealed that REST is regulated by the pluripotency factors OCT4, NANOG and SOX2. The goal of the present study was to evaluate the role of REST in hESCs. An inducible REST knockdown system was used to examine growth and differentiation over short and long-term culture. Interestingly, altering REST levels in multiple hESC lines did not result in loss of self-renewal, but

instead led to aneuploidy. During differentiation, reduced REST levels led to altered MAPK/ERK and WNT signaling, as well as upregulation of endoderm and mesoderm markers. Critical hurdles for the translation of the clinical potential of hESCs into practice are their tumorigenic capacity, and the inefficiency in tailoring lineage differentiation. Elucidating the role of REST in regulating cell fate and genetic stability of hESCs could enable development of robust methods to stably culture and tailor lineage differentiation of these cells for use in regenerative medicine applications.

The dissertation of Kaushali Anant Thakore-Shah is approved.

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2013

Dedication

This dissertation is dedicated to my maternal grandmother, Kamala Devi Desai, who refused to conform to the social restrictions imposed on child-widows, and left her family and hometown to get an education and become a teacher.

This dissertation is also dedicated to my maternal grandfather, Narendra Desai, a follower of Mahatma Gandhi, a school principal, a renaissance man who could quote Shelley and the *Bhagavad Gita* with equal ease, and also a courageous man, who defied tradition, family and society to marry a child-widow.

Together, my grandparents devoted their life to teaching, and molded many a life. They lived a life of simplicity, with dignity, integrity and generosity of spirit. I will always cherish my memories of them as loving grandparents.

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To my mother-in-law – I don't know how we w0uld have survived the last three weeks without you Mamma!

To my husband Biren – We did it!

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Shuang Hou, Juehua Yu, Kuan-Ju Chen, Mitch Andrï Garcia, Libo Zhao, Wei-Yu Lin, Xuan Xuan, **Kaushali Thakore-Shah**, Chen Shao, April D. Pyle, Guoping Fan, Hao Wang, Hsian-Rong Tseng. Catalytic delivery nanosubstrates for introducing functional biomolecules that are encapsulated in supramolecular nanoparticles. Nature Nanotechnology (In Review).

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PRESENTATIONS

Kaushali Thakore-Shah, Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research (BSCRC) at UCLA, Presentation entitled, “Elucidating the role of Repressor Element1 Silencing Transcription Factor (REST) in regulating Human Embryonic Stem Cell (hESC) fate.” September 28, 2012.

Kaushali Thakore-Shah, UCLA MB-IDP, Presentation entitled, “Elucidating the Role of REST in hESCs.” January 26, 2011.

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Kaushali Thakore-Shah, UCLA BSCRC, Presentation entitled, “Elucidating the Role of hESC Fate Networks in Controlling Pluripotency.” February 29, 2008.

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POSTERS

Thakore-Shah, K., Pyle, A. “Elucidating The Role Of REST/NRSF In Human Embryonic Stem Cells” UCLA BSCRC 7th Annual Stem Cell Conference: Stem Cells: Basic Biology to Translational Medicine. Poster Presentation: Feb 18, 2011. UCLA, Los Angeles, CA.

Thakore-Shah, K., Pyle, A. “Examining The Role Of REST/NRSF In hESCs” ISSCR 8th Annual Meeting. Poster Presentation: June 18, 2010. Moscone West, San Francisco, CA.

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Thakore-Shah, K., Peterson, C., Pyle, A. “Modulating Human Embryonic Stem Cell Fate.” The UCLA Institute for Stem Cell Biology and Medicine, JCCC, and UCLA Conference: Developmental Biology and Stem Cells. Feb 16, 2007. UCLA, Los Angeles, CA.

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Chapter 1. Introduction

1.1. Human Embryonic Stem Cells

Human embryo development begins at fertilization of an oocyte with a sperm to create a single-celled zygote in the oviduct, which then makes its way through the fallopian tubes to the uterus, dividing along the way. At about the eight-cell stage, the embryo compacts, and by the 16-cell stage, the compacted embryo is termed a morula. The outer cells of the morula divide to produce an outer rim of cells—the trophectoderm—and an inner core of cells, the inner cell mass (ICM). By embryonic days 5 to 6 the embryo develops a cavity called the blastocoel. At this stage, the morula becomes a blastocyst with an outer sphere of flattened trophectoderm cells, an ICM of small round cells, and the fluid-filled blastocoel. Ultimately, the cells of the ICM give rise to all the tissues of the embryo's body, as well as to the yolk sac, allantois, and amnion that support the developing embryo. Between days 5 to 7 post-fertilization, the blastocyst reaches the uterus. It has not yet implanted into the uterine wall and is therefore still a pre-implantation embryo (http://stemcells.nih.gov/info/scireport/pages/appendixa.aspx).

Human embryonic stem cells (hESCs) are derived from day 5 pre-implantation blastocysts of surplus embryos donated by couples undergoing in vitro fertilization therapy. Dr. James Thomson and his colleagues derived the first hESCs in 1998. From the thirty-six cleavage stage donated embryos that they started out with, fourteen ICMs were isolated, and the team was able to establish five hESC lines – H1, H7, H9, H13 and H14, originating from five separate embryos (Thomson 1998). Four of the five lines were derived from frozen embryos provided by Josef Itskovitz-Eldor, of the Rambam Medical Center in Haifa, Israel. The hESC line from the fifth, fresh embryo was derived from an

embryo donated in Wisconsin (http://stemcells.nih.gov/info/scireport/pages/appendixc.aspx).

To generate hESC cultures, the pluripotent cells of the ICM were separated from the trophectoderm by immunosurgery, the antibody-mediated dissolution of the trophectoderm (Thomson 1998). A normal day-5 human embryo *in vitro* consists of 200 to 250 cells. Most of the cells comprise the trophectoderm, and the ICM is composed of only 30 to 34 cells (Bongso et al. 1990). The ICMs were plated on feeder layers of mouse embryonic fibroblasts (MEFs) that had been gamma-irradiated to prevent their replication, and grown in medium supplemented with fetal bovine serum. After 9 to 15 days, ICM-derived outgrowths were dissociated into clumps either chemically or mechanically and replated in the same culture conditions. Individual colonies with a uniform undifferentiated morphology were selected by micropipette, mechanically dissociated into clumps of about 50 to 100 cells and replated. Once established and expanded, the cell lines were passaged by treatment with collagenase IV or by selection of individual colonies by micropipette (Thomson 1998). Several research groups have since published the derivation of new hESC lines using similar ICM isolation protocols (Cowan et al. 2004; Genbacev et al. 2005; Inzunza et al. 2005; Kim et al. 2005; Klimanskaya et al. 2005; Ludwig et al. 2006; Reubinoff et al. 2000; Richards et al. 2002). The hESCs proliferate in a stable undifferentiated state for extended periods in vitro, maintain the developmental potential to differentiate into derivatives of all three embryonic germ layers, and form teratomas when injected into immune-deficient mice, thus fulfilling the criteria for pluripotency.

HESCs provide a unique model system for understanding the early human developmental events critically involved in infertility, pregnancy loss and birth defects,

since experimental manipulations cannot be carried out in the intact human embryo due to ethical considerations. Knowledge of human development in the early post-implantation period has historically been based on histological sections of a few human embryos and on analogy to embryological studies of the mouse. However, early mouse and human development differ significantly in the timing of embryonic genome expression (Braude et al. 1988), in the arrangement of the germ layers at the time of gastrulation, in the formation, structure and function of the placenta and extraembryonic membranes (Benirschke, Kaufmann 1990; Luckett 1978), and in the formation of an embryonic disc in humans instead of the egg cylinder of the mouse (O'Rahilly, Müller 1987). Thus hESCs will provide insights into the differentiation and function of tissues that differ significantly between mice and humans. HESCs could also provide a potentially unlimited source of differentiated, non-transformed cells for studying the normal function and pathology of specific differentiated human cells. The sustained culture of many human tissue restricted stem or progenitor cells is difficult or infeasible, and hESCs offer a way to study mechanisms of tissue-specific differentiation and regeneration. The standardized production of large, purified populations of differentiated human cells such as cardiomyocytes, neurons, blood cells and corneal endothelial cells could provide a potentially limitless source of cells for drug discovery, toxicity testing, and regenerative therapies.

1.2. REST / NRSF

RE1-silencing transcription factor (REST), also called neuron-restrictive silencer factor (NRSF) is a member of the GLI-Krüppel family of zinc finger proteins that was originally identified as a negative regulator of neural differentiation through its ability to repress the expression of neural genes in non-neural tissue (Chong et al. 1995;

Schoenherr, Anderson 1995). REST binds to its target genes at a highly conserved 21 to 23 base cognate DNA binding site termed repressor element 1 (RE1) or neural-restrictive silencer element (NRSE) (Schoenherr et al. 1996). There are over 1900 RE1 sites in the human genome, spread across a diverse set of genes (Bruce et al. 2009; Johnson et al. 2007; Johnson et al. 2006). Johnson et al (2006) have demonstrated that there is widespread duplication of functional RE1s, principally located within or beside transposable elements (TEs) of most major classes, including long interspersed repeats (LINEs, principally LINE2s) and short interspersed repeats (SINEs, principally Alus). Approximately one in seven RE1s overlaps or flanks a LINE2, suggesting that LINE2 retrotransposition in particular has been an important driver of RE1 generation and insertion in the human lineage. The largest single family of RE1s, located in the coding region of a LINE2 element, has 28 members, with an apparently non-random distribution: 29% (8/28) are located within 1 Mb of a telomere, 25% (7/28) are within 1 Mb of a centromere and 43% (12/28) are located on Chromosome 7 (Johnson et al. 2006).

Although the major role of REST was initially believed to be in non-neuronal cells, it is now known to function in neural progenitor cells and neurons as well, where it mediates spatial and temporal expression of neuronal genes (Ballas et al. 2005). REST also appears to be essential for embryonic development. It is ubiquitously expressed in E8.5 and E9.5 mouse embryos, and in Rest null mice, growth retardation, widespread apoptosis and morphological changes are observed starting around E9.5. Rest null mice die at E11.5 (Chen et al. 1998). Several studies have identified additional aspects of REST function. For example, REST regulates a cardiac fetal gene expression program and reactivates this program in cardiac dysfunction (Bingham et al. 2007; Kuwahara et

al. 2003). In vascular smooth muscle cells, REST suppresses the expression of a critical potassium channel gene, and thus regulates both normal and diseased states of the cell (Cheong et al. 2005). REST is also implicated in the pathogenesis of multiple neurological diseases including Down syndrome and Huntington's disease. In Down syndrome, trisomic levels of DYRK1A, a REST regulator, results in reduced REST levels in embryonic neurons and increased levels in adult neurons (Canzonetta et al. 2008). This aberrant REST expression causes dysregulation of its target genes and contributes to the neurological changes seen in Down syndrome (Canzonetta et al. 2008; Lepagnol-Bestel et al. 2009). In Huntington's disease, the normal ability of Huntingtin protein to sequester REST in the cytoplasm of neuronal cells is abrogated, resulting in the translocation of REST to the nucleus and consequent repression of neuronal genes such as BDNF (Zuccato et al. 2003).

REST can function as either a tumor suppressor or an oncogene depending on the cellular context. In cells where REST expression normally results in repression of neuronal genes, REST functions as a tumor suppressor. Diminished REST expression is associated with transformation of human mammary epithelial cells, and its deregulation has been associated with several non-neural tumors including breast, colon, and small cell lung cancers (Coulson 2005a; Coulson et al. 2000; Wagoner et al. 2010; Westbrook et al. 2005). This tumor suppressor function of REST appears to be mediated via several mechanisms. One study showed that human mammary epithelial cells lacking REST exhibit increased PI3K signaling and are dependent upon this pathway for their transformed phenotype (Westbrook et al. 2005). A second study showed that REST knockdown in immortalized primary human cells induced oncogenic transformation via de-repression of the proto-oncogene TrkC (Mulligan et al. 2008). A third study showed

that REST knockdown in breast cancer cell lines resulted in increased expression of the oncogene TAC1, and that REST expression was inversely proportional to tumor cell aggression (Reddy et al. 2009). A fourth study identified the E3 ubiquitin ligase β-TRCP as an upstream regulator of REST, and showed that β-TRCP overexpression causes oncogenic transformation of human mammary epithelial cells via degradation of REST protein, thus validating the tumor suppressor role of REST (Westbrook et al. 2008). However, in neuronal cells where REST is not normally expressed, or functions as an activator, abnormal activation of REST's transcriptional repression program results in tumorigenesis. An oncogenic role for REST has been established in malignancies of the brain, namely, medulloblastoma, neuroblastoma, and gliobalstoma, where REST expression is upregulated (Conti et al. 2012; Lawinger et al. 2000; Palm et al. 1999; Su et al. 2006). A study examining REST's oncogenic role in medulloblastoma formation showed that ectopic expression of REST and c-Myc in neural stem cells caused cerebellum-specific tumors by blocking neuronal differentiation and thus maintaining the "stemness" of these cells (Su et al. 2006). Another study showed that REST knockdown reduces self-renewal potential of tumorigenic-competent glioblastoma cells and induces neuronal differentiation and cell death programs (Conti et al. 2012). Additionally, these REST-depleted glioblastoma cells were shown to have elevated expression of the REST target microRNA miR-124, accompanied by reduced expression of the phosphatases SCP-1 and PTPN12, which have previously been shown to be miR-124 targets (Sun et al. 2011; Visvanathan et al. 2007). SCP-1 is known to play a crucial role in suppression of neurogenic differentiation and PTPN12 has been shown to be involved in promotion of migration, invasion and metastasis of transformed cells

(Visvanathan et al. 2007; Zheng et al. 2011). Thus one mechanism by which REST executes its oncogenic function might be through up-regulation of miR-124 targets.

REST displays differential regulation depending on the developmental stage, cell type, target gene and specific stimuli. For example, in non-neuronal cells and neural progenitors, REST inhibits expression of the brain specific microRNA miR-124a, thus allowing the persistence of non-neuronal transcripts. As progenitors differentiate into mature neurons, REST leaves miR-124a gene loci, and non-neuronal transcripts are degraded selectively. Thus, the combined transcriptional and posttranscriptional consequences of REST action maximize the contrast between neuronal and non-neuronal cell phenotypes (Conaco et al. 2006). Additionally, variations in the assembled co-repressor complexes, the affinity of REST protein for the RE1 site and the availability of REST result in a distinct set of epigenetic marks in neuronal cells, differentiated non-neuronal and ES cells.

The initial step in REST mediated repression is the recruitment of REST to RE1 sites via its DNA binding domain containing eight zinc-finger motifs (Chong et al. 1995). The location or orientation of the RE1 site relative to the enhancer and promoter of the target gene do not affect the repressive ability of REST (Thiel et al. 1998). In addition to the DNA binding domain, REST contains two distinct repressor domains, one located in the N-terminus and the other located in the C-terminus of the protein (Tapia-Ramirez et al. 1997; Thiel et al. 1998). Although the N- and C-terminal domains of REST can function independently, together, they provide multiple layers of control, and allow REST to mediate both short-term repression and long-term silencing (Ballas et al. 2005). Repression through the N-terminus domain requires direct interaction of the co-repressor SIN3A/B with this domain (Grimes et al. 2000; Huang et al. 1999; Roopra et

al. 2000), while repression through the C-terminus domain requires interaction with the co-repressor CoREST (Andres et al. 1999).

SIN3 bound to the N-terminal repressor domain of REST recruits the histone deacetylases HDAC1 and HDAC2, which deacetylate lysine residues of nucleosomal core histones in the promoter region, thus limiting accessibility of DNA to transcription factors (Roopra et al. 2004). Retinoblastoma associated proteins RbAp46 and RbAp48 are also part of the SIN3 complex, and appear to function as a molecular bridge between histone metabolic enzymes and core histones (Parthun et al. 1996; Zhang et al. 1998). Additional factors in the core SIN3 complex include SIN3a associated proteins SAP18 and SAP30, which act as bridging peptides to recruit factors such as the RB associated protein RBP1 and the tumor suppressor p33ING1b (Fleischer et al. 2003; Zhang et al. 1997). RBP1 can in turn recruit RB family members to the SIN3 complex, and p33ING1b can recruit the BRG1 based hSWI-SNF chromatin remodeling complex (Kuzmichev et al. 2002). Although multiple cofactors can be recruited to the RE1 site, not all of them are recruited at all genes. Thus the co-repressor complexes can tailor the level of repression depending on developmental stage and cell type. The SIN3/HDAC complex is associated with a dynamic mode of repression that can alternate between repression and activation, and plays a direct role in regulating differentiated neuronal phenotype via chromatin modification (Roopra et al. 2000).

CoREST is associated with the histone deacetylases HDAC1 and HDAC2, the histone H3–K9 methylase G9a, the histone H3–K4 demethylase LSD1, the NADH-sensitive co-repressor CtBP, the BRG1 based hSWI-SNF chromatin-remodeling complex, and the methyl CpG-binding protein MeCP2 (Andres et al. 1999; Battaglioli et al. 2002; Lunyak et al. 2002; Roopra et al. 2004; Shi et al. 2004).

REST mediated repression requires the coordinated activity of histone-deacetylase, histone-demethylase and histone-methylase enzymes. Once REST and its co-repressor complexes are at the RE1 site, the chromatin-remodeling enzyme BRG1 recognizes acetylated H4–K8 residues in the RE1 region, and repositions nucleosomes with respect to DNA to stabilize REST binding (Ooi et al. 2006). HDACs then remove acetyl groups from H3 and H4 lysine residues (Roopra et al. 2000). H3–K9 de-acetylation allows G9a to mono- or dimethylate the same residues, and also stimulates LSD1 to remove di- and monomethylation marks from H3–K4 (Lee et al. 2005; Roopra et al. 2004). Thus epigenetic marks associated with transcriptional activation, namely, histone hyper-acetylation and H3–K4 methylation are removed and replaced by epigenetic marks associated with gene repression, namely, histone hypo-acetylation and H3–K9 methylation. H3–K9 methylation also stimulates recruitment of the heterochromatic protein HP1, which can mediate chromatin condensation (Lunyak et al. 2002; Roopra et al. 2004; Wood et al. 2003).

Embryonic stem (ES) cells retain the potential to differentiate into derivatives of all three germ lineages, and therefore must maintain somatic cell-specific genes in a transcriptionally repressed, but activation-permissive state. The activation-permissive state is achieved at least partly, by global enrichment in di- and tri- methylation of H3–K4, as well as in acetylation of histones H3 and H4 (Kimura et al. 2004). For neuronal genes, there is an additional modification: the H3–K9 methylation and DNA methylation associated with RE1 sites in non-neuronal differentiated cells is absent in ES cells (Ballas, Mandel 2005; Ballas et al. 2005). Thus, in spite of the presence of REST and its co-repressor complexes at their RE1 sites, REST target genes in ES cells are maintained in an activation-permissive state.

During embryonic neuronal differentiation, REST mediated repression is maintained at the neural stem cell state, but the transition to neuronal progenitor cell is accompanied by proteosomal degradation of unbound REST protein, and terminal differentiation is accompanied by transcriptional repression of REST (Ballas, Mandel 2005). For neuronal genes that are constitutively expressed at steady levels in mature cortical neurons, REST is absent from the RE1 site, chromatin is relaxed, and transcription is activated. For genes that have a dual expression mode, CoREST and MeCP2 complexes bound to mCpGs in the promoter region provide additional regulatory control. Transition to mature cortical neurons is accompanied by loss of the REST complex from the RE1 site, but the mCpG site associated CoREST and MeCP2 complexes stay bound. This arrangement allows low-level gene expression with potential for up-regulation. When the neuron receives a stimulus such as membrane depolarization, the CoREST complex remains bound to chromatin, but the MeCP2 complex is removed, allowing higher levels of expression (Ballas, Mandel 2005).

During differentiation of adult neural stem cells to mature neurons, a small, noncoding double-stranded (ds) RNA plays a critical role in mediating neuronal differentiation. The sequence defined by this dsRNA is RE1, and it can trigger expression of neuron-specific genes through interaction with REST protein. REST remains on neuronal gene chromatin, but the dsRNA-REST interaction converts REST from a repressor to an activator by dismissal of corepressors and recruitment of coactivators (Kuwabara et al. 2004).

In differentiated non-neuronal cells, REST is available at higher levels than in ES cells and in neural progenitor cells, and the repressive epigenetic modifications are more stringent. In addition to histone hypo-acetylation, the RE1 regions of differentiated non-

neuronal cells also exhibit elevated H3–K4 demethylation, DNA hyper-methylation, and elevated levels of H3–K9 di-methylation (Ballas et al. 2005). HP1 mediates condensation of the targeted chromatin, resulting in stable gene silencing (Ballas, Mandel 2005). Thus, unlike the scenario in ES cells, REST target genes in differentiated non-neuronal cells are not maintained in an activation-permissive state (Ballas et al. 2005).

In conclusion, REST regulates a large number of genes, depending on the cellular context, on the amount of REST protein present in the cell, and on the affinity of the REST protein complex toward its specific target gene in the given cellular environment. By using a variety of combinatorial mechanisms to differentially influence the expression of each of its many target genes, REST plays a role in regulating both normal and abnormal development.

Chapter 2. REST Is Not Required For hESC Self-Renewal, But Improves Survival

2.1. Introduction

One of the essential characteristics of pluripotent stem cells is continuous self-renewal, or the ability to go through numerous cycles of cell division while maintaining the undifferentiated state. A human embryonic stem cell is also defined by the expression of several transcription factors and cell surface proteins. The transcription factors OCT4, NANOG, and SOX2 form the core regulatory network that ensures the suppression of genes that lead to differentiation and the maintenance of pluripotency (Boyer et al. 2005). The cell surface antigens most commonly used to identify hESCs are the globoseries glycolipids stage specific embryonic antigen 3 and 4 (SSEA-3 and SSEA-4) and the high molecular weight glycoproteins TRA-1-60 and TRA-1-81. Additional molecular markers include alkaline phosphatase activity, expression of LIN28 (an RNA-binding protein that is highly expressed in hESCs and is important for their growth and survival), DNMT3b (a DNA methyltransferase required for de novo DNA methylation during development), the TGF beta superfamily member GDF3 (a growth factor implicated in cell renewal as either a nodal agonist or a BMP antagonist) and TDGF-1 (a co-receptor for the stem cell maintenance factor nodal) (Darr, Benvenisty 2009; Hough et al. 2009; Peng et al. 2011).

Although the first hESCs were derived in 1998, the regulation of hESC self-renewal remains to be fully characterized. A commonly used method for maintaining undifferentiated hESCs in culture is to grow them on a feeder layer of mouse embryonic fibroblasts (MEFs) in media containing knockout serum replacement (KOSR) and basic

fibroblast growth factor (bFGF or FGF-2). Feeder-free propagation of hESCs is feasible on Matrigel-coated plates and conditioned medium (medium conditioned by overnight incubation with feeder cells). Matrigel (BD Biosciences) is a solubilized basement membrane matrix preparation of mouse sarcoma (Engelbreth-Holm-Swarm (EHS) tumor) origin, which contains extracellular matrix proteins such as laminin, collagen IV, heparan sulfate proteoglycans, and entactin/nidogen. BD Matrigel Matrix also contains TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, and other growth factors that occur naturally in the EHS tumor. Thus Matrigel is not a well-defined matrix, and has significant batch-to-batch variations that can produce variability in experimental results (Hughes et al. 2010; Vukicevic et al. 1992).

Several groups have developed serum-free, xeno-free or defined media conditions for culturing hESCs, some of which, such as mTeSR1 (serum-free complete medium, STEMCELL Technologies Inc), TeSR2 (serum-free, xeno-free complete medium, STEMCELL Technologies Inc) and STEMPRO (defined, serum-free, xeno-free complete medium, Invitrogen), can sustain hESCs in feeder-free conditions, and are commercially available. To replace Matrigel with defined alternatives, several research groups have used extracellular matrix proteins such as laminin (Beattie et al. 2005; Miyazaki et al. 2012), vitronectin (Braam et al. 2008), collagen I (Jones et al. 2010), fibronectin (Amit et al. 2004), a combination of laminin and vitronectin (Heng et al. 2012) and a combination of human collagen IV, vitronectin, laminin and fibronectin (Ludwig et al. 2006). Commercially available growth substrates/surfaces include CELLstart (Life Technologies), Geltrex (designed for use with STEMPRO, Invitrogen), Synthemax (Corning) and Nunclon Vita Stem Cell Culture Surface (Thermo Scientific).

Studies in mESCs have demonstrated that Rest is directly regulated by the core pluripotency transcription factors Oct4, Nanog and Sox2, that Nanog is a direct Rest target, and that 107 genes including Rest itself are targets of all four factors (Johnson et al. 2008; Loh et al. 2006). A study examining the role of Rest in mESCs showed that attenuation of Rest expression compromises the self-renewal potential of mESCs and leads to up-regulation of differentiation markers (Singh et al. 2008). Furthermore, just as in mESCs, OCT4, NANOG and SOX2 have been shown to directly and positively regulate REST in hESCs as well (Boyer et al. 2005). Hence it was expected that REST would be important for hESC self-renewal, and that knocking down REST in hESCs would lead to differentiation. The data proved otherwise.

