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ROLE OF BRIGHT / ARID3A IN MOUSE DEVELOPMENT, SOMATIC CELL REPROGRAMMING, AND PLURIPOTENCY

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by

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Dedication

To my Mom and Dad, who have never given me anything but the best.

In memory of Auntie Sharon and Uncle Jack, who never gave me anything but love.

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ROLE OF BRIGHT / ARID3A IN MOUSE DEVELOPMENT, SOMATIC CELL REPROGRAMMING, AND PLURIPOTENCY

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The University of Texas at Austin, 2012

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Bright/ARID3A was initially discovered for its role in immunoglobulin heavy chain transcription in the mouse. Bright has also been implicated as a target of p53 and as an E2F binding partner. We have previously shown that Bright is necessary for hematopoietic stem cell development in the embryo. In this work, we show that Bright has a much broader role in development than previously appreciated. Loss of Bright in mice usually results in embryonic lethality due to lack of hematopoietic stem cells. Rare survivor mice initially appear smaller in size than either wildtype or heterozygous littermates, but as they age, these differences diminish. We show that adult Bright null mice have age-dependent kidney defects. Previous work in the adult mouse has not indicated a role for Bright in kidney function. We observed an increase in cellular proliferation in Bright null kidneys, indicating a possible mechanism behind our observation. Loss of Bright has recently been implicated in causing developmental plasticity in somatic cells. Our data indicate that loss of Bright is sufficient to fully reprogram mouse embryonic fibroblasts (MEFs) back to a pluripotent state. We term these cells Bright repression induced pluripotent stem cells (BriPS). BriPS derived from Bright knockout MEFs can be stably maintained in standard embryonic stem cell culture conditions, they express pluripotency markers, and can form teratomas *in vivo*. We

further show that Bright is active during embryonic stem cell differentiation. Bright represses key pluripotency genes, suggesting the mechanism of reprogramming may be Bright's direct repression of key pluripotency factors in somatic cells. Recent advances in inducing pluripotency in somatic cells (iPS cells) have created a new field of disease modeling, increased our knowledge of how pluripotency is regulated, and introduced the hope that they can be adapted to treat disease. However, current methods for producing iPS involve overexpression of potentially oncogenic transcription factors, leaving a large gap between the lab and the clinic. Our results mark the first demonstration of an alternative method to reprograming somatic cells that is not mediated by overexpression of pluripotency factors.

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Chapter 1

Introduction

1.1 REGENERATIVE MEDICINE

The aim of regenerative medicine is to replace injured or diseased tissues. Regenerative medicine uses either embryonic or adult stem cells and differentiates them *in vitro* into various cell types that are injected into injured tissues, where they can proliferate and repair or replace the damage. The field of regenerative medicine has seen many breakthroughs in the past 10 years. There is a huge effort aimed at bringing regenerative medicine treatments from the lab to the clinic. Currently, a wide range of diseases are being targeted for treatment including stroke, spinal cord injury, Parkinson's disease, Alzheimer's disease, diabetes, and blindness.

1.1.1 Embryonic Stem Cell Clinical Trials

In October 2010, the GERON Corporation conducted the first clinical trials using human embryonic stem (hES) cells in the United States. The application sent to the FDA to gain approval was the longest ever submitted (Eastin, 2009). GERON made an

oligodendrocyte progenitor cell line from hES cells to inject them into recent spinal cord injuries to aid in recovery. Due to financial constraints, GERON halted their Phase I trial in November 2011 (Walsh, 2011). Since this first trial, several other clinical trials involving hES-derived cells have begun in both the U.S. and Europe. In the U.S., Advanced Cell Technology has begun two phase I/II trials in the US and one in Europe to treat Strargardt's macular dystrophy, age-related macular degeneration, and macular degeneration using retinal pigment epithelial cells derived from hES cells (www.ACTCblog.com). Additionally, there are more clinical trials in the U.S. and Europe that use cell lines derived from tissue-specific stem cells (www.eurostemcell.org). Early results have not shown any safety or health issues (Normile, 2012; Paddock, 2012), encouraging more companies to develop stem cell-based treatments.

1.1.2 Potential Applications of Induced Pluripotent Stem Cell Technology

Publication of work from the Yamanaka lab in Kyoto University (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), followed shortly by work from the Thomson lab at the University of Wisconsin (Yu et al., 2007) created a new field of research almost overnight. They demonstrated that it was possible to reprogram fully differentiated human cells to a pluripotent, ES-like state termed induced pluripotent stem cells (iPS). This opened the possibility of creating therapeutic iPS cells directly from the patient.

The potential of hES cells in the clinic has been understood long before science was able to deliver on that potential. The issues that may limit using hES cells as a therapeutic, such as possible immune rejection and ethical concerns, have made iPS cells

an attractive alternative. iPS appear to have the same ability as ES cells to differentiate *in vitro*, so some surmise iPS can replace hES therapeutically. The underlying problem is whether iPS cells can be reliably and efficiently produced without any gene mutations or integration occurring during the reprogramming process (Okita et al., 2008; Carey et al., 2009; Gonzalez et al., 2009; Kaji et al., 2009). While huge strides have been made in deriving integration-free iPS, a reliable and robust method of reprogramming that is adaptable to a clinical setting has yet to be developed.

An interesting and beneficial field of research that has arisen from the discovery of iPS cells is the creation of disease-specific iPS cell lines (Park et al., 2008), such as for Huntington's disease and Down's syndrome. Disease-specific iPS cells can be used to study and model disease development and progression in a way never before seen, from pluripotent, ES-like cells through differentiation to the adult cell. This research is not only valuable in understanding disease progression and potentially developing therapies, but could provide a basis for autologous cell therapies where the genetic mutation(s) have been corrected before transplantation back into the patient.

In the past, it has been difficult to predict how quickly current promising basic research will be translated into practical applications. However, regenerative medicine's rapid development suggests approved stem-cell-based therapies will soon appear. The possibilities seem endless, but only through careful and diligent research will the full potential of ES and iPS cells be reached.

1.2 BRIGHT'S ROLE IN REPROGRAMMING AND POTENTIAL APPLICATIONS

Bright/ARID3A (**B**-cell regulator of **IgH T**ranscription) was first discovered as a regulator of immunoglobulin transcription in B-cells (Herrscher et al., 1995). Bright acts as a transcription factor that binds sequence-specific AT-rich regions (see Chapter 2 for further detail). In this work we show the effect loss of Bright expression has in the adult mouse and somatic cells. Previously we have shown Bright null (Bri-/-) mice are typically embryonic lethal by E12.5 due to failed hematopoiesis (Webb et al., 2011). Bri-/- animals that survive the embryonic lethality are, however, able to thrive. These rare survivors show an early developmental delay (most notably in the B-cell lineage), which is overcome with age. We show here that Bright null adults have morphologically normal early organ development, but aged mice appear to have kidney defects, possibly indicating a novel role for Bright in kidney maintenance.

Loss of Bright has a dramatic effect on somatic cells. Multiple cell lineages from Bright null animals have shown a remarkable developmental plasticity *in vitro*. In culture, loss of Bright, through germline deletion, dominant negative inhibition, or shRNA-mediated knockdown, can induce pluripotency related genes and partially reprogram somatic cells to an ES-like state. These cells are capable of differentiating into different cell lineages, but cannot form teratomas *in vivo* (An et al., 2010). This intriguing phenotype led us to further investigate the extent of Bright's reprogramming capabilities. We show in this work that loss of Bright in mouse embryonic fibroblasts (MEFs) can fully reprogram these cells to a pluripotent state. Moreover, we show that Bright is an important regulator of normal mouse embryonic stem cell differentiation. These data

suggest that Bright may have a previously unappreciated role in early differentiation. Most strikingly, our research indicates that Bright repression alone can mediate complete cellular reprogramming. Further improvements in this reprogramming technique may lead to the clinical use of iPS to treat a wide range of diseases.

1.3 OUTLINE OF DISSERTATION

Efficient, safe reprogramming of somatic cells to an undifferentiated state may hold the key to treatments for a huge spectrum of disease. Injured tissues could be repaired using cells from the patient that are reprogrammed and injected to the site of injury. Future therapies may be discovered using disease specific iPS cells, allowing scientists to model disease progression in previously impossible ways. It may also be possible to take a patient's own cells, correct a genetic defect, and use them as a therapy for that patient. These goals cannot be reached without developing a safe, effective, and robust reprogramming methodology. The purpose of this work is to progress the development of an alternative somatic cells reprogramming method that could usher in iPS cell based-therapies.

The promising observation that loss of Bright produces a developmentally plastic state in cultured primary cells provides the foundation for this work. To further understand Bright mediated reprogramming, I will present my work as follows. Background on Bright/ARID3A and pluripotency are presented in Chapter 2. Chapter 3 focuses on a novel role for Bright in kidney development. Results concerning the effect loss of Bright has on somatic cell reprogramming are detailed in Chapter 4. In Chapter 5,

I will focus on the role Bright plays in normal embryonic stem cells. Finally, conclusions from Chapters 3, 4 and 5 and future directions are presented in Chapter 6.

Chapter 2

Background

2.1 HEMATOPOIETIC DEVELOPMENT

The role of the hematopoietic system is as broad as its development is complicated. Simply, it is "the bodily system of organs and tissues, primarily the bone marrow, spleen, tonsils, and lymph nodes, involved in the production of blood" (The American Heritage Dictionary). The hematopoietic system is responsible for the flow of nutrients, hormones, and oxygen through the blood stream, defense against foreign agents, and transportation of waste (Fox, 2001; Silverthorn, 2006).

The immune system defends the body against foreign bodies, disposes of dying cells, and targets mutated cells for destruction. The immune response against foreign bodies is divided into two types of defense that are separate, but cross-regulate one another (Borghesi and Milcarek, 2007). The innate immune response is nonspecific and does not confer memory. It provides an immediate defense mechanism by removing foreign substances, recruiting immune cells, and initiating an inflammatory response. In contrast, the adaptive immune response is mediated by lymphocytes, which specifically

target foreign invaders and maintains a memory of them to mount a quicker, more thorough response the next time the organism is exposed. The first response after initial exposure to an invader is the primary response and is significantly slower with a lower affinity than the organism's responses to subsequent exposures, termed the secondary response. The response speed is increased because long-lived memory cells can more rapidly mount a defense against previously encountered pathogens (Fox, 2001; Silverthorn, 2006; Lai and Kondo, 2008).