2.2. Results

In order to evaluate the role of REST in hESC maintenance, the inducible Tet-On TRIPZ vector (Open Biosystems, http://www. openbiosystems.com), in which doxycycline activates the expression of a TurboRFP reporter in addition to the shRNAmir (shRNA adapted to a microRNA backbone), was utilized. REST shRNAmir vector was used to knockdown REST, and a scrambled Non-Target shRNAmir vector was used as a control. As shown in **Figure 2-1**, largely homogeneous RFP positive colonies were obtained in both the Non-Target control (NT) and REST knockdown (REST KD) hESC lines (H9 is shown). Since REST is a transcription repressor, expression of REST as well as its direct targets was evaluated via qPCR to verify REST KD. As can be seen in **Figure 2-2**, REST expression is down-regulated, and expression of REST targets SYP, SYT4 and TRKC is up-regulated. Immunofluorescence analysis with REST antibody verified REST KD at the protein level (**Figure 2-3**). QPCR (**Figure 2-4**A) and Western blot analysis (**Figure 2-4**B) were performed to determine whether

REST KD results in decreased expression of OCT4, NANOG and SOX2, and in both cases there was no change in expression of these signature pluripotency markers. FACS analysis for cell surface pluripotency markers showed no decrease in expression of SSEA4 and TRA 1-81 in H1 REST KD hESCs compared to control, confirming that REST is not required for maintenance of hESCs (**Figure 2-5**). **Figure 2-6** is a quantitative representation of FACS data from three independent experiments in both H9 and H1 hESCs showing that REST KD does not result in reduced TRA 1-81 expression. REST KD hESCs often appeared to have better survival than NT hESCs after passaging, and this observation was validated by the apoptosis assay that indicated lower levels of apoptotic cells among both H9 and H1 REST KD hESCs, compared to NT (**Figure 2-7**). In summary, the data show that REST is not required for hESC maintenance, but may play a role in regulation of hESC survival.

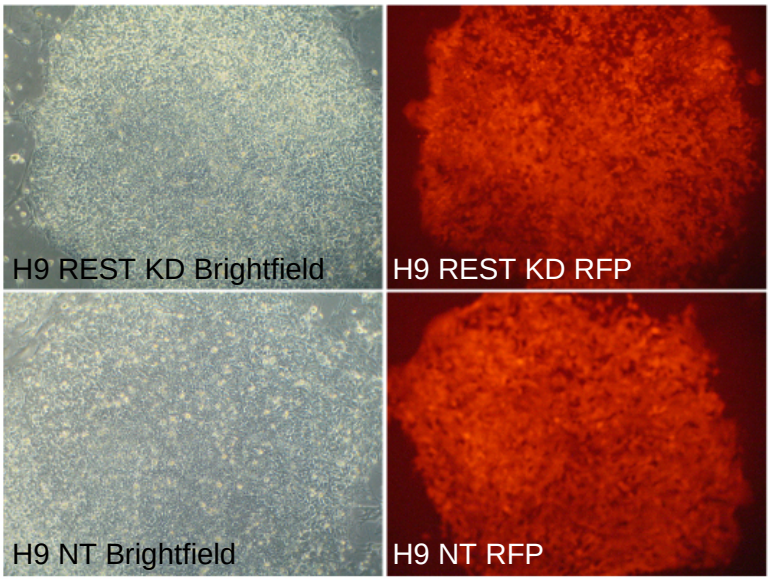


Figure 2-1: REST KD in H9 hESCs. RFP serves as a reporter for shRNA expression.

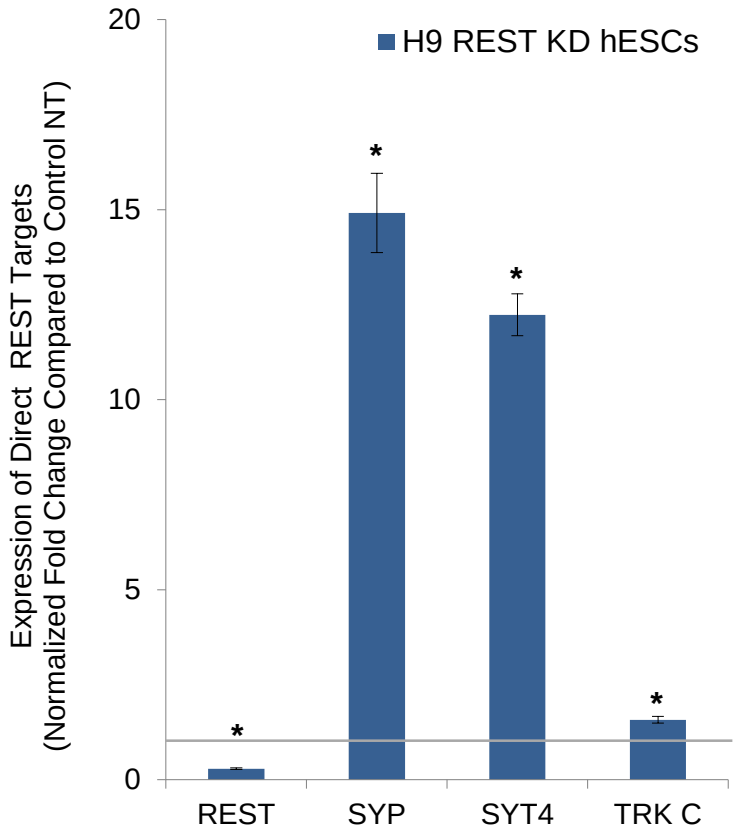


Figure 2-2: REST expression is decreased, and expression of REST target genes is increased upon REST KD. H9 hESCs are shown but similar results were seen for H1 and UCLA1 hESCs. REST levels are statistically significantly reduced compared to control NT hESCs ($p<0.0001$). Direct REST target genes (SYP, SYT4 and TRKC) are statistically significantly increased compared to control NT hESCs ($p<0.004$).

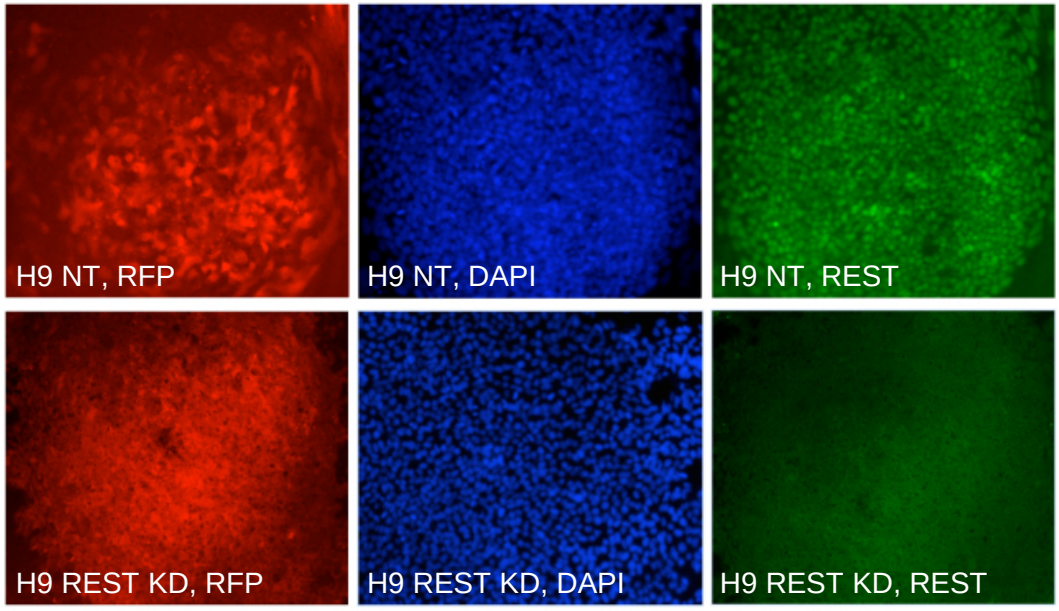


Figure 2-3: Immunofluorescence analysis demonstrates reduced REST protein expression in REST KD H9 hESCs.

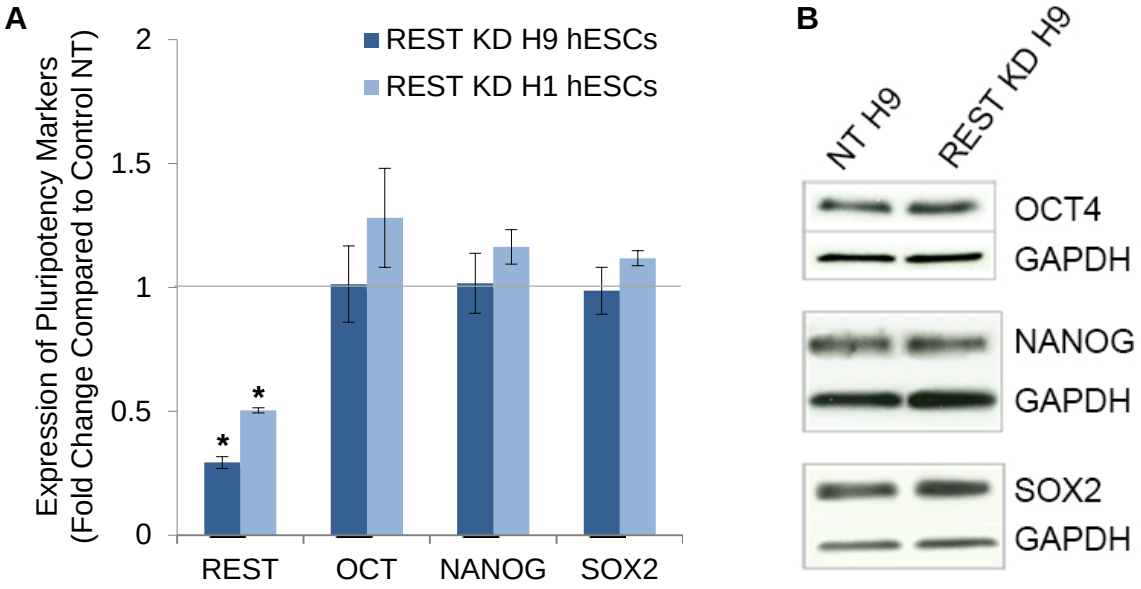


Figure 2-4: There is no change in pluripotency marker expression upon REST KD. A: Expression of pluripotency markers using qPCR. REST levels are statistically significantly knocked down in H9 and H1 REST KD hESCs ($p<0.0001$ in H9 and $p<0.0001$ in H1), and there is no change in the expression of OCT4, SOX2 or NANOG, compared to control NT. B: Western blot shows no change in protein levels of OCT4, SOX2 or NANOG in H9 REST KD hESCs compared to control NT.

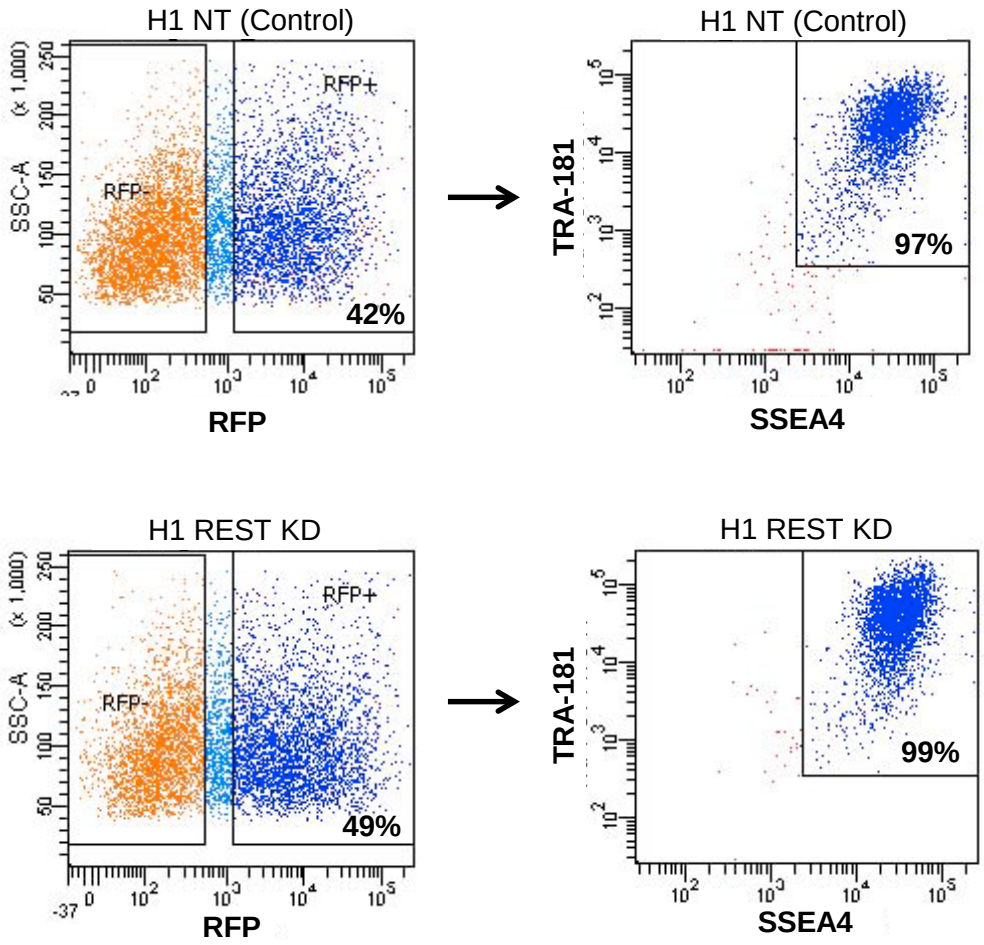


Figure 2-5: A representative FACS plot for SSEA4 and TRA 1-81 expression in H1 cells shows no change in cell surface pluripotency marker expression in REST KD hESCs. Cells with high RFP expression were gated for TRA-1-81, SSEA4 double positive expression. There is no decrease in percentage of TRA 1-81, SSEA4 double positive REST KD cells compared to control NT.

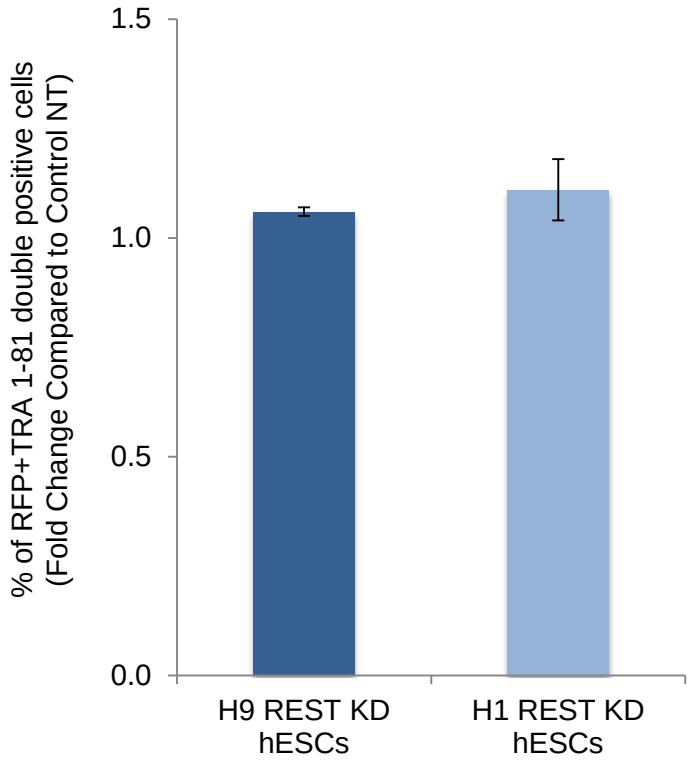


Figure 2-6: Quantitative representation of FACS analysis from three independent experiments in H9 and H1 hESCs shows that there is no decrease in TRA-1-81 expression in H9 or H1 REST KD hESCs compared to control NT.

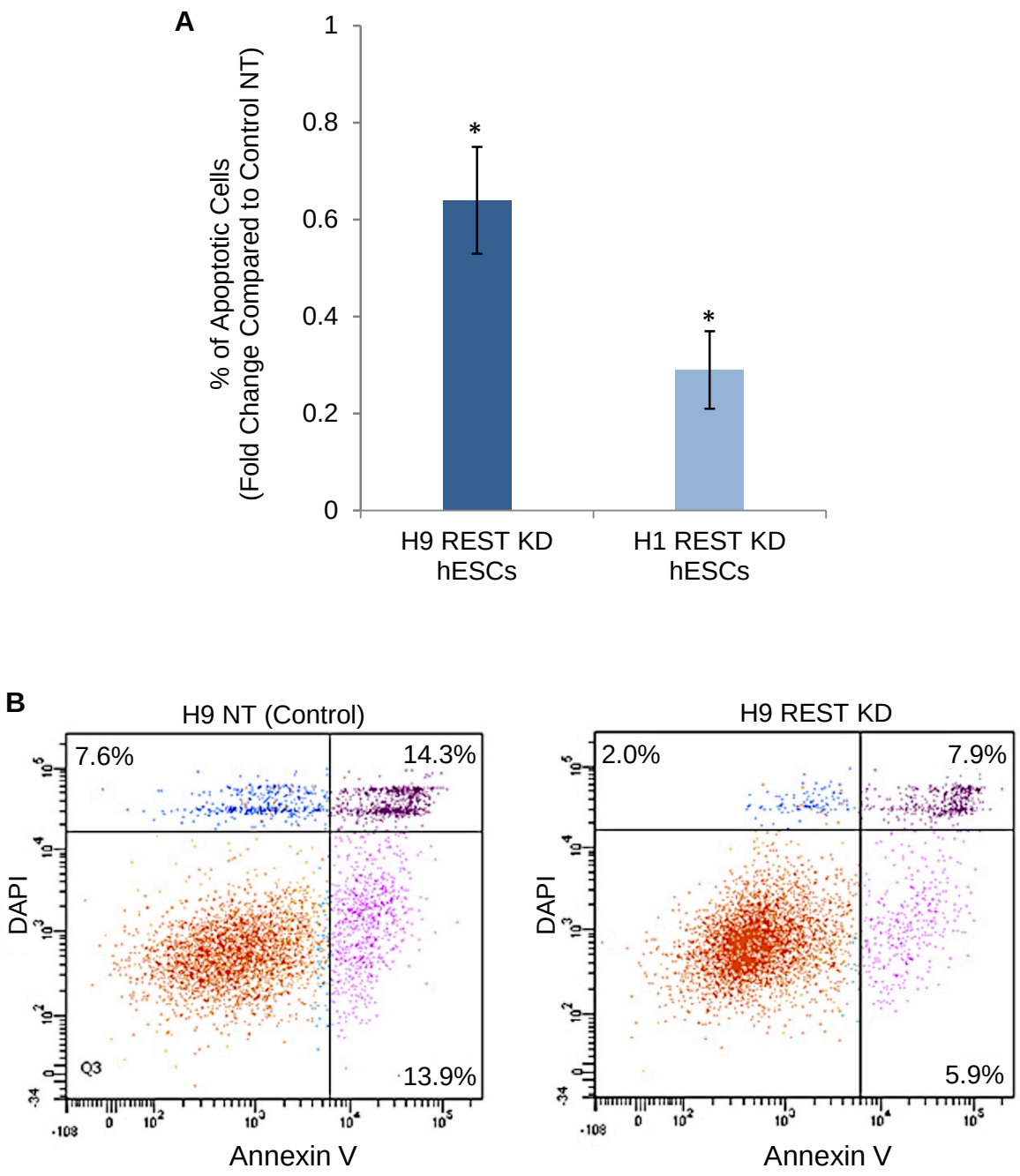


Figure 2-7: REST KD cells have increased survival. A: FACS analysis for Annexin V shows that REST KD hESCs have statistically significant improvements in survival as demonstrated by reduced levels of apoptotic cells ($p<0.032$ in H9 and $p<0.002$ in H1) compared to control NT hESCs. B: Representative FACS plot for apoptosis measurement in H9 hESCs. Cells in early apoptosis are positive for Annexin V only, cells in late apoptosis are positive for both Annexin V and DAPI, and dead cells are positive for DAPI only.

2.3. Discussion

The data clearly demonstrates that reduced REST expression does not affect hESC maintenance, in contradiction to the mESC study by Singh et al. (2008), but in agreement with several subsequent studies which showed that REST is not required for mESC maintenance (Buckley et al. 2009; Covey et al. 2012; Jorgensen, Fisher 2010; Jorgensen et al. 2009a; Jorgensen et al. 2009b; Yamada et al. 2010). Recently, the authors of Singh et al. (2008) have conceded that their earlier finding cannot be recapitulated when their REST deficient mESCs are grown in the presence of either feeder cells or laminin, or grown for more than two passages on the gelatin coated plates used in their earlier study (Singh et al. 2012b).

The present study also demonstrates that reduced REST expression enhances hESC survival, but the mechanisms by which REST expression affects survival are not clear. Pyle et al. (2006) have shown that TRKC, a receptor for the nerve growth factor-related (NGFR) family of neurotrophins, improves hESC survival via activation of the PI3K/AKT pathway. TRKC is a REST target, and it is slightly upregulated in REST KD cells (**Figure 2-2**), but there is no change in PI3K/AKT activation in REST KD cells compared to NT controls (Chapter 5, **Figure 5-1**C). Wang et al. (2009) have observed that hESCs are subject to constant anoikis (a subtype of apoptosis induced by detachment of adherent cells from the extracellular matrix), which is escalated in the absence of bFGF. The authors further demonstrated that bFGF represses anoikis and apoptosis in hESCs by inhibiting caspase activities via activation of ERK and AKT and down-regulation of Bcl-2-interacting mediator of cell death (BIM). Since REST KD results in up-regulation of the MAPK/ERK pathway (see Chapter 5), it would be

worthwhile to investigate whether elevated ERK activation contributes to increased survival of REST KD hESCs.

2.4. Future Directions

Wang et al. (2009) emphasized that they were only able to uncover the anti-anoikis effect of bFGF by using the defined medium mTeSR1 and the basement membrane matrix Matrigel for growing the hESCs, perhaps because the KOSR in undefined medium contains components that favor cell attachment, thus masking the anti-anoikis effect of bFGF. Other groups have shown that Matrigel too contains known and unknown growth factors and other proteins that can introduce experimental variability (Hughes et al. 2010; Vukicevic et al. 1992). Hence future experiments should be carried out in chemically defined culture conditions, so as to elucidate the effect of REST activity on survival without the confounding contributions of multiple unknown and variable factors associated with KOSR, MEFs, and Matrigel. REST KD and NT hESCs grown in defined culture conditions in the presence and absence of MAPK/ERK and/or PI3K/AKT inhibitors should be evaluated for changes in anoikis and apoptosis as described in Wang et al. (2009). Performing microarray-based gene expression analysis on REST KD and NT hESCs from three independent lines grown in defined culture conditions might also help identify additional pro- and anti-apoptotic pathways that may be differentially affected by REST levels.

Chapter 3. Decreased REST Expression Leads to Endoderm / Mesoderm Differentiation Bias in REST KD EBs

3.1. Introduction

One of the most important properties of pluripotent cells is their potential to differentiate into derivatives of all three embryonic germ layers. The differentiated derivatives of hESCs could be used for identification of gene targets for new drugs, testing the toxicity or teratogenicity of new compounds, and for regenerative transplantation therapy (Amit et al. 2000). In order to test the differentiation potential and thus pluripotency of hESCs in vivo, a suspension of hESCs is injected into the testis of immunodeficient mice, and the mice are then monitored for formation of a benign tumor known as a teratoma. The teratomas are usually harvested at 6–8 weeks, fixed, sectioned, stained with hematoxylin and eosin (H&E), and examined for presence of tissues from all three germ layers. To test differentiation potential in vitro, hESCs colonies are harvested by dispase or collagenase treatment and grown under non-adherent conditions in media with no growth factors added. This leads to the formation of differentiated rounded aggregates called embryoid bodies (EBs) (Doetschman et al. 1985; Martin 1981). The EBs can be grown for several days to weeks at which point the differentiation status can be determined by testing for expression of markers from the different germ layers. Commonly used markers are: GATA4, FOXA2, SOX17, ALBUMIN, PDX1, CXCR4 and AFP for endoderm; BRACHYURY, DESMIN, CD34, RUNX1, VEGFR2, MYH6, and MSX1 for mesoderm; and TUJ1, GFAP, PAX6, SEMA3A, NEUROD1, MAP2 and OLIG2 for ectoderm (Cai et al. 2006; Melchior et al. 2008; Takahashi et al. 2007).

A study examining REST function in neural stem cells (NSCs) used the REST-VP16 transgene, in which the repressor domains of REST had been replaced with the activation domain of herpes simplex virus protein VP16 (Su et al. 2004). Activation of REST targets by the REST-VP16 transgene in NSCs was sufficient to cause neuronal differentiation (Su et al. 2004), thus establishing a role for REST in repressing neuronal differentiation. A study examining REST function in neural differentiation of mouse embryonic stem cells (mESCs) showed that modulation of REST protein levels could regulate the transition from a pluripotent stem cell to a neural progenitor cell and from progenitor to mature neuron (Ballas et al. 2005). A study attempting to elucidate the role of REST in self-renewing mESCs ostensibly showed that REST plays a crucial role in maintaining mESC self-renewal and pluripotency (Singh et al. 2008). Using mESCs with heterozygous deletion of REST as well as mESCs with siRNA mediated REST KD, the study showed that attenuation of REST expression caused a loss of self-renewal and led to the expression of differentiation markers for multiple lineages. Conversely, exogenously added REST maintained expression of self-renewal markers in EBs. The study also stated that REST binds to the gene chromatin of a set of miRNAs that potentially target self-renewal genes. Additionally, it demonstrated that one of these miRNAs, miR-21, specifically suppresses the self-renewal of mESCs, corresponding to the decreased expression of Oct4, Nanog, Sox2 and c-Myc. Thus, the authors claimed, “REST is a newly discovered element of the interconnected regulatory network that maintains the self-renewal and pluripotency of mouse ES cells” (Singh et al. 2008). However, several other groups have tried and failed to recapitulate the findings of Singh et al. (2008), and the role of REST in mESCs has been a topic of much debate (Buckley et al. 2009; Jorgensen, Fisher 2010; Jorgensen et al. 2009a; Jorgensen et al. 2009b).

Furthermore, the Yamada group showed that while Rest is not required for maintenance of mESC pluripotency, it plays a role in the suppression of pluripotency genes upon the early differentiation. The authors showed that REST overexpression caused decreased expression of Nanog, Oct4 and Fgf5 (an epiblast marker), increased expression of endoderm marker Gata4, and rapid differentiation (Yamada et al. 2010). Conversely, REST knockout resulted in increased Nanog expression during early differentiation. Two years later, the same group used Rest conditional knockout mice to demonstrate that Rest plays a role in suppressing the expression of neuronal genes in cultured murine neuronal cells in vitro, as well as in non-neuronal cells outside of the central nervous system, but that it is dispensable for embryonic neurogenesis in vivo (Aoki et al. 2012). A study by the Ballas group showed that while REST-null mESCs remain pluripotent and generate teratomas with derivatives of all three germ layers, NSCs lacking REST have reduced self-renewal capacity owing to reduced cell cycle kinetics and precocious neuronal differentiation (Covey et al. 2012). Additionally, REST null NSCs had enhanced potential for neuronal and oligodendrocytic differentiation and diminished potential for astrocytic differentiation. Thus REST promotes NSC self-renewal while restricting the generation and maturation of neurons and oligodendrocytes (Covey et al. 2012).

At the start of this project, the Singh et al. (2008) study that claimed an essential role for REST in maintaining mESC self-renewal was the preeminent publication in the REST/ES cell field. Furthermore, it was known that in both, mESCs and hESCs, the core transcriptional regulators of pluripotency, OCT4, SOX2 and NANOG directly and positively regulate REST (Boyer et al. 2005; Johnson et al. 2008; Loh et al. 2006). Based on these studies, we expected that REST would be important for hESC

maintenance. However, similar to the studies by Yamada et al. (2010) and Covey et al. (2012), the data demonstrates that while REST is not essential for maintenance of self-renewal, it may play a role in regulating lineage differentiation in hESCs.

3.2. Results

REST KD and NT H9 hESCs were able to form teratomas when injected into immunodeficient mice. H&E staining of teratoma sections revealed tissues from all three germ layers in REST KD as well as NT teratomas, thus demonstrating that REST KD did not diminish the differentiation potential of the hESCs (**Figure 3-1**). In order to get a quantitative measure of the role of REST in differentiation, lineage marker expression analysis was performed on EBs from three independent hESC lines (H1, H9 and UCLA1). Q-PCR was used to compare the expression of at least three makers from each of the three germ layers. As can be seen in **Figure 3-2**, **Figure 3-3**, and **Figure 3-4**, REST KD EBs had aberrant differentiation compared to control NT EBs. In general, there was an increase in expression of endoderm or mesoderm, and a decrease in expression of ectoderm markers. Importantly, no increase in endoderm or mesoderm markers was seen without addition of doxycycline (Dox), i.e., when the inducible promoter for the shRNA was not activated (**Figure 3-5** and **Figure 3-6**). To verify the differentiation bias at the protein level, FACS analysis was used to evaluate the expression of three candidate lineage markers (SOX17, BRACHYURY and PAX6) representing each germ layer in REST KD and control NT EBs at day 5. The FACS analysis corroborated the q-PCR data showing that REST KD EBs had increased percentages of SOX17 (endoderm) and BRACHYURY (mesododerm) expressing cells across all three cells lines (**Figure 3-7** and **Figure 3-8**). PAX6 was decreased in H1 and H9 REST KD EBs and increased in UCLA1 REST KD EBs (also seen in qPCR). In

summary, this data shows that REST plays a role in regulating the early stages of EB differentiation. Surprisingly, REST KD biases EB differentiation towards endoderm or mesoderm at the expense of ectoderm.

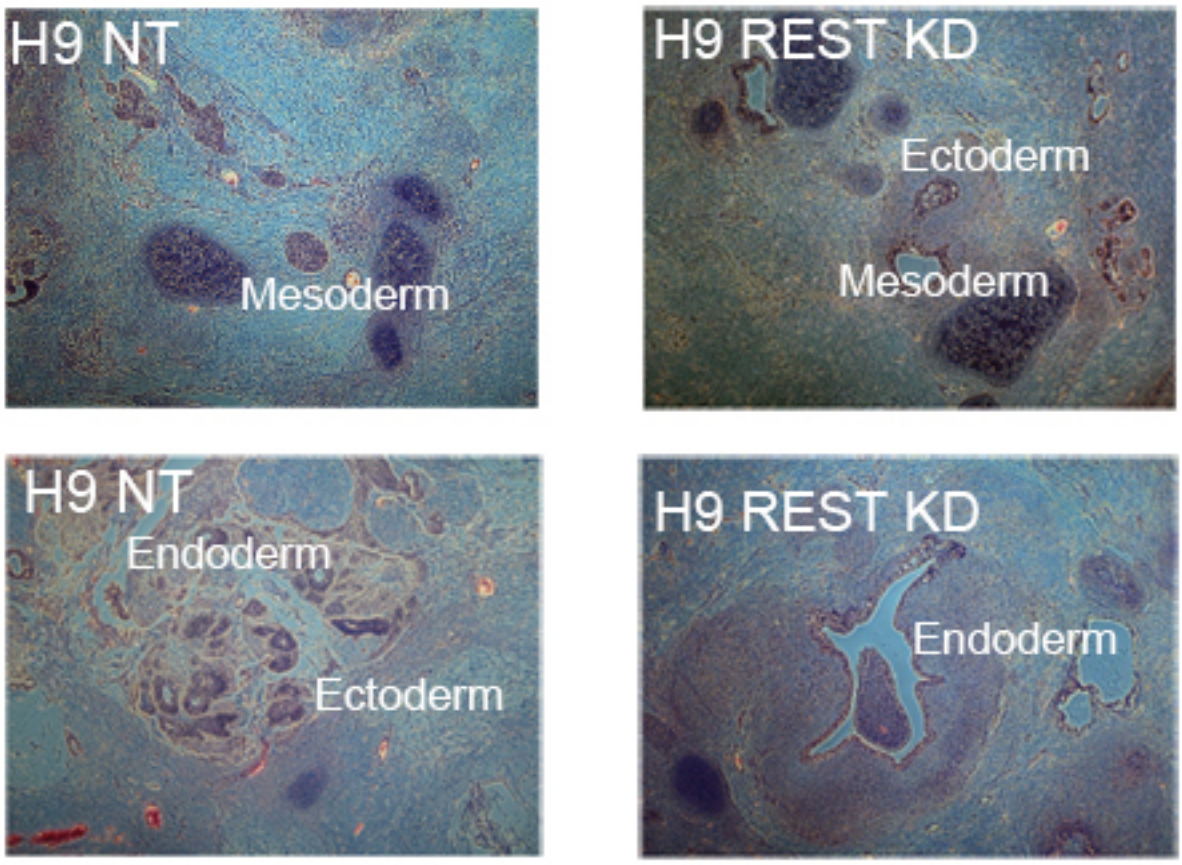


Figure 3-1: REST KD and NT hESCs are pluripotent and form teratomas in vivo. H&E staining reveals differentiation into all three germ layers, shown with labeling in white.

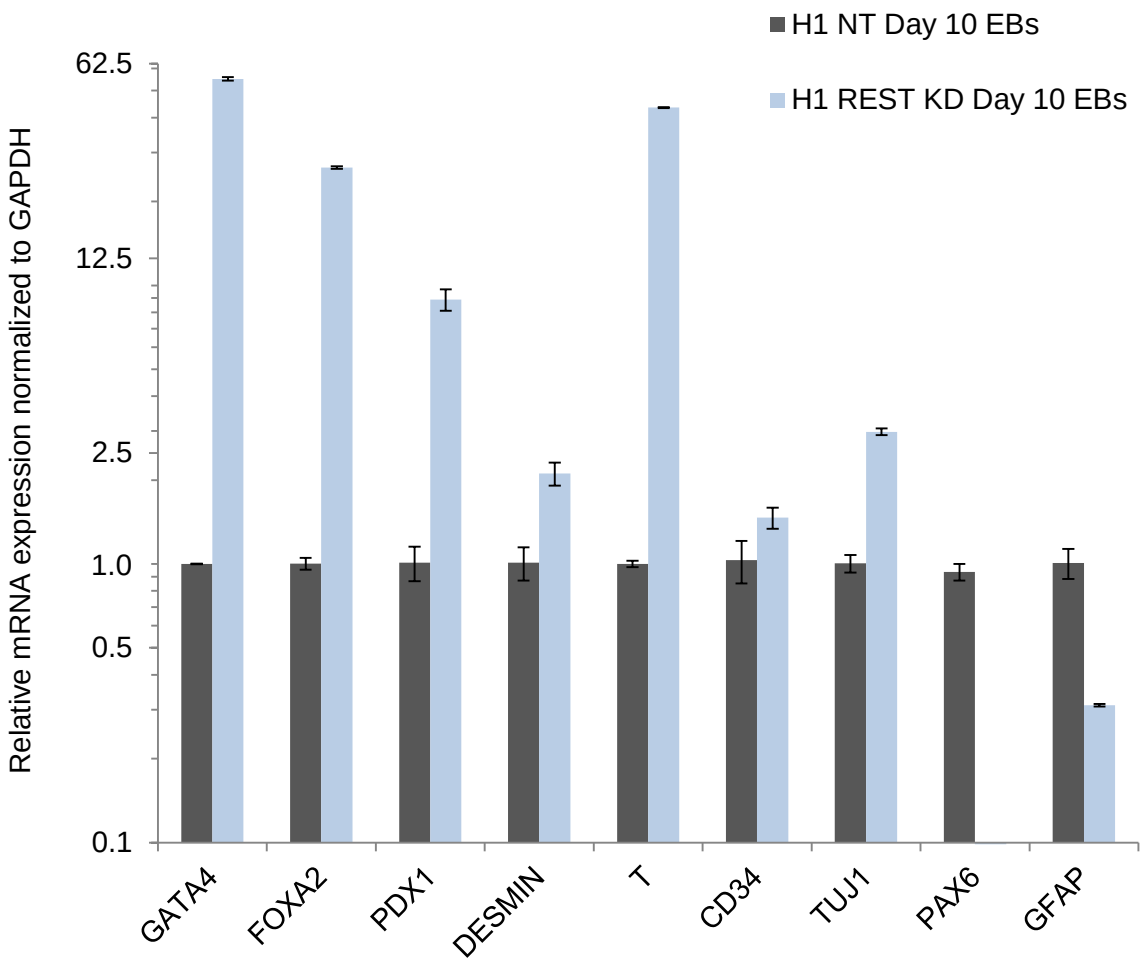


Figure 3-2: Representative graph of lineage marker analysis for H1 REST KD and control NT Day 10 EBs. The average ± standard error of the mean (SEM) of two to three technical replicates is shown.

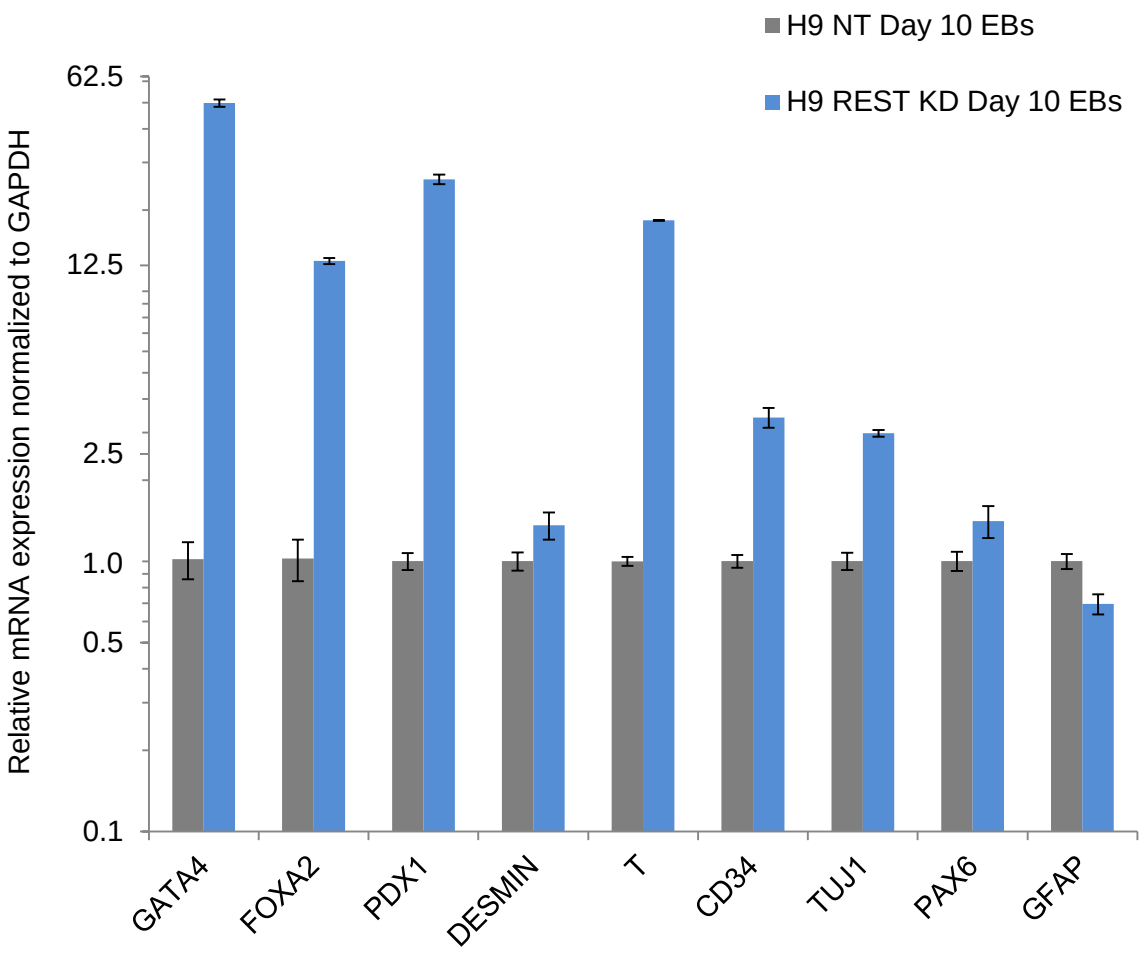


Figure 3-3: Representative graph of lineage marker analysis for H9 REST KD and control NT Day 10 EBs. The average ± SEM of two to three technical replicates is shown.

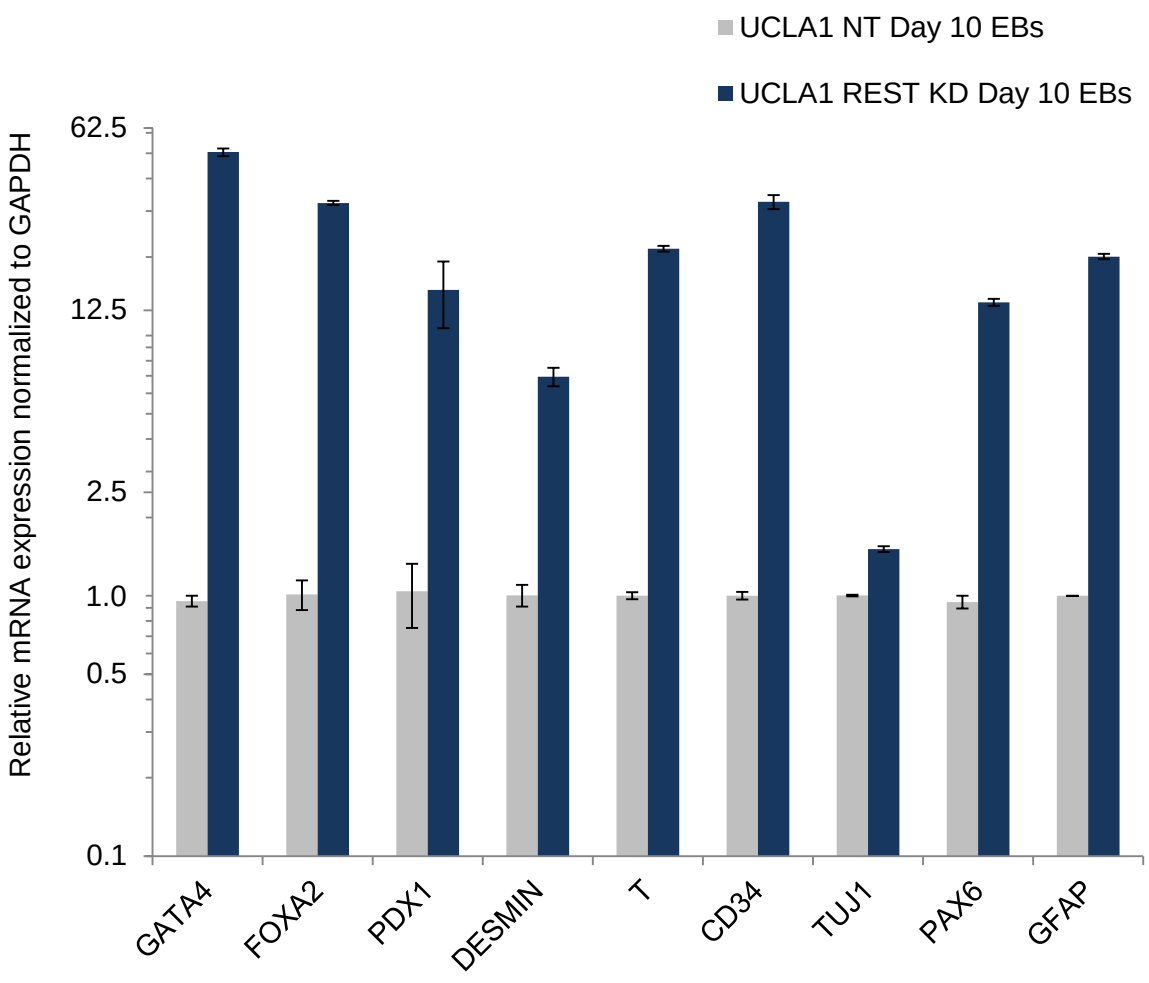


Figure 3-4: Representative graph of lineage marker analysis for UCLA1 REST KD and control NT Day 10 EBs. The average ± SEM of two to three technical replicates is shown.

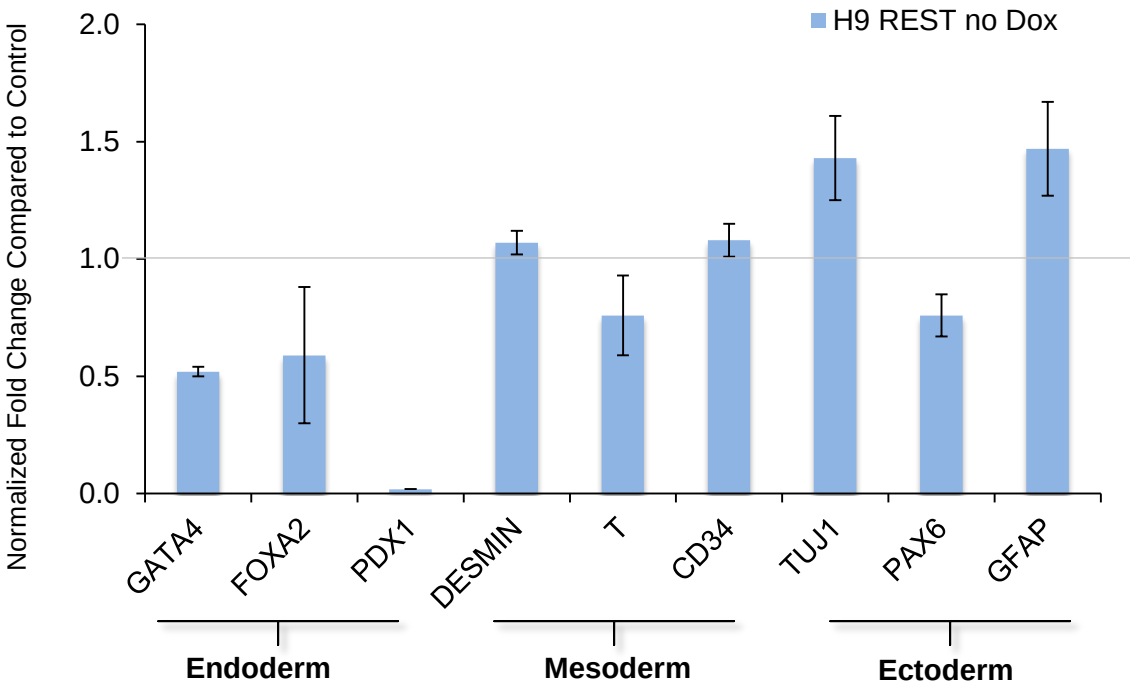


Figure 3-5: Q-PCR evaluation of in vitro differentiation potential in the absence of Dox. A representative graph with internal error bars shows expression fold change in H9 REST (no Dox) day5 EBs compared to H9 NT (No Dox) day5 EBs. The endoderm/mesoderm differentiation bias seen in REST KD EBs is not observed in the absence of Dox.

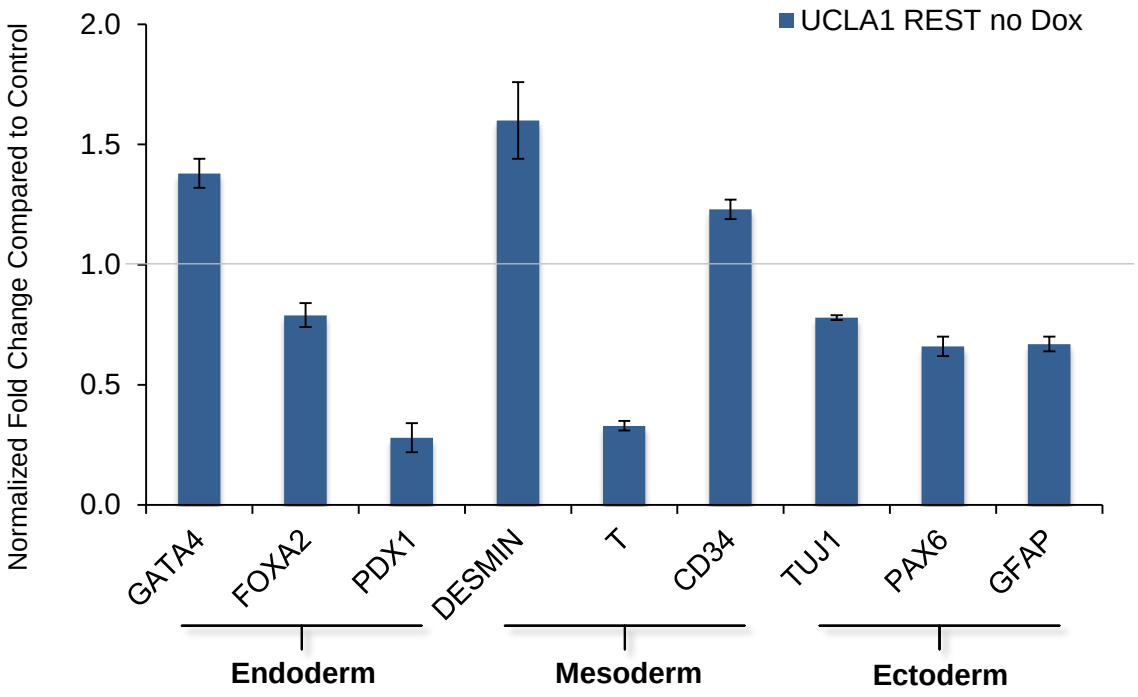


Figure 3-6: A representative graph with internal error bars shows expression fold change in UCLA1 REST (no Dox) EBs compared to UCLA1 NT (No Dox) EBs. The increased differentiation seen in REST KD EBs is not observed in the absence of Dox.