2.1.1 Embryonic Hematopoietic Stem Cell Development

The hematopoietic system is 'built' in several stages during development. Initial hematopoiesis, known as primitive or embryonic hematopoiesis, begins in the aortal-gonad-mesonephros (AGM), placenta, and yolk sac of the developing embryo. Primitive hematopoiesis produces red blood cells for oxygen transport, but early hematopoietic progenitor cells are not capable of long-term reconstitution of the hematopoietic system. In the mouse, around E8.5 (embryonic development day post-fertilization) when circulation is well established, precursors of definitive hematopoietic stem cells (HSC) begin to circulate, seeding the fetal liver by E12.5 (J. Palis et al. 1999; Sugiyama et al. 2011; Orkin and Zon 2008; Müller et al. 1994; James Palis et al. 2010). The shift of erythropoiesis to the fetal liver occurs at this point. As determined by transplantation assays into irradiated host mice, HSC arise after E10 (Müller et al. 1994), indicating that HSC are present in the early sites of hematopoiesis such as the yolk sac. The exact order and location of HSC maturation is not clear, but definitive hematopoiesis occurs in the fetal liver (James Palis et al. 2010), where the HSC expand and produce a large amount

of progenitors. One key feature of the switch from primitive to definitive hematopoiesis is globin switching. Early erythrocytes produce embryonic globin, which bind oxygen with greater affinity than adult hemoglobin. When definitive hematopoiesis begins, the erythrocytes begin producing adult hemoglobin and cease making the embryonic form (Bauer and Orkin 2011; Wilber, Nienhuis, and Persons 2011; Sankaran et al. 2009).

2.1.2 Adult Hematopoiesis

The development of the immune system begins in the embryo and continues throughout the life of the organism. Post-natal expansion of the hematopoietic system is necessary for the health of the organism. During mammalian early infancy, the immune system is supplemented by maternal antibodies that cross the placental barrier during development and come from breastfeeding post-natally while the immune system continues to develop (Eidelman et al., 2012). As the infant matures, the immune system becomes fully competent.

In mammals, hematopoiesis (**Figure 2.1**) is almost exclusively restricted to the bone marrow, where HSC reside. HSC produce several kinds of progenitor cells. The common lymphoid progenitor (CLP) produces T and B lymphocytes as well as natural killer cells. The common myeloid progenitor (CMP) can differentiate into erythrocytes, megakaryocytes/platelets, macrophages, neutrophils, eosinophils, and basophils (Cantor and Orkin, 2002; Krause, 2002; Larsson and Karlsson, 2005). Interestingly, dendritic cells are capable of developing from either CLP or CMP (Lipscomb and Masten, 2002).

2.1.3 B-cell Development

B-cells are a type of lymphocytes responsible for secretion of antibodies, which bind to foreign bodies. Naïve B-cells develop in the bone marrow where they undergo heavy chain VDJ and light chain VJ recombination. B-cells have unique B-cell receptors (BCR) that arise from these recombination events (Hardy and Hayakawa, 2001; Bianco, 2011). From there the B-cells migrate to secondary locations where they undergo further maturation in a stepwise fashion (**Figure 2.2**). Most B-cells undergo apoptosis, but some are activated when they specifically bind an antigen with their BCR. The B-cell will present the antigen on its cell surface bound to the major histocompatibility complex II (MHC II) molecules. This antigen/MHC II complex is recognized by helper T cells (Janeway et al.). The helper T cells then stimulate the B-cells to proliferate and either form plasma cells that secrete copious amounts of antibodies or form memory cells that contribute to the acquired immunity response. This process is termed clonal expansion (Silverthorn, 2006).

Antibodies are divided into 5 classes, IgG – produced in the secondary response, IgA- found in mucosal secretions and serum, IgE- secreted or on the surface of mast cells and associated with parasitic and allergic response, IgM – associated with the primary immune response, and IgD – found on the surface of B-cells and helpful in, but not required for B-cell activation (Geisberger et al., 2006; Silverthorn, 2006; Montecino-Rodriguez and Dorshkind, 2012).

2.2 ARID DOMAIN

ARID (AT-rich interaction domain) is an evolutionarily conserved DNA binding domain that has been identified in all higher eukaryote genomes thus far (Wilsker et al., 2005). The ARID domain is ~100 aa in length and binds to the major groove through a modified helix-turn-helix motif (Yuan et al., 1998; Iwahara and Clubb, 1999; Tu et al., 2001; Kim et al., 2004).

2.2.1 ARID Family of Proteins

ARID proteins all bind DNA, but only a subset, which includes Bright, binds DNA in a sequence specific manner (Wilsker et al., 2005). Expression patterns of ARID family members also vary in tissue specificity, from broad expression patterns to highly specific (**Table 2.1**). The ARID family is divided into 7 subfamilies, ARID 1-5 and JARID 1-2. ARID family proteins participate in a wide range of cellular functions including cell-cycle regulation, differentiation, chromatin remodeling, and development (Kortschak et al., 2000; Wilsker et al., 2002).

2.2.2 ARID3 Subfamily

Members of the ARID3 subfamily all share regions of homology adjacent to either side of the ARID domain (termed the extended ARID region or eARID) and a conserved C-terminal motif termed REKLES (for a conserved amino acid motif) (**Figure 2.3**) (Kortschak et al., 2000; Kim et al., 2007). The ARID3 subfamily consists of three

genes: ARID3A (Bright), ARID3B (BDP), and ARID3C (Brightlike). They are orthologs of the Drosophila DRI protein that also contains the conserved ARID, eARID, and REKLES domains. The Dri gene is important in anterior-posterior patterning and muscle development in *Drosophila melanogaster* embryonic development (Shandala et al., 1999). The REKLES domain is specific to this subfamily of proteins and is necessary for nuclear shuttling (Kim and Tucker, 2006), paralogous and self-association, and nuclear matrix targeting (Kim et al., 2007).

2.2.3 Bdp/ARID3B and Bright-like/ARID3C

Bdp/ARID3B was initially described as a binding partner of the Retinoblastoma protein (Numata et al., 1999). Bdp expression increases with mES differentiation (Wang et al., 2006). During embryonic development Bdp is expressed in the cranial and caudal mesenchyme, but expression in the cranial mesenchyme is downregulated by E10.5 (Takebe et al., 2006). By regulating proper cell motility and rearrangements, Bdp also ensures correct apical ectodermal ridge development (located at the distal edge of the limb bud which directs outgrowth of the limb) (Casanova et al., 2011). Bdp null mice die at E9.5 of neural crest defects as well as heart defects (Takebe et al., 2006; Webb et al., 2011). Normal expression in adult tissues appears restricted to testes, prostate, thyroid, and thymus (Takebe et al., 2006).

Brightlike/ARID3C is the smallest member of the ARID3 family. The Brightlike gene encodes two alternate splicing isoforms, one with and one without the REKLES domain (Tidwell et al., 2011). Only the Brightlike isoform that contains the REKLES

domain associates with Bright in B-cells and significantly co-activates Bright dependent IgH transcription (Webb et al., 1991; Tidwell et al., 2011).

2.2.4 Bright/ARID3A

Bright is the founding member of the ARID family of proteins (Herrscher et al., 1995) (**Figure 2.4**). It was first identified due to its ability to bind matrix-associated regions in the immunoglobin heavy chain (IgH) locus in B-cells (Webb et al., 1991; Webb, 2001). Bright is expressed in mES, and its expression increases during differentiation (Wang et al., 2006). In the developing embryo, Bright is broadly expressed in early development (E5.5-E8.5), but then becomes restricted to the fetal liver. Bright null embryos typically die by E12.5 due to failed erythropoiesis (Webb et al., 2011). In the adult mouse, Bright expression is restricted primarily to B-cells, where it is required for proper B-cell development (Webb et al., 1998, 2011; Nixon et al., 2004, 2008; Oldham et al., 2011). Bright also interacts with BTK and TFII in lipid rafts as part of the B-cell receptor (BCR) complex (Webb et al., 2000; Rajaiya et al., 2006; Schmidt et al., 2009).

Bright has been implicated in cell-cycle control pathways. Bright is sometimes termed E2FBP1, (E2F binding protein 1) because it was independently identified as an E2F binding protein in NEC14 cells, a human embryonic carcinoma cell line (Suzuki et al., 1998). Later work has shown that Bright is a direct target of p53 (Ma et al., 2003; Lestari et al., 2012). Overexpression of Bright overcomes Ras-induced senescence downstream of or independent of p53 (Peeper et al., 2002). Similarly, Bright depletion

induces promyelocytic leukemia protein (PML) dependent premature senescence through the $p16^{ink4A}$–Rb pathway (Fukuyo et al., 2011b). Bright cooperates with p53 to regulate $p21^{WAF1}$ expression. p53 knockdown decreased Bright expression, whereas overexpression of Bright increased p53 stability and knockdown of Bright decreased p53 stability (Lestari et al., 2012). Despite the role Bright plays in maintaining proliferation through dissociating PML bodies, it may also act as an intrinsic defense against viral infection such as herpes simplex virus 1 (Fukuyo et al., 2011a). These data indicate that Bright regulates cell cycle and senescence control.

Bright has recently been implicated in the TGF-beta pathway in *Xenopus* embryos and human lung fibrosis. In *Xenopus*, Bright was found downstream of the TGF-beta pathway in the emerging mesoderm as a cofactor in both the Activin/SMAD2 and BMP/SMAD1 signaling pathways (Callery et al., 2005). In the lungs, Bright enhances TGF-beta target genes and was bound by Id1, which acted to repress Bright (Lin et al., 2008). We have previously shown that loss of Bright induces developmental plasticity in multiple cell types (An et al., 2010), further indicating that Bright has more cellular functions in different tissue types beyond its role in hematopoiesis and B-cell development.

2.2.5 Bright Function in Adult Tissues

Bright is a well-established regulator of B cell development and early hematopoietic stem cell development (Webb et al., 1998, 2011; Nixon et al., 2004, 2008; Oldham et al., 2011). Little work has been done determining Bright's normal function in

adult tissues. Bright null embryos and rare survivors are smaller than either their wild type or heterozygous littermates (Webb et al., 2011). As the mice aged, these early differences normalized, and Bright null mice were indistinguishable from their littermates. Bright expression is restricted in the adult animal, but it is not completely repressed. We hypothesize that Bright might play a less obvious role in regulating adult tissues. We examined the histology of multiple organs of 6 week and >1 year old Bright null, heterozygous, and wild type animals. Our data indicate that Bright may be important for kidney growth and/or maintenance.

2.3 STEM CELLS

Stem cell is a term used to describe a wide variety of cell types during development and adulthood. Simply, stem cells have the ability to divide and produce daughter cells of different cell types as well as self-renew. Multicellular organisms all begin life as a single cell. This single cell divides and produces every cell type in the organism. Therefore, all cells in an organism have the genetic code for every protein, but only a fraction of these proteins are expressed in a given cell type. This allows cells to become specialized in their function. Red blood cells are fully differentiated cells that express hemoglobin, a protein which binds oxygen, allowing it to be transported to the rest of the body. Other tissue types do not express hemoglobin; they express only the proteins that are necessary to their own function. Differentiated cells do not express genes associated with proteins they do not need by acquiring a highly compacted chromatin structure. During development and into adulthood, a small population of stem cells maintains the ability to form multiple differentiated cell types. Stem cells retain the

ability to differentiate partially by having an ‘open’ chromatin structure. This ‘open’ chromatin allows genes to be accessible and therefore not ‘shut off’, as is the case in differentiated cells. Differentiated cells have a more ‘closed’ chromatin structure, restricting gene expression. Stem cells express a cohort of pluripotency related proteins that act to maintain repression of differentiation genes while promoting pluripotency, until the cell receives external cues to begin its differentiation program.

2.3.1 Early Embryonic Development

Fertilization occurs when a haploid sperm enters a haploid oocyte, creating a diploid cell. Global demethylation occurs across maternal and paternal genomes to ready the new genome before the first cell division occurs. In the mammal, embryonic development occurs in regulated steps that are highly similar across species. Initial oocyte development is controlled by maternally provided mRNA transcripts and proteins (Verlhac et al., 2010). Degradation of these proteins and initiation of the embryo’s transcriptional program occurs after fertilization (Knowles et al., 2003; Verlhac et al., 2010).

As the fertilized egg begins its early divisions, it comprises a solid ball of cells called a morula. The morula is composed of cells called blastomeres. The first differentiation event occurs between the 8 to 16-cell stage. The cells of the morula on the outer layer are fated to become trophectoderm (TE), while the inner cells are fated to become the inner cell mass, which in turn develops into the embryo and yolk sac. At this point, cell specification has begun but is reversible. The cells of the morula are totipotent,

meaning they have the ability to develop into both the extraembryonic and embryonic cell types. The morula next commits cells to their specific fate. The outer layer of cells, determined to be the TE that will develop into the placenta, are polarized and undergo compaction. As compaction occurs, the visibly distinct cells become obscured by the tightly joined TE cells. The outer TE cells begin to secrete vacuoles into the center of the morula that fuse, forming a large cavity into which fluid is pumped. At this developmental stage, the morula has matured into a blastocyst.

The internal cells of the blastocyst are the inner cell mass (ICM), which will develop into the embryo. Unlike the TE cells, ICM cells are not polarized, but are located as a single mass on one side of the blastocyst. These cells are pluripotent, capable of differentiating into all cell types of the embryo. Embryonic stem cells used in *in vitro* studies are solely derived from the inner cell mass (Marikawa and Alarcón, 2009; Chen et al., 2010) (**Figure 2.5**).

2.3.2 Stem Cell Potency

The 'potency' of a cell refers to its ability to divide and become a more differentiated cell type. Totipotent cells are able to develop into all embryonic and extraembryonic cell types. The zygote and the blastomeres of the morula are totipotent. Blastomeres begin to be committed to a cell fate at the 8-cell stage, but studies have shown that they do not lose totipotency until the 32-cell stage (Suwińska et al., 2008).

Pluripotent cells, which include embryonic stem cells (ESC), epiblast stem cells (EpiSC), primordial germ cells (pGC), and germ cells (GC), can differentiate into all cells of the adult. With the exception of germ cells, totipotent and pluripotent cell types are only found in the developing embryo. Embryonic stem cells (ES) are derived from the inner cell mass of the pre-implantation blastocyst. Mouse ES cells were first cultured in 1981 (Evans and Kaufman, 1981). This breakthrough opened the door to understanding early embryonic development and helped lead, ultimately, to the field of regenerative medicine.

Epiblast stem cells are found in the epiblast layer of the embryo. The epiblast is formed from the ICM layer closest to the trophectoderm. These cells can be derived from either pre- or post-implatation blastocysts (Najm et al., 2011). Interestingly, based on gene expression analysis, human ES cells appear more similar to mouse EpiSC than mouse ES (De Miguel et al., 2010).

Primordial germ cells are the precursors of the germ cells. pGCs are diploid and form in the developing embryo. During early embryogenesis they migrate to the yolk sac before they migrate to the gonadal ridge. Once PGCs reach the gonadal ridge, they proliferate, undergo meiosis or mitosis, and become the germ cells. Germ cells give rise to gametes of an adult organism (Ginsburg et al., 1990; Hajkova et al., 2002).

Tissue specific stem cells are present in adult tissues, and are capable of differentiating into multiple cell types specific to that tissue, making them multipotent. Well-studied adult stem cells include the mesenchymal and hematopoietic stem cells,

found in the bone marrow, as well as crypt cells found in the intestine. Also, neuronal stem cells have been isolated from several areas of the brain. These tissue specific stem cells are responsible for cellular turnover of a limited subset of cell types and are necessary for growth and replacing dead cells throughout the organism's lifespan (Mimeault and Batra, 2012; Oh and Humphries, 2012; Zapata et al., 2012).

2.3.3 Embryonic Stem Cells

Cells were first successful grown in culture in 1907 when Ross Harrison at Johns Hopkins University successfully cultured frog neural tubes (Harrison et al., 1907). Since then, there have been many milestones in developing cell culture systems as successful models for discovering the molecular machinery that drive cellular processes. The first human cells immortalized in culture were HeLa cells derived in 1951 (Gey et al., 1954). HeLa cells--present in almost all initial cell-culture research--have been used in the development of the Polio vaccine, AIDS and cancer research, drug development, and in space to test the effects of radiation (Skloot, 2011). Two groups in the US and UK derived the first pluripotent embryonic stem cells from mice (mES) in 1981 (Evans and Kaufman, 1981; Martin, 1981). The first human pluripotent stem cells (hES) were derived in 1998 by James Thomson (Thomson et al., 1998). Each of these breakthroughs allowed researchers to identify cellular genes and pathways that drive growth, senescence and maintain pluripotency. Ultimately, it has allowed researchers to manipulate all these characteristics in order to use ES cells as a research and therapeutic tool.

2.3.4 Reprogramming

The isolation and characterization of human embryonic stem cells has had a profound effect on developmental, cellular, and molecular biology. These fields of research have come together to help develop regenerative medicine. The promise of regenerative medicine is far reaching, from repairing cardiac muscle or spinal cord nerves after an injury to reversing Alzheimer's disease. However, employing hES cells raises ethical issues, since the embryo is typically destroyed in order to derive the cell lines. Potential immune system rejection may also pose a problem with the use of hES cells as a therapeutic.

Cell fusion and somatic cell nuclear transfer (SNCT) proved that reprogramming of a fully differentiated nucleus was possible (Campbell et al., 1996; Wakayama et al., 1998; Byrne et al., 2007; Hasegawa et al., 2010). This is achieved through either fusion of the somatic cell with an embryonic stem cell or injection of the somatic nucleus into an enucleated oocyte, respectively. Molecular studies in mouse and human ES cells gave researchers insight into which genes were important for pluripotency. Using this knowledge, Shinya Yamanaka and colleagues hypothesized that overexpression of one or a combination of pluripotency factors would lead to cellular reprograming. In 2006, they successfully reprogrammed mouse fibroblasts to a pluripotent state using overexpression of just four factors:, Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). Yamanaka termed these cells induced pluripotent stem cells (iPS). In 2007, both the Yamanaka and Thomson labs successfully reprogrammed human fibroblasts into iPS using similar gene combinations. Yamanaka reprogrammed human fibroblasts using the

previous combination of Oct4, Sox2, Klf4, and c-Myc, while Thomson used Oct4, Sox2, Nanog, and Lin28. The somatic cell reprogramming technique has the potential to lead to personalized regenerative medicine that circumvents both the ethical and technical issues associated with hES cells.

Reprogramming MEFs into iPS has now become a common laboratory technique, as it is a relatively straightforward procedure. It is most commonly achieved through integration of the transcription factors into the genome using a retroviral vector. The integration proves problematic, as virally altered cells are not viable candidates for human therapeutics. Alternative techniques have been successfully reported that do not modify the target cell's genome, lending hope to the idea of adapting the reprogramming method to the clinic (Okita et al., 2008; Carey et al., 2009; Gonzalez et al., 2009; Kaji et al., 2009; Parameswaran et al., 2011; Wu and Hochedlinger, 2011).

2.3.5 Key Transcription Factors of Pluripotency

There are a large number of genes preferentially expressed in pluripotent stem cells. Of these genes, some are considered "key" regulatory transcription factors. The central 'triad' of transcription factors that maintains pluripotency and represses differentiation is Oct4/Pou5f1, Sox2, and Nanog (O/S/N) (Silva and Smith, 2008; Silva et al., 2009). These three transcription factors interact with each other as part of larger protein complexes that regulate pluripotency by repressing differentiation genes and expressing other pluripotency genes, including themselves (**Figure 2.6**), (Guenther, 2011; Ng and Surani, 2011; Young, 2011; Sterneckert et al., 2012).

Although O/S/N work together to maintain pluripotency, they also promote its end. Oct4 and Sox2 control expression of FGF4 (Yuan et al., 1995), which induces differentiation. *In vivo*, pluripotent cells are all destined to differentiate to some degree; only under strict cell culture conditions do cells maintain pluripotency indefinitely.

2.3.6 Bright's Role in Reprogramming and Stem Cells

Loss of Bright can induce partial reprogramming in multiple cell lines (An et al., 2010), indicating that repression of Bright may be a viable approach for producing reprogrammed cells. We hypothesize that loss of Bright could fully reprogram somatic cells under the correct culture conditions. To test this hypothesis, in Chapter 4, we derive Bright null mouse embryonic fibroblasts and determine their reprogramming capabilities *in vitro*. Our data indicate that Bright null MEFs are capable of fully reprogramming to pluripotent cells.

Bright is a well-known regulator of B-cell development, but its role in stem cells is not well defined. Bright is expressed in embryonic stem cells, and its expression increases with differentiation. We hypothesize that Bright plays a role in the early differentiating decision of embryonic stem cells. To test this hypothesis, in Chapter 5, we analyze the protein expression patterns of Bright null mES cells. Further, we analyzed the ability of Bright null mES cells to differentiate *in vitro*. Our data indicates that Bright is important for correctly timed differentiation.