3.3. Discussion

The differentiation data from H1, H9 and UCLA1 lines demonstrates unequivocally that reduced REST expression does not result in loss of pluripotency. REST KD hESCs express all of the traditional pluripotency markers and can differentiate into derivatives of all three germ layers. However, REST KD embryoid bodies (EBs) express higher levels of endoderm and/or mesododerm markers as compared to control NT EBs. This differentiation bias was not observed in the absence of doxycycline, i.e., when REST was not knocked down. Interestingly, while we observed elevated expression of the endoderm marker GATA4 in day 5 and day 10 REST KD EBs, three mESC studies have observed discordant effects of REST KD on GATA4 expression. Jorgensen et al. (2009b) did not observe a difference in Gata4 expression between WT

and Rest-/- EBs at days 2, 4 and 6, whereas Singh et al. (2008) observed increased Gata4 expression in day 3 Rest+/- EBs compared to WT control EBs. Yamada et al. (2010) observed significantly lower expression of Gata4 in the Rest -/- EBs in comparison to the control isogenic EBs, and conversely, an increased number of cells in the periphery of Rest overexpressing EBs that stained positive for Gata4. It is not clear at what day Yamada et al. (2010) evaluated Gata4 expression in these experiments. However, in another experiment where conditional Rest knockout mESCs were cultured under differentiation culture conditions and treated with doxycycline for 24 hr starting at 24 hr to induce Rest knockout, decreased expression of Gata4 was not detectable at 3 days after doxycycline treatment, and later time points were not presented (Yamada et al. 2010). None of these mESC studies (Jorgensen et al. 2009b; Singh et al. 2008; Yamada et al. 2010) presented data evaluating additional endoderm markers under differentiation conditions, nor did they evaluate Gata4 expression in multiple mESC lines. In contrast, we evaluated GATA4, FOXA2 and PDX1 expression in three different hESC lines, at two time points (day 5 and day 10) per line. Additionally, protein expression for a fourth endoderm marker, SOX17, was evaluated at day 5 in three lines. Based on my results, I am confident that REST KD leads to elevated endoderm marker expression in hESCs.

Since REST is a known transcriptional repressor of neuronal genes, we were surprised that REST KD did not bias differentiation towards the neuroectoderm lineage. However, a previous hESC study has shown that neuroectoderm differentiation is inhibited byFGF/ERK signaling (Greber et al. 2011), and, as Chapter 5 will demonstrate, FGF/ERK signaling is elevated in REST KD hESCs and Ebs. Another study has demonstrated that activation of FGF/ERK signaling in differentiating hESCs promotes

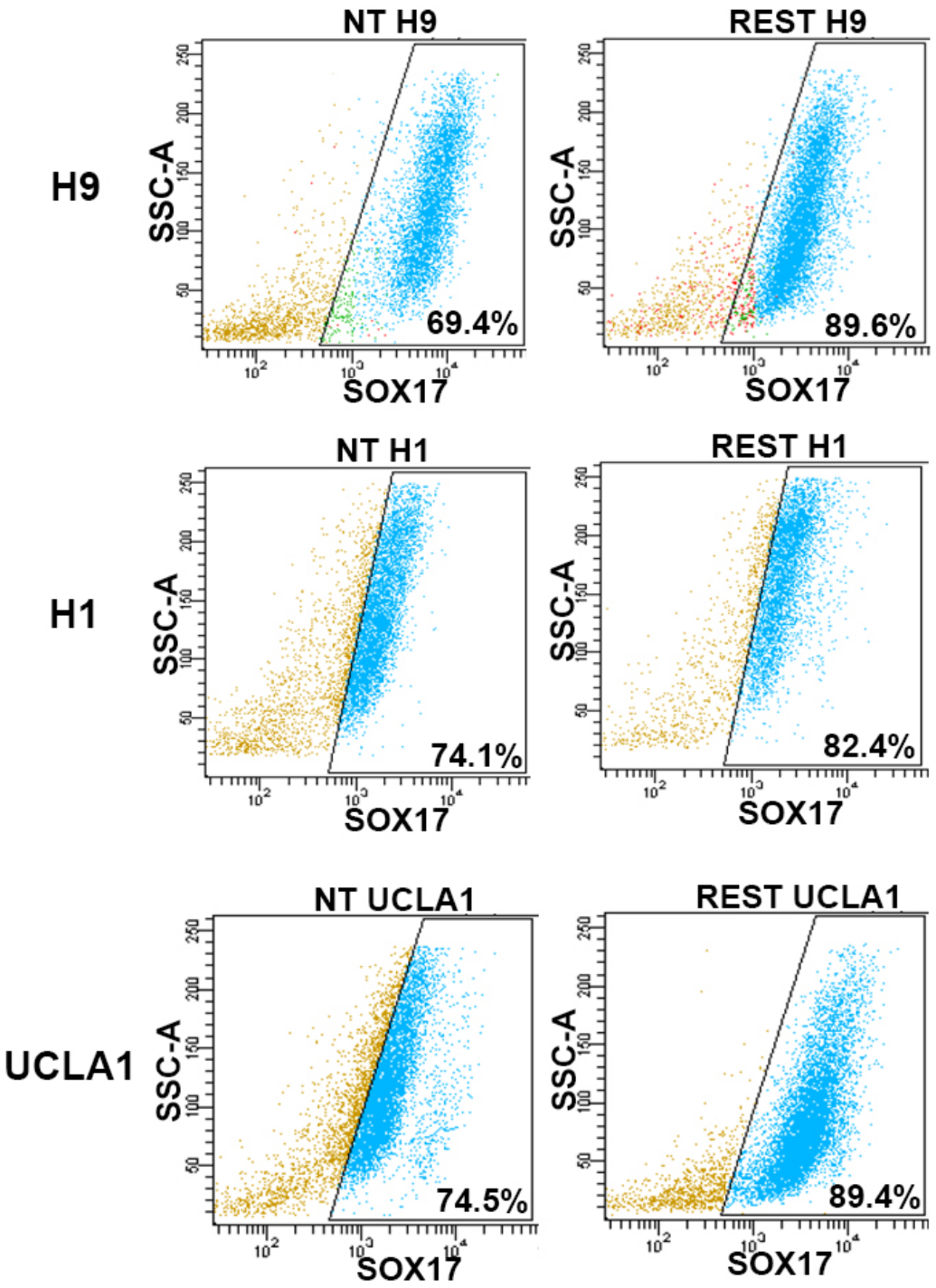
mesendoderm differentiation, marked by elevated Brachyury expression (Yu et al. 2011). In mouse embryos, inhibition of FGF/ERK signaling from the 8-cell to expanded blastocyst stage completely eliminates the Gata6-positive primitive endoderm (PE) cells, whereas addition of exogenous Fgf4 shifts all ICM cells to a PE fate (Yamanaka et al. 2010). Stavridis et al. (2010) have shown that inhibition of FGF/ERK signaling by retinoic acid in differentiating mESCs leads to neural differentiation, and Greber et al. (2010) showed that FGF/ERK signaling inhibits neuroectoderm differentiation in the pluripotent stem cells derived from early post-implantation mouse epiblast (EpiSCs).

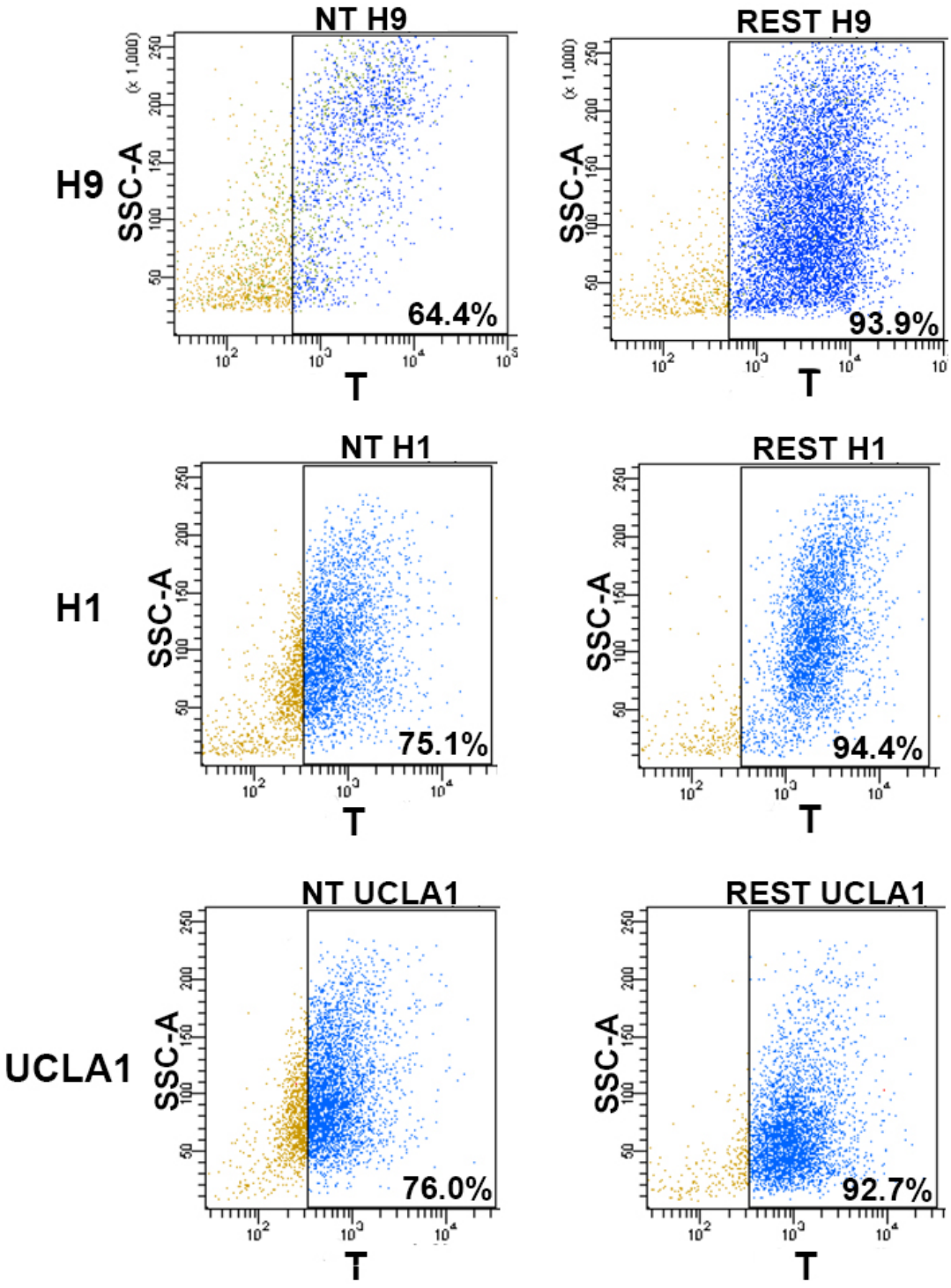
Importantly, members of the ERK superfamily are REST target genes, and provide mechanistic candidates for activation of the changes in lineage differentiation seen in REST KD hESCs. Previous work has also shown that levels of WNT activation are critical to balancing lineage differentiation bias, with high levels of WNT signaling activating mesoderm and/or endoderm, and lower levels being sufficient to generate ectoderm (Blauwkamp et al. 2012). Verifying the importance of balancing WNT signals during lineage specification, UCLA1 REST KD EBs had decreased β-CATENIN expression and slightly increased expression of the neuroectoderm marker PAX6 compared to NT Day 5 Ebs (see Chapter 5). In contrast there was no increase in PAX6 in H1 or H9 REST KD EBs, which had elevated expression of β CATENIN. Thus activation of MEK and WNT signaling in REST KD cells likely play key roles in the lineage bias seen in the EB studies.

3.4. Future Directions

All future experiments should be carried out in chemically defined culture conditions (Bone et al. 2011; Parsons et al. 2012; Yao et al. 2006; Zhou et al. 2010), so as to elucidate the effect of REST activity on the molecular mechanisms underlying

differentiation without the confounding contributions of multiple unknown and variable factors associated with KOSR, MEFs, and even Matrigel (See Chapter 2 introduction). In order to tease apart the contributions of REST KD and aneuploidy to the differentiation profile of REST KD cells, new experiments should be set up where REST KD is induced at the beginning of the differentiation process in karyotypically normal lines. In order to gain a better understanding of the effect of REST activity on the differentiation potential towards each of the three germ lineages, the differentiation profile should be evaluated in vivo via teratoma assays, and in vitro via EB formation and directed differentiation experiments. Teratoma quantification assays (Alva et al. 2011) would help evaluate whether REST KD cells show a differentiation bias in vivo. In order to elucidate the role of ERK and WNT signaling in regulating differentiation of REST KD cells, the in vitro differentiation experiments should be carried out with and without inhibition of ERK and/or WNT pathways. Microarray based gene expression analysis of the in vitro differentiation experiments would help elucidate the downstream consequences of REST KD on the differentiation potential of hESCs in the presence and absence of exogenous lineage specific differentiation signals (directed differentiation v/s EBs), and with or without concurrent activation of ERK and/or WNT signaling.


H9
NT H9
REST H9
SSC-A
SOX17
69.4%
89.6%
H1
NT H1
REST H1
74.1%
82.4%
UCLA1
NT UCLA1
REST UCLA1
74.5%
89.4%


NT H9
REST H9
H9
SSC-A
(x 1,000)
T
64.4%
93.9%
NT H1
REST H1
H1
75.1%
94.4%
NT UCLA1
REST UCLA1
UCLA1
76.0%
92.7%

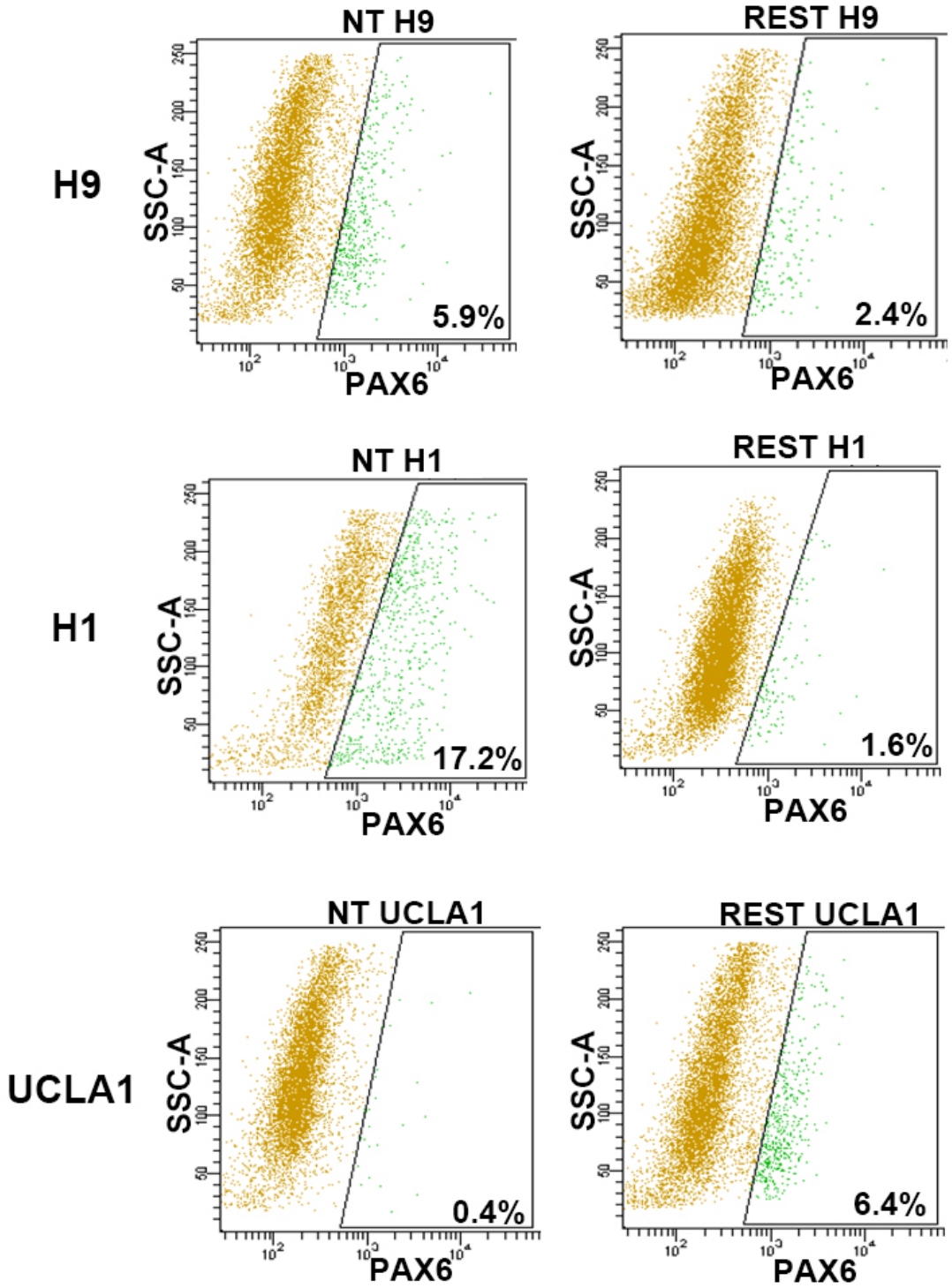
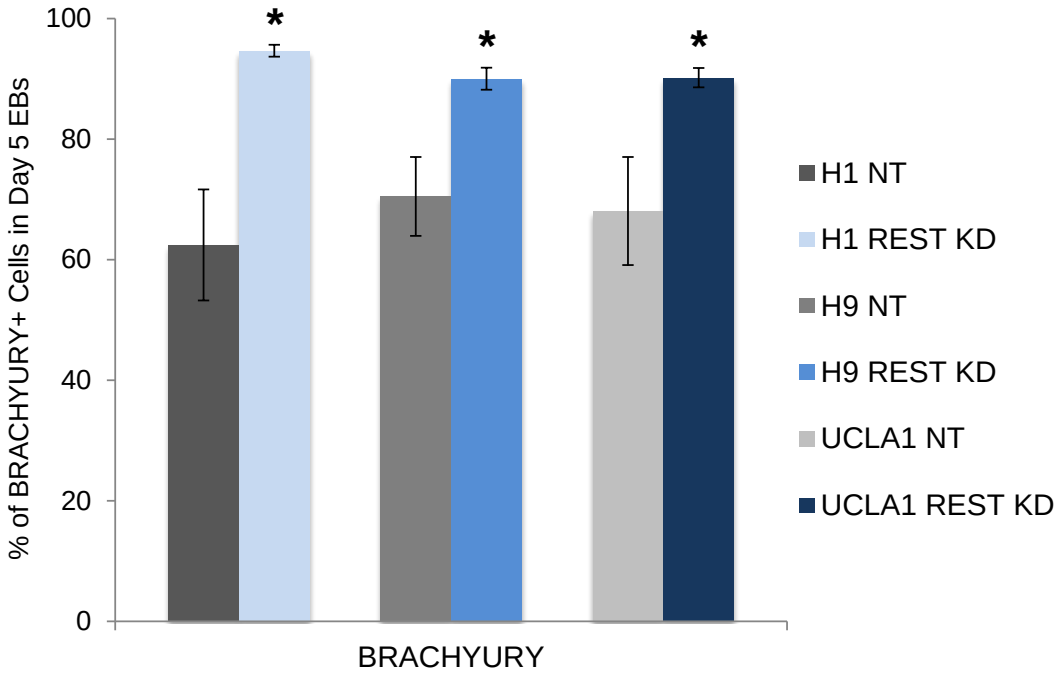
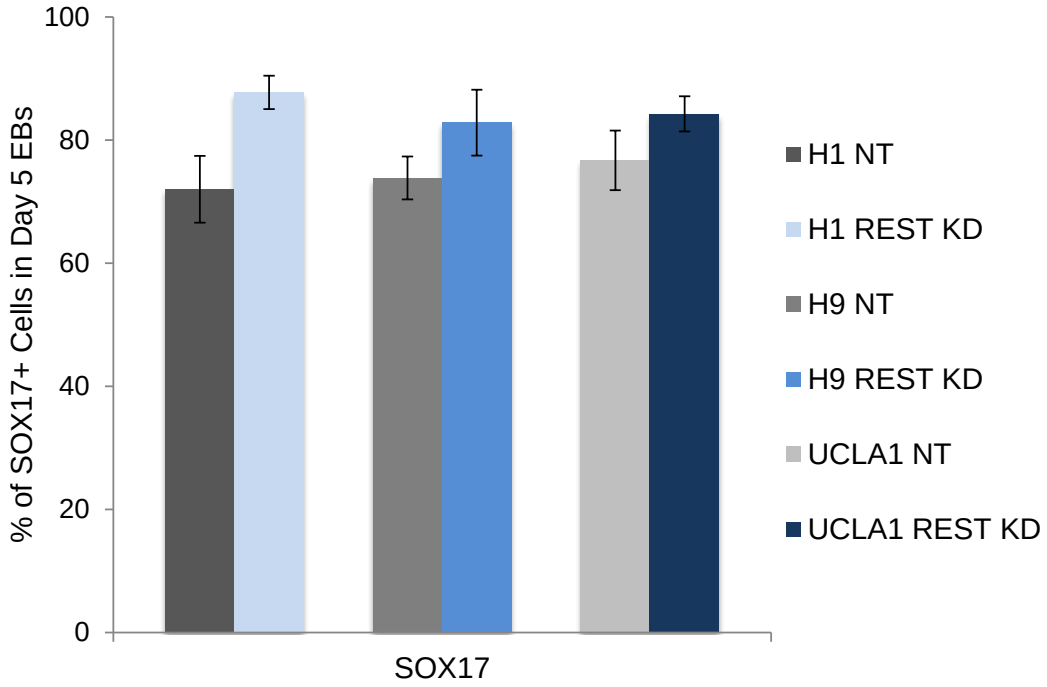


Figure 3-7: FACS analysis for protein expression in Day 5 EBs across three independent (H9, H1 and UCLA1) revealed increased expression of endoderm marker SOX17 and mesoderm marker BRACHYURY (T) in REST KD EBs compared to NT EBs. PAX6 (ectoderm marker) expression was lower in H9 and H1 REST KD EBs compared to NT EBs, but higher in UCLA1 REST KD EBs compared to NT EBs.

A


100
80
60
40
20
0
% of BRACHYURY+ Cells in Day 5 EBs
*
*
*
BRACHYURY
H1 NT
H1 REST KD
H9 NT
H9 REST KD
UCLA1 NT
UCLA1 REST KD

B


100
80
60
40
20
0
% of SOX17+ Cells in Day 5 EBs
SOX17
H1 NT
H1 REST KD
H9 NT
H9 REST KD
UCLA1 NT
UCLA1 REST KD

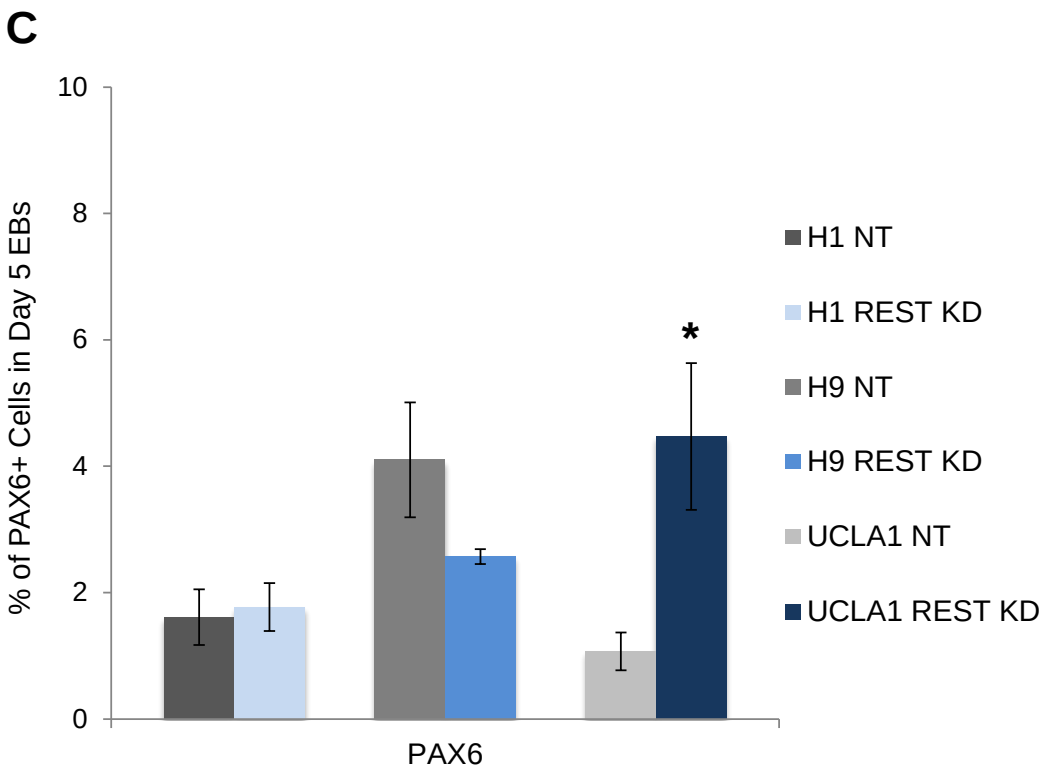


Figure 3-8: Quantitative representation of FACS analysis for lineage markers in Day 5 EBs across three lines. Significant changes, calculated using an unpaired students t-test are shown with a single asterisk (*). **A**: Percentage of BRACHYURY+ cells is significantly higher in REST KD cells compared to control NT in all three lines ($p=0.05$ for H1, $p=0.009$ for H9, and $p=0.04$ for UCLA1). **B**: Percentage of SOX17+ cells is not significantly changed between NT and REST KD cells for any of the lines. **C**: Percentage of PAX6+ cells is significantly increased in UCLA1 REST KD cells compared to NT ($p = 0.05$).