2.4 FIGURES AND TABLES

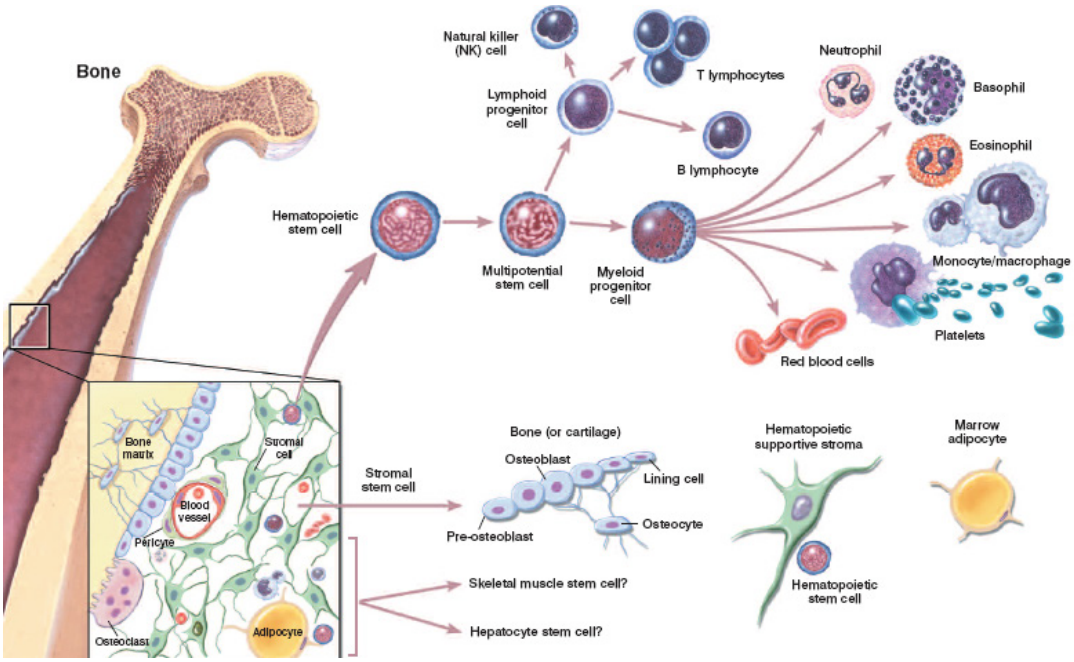

FIGURE 2.1 ADULT HEMATOPOIESIS. Hematopoietic stem cells reside in a specialized niche in the bone marrow in the adult. The hematopoietic stem cells can self-renew as well as produce the progenitors that differentiate into all cell lineages of the blood. (http://stemcells.nih.gov/info/basics/basics4.asp, © Terese Winslow, assisted by Lydia Kibiuk)

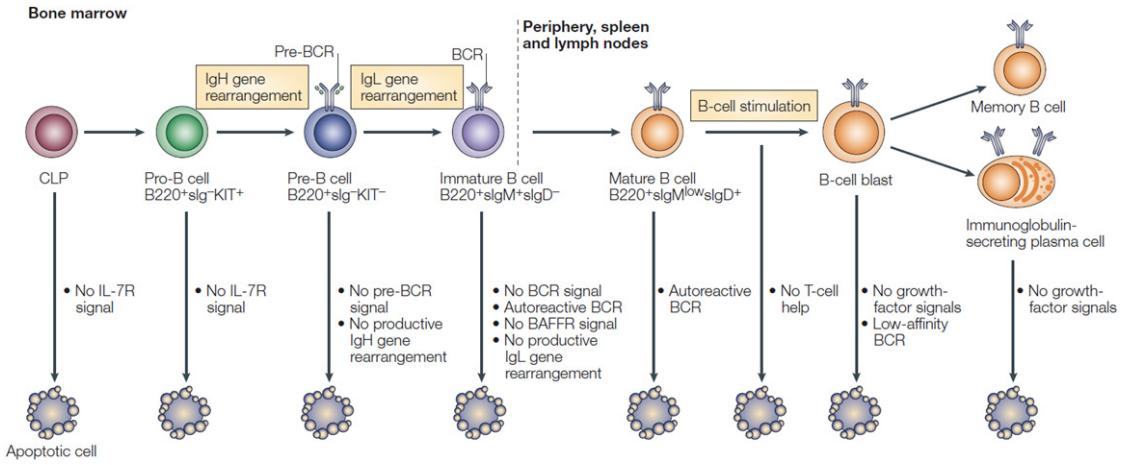

FIGURE 2.2 B-CELL DEVELOPMENT. B-cell development starts with the common lymphoid progenitor (CLP) in the bone marrow. Pro- and Pre-B-cells undergo heavy and light chain (VDJ) recombination in the bone marrow. At each stage of B-cells' development, cells may undergo apoptosis if the clones are incorrectly formed. Figure indicates cell surface markers present on B-cells and the nature of apoptotic signals at each stage of development. (Strasser, 2005)

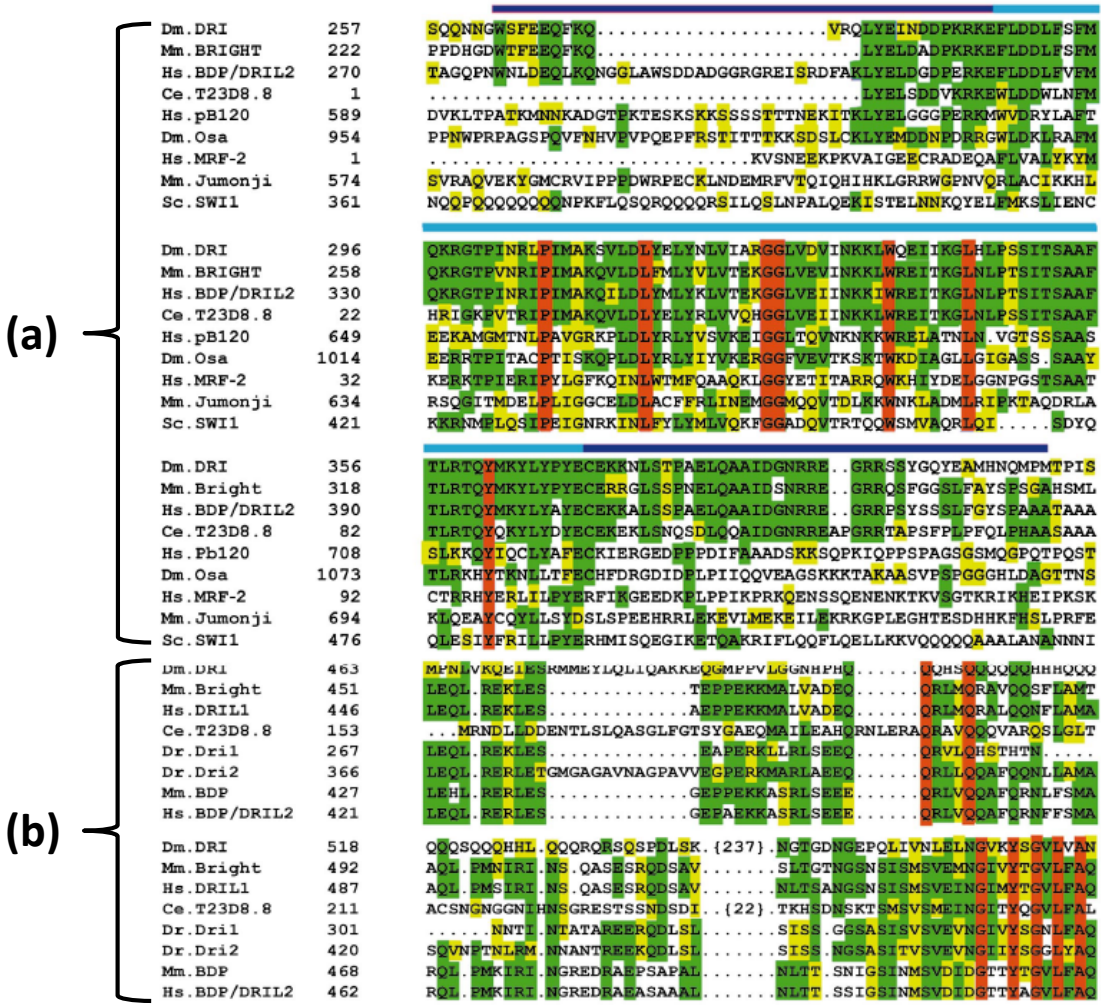

FIGURE 2.3 CONSERVED ARID SEQUENCE. **(a)** Protein sequence alignment of the extended A-T rich interaction domain (eARID) sequence of Dri, Bright, and Bdp to other members of the ARID family of proteins. Light blue bars indicates core ARID region, while dark blue bars indicates extended ARID region. **(b)** Protein sequence alignment over the REKLES domain of eARID family members. Alignment created using the multiple-sequence alignment program (MAP). Invariant and conserved residues are indicated by a red or dark-green background, respectively. Similar residues are indicated by a yellow background. Abbreviations: Ce., Caenorhabditis elegans; Dm., Drosophila melanogaster; Dr., Daniorerio; Hs., Homo sapiens; Mm., Mus musculus; Sc., Saccharomyces cerevisiae. Adapted from (Kortschak et al., 2000)

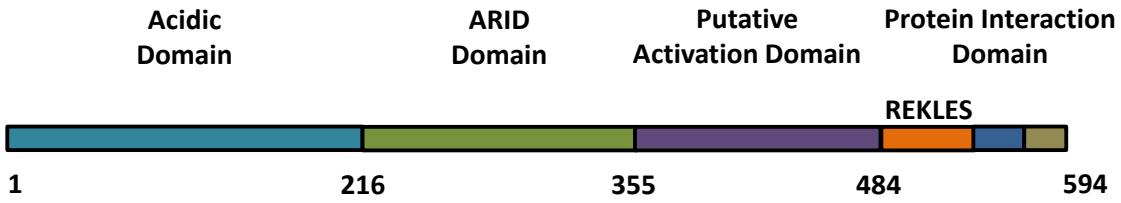

FIGURE 2.4 SCHEMATIC OF BRIGHT PROTEIN. Known domains of the Bright protein are indicated by colored bars, labels are above the bars. Amino acid numbers are indicted below the bars. The ARID domain binds DNA. The REKLES domain is required for protein-protein interactions and nuclear cytoplasmic shuttling.

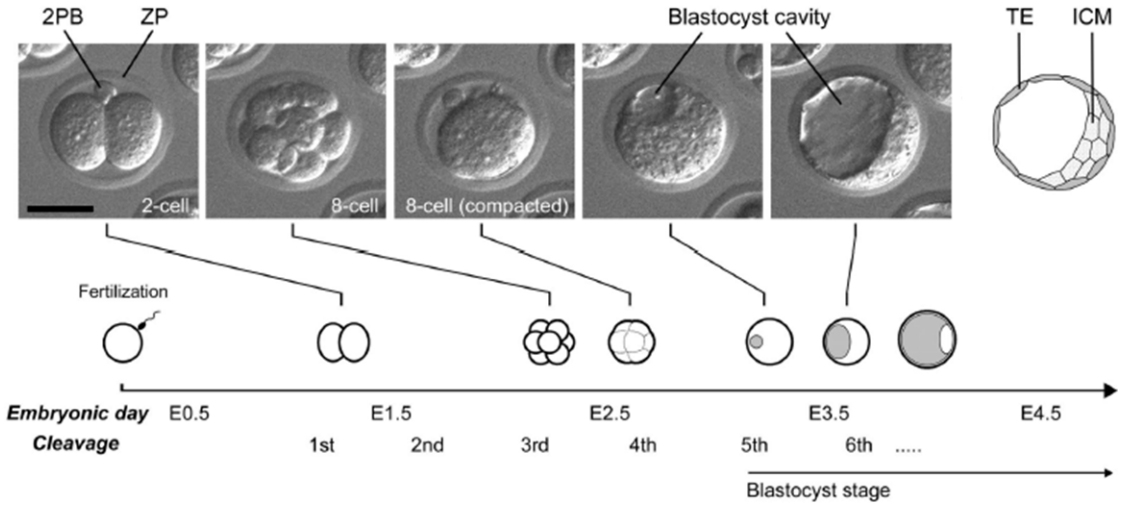

FIGURE 2.5 BLASTOCYST DEVELOPMENT. Brightfield images of the mouse embryo during the first three days of development after fertilization. Abbreviations: 2PB-second polar body; ZP-zona pellucida; TE-trophoectoderm; ICM-inner cell mass. Scale bar is 50 μm. Adapted from (Marikawa and Alarcón, 2009)

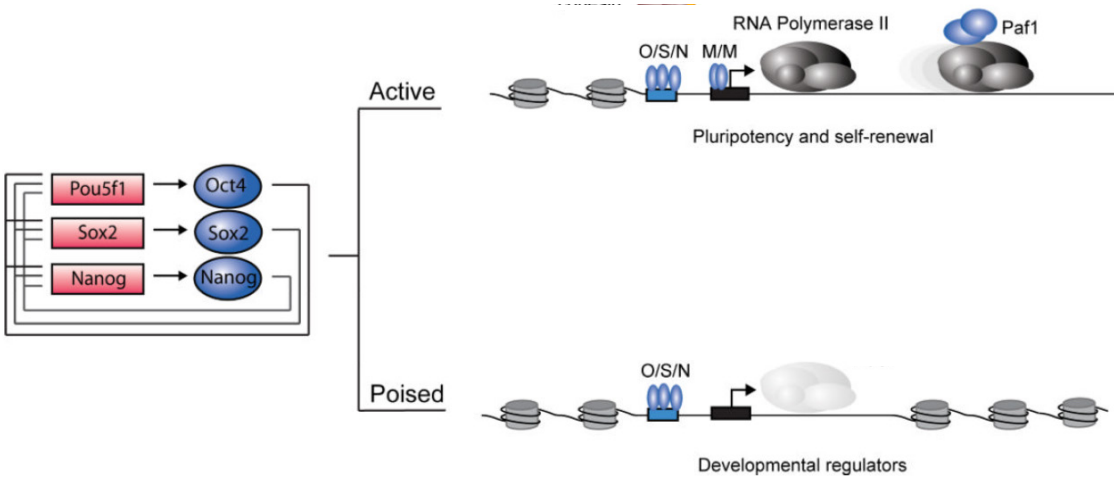

FIGURE 2.6 CORE PLURIPOTENCY GENES. The core 'triad' Pou5f1/Oct4, Sox2, and Nanog (O/S/N) act in coordination with additional transcription factors c-Myc and Max (M/M) to activate genes necessary for maintaining pluripotency. These genes also interact to repress differentiation promoting genes. Pluripotency related genes Pou5f1/Oct4, Sox2, and Nanog are represented as red boxes and proteins as blue balloons. Adapted from (Young, 2011)

	M_r Length[a]	Human chromosome	Tissue distribution
p270 (SMARCF1) (B120) (BAF250)	270,000 2285 aa	1p36.1–p35	Broad. Northern Blots show similar levels of expression in the full range of tissues tested: spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood lymphocytes, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (12).
KIAA1235	245,000 1711 aa	6q25.1–q25.3	Broad (18).
RBP1 (RBBP1)	200,000 (observed) 143,000 (predicted) 1257 aa	14q22.3	Broad with some specialization. RBP1 is expressed in all tissues examined by Northern blot, although the level of expression among different tissues is not constant (results with specific tissues were not reported; Ref. 20).
RBP1L1 (BCAA)	1311 aa	1q42.1–q43	Restricted. Among normal tissues, RBP1L1 is well expressed only in testis, but high expression was seen in all cancer tissues examined of breast ovary, lung, colon, and pancreatic origin (25).
RBP2 (RBBP2)	195,000 1722 aa	12p11	Broad with some specialization as indicated for RBP1 (20).
SMCY	1538 aa	Yq11	Specific to males, but RT-PCR indicates similar levels of expression in the full range of tissues tested: brain, kidney, liver, lung, muscle, spleen, and heart (35).
SMCX (XE169)	1560 aa	Xp11.22–p11.21	RT-PCR indicates similar levels of expression in the full range of tissues tested: brain, kidney, liver, lung, muscle, spleen, and heart (35).
PLU-1	1544 aa	1q32.1	Restricted. In normal tissues, Plu-1 is well expressed only in testis, but it is consistently up-regulated in breast cancers (27).
jumonji (JMJ)	160,000 1266 aa	6p24–p23.	Specialized. Abundant in brain, heart, skeletal muscle, kidney, and thymus but hard to detect in lung, liver, or spleen (29).
MRF-1	Unknown	2p11.1	Not reported.
MRF2	83,000 743 aa in mouse	10q11.22	The expression profile of MRF2 is not reported. Expression of Desrt (murine MRF2) is broad with some specialization. A Northern blot shows abundant expression in brain, kidney, and lung; moderate expression in heart, small intestine, and muscle; and no detectable signal in liver, spleen, large intestine, or skin (33).
Bdp (DRIL2)	61,000 560 aa	15q24	RNA was detected in a broad range of tissues but was abundant in placenta, testis, and leukocytes (32).
Bright (DRIL1)	75,000 (observed) 593 aa	19p13.3	Restricted. A ribonuclease protection assay shows message accumulation in mature B cells but not in T cells or immature B cells; in mouse tissues, expression was detected in testis but not in brain, kidney, lung, liver, spleen, or thymus (1).

[a] The total number of amino acids (aa) of the major form of each of the 13 human ARID-containing proteins is shown here. Where endogenous full-length protein has been observed, the relative migration rate (M_r) reported is indicated.

TABLE 2.1 **HUMAN ARID PROTEINS.** Table gives name, alternative names, size, chromosomal location, and known tissue distribution for all human ARID proteins. (Wilsker et al., 2002)

Chapter 3

Loss of Bright in the Adult Mouse

3.1 INTRODUCTION

Bright is a transcription factor that is broadly expressed during early development. Its expression becomes restricted first to the fetal liver, and then primarily to B cells later in development and in the adult. In the mouse, Bright is required for hematopoietic stem cell (HSC) development, which occurs in the fetal liver at E12.5 (Webb et al., 2011). Bright is a well-established regulator of B-cell development in the adult mouse (Nixon et al., 2008). The hematopoietic system develops as rare HSCs arise in the embryonic yolk sac and the aorta–gonad–mesonephros (AGM), seed the fetal liver, and then circulate to the bone marrow of adult mammals. Fetal and adult HSC progenitors then become progressively dedicated to differentiation into erythrocytes, myeloid cells and lymphocytes.

We have shown that the loss of Bright engenders a defect in hematopoietic stem cell development during embryogenesis (Webb et al., 2011), causing nearly all Bright null (Bri-/-) animals to die by E12.5. Analysis of the fetal livers of Bri-/- mice showed a drastic loss of hematopoietic stem cells (HSC), common lymphoid progenitors (CLP), and myeloid lineage progenitors (MLP). Rare survivor mice are typically smaller then either their wildtype (WT) or heterozygous (Bri +/-) littermates, but will grow to full size as they reach maturity. The surviving mice are viable and do not have shortened lifespans. Early Bright embryonic expression patterns show fairly ubiquitous expression in early development, but it becomes more restricted as development progresses, indicating that Bright likely plays a role in early embryonic development. We investigated E9.5 embryos to determine whether the observed loss of HSCs were due to defects present earlier in development than had initially been investigated.

Early loss of Bright and the subsequent embryonic death of most Bright -/- mice inhibited discovering other non-lethal phenotypes that could be present in Bri-/- animals. While in the adult, Bright expression is highest in B-cells, Bright is also present in other tissues in the adult animal (Su et al., 2004). In order to discover whether Bright null animals contain other non-lethal defects, we conducted a histological study of adult animals using 6 week and > 1 year old animals.

3.2 MATERIALS AND METHODS

3.2.1 Mouse Husbandry

All mice were housed under disease-free conditions in the barrier facilities in the Mouse Genetic Engineering Facility at the University of Texas at Austin. Bright null mice were created using homologous recombination into the Bright gene into SM1-129SVJ embryonic stem cells which were injected into C57BL/6 blastocysts (Webb et al., 2011). Animals were backcrossed at least 4 generations onto the C57BL/6 strain and lines were maintained with heterozygous animals, due to the embryonic lethality observed.

3.2.2 Histology

Tissues were harvested from mice at indicated timepoints using humane techniques as defined by the University of Texas at Austin Institutional Animal Care and Use Committee (http://www.utexas.edu/research/rsc/iacuc/policies_index.html). Tissues were prepared in 5% paraformaldehyde solution for a minimum of 24 hours. The tissues were then dehydrated using 30, 50, 70, 95, and 100% ethanol (EtOH). Samples were stored in 100% EtOH at 4°C until processing. The tissues samples were embedded at the Histology and Tissue Processing Facility Core located at The Virginia Harris Cockrell Cancer Research Center at The University of Texas MD Anderson Cancer Center, Science Park facility (http://sciencepark.mdanderson.org/resources/fcores/histology/).

Embedded tissues were sectioned and stained for Ki67 or Hematoxylin and Eosin (H&E) at the core facility.

3.2.3 Proliferation Analysis

Tissues sections stained for Ki67 were imaged using a light microscope under 10x magnification. The entire section was imaged with no overlap. For the spleen, a grid was placed on top of the section image and ten squares were randomly chosen, counted from each picture, and averaged. Whole images of the kidney were counted. The mean from the entire organ was plotted. The variance was determined by F-test; the P-value was determined by T-test; and the standard error of the mean was calculated. All calculations and statistical tests were performed in Microsoft Excel.