Chapter 4. REST KD hESCs Have Increased Propensity To Become Genetically Unstable Over Long-Term Culture

4.1. Introduction

Human embryonic stem cell (hESC) lines provide a valuable resource for developmental studies, disease modeling and regenerative therapies. However, the preservation of genomic integrity in culture is a major constraint in the use of hESCs and their derivatives for therapeutic purposes, as long-term culture of hESCs is associated with the accumulation of karyotypic abnormalities (Baker et al. 2007; Draper et al. 2004). The most common recurrent karyotypic abnormalities in hESCs involve partial or whole gains of chromosomes 1, 12, 17, 20 or X (The International Stem Cell Initiative 2011). Years before the isolation of hESCs, embryonal carcinoma (EC) cell lines derived from human teratocarcinomas provided an important in vitro model for pluripotency. EC stem cells always contain amplified regions of chromosome 12p and, often, gains of chromosomes 1, 17 and X (Rodriguez et al. 1993; Skotheim et al. 2002; Wang et al. 1980). Gain of chromosome 20q has been observed in yolk sac carcinoma and non-seminomatous germ cell tumors, which contain EC cells (Looijenga et al. 2000; Mostert et al. 2000; Schneider et al. 2001). Several genes that control self-renewal, differentiation and apoptosis, including STELLAR, NANOG, GDF3, NT3, DPPA3, GRAP, BCL2L1 and BIRC5 are located on these commonly amplified chromosomal regions (Baker et al. 2007; Clark et al. 2004; Pyle et al. 2006; The International Stem Cell Initiative 2011). Thus these recurrent karyotypic abnormalities in hESCs may be tolerated because they confer a growth advantage to pluripotent cells in culture.

Genetic change that confers a growth advantage has obvious parallels with malignant transformation. Chromosomal instability is frequently found in solid tumors, and is believed to play a crucial role in the development of many cancers. REST is known to exert a tumor suppressor activity in epithelial cells and an oncogenic activity in neural cells. Inactivation of REST in human epithelial cells leads to transformation and tumor formation (Westbrook et al. 2005), and attenuated REST activity is associated with many epithelial cancers, including prostate, colon, lung, and breast cancers(Coulson 2005b). In contrast, elevated levels of REST are associated with malignancies of the brain (Fuller et al. 2005; Su et al. 2006). Given this known involvement of REST in tumorigenicity, and the propensity of cancer cells as well as hESCs to exhibit karyotypic abnormalities, it seemed prudent to ask whether altering REST levels in hESCs would affect their genomic stability.

4.2. Results

In order to determine whether REST played a role in regulating genomic integrity, stable REST Knockdown (KD) and Control Non-target (NT) lines were generated for H9, H1, HSF1 and UCLA1 hESCs. REST KD and Control NT hESCs were maintained in culture for at least 6 weeks prior to shipping to Cell Line Genetics (Madison, Wisconsin http://www.clgenetics.com) for karyotype analysis. REST KD resulted in karyotypic abnormalities in all four hESC lines tested (H9, H1, UCLA1 and HSF1), as shown in **Figure 4-1**, **Figure 4-3**, **Figure 4-5** and **Figure 4-7**. In total, some form of genetic instability was demonstrated in 6 out of 6 karyotype evaluations from REST KD hESCs (see **Table 4-1**). In contrast, karyotypic abnormality was demonstrated in only one of the NT hESCs, as can be seen in **Figure 4-2**, **Figure 4-4**, **Figure 4-6**, **Figure 4-8**, and **Table 4-1**. The types of abnormalities observed in REST

KD hESCs ranged from whole chromosome amplification of chromosome 12, which is often seen in hESCs (Baker et al. 2007; Draper et al. 2004), to complex chromosome rearrangements. This suggests that REST levels are critical for ensuring genomic stability in hESCs. To determine if REST KD results in immediate instability (within 96 hours), transient REST KD was performed using siRNA, and compared to control NT siRNA cells. Karyotype analysis revealed that in contrast to stable REST KD, short term REST KD does not immediately lead to karyotypic abnormalities (**Table 4-1**). This result suggests that REST knockdown in combination with long-term culture may predispose hESCs to genetic instability. In order to determine whether the differentiation bias observed in REST KD hESCs (see Chapter 3) is due to genetic instability, the BGO1V (V=variant or aneuploid) hESC line that has previously been reported to have karyotypic abnormalities, including amplification of chromosomes 12 and 17 (Zeng et al. 2004), was evaluated for differentiation potential. Lineage marker expression analysis demonstrated that BGO1V hESCs did not have mesoderm or endoderm differentiation bias compared to the control BGO1 hESCs, as shown in **Figure 4-9** (qPCR), **Figure 4-10** (FACS analysis), and **Figure 4-11**. This confirms previously published work demonstrating that aneuploidy does not predispose hESC lines to differentiate more efficiently or with a mesoderm/endoderm differentiation bias (Fazeli et al. 2011). In fact, the two aneuploid lines examined in that study exhibited reduced endoderm differentiation potential. Thus, the data suggest that REST KD hESCs have increased propensity to become genetically unstable over long-term culture, and the lineage bias seen in REST KD hESCs is not a fundamental feature of all aneuploid lines.

Interestingly, even though all four stable REST KD hESC lines were karyotypically abnormal, each cell line had a different aneuploidy. All of the 20 metaphases counted for H9 REST KD hESCs at p44 demonstrated a large pericentric inversion involving almost the entire chromosome 9, with breakage of the short arm at band p22 and the distal long arm at band q22.3. When the same line was karyotyped at p55, 19 cells demonstrated the same chromosome 9 inversion as seen at p44, while one cell demonstrated a non-clonal aberration, which was most likely a technical artifact. This aberration included an intra-chromosomal translocation in addition to the pericentric inversion of chromosome 9. For the H1 hESCs, two independent stable REST KD lines, denoted Version1 (V1) and Version 2 (V2), were generated. Out of the 20 metaphases counted for H1 REST KD V1, 19 cells demonstrated an abnormal male karyotype with trisomy 12, and one cell demonstrated a non-clonal chromosome aberration (45,Y,+12,-20), which was most likely a technical artifact. No normal cells were detected. For H1 REST KD V2, fifteen cells demonstrated an abnormal male karyotype with trisomy 12, while five cells had a duplication of the long arm of chromosome 1 from q21 to q42 in addition to trisomy 12. For HSF1 REST KD hESCs, two cells had an apparently normal male karyotype, but 16 cells demonstrated a complex rearrangement of chromosome 7 consisting of an extra copy of a segment from the short arm (p13→pter) translocated to the chromosome 7 long arm at band 32, resulting in partial loss of the distal 7 long arm (q32→qter). The two remaining cells demonstrated this same complex rearrangement of chromosome 7, but had additional non-clonal changes (-Y for one cell, and -20 for the other), which were most likely technical artifacts. For UCLA1 REST KD hESCs, all twenty cells demonstrated an abnormal female karyotype with an inverted duplication on the short-arm of

chromosome 1 from band p21.2 to p32.1, resulting in partial trisomy for the short arm of chromosome 1.

For each of the four hESC lines, H9, H1, HSF1 and UCLA1, a stable Non-Target (NT) control line was also generated by transducing the cells with a scrambled non-target shRNA sequence inserted in the same vector backbone as used for the REST KD cells. For the H9 NT hESCs and the H1 NT hESCs, all 20 metaphases demonstrated a normal karyotype. For the UCLA1 NT hESCs, 19 of the cells demonstrated the same inverted duplication on the short arm of chromosome 1 as seen in the UCLA1 REST KD hESCs, while one cell demonstrated a normal karyotype.

Karyotype analysis for HSF1 NT hESCs revealed that the line had been cross-contaminated with the H9 REST KD line. Although HSF1 is a male cell line, 19 of the cells from the HSF1 NT sample demonstrated an abnormal female karyotype. Moreover, these 19 female cells had the same pericentric inversion on chromosome 9 as was seen in the female H9 REST KD line. One cell from the HSF1 NT sample demonstrated a normal male karyotype. DNA fingerprint analysis was also performed on a sample of cells reserved at the time the HSF1 NT specimen was received at Cell Line Genetics. The DNA fingerprint confirmed the presence of two cell lines, with one major cell population, which was identified as matching the female H9 line, and one minor cell population, which was identified as matching the male HSF1 line.

In summary, for all the stable lines generated, all the NT controls with the exception of the UCLA1 NT line were normal. In contrast, all the REST KD lines were abnormal, each with a different chromosomal aberration. However, short term REST KD does not appear to be sufficient for invoking genomic instability, because cells with transient siRNA-mediated REST KD were normal. This suggests that stressful

conditions (in this case, lentiviral infection followed by long-term culture in the presence of puromycin for vector integration selection pressure, and doxycycline for shRNA induction) accompanied by reduced REST levels placed the cells at higher risk for aneuploidy.

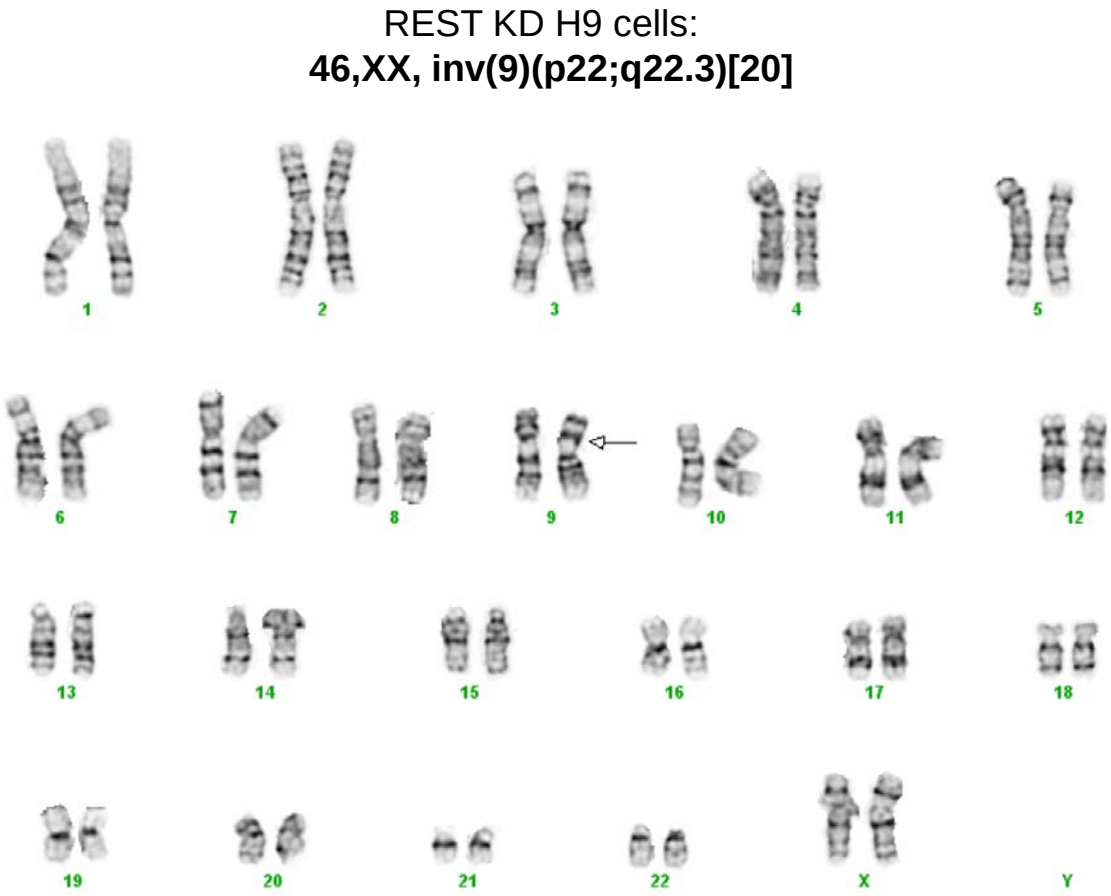


Figure 4-1: Karyotype for H9 REST KD hESCs shows that the cells have a large pericentric inversion on chromosome 9

Non-Target (control) H9 cells:
46,XX[20]

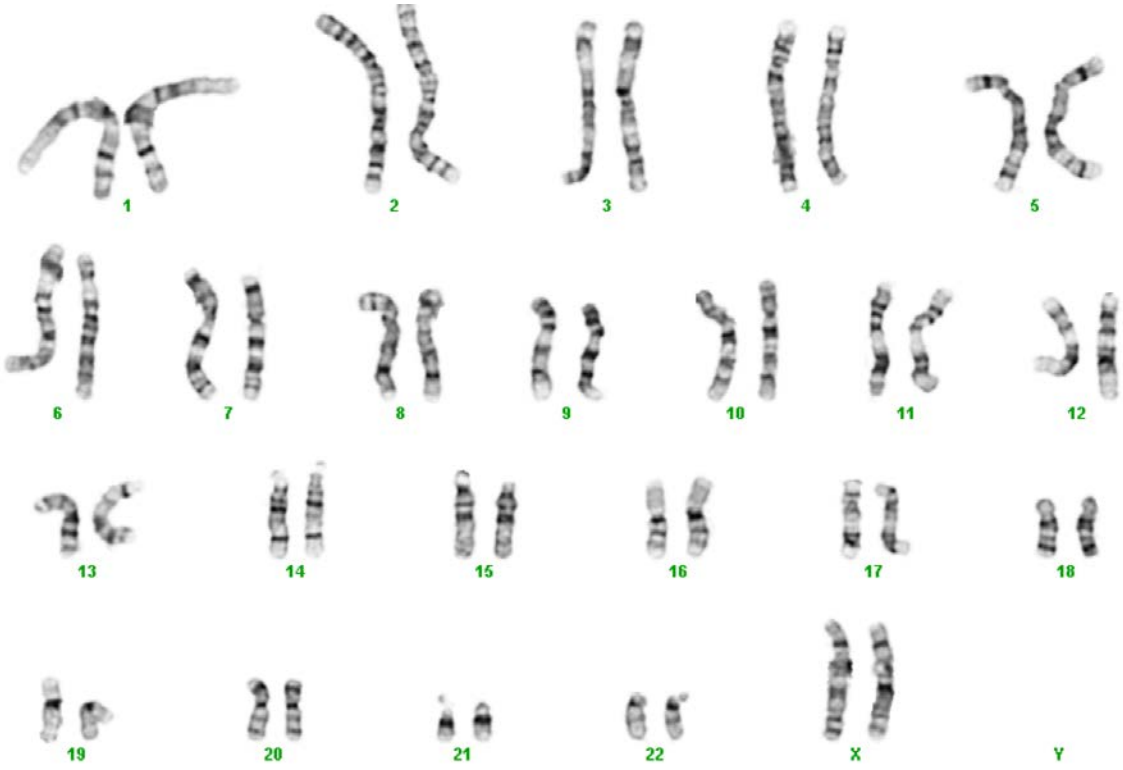


Figure 4-2: Karyotype for H9 Non-Target Control hESCs shows that the cells have a normal karyotype

REST KD H1 cells:
47,XY,+12[15]/47,XY,dup(1)(q21q42),+12[5]

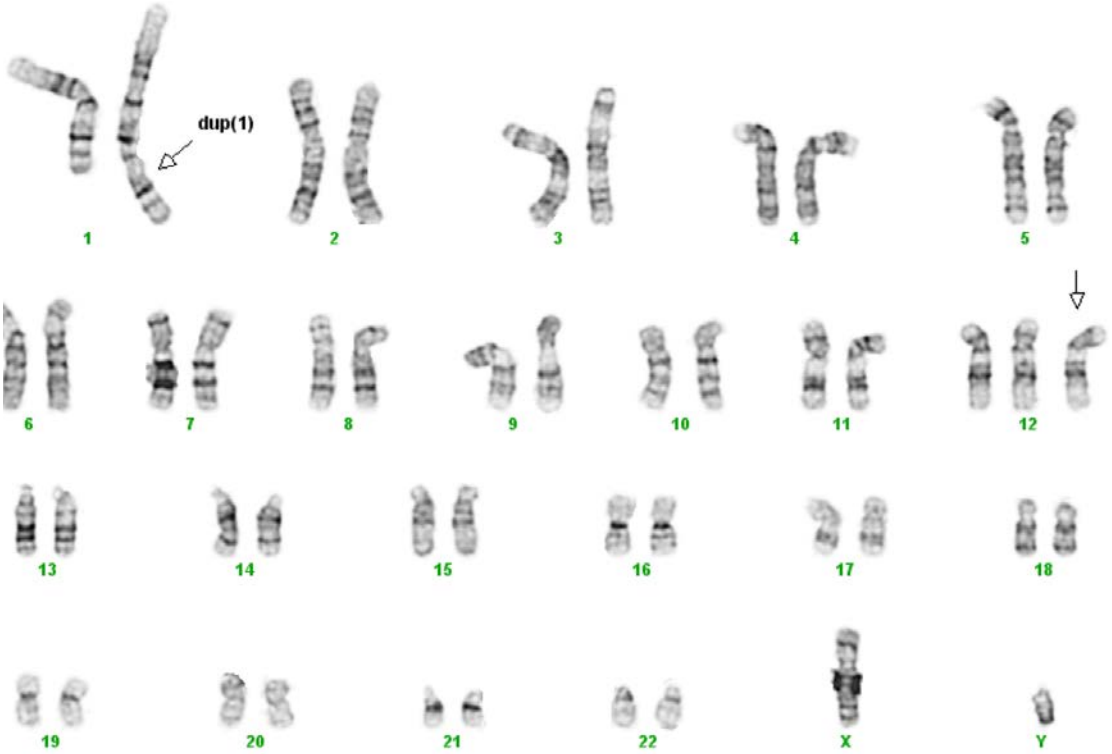


Figure 4-3: Karyotype of H1 REST KD hESCs shows that the cells have trisomy 12 and partial duplication of chromosome 1q

Non-Target (control) H1 cells:
46,XY[20]

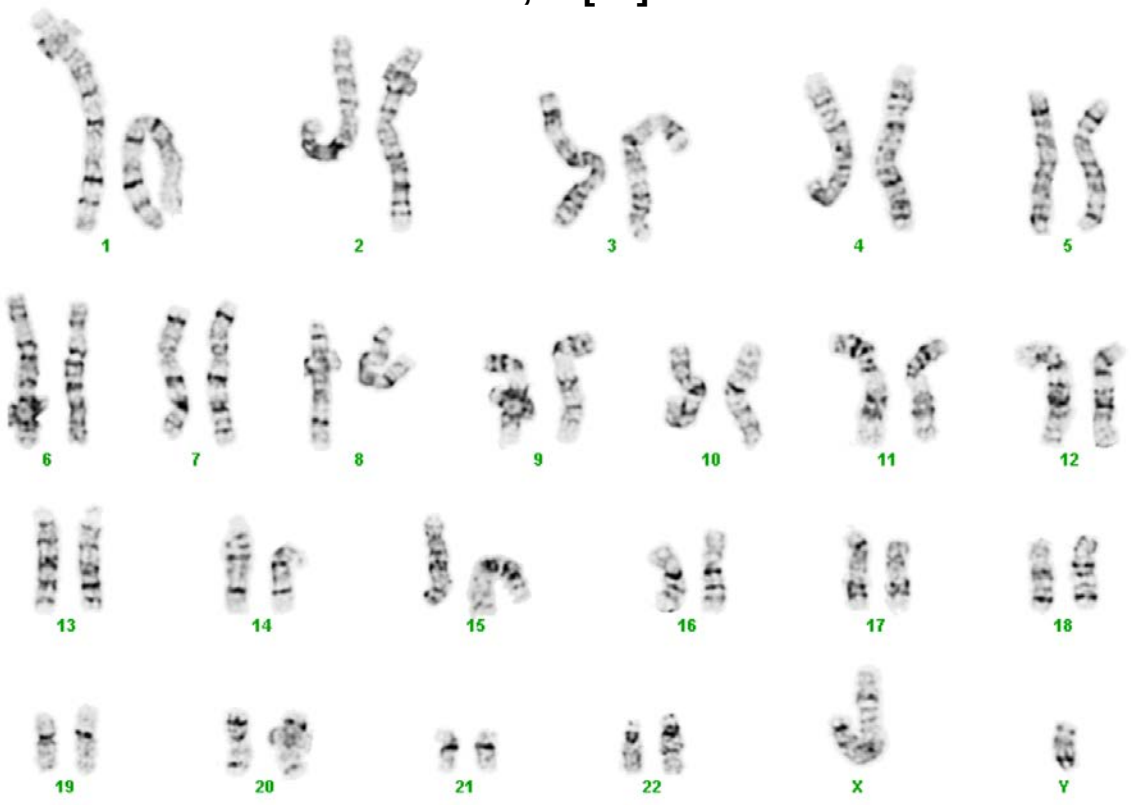


Figure 4-4: Karyotype for H1 Non-Target Control hESCs shows that the cells have a normal karyotype

REST KD HSF1 cells:

46,XY,der(7)dup(7)(pter→q32::p13→pter) del(7)(q32)[16]

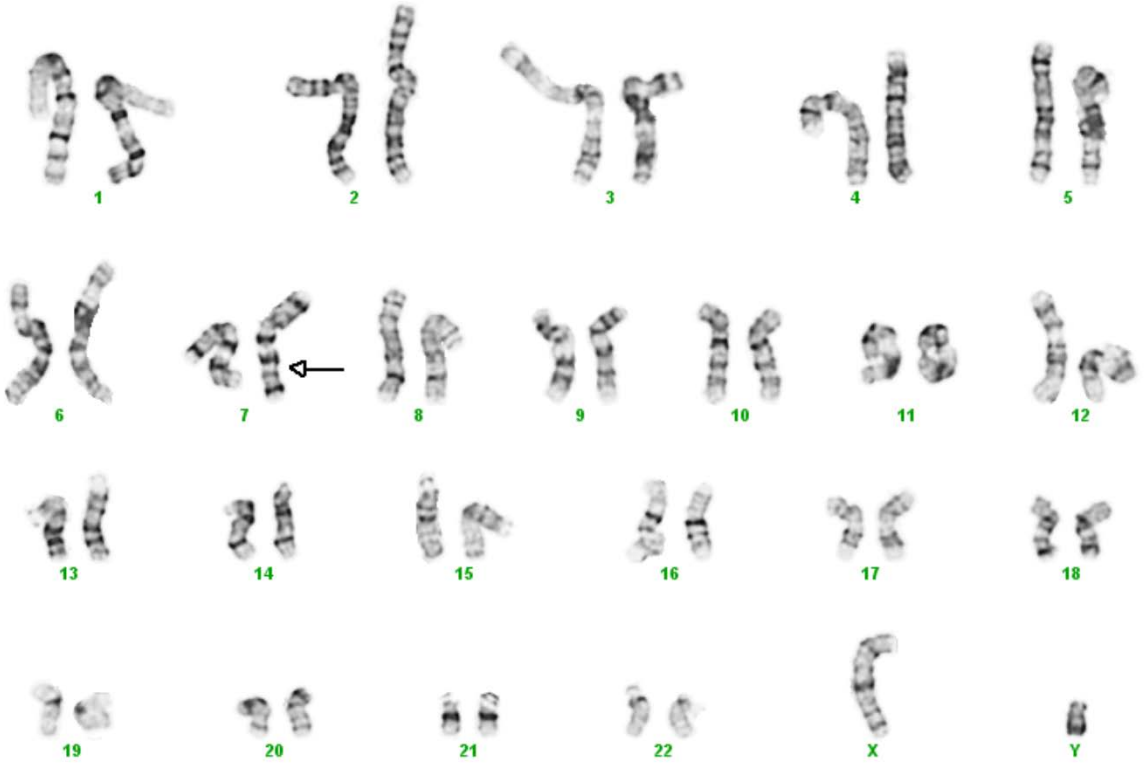


Figure 4-5: Karyotype of HSF1 REST KD hESCs shows that the cells have a complex rearrangement of chromosome 7

Non-Target (control) HSF1 cells:
46,XY[1]

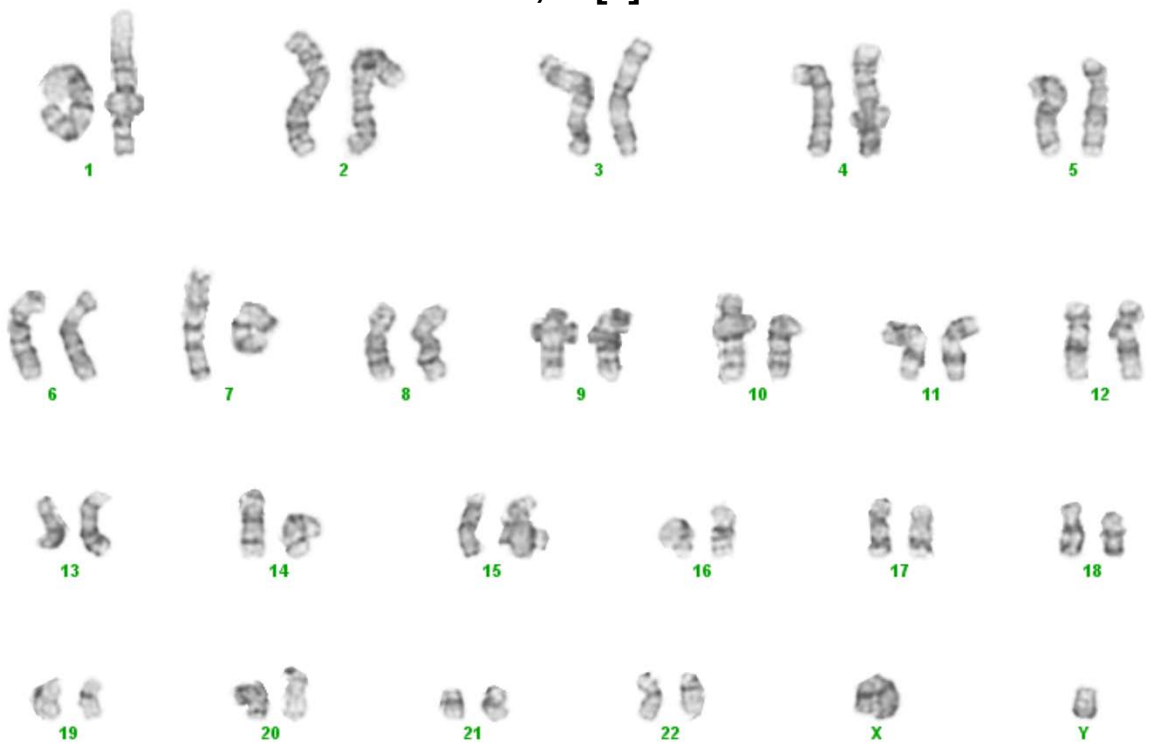


Figure 4-6: Karyotype for HSF1 Non-Target Control hESCs

REST KD UCLA1 cells:
46,XX,dup(1)(p32.1p21.2)[20]