3.3 RESULTS

3.3.1 Loss of Bright Does Not Induce Proliferative Differences or Induce Apoptosis in the Fetal Liver

Bri-/- embryos and neonates show a significant decrease in size. Bright binds to E2F and regulates cell growth (Suzuki et al., 1998; Fukuyo et al., 2004). We hypothesize that a change in cell proliferation may cause the smaller size phenotype of Bri-/- mice. Embryonic development 11.5 (E11.5) embryos from heterozygous-to-heterozygous matings were sectioned and stained for Ki67, a well-established marker of proliferation.

No differences in proliferation were observed (**Figure 3.1**), suggesting that the smaller size seen in Bri-/- animals is not due to less proliferation but to some other requirement for Bright in embryonic development.

Previous work done in the Tucker and Webb labs has shown that there were significant reductions in the hematopoietic stem cell (HSC) and B-cell populations--both in the fetal liver and 6 week-old animals (Webb et al., 2011). To determine whether cellular apoptosis at an early timepoint caused the loss of HSC in the fetal liver, E9.5 embryos were sectioned and stained using TUNEL to detect apoptotic cells and H&E to determine morphological features. The yolk sac is the site of primitive hematopoiesis beginning at E8.5. Definitive HSC progenitors arise in the yolk sac, migrate through the blood stream, and seed the fetal liver, which is the site of definitive hematopoiesis in the embryo by E12.5. H&E stains of E9.5 embryos show that Bri-/- has normal blood vessels and yolk sacs (**Figure 3.2 a-d**). Interestingly, the Bri-/- shows comparable numbers of erythrocytes compared to WT embryos (**Figure 3.2 a-d**). Normal blood vessel and yolk sac development, along with the presence of erythrocytes indicates that loss of HSC occurred after their initial development in the yolk sac.

Our evidence indicates that HSC are functional in the embryo before they migrate to the fetal liver. Lack of Bri-/- HSC in the fetal liver could be attributed to loss of these cells. To determine whether the absence of HSC in the fetal liver occurs due to apoptosis, we stained E9.5 embryos for TUNEL, a well-established method to determine apoptosis. TUNEL staining did not detect any apoptosis occurring at the either the yolk sac or

embryonic blood vessels (**Figure 3.2 e-f**), again supporting the contention that the loss of HSC seen in Bri-/- fetal livers occurs in the fetal liver and not earlier in development.

3.3.2 Loss of Bright Does Not Affect Organ Morphology in 6 Week-Old Mice

The typical size difference seen in Bri-/- mice diminishes in later adulthood. To study this phenotype further, histological analysis was performed on the spleen, thymus, kidney, liver, skin, lung, mammary gland, and skeletal muscle of 6 week-old Bri-/-, Bri +/-, and Bri+/+ littermates to determine whether changes in organ development occurred. Paraffin embedded sections from the above organs were stained for H&E and gross anatomical analysis was performed. There are no differences in formation, size, or cellular composition of the Bri-/- organs compared to either the Bri +/- or Bri+/+ littermate (**Figure 3.3**).

3.3.3 Proliferation Differences Occur in 6 Week-Old Bri-/- Kidneys but Not Spleen

While no obvious proliferative differences were observed in Ki67 staining of the developing embryo (**Figure 3.1**), that did not rule out proliferative changes that may occur later in development. To determine more thoroughly whether any proliferative differences in young Bri-/- mice arose, histological sections from Bri+/+ and Bri-/- spleens and kidneys were stained with the proliferative marker Ki67 (**Figure 3.4 a**).

The spleen is the site of blood filtration, as well as monocyte and erythrocyte reserves. In the adult, most hematopoiesis occurs in the bone marrow, while the spleen is the site for B-cell maturation. The spleen also has the ability to produce lymphocytes and maintain a small B-cell population to respond quickly to infection (Hardy and Hayakawa, 2001). Post sectioning and Ki67 staining, the number of proliferative cells were determined, and averaged (**Figure 3.4 b**). There are no no significant differences in proliferation.

The kidney filters waste out of the blood as well as maintains the body's electrolyte balance and blood pressure, amongst other functions. We chose the kidney as a potential target because we previously observed several Bri-/- mice were missing their right kidney. This phenotype was not present in all Bri-/- mice, but it led us to question Bright's role in the kidney. Post sectioning and Ki67 staining, all proliferative cells from the kidney were counted and averaged. Interestingly, there is a significant increase in proliferative cells in the Bri-/- kidney compared to the WT control (**Figure 3.4 c**).

3.3.4 Older Bright Knockout Mice Show Loss of Kidney Structure

Bright's effect on embryonic and early development includes a generally smaller size, loss of HSC in the fetal liver, loss of B cells in early adulthood, and an increase in proliferative cells in the kidney. To study the role Bright may play in later development, Bright-/- mice were allowed to mature, breed, and age. Bri-/- mice, both male and female, were fertile, although they were poor breeders and litter size tended to be smaller. Otherwise, they appeared to be normal, healthy animals into maturity. Bri+/+, Bri+/-, and

Bright-/-animals were allowed to age > 1 year. All animals appeared healthy at time of analysis. Animals were sacrificed; the thymus, skin, skeletal muscle, pancreas, kidney, and cardiac muscle fixed and stained for H&E; and gross anatomical analysis performed. There are no differences in formation, size, or cellular composition of the Bri-/- organs compared to their Bri+/+ or Bri+/- age-matched animals except for the kidney (**Figure 3.5**). The Bri-/- kidney shows a distinct loss of organization of the normal tubular structure, which may predict a loss of kidney function in adult Bri-/- animals.

3.4 DISCUSSION

This work seeks to determine whether loss of Bright affects adult tissues in mice. Bright knockout mice typically die at E12.5, but there are rare survivor mice (<1%). The Bri-/- mice die from impairment of hematopoiesis in the fetal liver (Webb et al., 2011). Hematopoiesis begins in the AGM and yolk sac of the developing embryo before the HSC migrate and seed the fetal liver. We show that primitive hematopoiesis appears to occur normally in the yolk sac. We also show that there is no apoptosis occurring at the yolk sac stage of hematopoietic development. These data indicate that the hematopoietic defect occurs during a later stage in development. From this and previous work, we concluded that loss of HSC in the fetal liver stems from a failure of HSC to proliferate or differentiate in the fetal liver.

Bri-/- mice are smaller than their wildtype and heterozygous littermates at birth, but as they age, they grow to normal size. Similarly, at 6 weeks, Bright knockout mice show reduced B-cell populations; at 6 months, B-cell populations are within normal

ranges (Webb et al., 2011). This indicates that a developmental delay is present in Bright null animals (most dramatically in hematopoietic development). The adult survivors can overcome this delay. Histological analysis from 6 week and > 1 year old mice show normal cellar structure in almost all the organs observed. The kidney appears normal in 6-week-old mice, showing a normal tubular structure and cellular composition, but as mice aged, their kidneys develop a marked loss of tubular integrity. At the time of harvest, no indication of disease was observed.

Ki67 proliferation studies of young Bri-/- kidney show a marked increase in proliferation not seen in the spleen. Bright is a known binding factor of the cell cycle protein EF2, a transcription factor that targets many cell cycle control proteins (Suzuki et al., 1998), and its overexpression rescues Ras-induced senescence in primary fibroblast (Peeper et al., 2002). Contrarily, we have shown that loss of Bright in primary cells prevents senescence *in vitro*. These conflicting data indicate that Bright's function in cell cycle, proliferation, and senescence is complex and likely context specific. Our observation in the kidney indicates that Bright plays a crucial role in regulating kidney development and proliferation. The increase in proliferation may be responsible for the loss of kidney structure. However, the proliferation increase may be unrelated, and Bright has multiple functions in the kidney. Further work will determine how loss of Bright affects proliferation in the kidneys of the adult mouse and other factors with which Bright interacts.

3.5 FIGURES AND TABLES

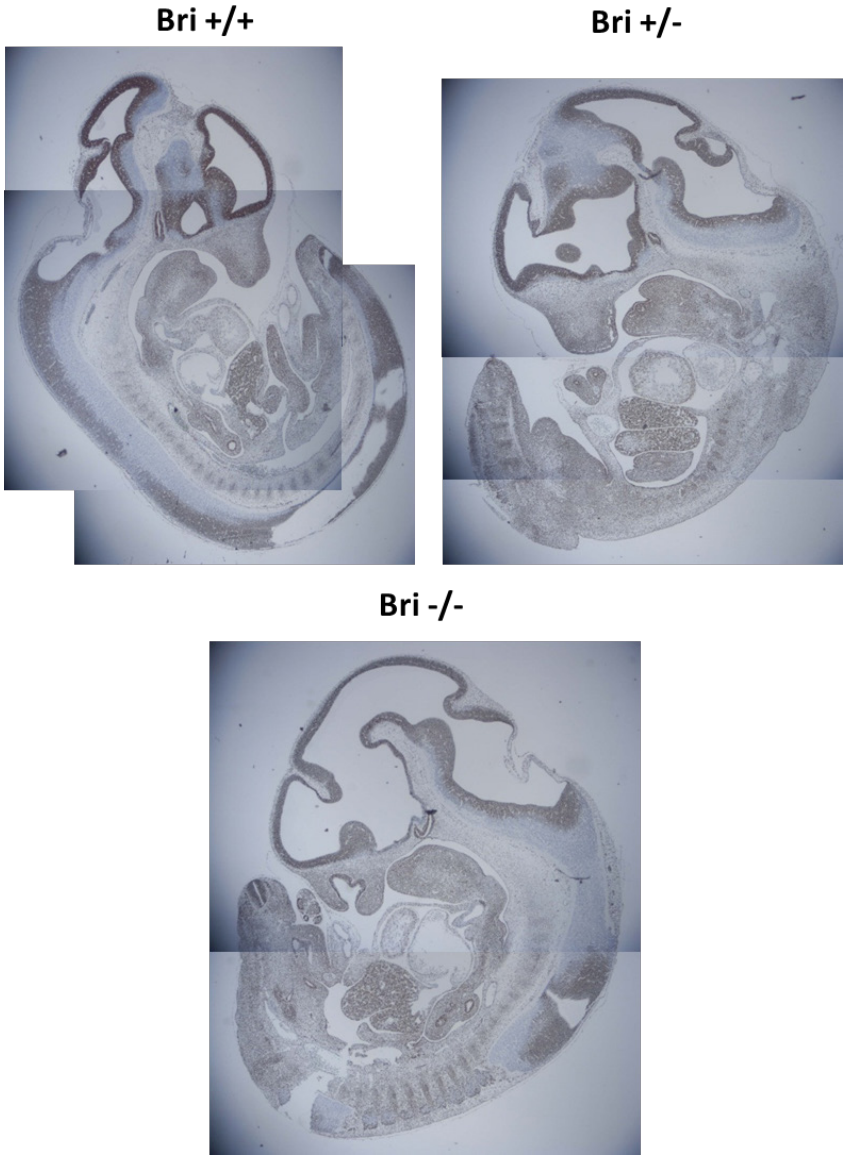

FIGURE 3.1 KI67 STAINING OF E11.5 EMBRYOS. Embryonic day 11.5 embryos that are Bright wildtype (Bri +/+), heterozygous (Bri +/-) and knockout (Bri -/-) were sectioned and stained for the proliferative marker Ki67. All three types of embryos showed similar levels and expression patterns of Ki67 staining.

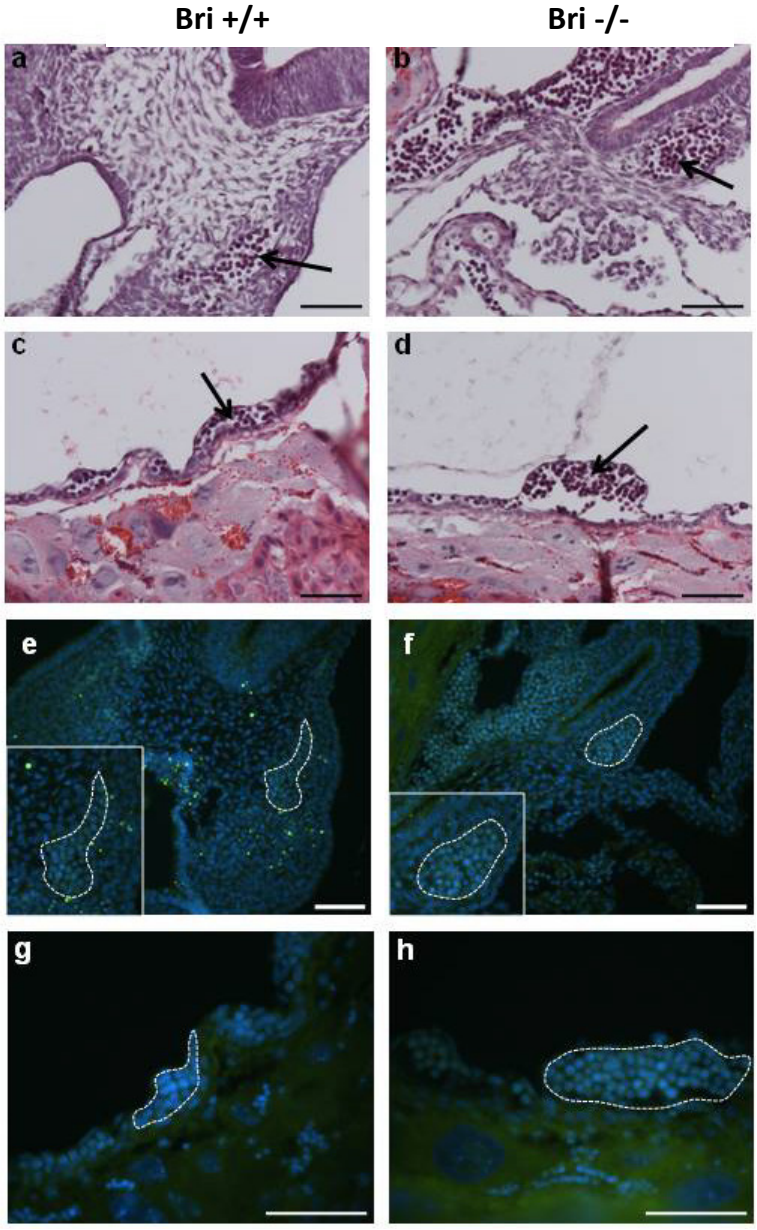

FIGURE 3.2 E9.5 BRIGHT-/- ERYTHROCYTES ARE NORMAL. Bright wildtype (Bri+/+) and knockout (Bri-/-) littermate embryos **(a, b)** and yolk sacs **(c, d)** contain blood vessels with comparable numbers of circulating erythrocytes. Erythrocytes found in wildtype and Bri-/- embryonic vessels **(e, f)** and yolk sac vessels **(g, h)** are non-apoptotic, as evidenced by a lack of TUNEL staining (blue=DAPI, green=TUNEL). Blood vessels are outlined for identification; scale bars=100μm.

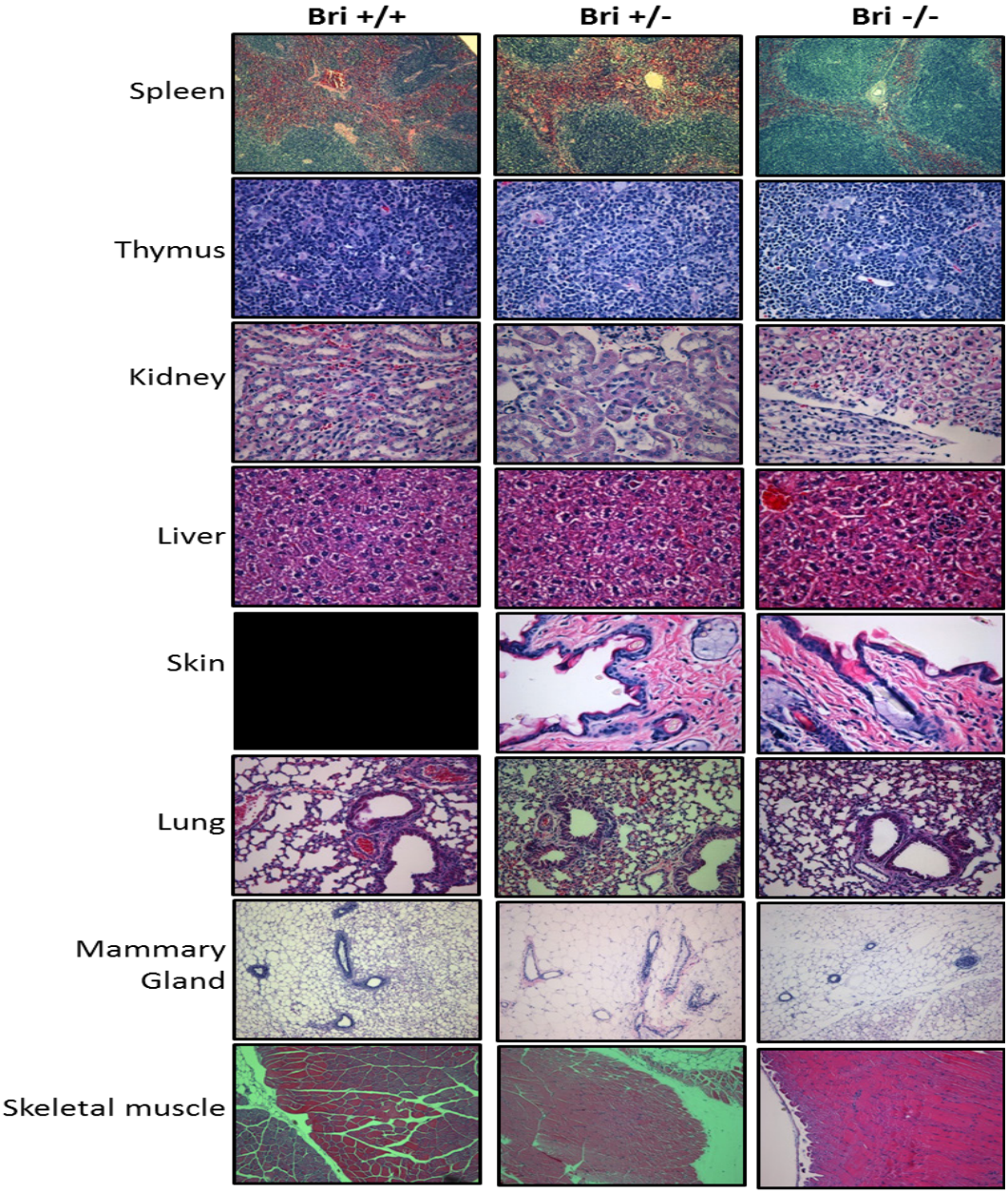

FIGURE 3.3 SIX WEEK-OLD BRIGHT -/- MICE SHOW NO STRUCTURAL ABNORMALITIES IN MOST MAJOR ORGANS. H&E staining of Bright wildtype (Bri+/+), heterozygous (+/-) and knockout (Bright -/-) littermates was performed on the spleen, thymus, kidney, liver, skin, lung, mammary gland, and skeletal muscle. No structural abnormalities were observed. Black box indicates no data available.