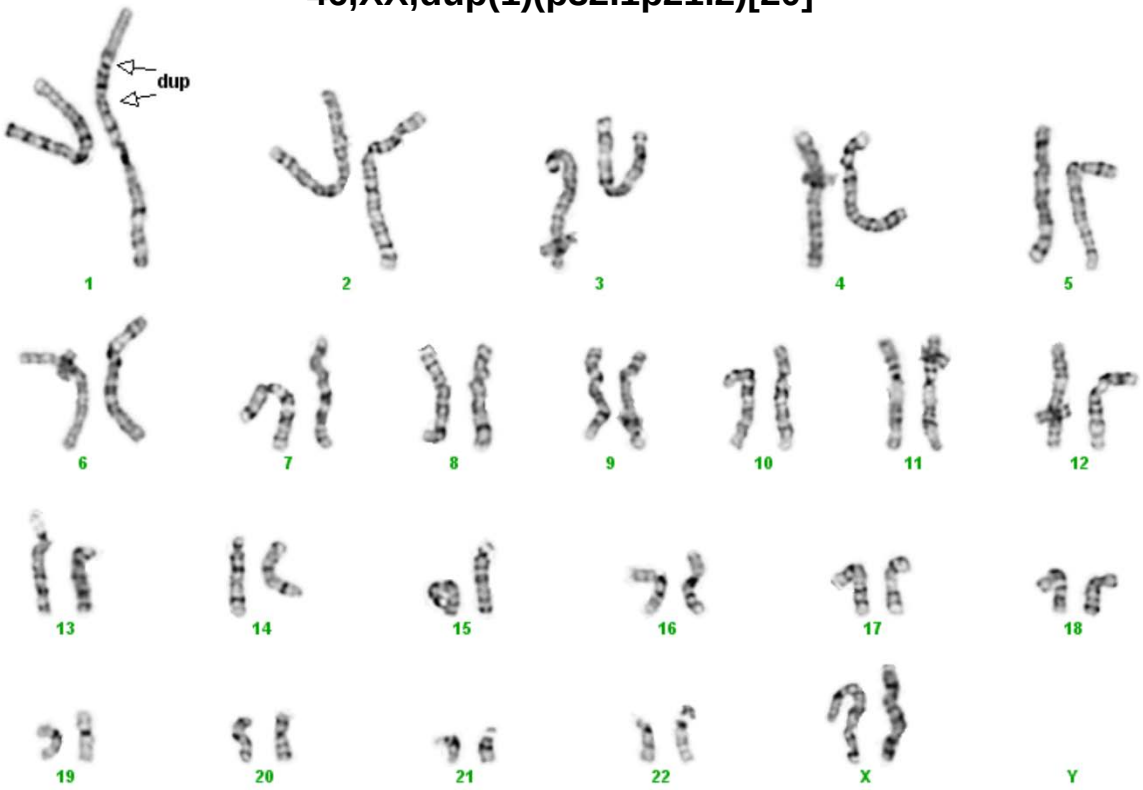


Figure 4-7: Karyotype of UCLA1 REST KD hESCs shows an inverted duplication resulting in partial trisomy of chromosome 1p

Non-Target Control UCLA1 cells:
46,XX,dup(1)(p32.1p21.2)[19]

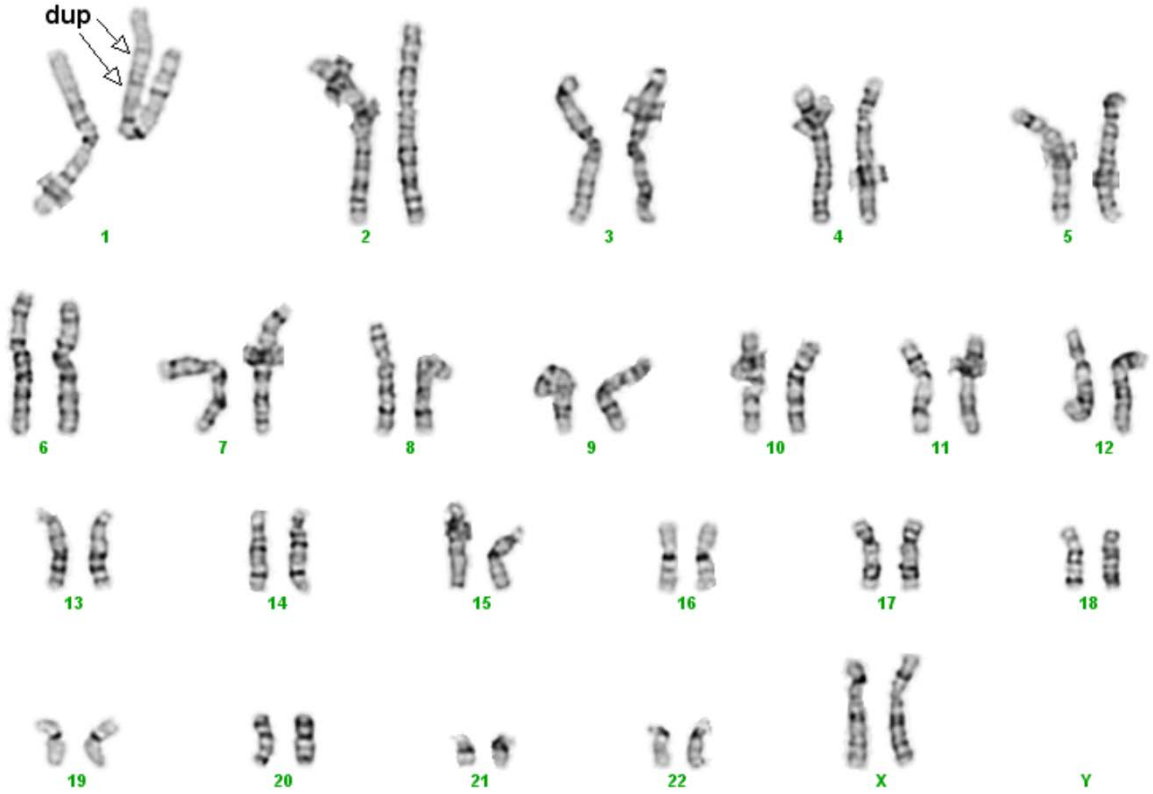


Figure 4-8: Karyotype for UCLA1 Non-Target Control hESCs shows the same inverted duplication of chromosome 1p as UCLA1 REST KD hESCs

Table 4-1: Karyotypes for REST KD and Non-Target Control hESCs

Cell Line	**Karyotype**
REST pTRIPZ H9 p44	46,XX,inv(9)(p22;q22.3)[20]
REST pTRIPZ H9 p55	46,XX,inv(9)(p22;q22.3)[19]
NT pTRIPZ H9 p55	46,XX[20]
REST pTRIPZ H1 V1 p54	47,XY,+12[19]
REST pTRIPZ H1 V2 p57	47,XY,+12[15] 47,XY,dup(1)(q21q42),+12[5]
NT pTRIPZ H1 p50	46,XY[20]
REST pTRIPZ HSF1 p48	46,XY,der(7)dup(7)(pter→q32::p13→pter) del(7)(q32)[16] / 46,XY[2]
NT pTRIPZ HSF1 p51	46,XY[1]
REST pTRIPZ UCLA1 p27	46,XX,dup(1)(p32.1p21.2)[20]
NT pTRIPZ UCLA1 p27	46,XX,dup(1)(p32.1p21.2)[19]/ 46,XX[1]
REST siRNA 288hr UCLA1 p23	46,XX (CNV Analysis)
NT siRNA 288hr UCLA1 p23	46,XX (CNV Analysis)

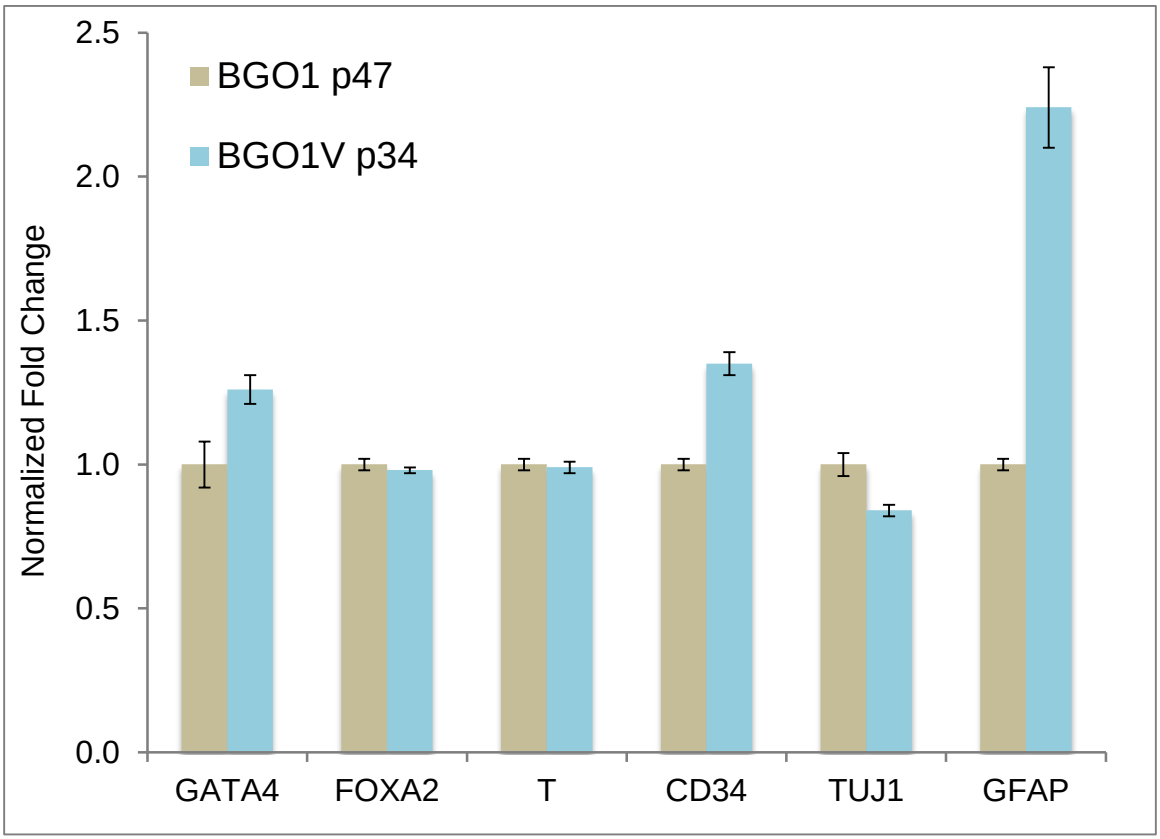


Figure 4-9: The aneuploid hESC line BGO1V does not exhibit endoderm/mesoderm bias compared to control BGO1 hESCs that are normal. Q-PCR for lineage markers in day 5 EBs

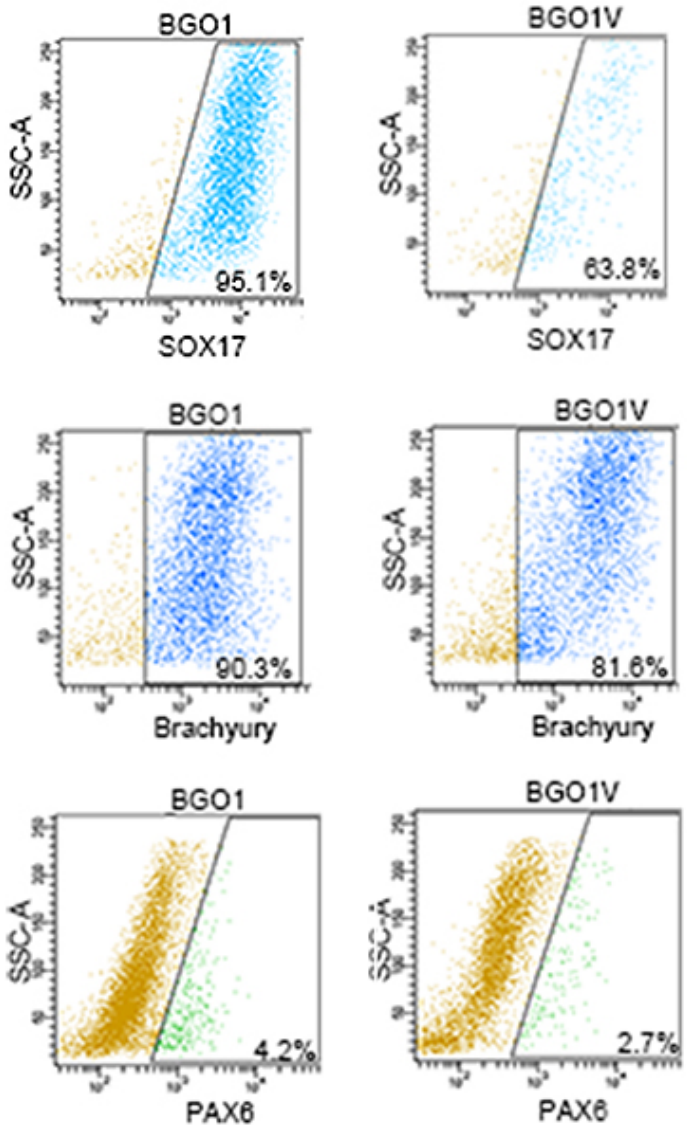


Figure 4-10: FACS analysis for protein expression of lineage markers in day 5 EBs shows that the aneuploid BGO1V line does not exhibit endoderm/mesoderm differentiation bias compared to control BGO1 line

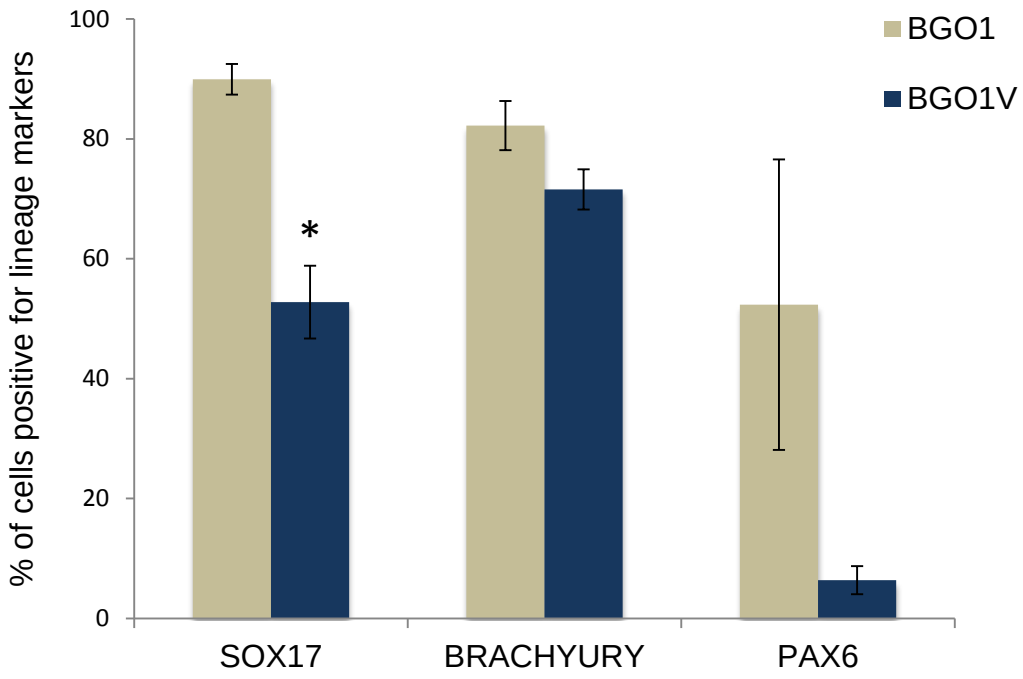


Figure 4-11: Quantitative representation of FACS analysis for lineage markers in BGO1 and BGO1V Day 5 EBs. Significant changes, calculated using an unpaired students t-test are shown with a single asterisk (*). Percentage of SOX17+ cells is significantly lower in the BGO1V line compared to BGO1 ($p=0.005$). Percentage of BRACHYURY+ and PAX6+ cells is not significantly altered between the two lines.

4.3. Discussion and Future Directions

Based on the fact that REST is known to have tumor suppressor or oncogenic roles in different human cancers (Coulson 2005b), it is not surprising that altering levels of REST expression in hESCs predisposes these cells to instability over long term culture. RAS/ERK/MAPK signaling, which is elevated in many cancers, and has previously been shown to play a role in regulating genetic stability in a number of systems (Duhamel et al. 2012; Sebolt-Leopold, Herrera 2004), is also elevated in REST KD cells (see Chapter 5). Importantly, members of the ERK superfamily are REST target genes, and provide mechanistic candidates for changes in genetic stability seen in REST KD hESCs. Understanding how REST and/or REST target genes control genetic stability could provide a route for preventing instability from occurring over long-term culture. It will also be important to determine if REST KD hESCs have alterations in epigenetic modifications, DNA damage response or cell division that may predispose these cells towards instability.

Chromatin regulators that shape the epigenetic landscape orchestrate integration of DNA repair, DNA replication, transcription and cell division, and thus influence the genetic integrity of the cell. REST mediated transcriptional regulation involves chromatin remodeling at the target gene to induce a repressive chromatin state. In the context of genomic instability seen in REST KD hESCs, it would be worthwhile to examine whether lower REST levels lead to an altered epigenetic landscape at target genes. It is quite likely that in the absence of REST binding at RE1 sites in REST KD cells, REST cofactors are not recruited to target genes, and hence the repressive chromatin state normally facilitated by these cofactors fails to materialize. In order to examine chromatin modifications, one approach would be to perform a ChIP-Seq

analysis as described by Zheng et al. (2009) to generate a profile of REST mediated histone modifications in hESCs, and compare REST KD cells with NT controls for changes in histone acetylation and methylation patterns.

Global DNA hypomethylation and consequent genomic instability are frequently seen in cancer as well as in various cell culture systems (Konkel, Batzer 2010). Dnmt3b-deficient MEFs exhibit aneuploidy, polyploidy, chromosomal breaks, and fusions (Dodge et al. 2005). The presence of two active X chromosomes in XX mESCs results in decreased levels of Dnmt3a, which leads to global hypomethylation , and consequently, instability of the XX karyotype (Zvetkova et al. 2005). Dnmt1 deficiency has also been shown to cause genome wide hypomethylation followed by enhanced microsatellite instability in mouse embryonic stem cells (Kim et al. 2004). It has been proposed that alterations in genome-wide methylation due to variable expression of the DNA methyltransferases (Bhattacharya et al. 2004; Brandenberger et al. 2004; Rao et al. 2004; Richards et al. 2004; Skottman et al. 2005; Sperger et al. 2003) may also contribute to chromosomal instability in hESC lines (Allegrucci, Young 2007). Elucidating the extent to which REST KD alters DNA methylation patterns in hESCs could be achieved by using methyl CpG-binding domain protein-based enrichment coupled with next-generation sequencing (MBD-seq), as described in Jin et al. (2012). It might also be useful to compare the methylation patterns in REST KD cells to the data from human embryonic carcinoma cells obtained by Jin et al. (2012), to determine whether there are similarities to the transformed cell.

DNA methylation promotes faithful chromosomal segregation during mitosis by suppressing mitotic recombination (Chen et al. 1998). Repetitive sequences have been found to be hotspots for recombination (Shiroishi et al. 1995; Wahls et al. 1990), and

many repetitive sequences are highly methylated (Yoder et al. 1997). The two types of repeat elements prevalent in the human genome are tandem repeats (TRs) and interspersed repeats that mainly include transposable elements (TEs). Some TRs seem to be generated from younger/more active TEs, and once initiated they are expanded with time via local duplication of the repeat units (Ahmed, Liang 2012). Approximately 45% of the human genome is derived from TEs, including DNA transposons, long terminal repeat (LTR) retrotransposons, and non-LTR retrotransposons. TEs have gone through bursts of active transposition during evolution, and most have been rendered inactive through mutation. Long interspersed element-2 (LINE2) retrotransposons were thought to be active about 200 million years ago, while many LINE1 retrotransposons and small interspersed element (SINE) retrotransposons of the Alu class continue to mobilize in the human genome and cause genomic instability through both insertion, and post-insertion-based mutagenesis (Johnson et al. 2006). The human genome contains over 500,000 copies of LINE1 elements and more than one million copies of Alu elements (International Human Genome Sequencing Consortium 2001).

Although recent studies that analyzed the retrotransposon transcriptome in a panel of human somatic tissues have determined that retrotransposons are less expressed, on average, than non-repetitive regions of the genome (Faulkner et al. 2009; Rangwala et al. 2009), LINE1 and Alu insertions have been implicated in many human diseases. Non-allelic homologous recombination (NAHR) between two inverted Alu elements has been implicated in familial hypercholesterolemia (Lehrman et al. 1985), and NAHR between LINE1 elements has been implicated in sporadic cases of phosphorylase kinase deficiency, Alport syndrome, and Ellis–van Creveld syndrome (Burwinkel, Kilimann 1998; Segal et al. 1999; Temtamy et al. 2008). A study designed to

examine local recombination rates around the youngest member of the Alu family, AluY, determined that the presence of a fixed AluY element enhances the local recombination rate by approximately 6% (Witherspoon et al. 2009). While the effect of each AluY element is relatively small, the presence of hundreds of thousands of these elements throughout the human genome implies that they exert a substantial effect on genome-wide recombination rates.

Johnson et al. (2006) have shown that at least 10% of RE1s in the human genome belong to evolutionarily related groups, and that these RE1s arose via transposon mediated duplication and insertion. Many RE1s are associated with LINE elements, with 190 RE1s associated with LINE2 elements alone. Most of the SINE elements associated with RE1s are of the Alu class. Additionally, a number of REST target genes have been found to contain groups of RE1s arranged in tandem (See **Table 4-2** and **Figure 4-12** for examples).

Table 4-2: Example of RE1 elements located within retrotransposon sequences. Adapted from Johnson et al. (2006).

Re1 ID	**Chromosome Location**	**Transposon Element**	**Number of RE1 repeats**
hum2528	Chr1 q21.2-q21.3	Alu	7
Hum2515	Chr1 q12	Alu	10
hum6034	Chr2 q21.1	LINE1	6
hum11432	Chr4 q26	LINE2	2
hum11438	Chr4 q26-q27	LINE2	4
hum17621	Chr7 p11.2	LINE2	1
hum17654	Chr7 q11.21	LINE2	1
hum21648	Chr9 p11.2	LINE2	1
hum34541	Chr16 p12.3	LINE2	1
hum36473	Chr17 p11.2	LINE2	5
hum41830	Chr20 q13.13	LINE2	1

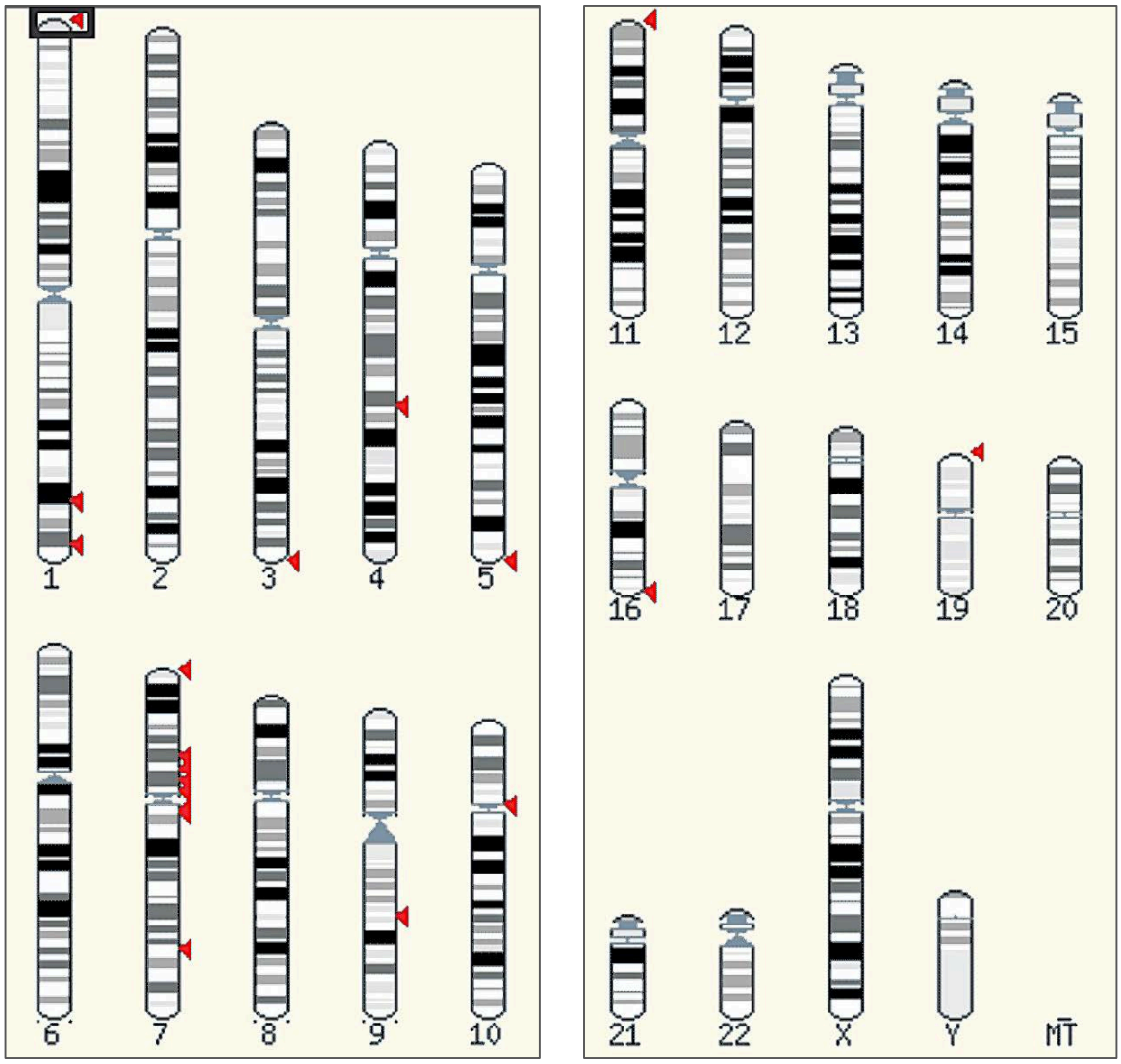


Figure 4-12: Duplication and dispersal of RE1s from a single parent site located in the sub-telomeric region of the Chromosome 1 p-arm (boxed). Adapted from Johnson et al. (2006).

Bioinformatic analysis of microarray gene expression data for genome-wide silencing of repetitive elements has determined that a complex interplay between multiple epigenetic mechanisms such as histone deacetylation by Hdac1, DNA methylation, Eset-mediated histone modification, Ring1B/Eed polycomb complex mediated H3K27 trimethylation and Lsd1-dependent H3K4 demethylation mediates retrotransposon repression in mouse embryonic stem cells and germ cells (Reichmann et al. 2012). Since REST employs similar mechanisms to repress target genes, it is possible that REST KD results in an open chromatin state at TE associated RE1s, and that this anachronistic epigenetic landscape induces activation or NAHR of otherwise latent TEs. Support for this hypothesis could be deduced from a spurt of recent studies on TE mediated genomic alterations in cancer, early human development and cultured stem cells, as elaborated below.

A variety of cancers are associated with aberrant repetitive element recombination or activation. In a review on the role of TEs in genomic instability of cancer cells, Konkel, Batzer (2010) have observed that as a result of their abundance and high sequence identity, TEs frequently mislead the homologous recombination repair pathway into NAHR, causing deletions, duplications, and inversions. This may be at least partially attributed to the commonly seen global hypomethylation as well as general epigenetic dysfunction of cancer cells (Konkel, Batzer 2010). Enhanced association of homologous recombination proteins with Alu repetitive sequences and consequent genomic rearrangements and instability are a defining characteristic of Barrett's adenocarcinoma cells (Pal et al. 2011). Detection of increased LINE1 copy number in patients' sera has been shown to be useful for detecting early-stage breast cancer (Sunami et al. 2008), and increased copy numbers of specific LINE1 and Alu

small fragment DNA markers are being used as diagnostic tools in colorectal cancer patients (Mead et al. 2011). Digital gene expression analysis of pancreatic, lung, kidney, ovary, colon, and prostate cancer tissue has revealed overexpression of the LINE1 retrotransposon accompanied by aberrant expression of neuroendocrine-associated genes proximal to LINE1 insertions (Ting et al. 2011). LINE1 hypomethylation and activation has also been observed in gastroenteropancreatic neuroendocrine tumours, ovarian carcinoma, and in female bladder cancer (Dammann 2009; Stricker et al. 2012; Wilhelm et al. 2010). A recent examination of 43 high-coverage whole genome sequencing datasets from five cancer types (i.e., colorectal, glioblastoma, multiple myeloma, ovarian and prostate) revealed 194 somatic TE insertions, where all the LINE1 and Alu insertions were observed in epithelial cancers and none in blood and brain cancers (Lee et al. 2012). Interestingly, epithelial cancers are associated with decreased REST activity (Coulson, 2005), and malignancies of the brain are associated with elevated REST activity (Conti et al. 2012; Fuller et al. 2005; Su et al. 2006).

During normal human development, de novo TE insertions occur in the parental germline and/or during early embryogenesis prior to the partitioning of the germ cell lineage (Macia et al. 2011; Ostertag et al. 2002). Alu elements have the highest estimated retrotransposition rate, with about 1 in 20 live births, followed by LINE1 with about 1 in 200 (van den Hurk et al. 2007). Macia et al. (2011) showed that hESCs also express endogenous LINE1 and Alu elements, and that expressed LINE1s are mostly located within genes. Apparently, the widespread chromatin modifications that are required to maintain the pluripotent state allows exposure of LINE1 promoters within specific genes to chromatin contexts permissive for LINE1 expression (Macia et al. 2011). Thus the epigenetic plasticity of hESCs that allows developmental and tissue

specific genes to be turned on in the presence of appropriate differentiation cues might also make these cells prone to genomic instability (Dean et al. 1998; Humpherys et al. 2001; Minoguchi, Iba 2008).

LINE1 retrotransposition has been demonstrated in hESC and hiPSC derived neural progenitor cells (NPCs) (Coufal et al. 2009; Muotri et al. 2010). Another study has documented LINE1 retrotransposition of a human LINE1 element both in cultured rat NPCs and in the brains of transgenic mice (Muotri et al. 2005). In this study, LINE1 retrotransposition was detected in virtually all regions of the mouse brain, including the striatum, cortex, hypothalamus, cerebellum, ventricles, amygdala, and hippocampus. In cultured rat NPCs, H3K9 acetylation, H3K4 methylation, and significant stimulation of LINE1 expression was observed after the induction of differentiation, accompanied by upregulation of the neuron-specific Synapsin gene (Muotri et al. 2005), which happens to be a direct REST target. It was also noted that the LINE1 activation occurred in Map2 expressing neuronal cells, but not in Gfap expressing glial cells. A subsequent study also noted that in the mouse brain, LINE1 expression was only detected in neurogenic regions, where it colocalized with NeuroD1 and β-tubulin III (Kuwabara et al. 2009). Characterization of post-integration sites revealed several insertions occurred in neuronally expressed genes.

Kuwabara et al. (2009) demonstrated that the neuronal specificity of LINE1 retrotransposition in neural progenitors was partially due to Sox2 down-regulation and Wnt-mediated coordinated activation of NeuroD1 and LINE1. The promoters of both, NeuroD1 and LINE1, contain overlapping Sox2 and T-cell factor/lymphoid enhancer factor (TCF/LEF) binding sites (Muotri et al. 2005). In neural stem cells Sox2 is partially responsible for inhibiting NeuroD1 and LINE1 expression by forming a

repressor complex with HDAC1 and associating with these promoters (Muotri et al. 2005). As the neural stem cell commits to the neuronal lineage, Sox2 expression decreases, and the repressor complexes dissociate from the NeuroD1 and LINE1 promoters. Concurrently, a β-catenin activator complex binds to the TCF-LEF sites (Kuwabara et al. 2009). These factors result in the simultaneous induction of NeuroD1 and LINE1 expression in neurogenic NPCs. Interestingly, NeuroD1 is also a REST target gene (Wu, Xie 2006).

Studies by the Mandel group (Ballas, Mandel 2005; Ballas et al. 2005) have shown that while REST continues to repress neuronal gene expression during differentiation into glial and non-neural lineages, REST levels are downregulated during neuronal differentiation, allowing neuronal genes such as NeuroD1 and Synapsin to be expressed. Given that there is up-regulation of LINE1 expression during neuronal, but not glial differentiation (Kuwabara et al. 2009; Muotri et al. 2005), and that many RE1 sites are associated with LINE family sequences (Johnson et al. 2006), it would be worthwhile to examine whether there is aberrant retrotransposition or NAHR of LINE family TEs when REST KD lifts the epigenetic repression imposed at target RE1s in hESCs. The fact that genomic instability is seen in hESCs with stable, lentivirus mediated REST KD and not after transient REST KD, suggests that stress, in addition to REST KD might play a role in inducing genomic instability. Several reports have shown that stress can evoke profound changes in the epigenetic framework, and loosen the epigenetic constraints on transposons, resulting in increased homologous recombination frequency and chromosomal rearrangements (Fedoroff 2012; Ito et al. 2011; Kathiria et al. 2010). Since the controls for REST KD shRNA hESCs, which were derived using the same vector backbone and the same experimental conditions did not

usually demonstrate genomic instability, it is unlikely that stress alone induced the genomic instability seen in REST KD hESCs.

One approach to evaluate whether TEs associated with RE1s play a role in inducing aneuploidy in REST KD hESCs, would be to start by examining the full list of RE1s and RE1 associated TEs from Noel Buckley's group (Johnson et al. 2006). High-resolution array comparative genomic hybridization (aCGH) could be used for precise mapping of breakpoint boundaries for the chromosomal rearrangements on Chromosome 9 in REST KD H9 hESCs and on Chromosome 7 in REST KD HSF1 hESCs. A comparison of the data from this aCGH analysis and the data from Johnson et al. (2006) would help determine whether there are any RE1 associated TEs in the vicinity of the breakpoints on Chromosome 9 and Chromosome 7. Sequencing the breakpoint and flanking regions and then using RepeatMasker (http://www.repeatmasker.org/) to identify repeat sequence elements would help validate the aCGH findings and/or identify other TE/repeat elements in the region. The mechanism for the chromosomal rearrangement might also be deduced from the sequencing information. For example, if there is repeat sequence identity between the two breakpoints on chromosome 9 of REST KD cells, it would suggest NAHR. In contrast, dissimilarities at breakpoint junctions would rule out homology-dependent mechanisms.

Another crucial avenue for future studies is to examine whether altered DNA damage response plays a role in genomic instability of REST KD cells. The cell cycle in hESCs is shortened in comparison to somatic cells, with an abbreviated G1 phase (Becker et al. 2006). Rapid progression through successive rounds of DNA replication and mitotic division may expose hESCs to increased risk of replication errors, which are the most common source of DNA double strand breaks (DSB) in proliferating cells, and

unrepaired DSBs can lead to chromosomal aberrations and genomic instability (Becker et al. 2006). There are two major pathways for DSB repair in mammalian cells, homologous recombination repair (HRR) and non-homologous end joining (NHEJ) (Valerie, Povirk 2003). In hESCs, HRR appears to be the primary means of DSB repair, although NHEJ is also used (Adams et al. 2010a; Adams et al. 2010b). HRR in hESCs is predominantly ATR dependent, and can be detected by RAD51 foci formation (Adams et al. 2010a), while NHEJ is XRCC4 dependent (Adams et al. 2010b), and can be detected by XRCC4 and γ-H2AX co-localization. Additionally, a study attempting to elucidate factors that contribute to the accumulation of chromosomal abnormalities in hESCs demonstrated that during prolonged culture, expression of apurinic/apyrimidinic endonuclease 1 (APE1), the major nuclease required for base excision repair (BER) was reduced, causing a significant decreased in BER (Kruta et al. 2013). Given the importance of efficient DNA repair in maintaining genomic integrity, it would be important to determine whether any of these DNA repair pathways are compromised in REST KD hESCs.

Repair of DNA damage would be meaningless if chromosomes were not properly segregated and equally inherited during cell division, as abnormal mitosis can lead to the generation of aneuploidy. A number of mitotic defects are known to generate aneuploidy, including atypical mitotic spindle assembly, inefficient chromosome congression, abnormal microtubule dynamics, cohesion and condensation defects and supernumerary centrosomes (Compton 2011; Gordon et al. 2012; Holland, Cleveland 2012; Schvartzman et al. 2010). Guardavaccaro et al. (2008) have shown that timely degradation of REST during G2 permits the optimal activation of the spindle checkpoint, and consequently it is required for the fidelity of mitosis. Staining

centrosomes with γ-tubulin, kinetochores with CREST and DNA with Hoechst 33343 and histone H3 for condensed chromosomes would help visualize mitotic events and determine whether there are mitotic aberrations in REST KD hESCs.

An intricate network of regulatory pathways ensures that each cell cycle event is performed correctly and in proper sequence (Meloche, Pouysségur 2007). The decision of cells to replicate their genetic material and divide is influenced by extracellular signals, and one of the key signal transduction pathways responsible for integrating these signals is the MEK/ERK pathway (Meloche, Pouysségur 2007). Alterations in FGF/MEK/ERK signaling are very common in human malignancies (Cronauer et al. 2003; Kwabi-Addo 2004; Murphy et al. 2010), and have been shown to play a role in regulating genetic stability (Duhamel et al. 2012; Sebolt-Leopold, Herrera 2004). A recent study also showed that elevated FGF-2 levels play a role in supernumerary mitotic spindle poles and aberrant mitoses (pseudobipolar or multipolar), ultimately leading to aneuploidy (Cuevas et al. 2012). Thus, it would be important to examine whether elevated FGF/MEK/ERK signaling plays a role in genomic instability of REST KD cells. In order to accomplish this, new hESC lines with stable REST KD would have to be generated while simultaneously inhibiting the MEK/ERK pathway with varying concentrations of a specific MEK1/MEK2 inhibitor like U0126. After culturing the cells for 6 weeks, they should be karyotyped to determine whether the combinatorial effect of attenuated REST activity and particular level(s) of FGF/ERK signaling predispose REST KD cells to aneuploidy. Finally all proposed experiments should be carried out in chemically defined culture conditions (Bone et al. 2011; Parsons et al. 2012; Wang et al. 2009; Yao et al. 2006; Zhou et al. 2010), so as to elucidate the effect of REST activity on genomic stability without the confounding contributions of multiple unknown and

variable factors associated with KOSR, MEFs, and even Matrigel (See Chapter 2 introduction).

Chapter 5. REST KD Cells Have Altered FGF/ERK and WNT Signaling

5.1. Introduction

Several signaling pathways have been implicated in regulating hESC self-renewal and pluripotency, including FGF, Activin-A/TGF-β, BMP4 and WNT. Concentration or duration of signaling from one pathway can influence whether or when another pathway is activated, and it is the cumulative signaling landscape, not any one pathway in isolation, that determines whether hESCs self renews or differentiates toward a specific germ layer (Singh et al. 2012b; Sumi et al. 2008; Warmflash et al. 2012).

Fibroblast growth factor-2 (FGF2) is widely used to culture hESCs, and a high concentration of FGF2 (100 ng/ml) can support undifferentiated growth in the absence of supporting mouse embryonic fibroblasts (Levenstein et al. 2006). In addition, FGF and FGF receptors (FGFRs) are endogenously expressed by hESCs (Dvorak et al. 2005; Kang et al. 2005). FGF/FGFR stimulation activates two signaling pathways, the mitogen activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) pathway, and the phosphoinositide 3-kinase (PI3-K) pathway (Armstrong et al. 2006; Dvorak et al. 2005; Eiselleova et al. 2009; Li et al. 2007).

MAPK/ERK activity leads to phosphorylation of transcription factors such as c-FOS, c-JUN, and c-MYC. MAPK/ERK signaling has been shown to promote hESC adhesion, survival and proliferation (Kang et al. 2005; Na et al. 2010), but there are contradictory reports on the effect of MAPK/ERK signaling on pluripotency. Li et al. (2007) reported that blocking MAPK results in loss of OCT4 and NANOG, whereas Ding et al. (2010) did not observe any effect of MAPK inhibition on OCT4 and TRA-1-60 levels. One mechanism by which ERK signaling tips the balance in favor of self-renewal

is by inducing inhibitory phosphorylations in the linker regions of the BMP signal transducers Smad1/5/8, and thus suppressing BMP mediated differentiation (Kretzschmar et al. 1997; Xu et al. 2008). ERK activation also leads to phosphorylation and inactivation of GSK-3β, followed by downstream potentiation of β-catenin /TCF-mediated transcription (Almeida et al. 2005; Ding et al. 2005).

Activation of PI3K leads to phosphorylation of AKT, which regulates multiple downstream effectors such as MDM2, mTOR, NF-κB and CASPASE-9. PI3K/AKT signaling has been shown to promote hESC pluripotency, proliferation and survival (McLean et al. 2007; Storm et al. 2007; Watanabe et al. 2006), and several studies have shown that inhibition of PI3K signaling results in down-regulation of pluripotency factors markers OCT4, NANOG and SOX2 (Alva et al. 2011; Armstrong et al. 2006; Ding et al. 2010). Additionally, PI3K has been shown to regulate hESC pluripotency by suppressing the ability of Activin A to promote differentiation (McLean et al. 2007). Singh et al. (2012a) showed that PI3K/AKT signaling suppresses MAPK/ERK activity, which leads to high GSK3β activity and low β-Catenin activity.

Activin-A/TGF-β signaling is believed to be essential for the self-renewal of hESCs (Beattie et al. 2005; Xiao et al. 2006). Activin-A/TGF-β signaling functions by activating Smad2/3, which maintains pluripotency through activation of NANOG transcription (Vallier et al. 2009; Xu et al. 2008). Activin-A/TGF-β signaling also suppresses BMP signaling by competing for common pathway elements such as Smad4 (Avery et al. 2010; Candia et al. 1997), and thus inhibits BMP mediated differentiation. However, McLean et al. (2007) have shown that Activin-A plays a prominent role in promoting initial differentiation events in the epiblast and in hESCs. In developmental

models such as Xenopus, Activin-A signaling is required for the establishment of mesoderm and endoderm lineages (Wardle, Smith 2006). Singh et al. (2012a) postulate that in the presence of robust AKT activity, moderate Activin/Smad2/3 activity is unable to induce mesendoderm gene expression, thereby maintaining hESCs in their self-renewal state.

BMP is a differentiation pathway in hESCs, and even relatively low doses cause differentiation to extraembryonic or mesodermal fates. When the FGF pathway is active, it cooperates with BMP signaling to induce mesoderm, but in the absence of FGF signaling, BMP induces trophectoderm (Yu et al. 2011).

The canonical WNT pathway is associated with early cell fate decisions made by pluripotent cells during gastrulation, but its role in regulating hESC self-renewal is still unclear. Activation of canonical WNT signaling by treatment with the GSK-3β inhibitor BIO was able to maintain hESC pluripotency over short time scales (4–5 days) in the absence of feeder-conditioned media (Sato et al. 2004), and a recent study has demonstrated that canonical WNT receptor Frizzled-7 is necessary for hESC self-renewal (Melchior et al. 2008). Blauwkamp et al. (2012) showed that self-renewing hESCs are heterogeneous with respect to endogenous WNT signaling activity, and that the levels of WNT activation are critical to balancing lineage differentiation bias, with high levels of WNT signaling inducing mesoderm and/or endoderm, and lower levels being sufficient to generate ectoderm. Davidson et al. (2012) showed that OCT4 functionally represses endogenous WNT/β-Catenin signaling in self-renewing hESCs, and that activation of β-Catenin signaling leads to induction of mesoderm lineage transcripts and an eventual loss of self-renewal after several passages. Sumi et al.

(2008) showed that while elevated WNT/β-Catenin signaling promotes differentiation, basal β-Catenin activity under self-renewing conditions maybe important for sustaining pluripotency. Notably, this study demonstrated that the orchestrated balance of WNT/β-Catenin, PI3K/AKT, Activin-A/TGF-β, and BMP signaling pathways determines cell fate decisions (Sumi et al. 2008).

5.2. Results

In order to examine whether REST levels influence pluripotency signaling in hESCs or EBs, four of the pluripotency signaling networks (Activin-A/TGF-β, AKT, ERK, and WNT signaling) were evaluated in H9 hESCs. Western blot analysis for Activin-A/TGF-β pathway activation revealed increased levels of pSMAD2/3 in H9 REST KD hESCs (**Figure 5-1**B), indicating up-regulation of Activin-A/TGF-β signaling pathway. In order to evaluate the status of AKT signaling after REST KD, FACS analysis for TRA1 81, pAKT (Ser473) double positive cells was performed in H9 hESCs. As shown in **Figure 5-1**C, there was no statistically significant difference between REST KD and NT H9 hESCs. Western blot analysis for MAPK/ERK pathway activation revealed increased levels of pMEK 1/2 in H9 REST KD hESCs (**Figure 5-1**A), and qPCR for c-FOS, a key transcription factor downstream of the FGF/ERK pathway (Kang et al. 2005), showed increased c-FOS expression in H9 (**Figure 5-3**A) as well as in UCLA1 (**Figure 5-3**B) REST KD hESCs, demonstrating that the MAPK/ERK signaling pathway is up-regulated after REST KD. Western blot analysis was also performed for MAPK/ERK pathway activation in day 5 REST KDs EBs. The results demonstrate that increased activation of MAPK/ERK signaling is maintained after differentiation in H1 (**Figure 5-2**A), H9 (**Figure 5-2**B), and UCLA1 (**Figure 5-2**C) day 5 REST KDs EBs. In

order to evaluate WNT signaling, qPCR was performed for canonical WNT pathway genes AXIN2, β-Catenin and TROY, in REST KD hESCs as well as EBs, across three lines. While there was no significant change in expression of any of the genes in REST KD hESCs, increased expression of AXIN2 was observed in Day 5 REST KD EBs across H1 (**Figure 5-4**A), H9 (**Figure 5-4**B), and UCLA1 (**Figure 5-4**C) lines. β-Catenin and TROY levels were elevated in H1 (**Figure 5-4**A), and H9 (**Figure 5-4**B) REST KD EBs, but not in UCLA1 REST KD EBs (**Figure 5-4**C).

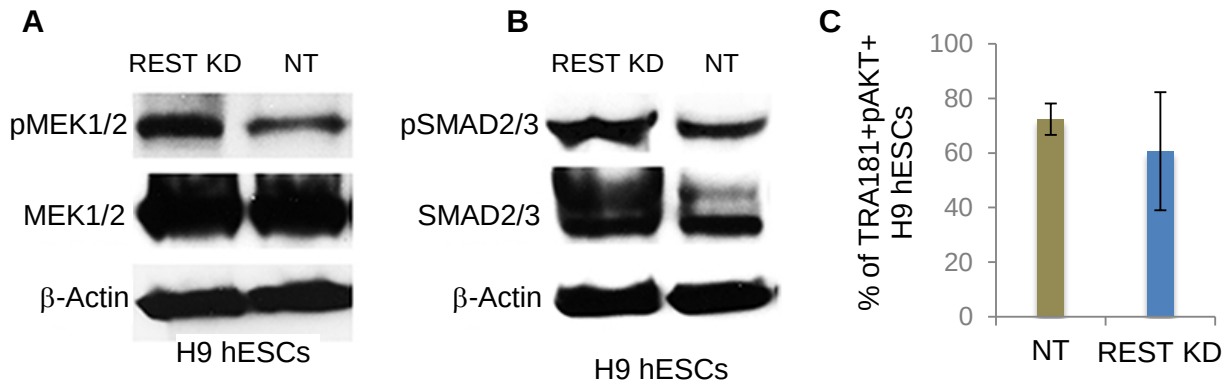


Figure 5-1: REST KD alters MAPK/ERK and Activin-A/TGF-β signaling, but not AKT signaling. **A, B:** Western blots showing that H9 hESCs have an increase in pMEK1/2 (S217/221) and pSMAD2/3 (S465/467) compared to control NT H9 hESCs. MEK1/2, SMAD2/3 and β-ACTIN were used as a loading control. **C:** Quantitative representation of FACS data from 3 independent experiments shows no statistically significant difference in percentage of TRA1-81, pAKT (Ser473) double positive hESCs between REST KD and NT H9 hESCs.

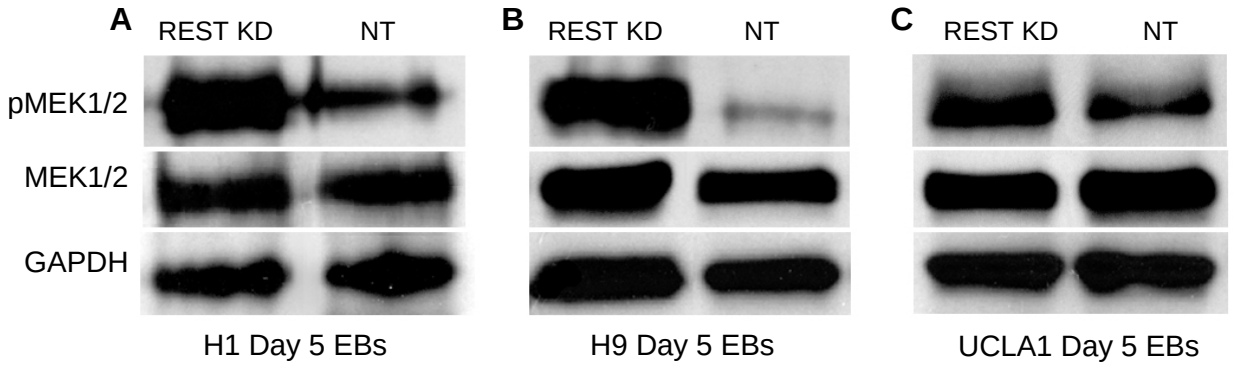


Figure 5-2: Western blots of day 5 EBs showing increased pMEK 1/2 in REST KD EBs from all three lines, H1 (**A**), H9 (**B**) and UCLA1 (**C**). MEK and GAPDH were used as loading controls.

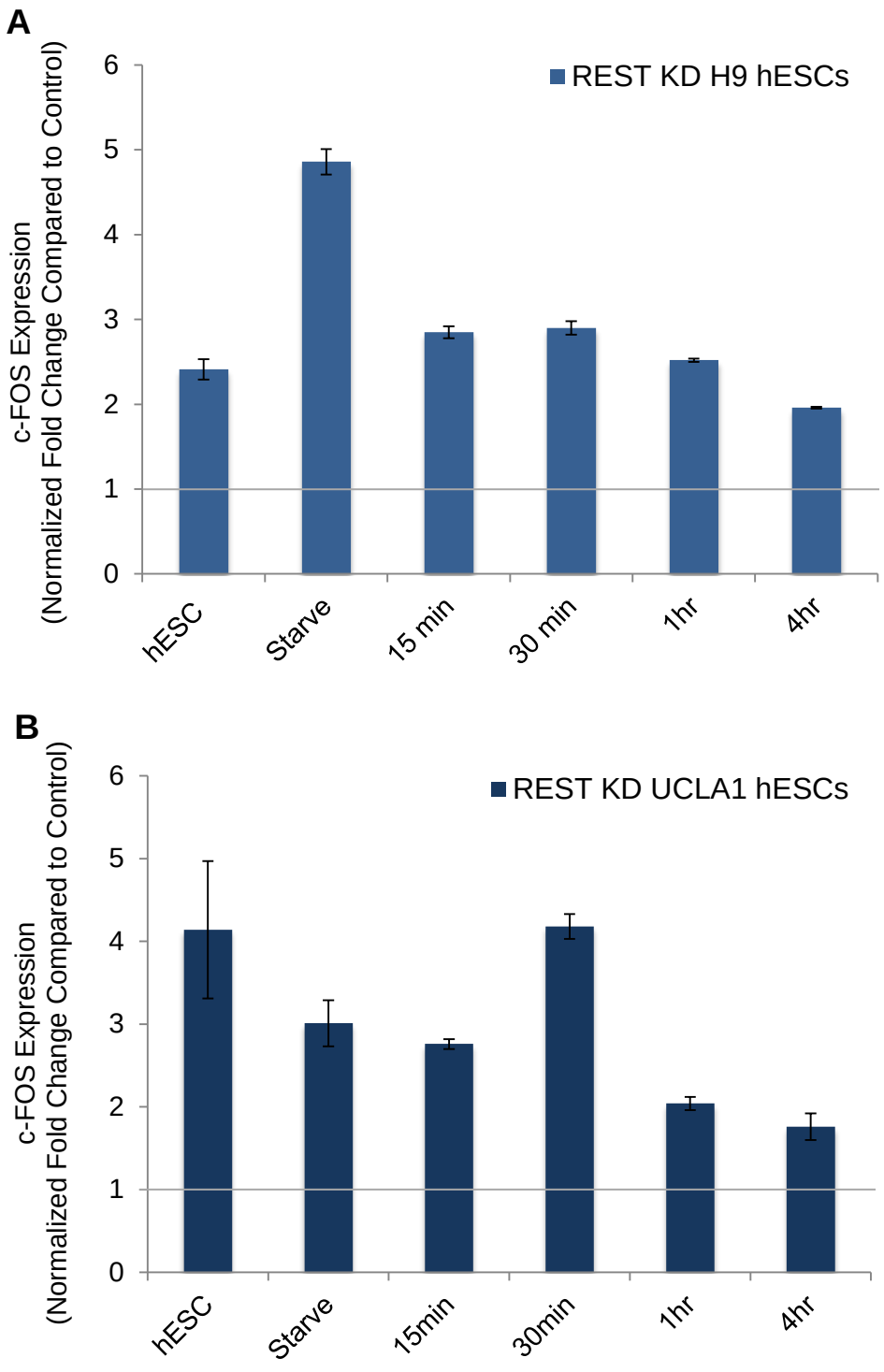


Figure 5-3: QPCR analysis for c-FOS, a downstream target of FGF/ERK signaling. In all conditions tested in both H9 (**A**) and UCLA1 (**B**) hESCs, c-FOS expression was higher in REST KD compared to control NT cells (hESC = normal hESC medium; starve = serum starvation for 18 hours and no bFGF; 15 min, 30 min, 1 hr, 4 hr = serum starvation for

18 hours followed by treatment with hESC media containing 10 ng/ml bFGF for indicated time periods). Representative graphs using internal error bars are shown.

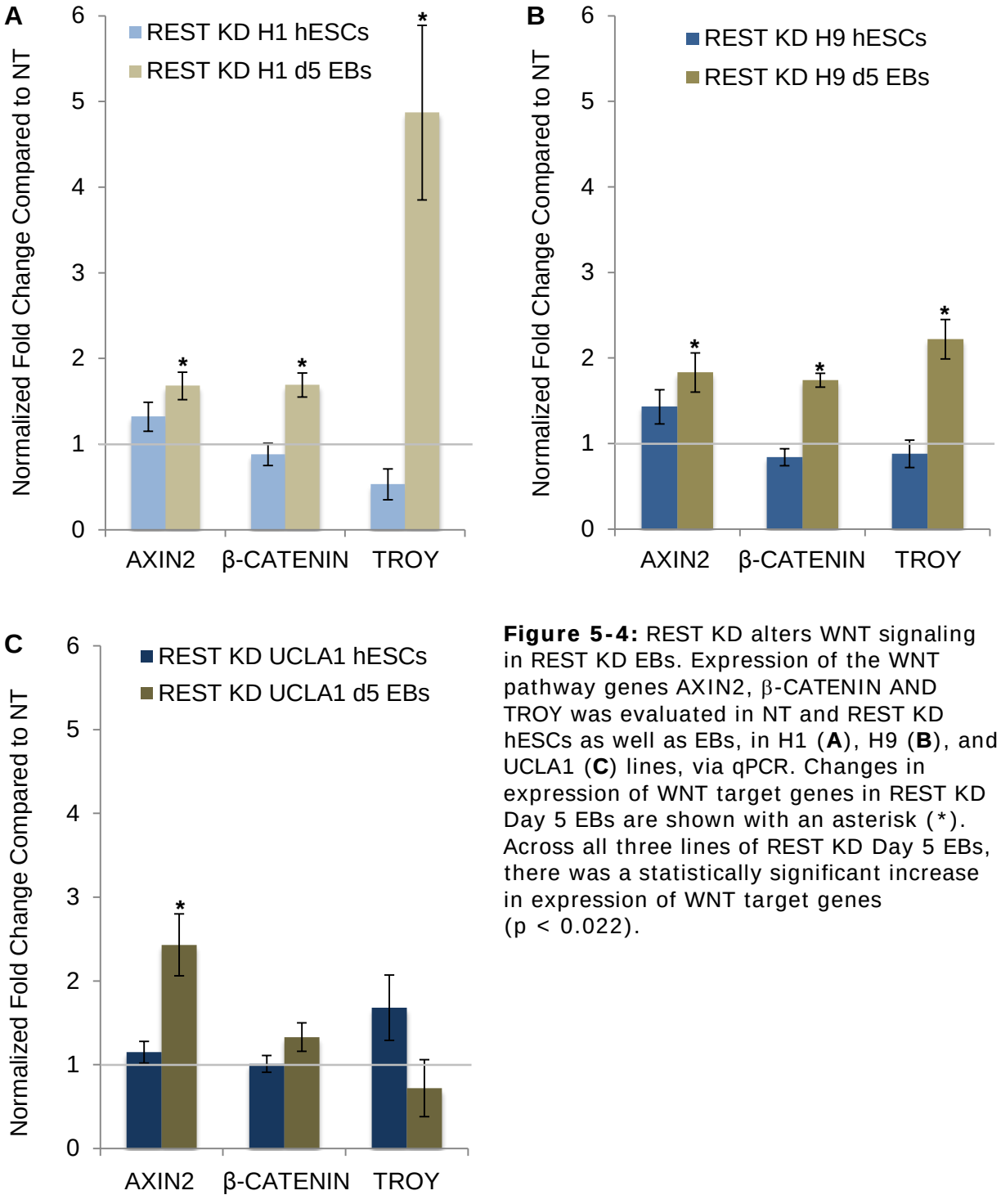


Figure 5-4: REST KD alters WNT signaling in REST KD EBs. Expression of the WNT pathway genes AXIN2, β-CATENIN AND TROY was evaluated in NT and REST KD hESCs as well as EBs, in H1 (**A**), H9 (**B**), and UCLA1 (**C**) lines, via qPCR. Changes in expression of WNT target genes in REST KD Day 5 EBs are shown with an asterisk (*). Across all three lines of REST KD Day 5 EBs, there was a statistically significant increase in expression of WNT target genes ($p < 0.022$).

5.3. Discussion

REST KD hESCs were found to have a slight increase in pSMAD2/3, but no significant change in pAKT, even though they did demonstrate increased survival compared to control NT hESCs. Increased MAPK/MEK/ERK activity was detected in REST KD hESCs as well as REST KD Day 5 EBs. It has recently become clear that the balance of each of these signaling pathways is key to regulating the pluripotent state in hESCs (Singh et al. 2012a; Sumi et al. 2008; Warmflash et al. 2012). Thus, the increase in pMEK 1/2 in REST KD hESCs is counterbalanced by no significant change in pAKT levels, which enables support of self-renewal in REST KD hESCs. Finally, increased WNT signaling was seen in REST KD Day 5 EBs, but not in REST KD hESCs. The three genes used to evaluate WNT signaling were AXIN2, β-Catenin and TROY. AXIN2 and TROY are direct targets of canonical WNT/β-Catenin signaling (Buttitta et al. 2003; Hisaoka et al. 2006; Jho et al. 2002; Tam et al. 2012). AXIN2 also participates in a negative feedback loop by promoting the phosphorylation and consequent degradation of β-Catenin (Jho et al. 2002), and this might explain why UCLA1 REST KD EBs, which show the highest AXIN2 expression among the three cell lines, have decreased TROY expression, whereas H1 and H9 REST KD EBs have elevated TROY expression. The differences in WNT signaling across the three lines verify the importance of balancing WNT signals during lineage specification. Blauwkamp et al. (2012) have demonstrated that while high levels of WNT signaling activating mesoderm and/or endoderm, lower levels are sufficient to generate ectoderm. UCLA1 did show a slight increase in expression of the neuroectoderm marker PAX6 and had decreased TROY expression in

Day 5 EBs. In contrast there was no increase in PAX6 in H1 or H9 REST KD EBs and these had elevated expression of β-Catenin and TROY.

In summary, REST KD hESCs and EBs have altered pluripotency signaling, with REST KD EBs having increased MEK and WNT signaling during early differentiation. Elevated MEK and WNT signaling in REST KD cells likely plays a key role in the lineage bias seen in the EB differentiation analysis.

5.4. Future Directions

Future experiments should focus on identifying the mechanisms by which REST KD leads to alterations in ERK and WNT signaling. Members of the ERK superfamily are REST targets (http://bioinformatics.leeds.ac.uk/RE1db_mkII/), as are several WNT pathway genes (Johnson et al. 2008). Culturing REST KD and NT hESCS in defined media conditions, with and without inhibitors to each of these pathways, followed by microarray expression analysis would help identify the REST target genes that mediate the signaling alterations observed in REST KD cells.

Chapter 6. Materials and Methods

- **Cell Culture:** hESCs used in this study were from lines H1 between 35–70 passages, H9 between 35–70 passages and UCLA1 between passages 20–42 (Diaz Perez et al. 2012). hESCs were grown on gelatin-coated plates with mitomycin-C treated Mouse Embryonic Fibroblasts (MEFs) in hESC medium consisting of Dulbecco's Modified Eagle Medium-F12 (DMEM/F-12, Invitrogen) supplemented with 20% Knockout serum (Invitrogen), 1 mM non-essential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% Penicillin/Streptomycin (Hyclone) and 4 ng/ml basic Fibroblast Growth Factor (Biological Resources Branch, National Cancer Institute). hESCs were routinely passaged every 5–7 days at a ratio of 1:2 or 1:3 depending on cell density. Briefly, hESCs were incubated with a sterile filtered collagenase IV (Invitrogen) solution (1 mg collagenase/mL of DMEM/F-12) for five minutes at 37°C, physically dissociated into small clumps using a 5 ml pipette, collected in a conical tube, and centrifuged at 1,000 rpm for 5 minutes. The cell pellet was re-suspended in hESC media, and the cells were plated onto MEF plates. hESCs used for RNA or protein collection were grown on Matrigel (BD Biosciences) coated plates and fed MEF conditioned medium (CM) to reduce MEF contribution.

- **Development of Inducible REST KnockDown (KD) shRNA System in hESCs:** Non-target control hESCs (NT) and REST knockdown hESCs (REST KD) were generated using the TRIPZ vector (Open Biosystems, http://www.openbiosystems.com), a lentiviral inducible RNAi system with microRNA-adapted shRNA (shRNAmir). The shRNA sequence is "TCGATTAGTATTGTAGCCG", and it

targets exon 3 of REST mRNA transcript variants 1 and 2. The TRIPZ vector contains a puromycin drug resistance marker, and a Tet-On system that induces expression of the shRNAmir and TurboRFP in the presence of doxycycline. To make stable lines, hESCs were harvested with trypsin, and plated onto a matrigel coated 24 well plate at a density of 5 × 104 cells/well using CM supplemented with 10 μM of the ROCK inhibitor HA 1077 (Damoiseaux et al. 2009). The day after cell plating, lentivirus containing non target shRNA (NT) or REST shRNA (REST KD) was added to the cells at a final virus concentration of 3 5 × 107 TU/ml CM. Polybrene was added to the media at a final concentration of 8 μg/ml to aid transduction. The plates were spinoculated at 2,000 rpm at 37°C for four hours, then transferred to the incubator for overnight incubation. The next morning, the transduction cocktail was replaced with fresh CM. 48 hours after transduction, and daily for 4 weeks thereafter, cells were fed CM containing 1 μg/ml puromycin for selection. NT and REST KD hESC cells were treated with collagenase and transferred onto feeders when ready for splitting. 1 μg/ml doxycycline was added to the media to turn on expression of the shRNAmir and TurboRFP. RFP positive colonies were manually scraped to obtain a homogenous population of RFP expressing cells.

- **siRNA Transfection:** SMARTpool siRNA and transfection reagents were purchased from Dharmocon (http://www.thermoscientificbio.com/rnai-and-custom-rna-synthesis/sirna/). 5 μM siRNA stocks were prepared for REST and Control Non-Target (NT) siRNA by resuspending each siRNA in 1x siRNA buffer. Stocks were stored at –20°C. One day prior to transfection, hESCs were harvested

with trypsin, and plated onto matrigel coated 6 well plates at a density of 2 × 105 cells/well using CM supplemented with 10 μM of the ROCK inhibitor HA 1077. On the day of the transfection, two tubes were setup for each siRNA/hESC well being transfected. 20 μl of the 5 μM siRNA stock (REST/NT) was added to 180 μl of serum-free medium (Optimem) in tube1, and mixed by gently tapping the tube. The corresponding tube2 was prepared by adding 8 μl of DharmaFECT1 reagent to 192 μl of Optimem, and mixed by gentle tapping. Tube1 and tube2 were incubated at room temperature for 5 minutes. Contents of each tube1 (siRNA) was added to corresponding tube2 (DharmaFECT), mixed gently by pipetting up/down, and incubated at room temperature for additional 20 minutes. 1600 μl of CM was added to each siRNA-DharmaFECT mix, to obtain 2ml of transfection cocktail with final siRNA concentration of 50 nM. Culture medium from the hESC plates was removed, and 2 ml of either NT or REST transfection cocktail was added to each well. After overnight incubation, the transfection cocktail was replaced by fresh CM. The hESCs were grown for additional 24 hours, before re-transfecting each well. 96 hours after the first transfection and 48 hours after the second transfection, the cells were harvested and processed for mRNA analysis or genomic DNA analysis.

- **Cell death/ Cell Survival analysis:** NT and REST KD hESC cells were trypsin harvested and plated at a density of 1X105 cells/well of Matrigel coated 6 well plates, using CM supplemented with 10 μM of the ROCK inhibitor HA-1077. Cells were cultured in CM (without the ROCK inhibitor) for 5–7 days. Cell death was measured

by staining with Annexin V (FITC Annexin V Apoptosis Detection Kit, BD Pharmingen) followed by flow cytometry analysis.

- **Embryoid Body (EB) Formation:** Confluent hESCs colonies were detached from the feeder layer by incubating cells with 1 mg/ml collagenase for 30–60 minutes at 37°C. The detached colonies were washed off the plate, collected in a conical tube, and allowed to pellet. After aspirating the supernatant, the colonies were resuspended in EB medium (hESC medium without bFGF) and plated in ultra low attachment plates (Corning). EB media was replaced every other day.

- **Teratomas:** For teratoma formation, two confluent wells of hESCs were harvested as previously described (Alva et al. 2011). Cell pellets were resuspended in 50 µl of PBS and injected into the testis of 4–8-week-old SCID beige mice (Charles River) according to UCLA-approved Animal Research Committee protocols. After 8 weeks, teratomas were isolated and fixed in 4% PFA for 24 hours. Fixed teratomas were embedded and processed by the Translational Pathology Core Laboratory at the David Geffen School of Medicine at UCLA. Teratoma sections were stained with H&E.

- **Karyotype Analysis:** Karyotype analysis was performed on long-term cultured cells by Cell Line Genetics (Madison, Wisconsin, http://www.clgenetics.com). Cells were prepared and shipped according to Cell Line Genetics procedures. Stable lines were generated as described in the “Cell Culture and Development of Inducible REST KnockDown (KD) System in hESCs” methods section above. REST KD and Control

NT hESCs were maintained in culture for at least 6 weeks prior to shipping for karyotype analysis. hESCs were passaged onto feeders in T-25 flasks, and live cultures were sent for analysis of at least 20 metaphase spreads per sample.

- **Western blot:** Western blotting was performed using a Criterion system with pre-cast Tris-HCl gels (Bio-Rad). The following primary antibodies were used at 1:1000 dilution: OCT4, NANOG, SOX2, pSMAD, SMAD, pMEK, and MEK (Cell Signaling), GAPDH (Abcam) and β-Actin (Santa Cruz Biotechnology). HRP-conjugated secondary antibodies (Promega, 1:5000 dilution) were detected by enhanced chemiluminescence (GE Lifesciences).

- **Immunocytochemistry:** Slides were fixed with 4% PFA in PBS at room temperature for 15 minutes, washed thrice with PBS, and blocked for an hour in blocking buffer (0.3% Triton-X 100, 10% normal goat serum in PBS) prior to staining. Slides were incubated for 2 hours at room temperature with anti-REST (Millipore) primary antibody diluted 1:100 in antibody dilution buffer (0.1% Triton-X 100, 1% normal goat serum in PBS). After washing thrice with PBS, slides were incubated for 1hr in the dark at room temperature with Goat anti-rabbit FITC-conjugated secondary antibody (Pierce) diluted 1:500 in antibody dilution buffer. Slides were mounted in Vectashield Mounting medium for Fluorescence with DAPI (Vector Labs).

- **Fluorescence-Activated Cell Sorting Analysis:** For staining hESCs with an extracellular stem cell marker, cells were treated with trypsin to dissociate into single

cells, rinsed with PBS, and blocked in FACS buffer (0.5% BSA in PBS) for 10 minutes at 4°C. Cells were incubated with TRA-1-81 (1:100, Santa Cruz Biotechnology) primary antibody for 30 minutes at 4°C, washed with FACS buffer, and then incubated with Cy5-conjugated goat anti-mouse secondary antibody (1:250, Jackson ImmunoResearch) for 20 minutes at 4°C. To detect intracellular markers of differentiation, EBs were dissociated into single cells with trypsin treatment, rinsed with PBS, fixed with 2% PFA for 10 minutes at 37°C and then permeabilized using 90% methanol for 30 minutes at 4°C. After washing twice with FACS buffer and blocking for 10 minutes, cells were incubated with one of the following antibodies for 1hr at room temperature: the Alexa Fluor 488 conjugated mouse anti-human antibodies PAX6 or SOX17 (1:20 dilution, BD Biosciences), or APC-conjugated mouse monoclonal anti-human Brachyury (1:10 dilution, R&D Systems). FACS acquisition was performed using a LSR II FACS sorter and analyzed using FACS DIVA software (BD Biosciences).

- **Quantitative Real Time PCR (qPCR):** RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (BioRad). RT-PCR was performed using FastStart Universal SYBR Green Master Mix (Roche). ViiA7 RT-PCR system was used for RT-PCR run (Applied Biosystems). Primer sequences are shown in **Table 6-1**.

Table 6-1: Primers sequences

Gene	Forward primer	Reverse primer
GAPDH	TGCCAAGGCTGTGGGCAAGGTCATCCCT	ACGGCAGGTCAGGTCCACCACTGACACG
REST	CGACATGCAAGACAGGTTCACAAT	AGCTGCATAGTCACATACAGGGCA
OCT4	ACATCAAAGCTCTGCAGAAAGAACT	CTGAATACCTTCCCAAATAGAACCC
SOX2	CTTTTGTTCGATCCCAACTTTC	ATACATGGATTCTCGGCAGAC
NANOG	CAGCTGTGTGTACTCAATGATAGATTT	ACACCATTGCTATTCTTCGGCCAGTTG
GATA4	TACATCAGCTTCCGGAACCACCAA	ATCCAGCATTGAGCAAAGGGCTC
FOXA2	GGAGCGGTGAAGATGGAA	TACGTGTTCATGCCGTTCAT
BRACHYURY	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG
CD34	TGAAGCCTAGCCTGTCACCT	CGCACAGCTGGAGGTCTTAT
TUJ1	GTACGAAGACGACCAGGAGG	GGGTTTAGACACTGCTGGCT
GFAP	GGGAGCTTGATTCTCAGCAC	CTGGGGTTAAGAAGCAGCAG
SYP	CTTTAAGCGAGGCAGAATGG	GCCTTGCTCAAGATCTGTCC
SYT4	GGGCTGAGTTGAGAACACTGTGGC	ACTGAGGCTTGTGACAGGCAGTGA
TRKC	TGCAGTCCATCAACACTCACCAGA	TGTAGTGGGTGGGCTTGTTGAAGA
AXIN2	ACAACAGCATTGTCTCCAAGCAGC	GCGCCTGGTCAAACATGATGGAAT
β-CATENIN	TGCAGTTCGCCTTCACTATGGACT	GATTTGCGGGACAAAGGGCAAGAT
FZD2	GGAAGCATTCGCCTTTGAGCACTT	AACTGCTAACCTGAACGCCAGAGA
TROY	TGTCTAAGGAATGTGGCTTCGGCT	TCACTGGTGGCTGAACAATTTGCC

Chapter 7. Summary

The present study clearly demonstrates that reduced REST expression does not result in loss of pluripotency. REST KD hESCs express all of the traditional pluripotency markers and can differentiate into cells from all three germ layers. However, REST KD embryoid bodies (EBs) express higher levels of endoderm and/or mesoderm markers as compared to control NT EBs. This differentiation bias was not observed in the absence of doxycycline, i.e., when REST was not knocked down. In addition, all four stable REST KD lines tested had karyotypic abnormalities. However, transient REST KD using siRNA did not result in aneuploidy. This suggests that long term-cultured hESCs with lower REST levels may be at higher risk for aneuploidy.

REST KD hESCs were found to have a slight increase in pSMAD2/3, but no significant change in pAKT, even though they did demonstrate increased survival compared to control NT hESCs. Increased MAPK/ERK activity was detected in REST KD hESCs as well as REST KD Day 5 EBs, while significantly increased WNT signaling was detected in REST KD Day 5 EBs but not in REST KD hESCs.

Critical hurdles for translation of the clinical potential of hPSCs into practice are their tumorigenic capacity, and the inefficiency in tailoring lineage differentiation. It is striking that altering REST levels in hESCs plays a role in regulating both differentiation and genetic stability. Interestingly, activation of MAPK/ERK signaling, which is elevated in REST KD cells, has previously been shown to play a role in inhibiting neuroectoderm differentiation (Greber et al. 2011), as well as in regulating genetic stability in a number of systems (Duhamel et al. 2012; Sebolt-Leopold, Herrera 2004). Importantly, members of the ERK superfamily are REST target genes, and provide mechanistic candidates for

activation of the changes in cell growth, genetic stability and lineage differentiation seen in REST KD hESCs. Elucidating the role of REST in regulating cell fate of hPSCs could enable development of robust methods to stably culture and tailor lineage differentiation of these cells for use in regenerative medicine applications.

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