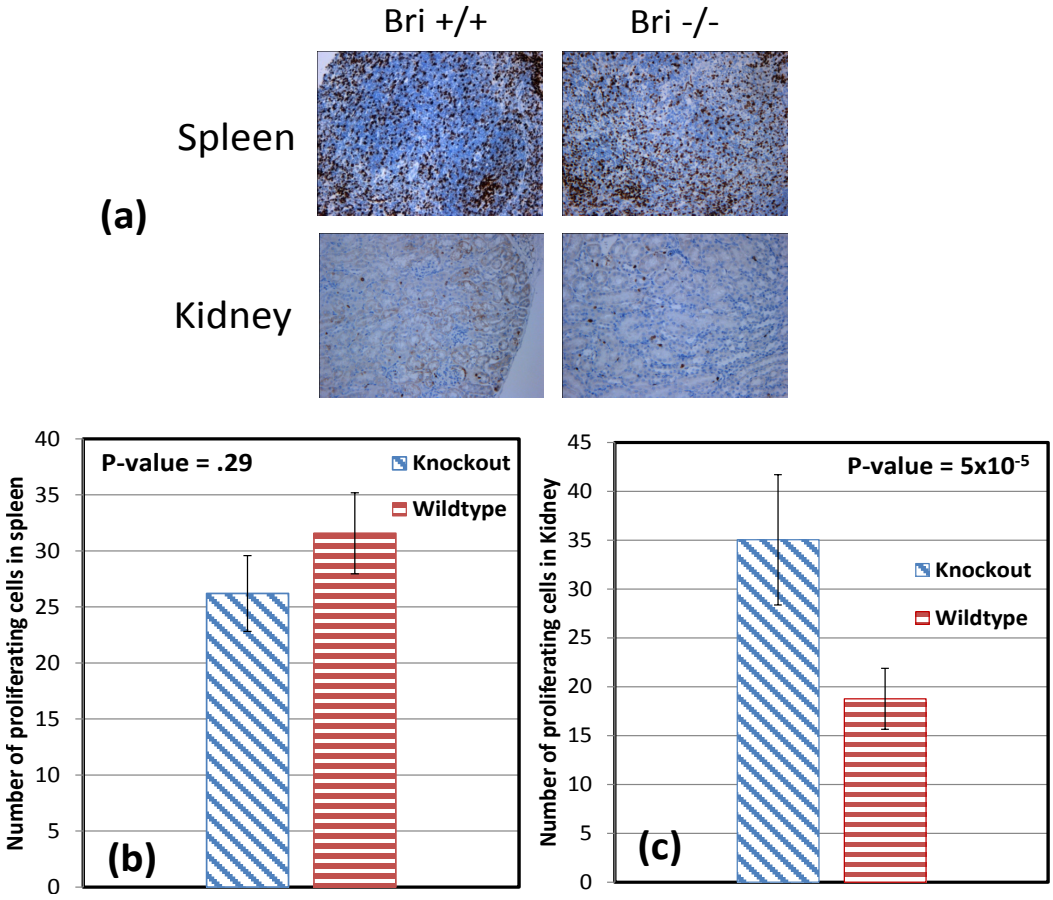

FIGURE 3.4 BRIGHT KNOCKOUT KIDNEY BUT NOT SPLEEN IS MORE PROLIFERATIVE. **(a)** Spleen and kidney of 6 week-old Bright wildtype (Bri+/+) and knockout (Bright-/-) littermates were stained for the proliferative marker Ki67 (brown) and the nuclei counterstained with eosin (blue). **(b)** The number of proliferating cells in Bright wildtype (Bri +/+) and knockout (Bright -/-) spleens were averaged by randomly counting ten equally sized squares from each stain. The average number of positive cells for each section was averaged from each square counted; variance was determined by F-test and found to be equal. Student's T-test was performed to determine the P-value. All calculations were performed in Microsoft Excel. **(c)** The number of proliferating cells in Bright wildtype (Bri +/+) and knockout (Bright -/-) kidney were counted for the entire section. The numbers of positive cells were averaged; the variances were determined by F-test and found to be equal. Student's T-test was performed to determine P-value.

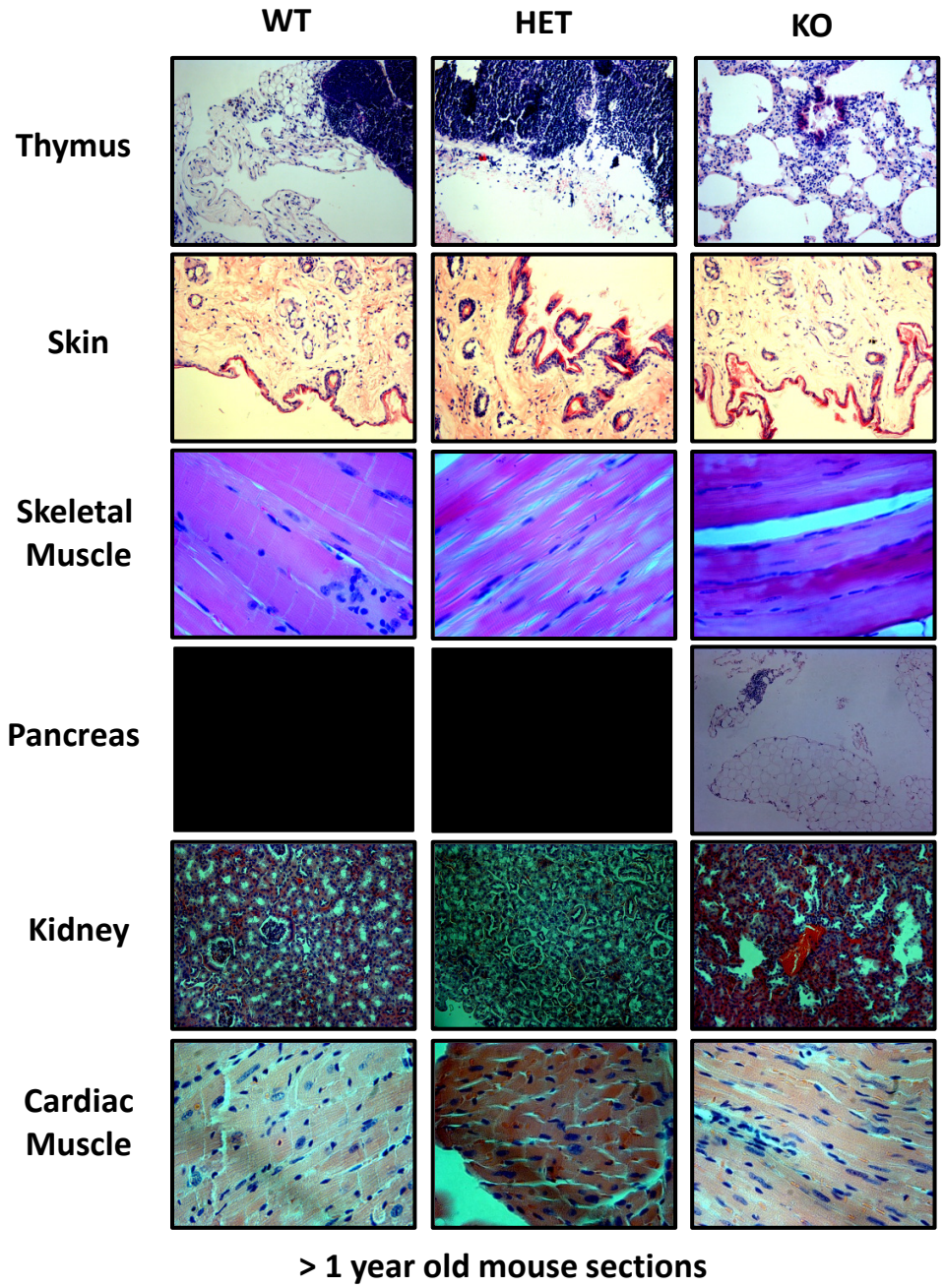

FIGURE 3.5 BRIGHT KNOCKOUT KIDNEY LOSES STRUCTURE WITH AGE. H&E staining of Bright wildtype (Bri+/+), heterozygous (+/-) and knockout (Bright -/-) age matched mice was performed on the thymus, skin, skeletal muscle, pancreas, kidney, and cardiac muscle. Structural abnormalities were observed only in the kidney. Black box indicates no data available.

Chapter 4

Loss of Bright Induces Spontaneously Pluripotent Cells

4.1 INTRODUCTION

Improving induced pluripotent stem cell (iPS) generation and efficiency has resulted in a number of advances that increase efficiency and reduce the number of factors needed to reprogram somatic cells (Kim et al.; Silva et al., 2008; Ichida et al., 2009; Kaji et al., 2009; Patel and Yang, 2010; Nemajerova et al., 2012). These advances have made iPS cells readily available to researchers, but thus far, have not made iPS cells viable for clinical use. We have previously observed that the loss of Bright results in profound developmental plasticity of both mouse and human somatic cells (An et al., 2010). Cells silenced for Bright expression by germline deletion, transgenic dominant negative inhibition, or shRNA repression shared morphological and other features with mouse embryonic stem (mES) cells. These observed similarities with mES prompted us to test whether Bright might serve as a regulator of reprogramming. We utilized Bright null mouse embryonic fibroblasts to determine the extent of Bright repression mediated reprogramming.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

All cells were cultured in 5% CO_2 at 37°C in media containing 100U/ml Penicillin G, and 100µg/ml Streptomycin Sulfate. MEFs were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS). Clones from Bright KO-MEFs were picked under sterile conditions and transferred to mitotically inactivated MEF feeder cell lines in ES cell culture media. Embryonic stem cells were cultured in 20% FBS (ES grade, HyClone), 0.07% 2-mercap, 1x non-essential amino acids (Gibco), and 1x nucleosides (Gibco) on STO feeder cells that were mitotically inactivated with Mitomycin C (10µg/ml)

4.2.2 Immunocytochemistry

Cells were plated on chamber slides for two days under standard ES cell conditions. Alkaline phosphatase presence was detected using the Vector alkaline phosphatase kit (SK-5100). Immunostaining was performed using Nanog, Sox2, Oct4, and SSEA-1 specific antibodies and corresponding fluorescently tagged secondary antibodies. Cells were washed in 4°C PBS, fixed in -20°C 1:1 Acetone:Methanol for 20 minutes, allowed to dry for 10 minutes. Cells were then washed 3 times in PBS and permeabilized in 0.1% Triton-X in PBS and blocked in 10% normal goat serum for 1 hour at room temperature. Cell were incubated in primary antibody overnight at 4°C, washed 3 times in PBS, and then incubated with secondary at room temperature for 1

hour. Cells were washed three times in PBS, and finally slides were mounted using mounting solution containing DAPI (Vectashield). Slides were stored at 4°C in the dark.

4.2.3 Teratoma

Near confluent cells were lightly trypsinized (5% trypsin/1% EDTA) and washed twice in PBS. 100ul containing ~350,000 cells were injected subcutaneously into the flanks of NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice. Mice were palpated for tumor growth and sacrificed when visible tumors were 1-2 cm across the longest diameter. Tumors were embedded at the Histology and Tissue Processing Facility Core located at The Virginia Harris Cockrell Cancer Research Center at The University of Texas MD Anderson Cancer Center, Science Park facility (http://sciencepark.mdanderson.org/resources/fcores/histology/). Embedded tissues were sectioned and stained for Hematoxylin and Eosin (H&E) at the core facility and analyzed by a trained pathologist at M.D. Anderson – Science Park Histology and Tissue Processing Facility Core.

4.2.4 Microarray Analysis

Cells were harvested by trypsin digestion. Total RNA was isolated (Qiagen RNAEasy). On-column DNase digestion was performed (Qiagen) to remove genomic DNA contamination. RNA was reversed transcribed (Invitrogen). Labeling with cy3 random nonamers and array hybridizations were performed by following the Nimblegen

expression array protocol. Alignment and data normalization were done using Nimblescan provided from Nimblegen.

4.2.5 Immunoprecipitation

Cells were harvested as in western blots or cell fraction as indicated. Lysate was pre-cleared with 100ul Protein G Agarose beads (GE) at 4°C > 4 hours. Lysate was incubated with 2µg of α-Bright rabbit polyclonal antibody overnight at 4°C. Protein G Agarose beads were incubated with the protein-antibody complex at 4°C > 4 hours. Beads were washed three times in TBS-T and once in TBS. 2X Sample buffer (125mM Tris pH 6.8, 2% SDS, 16% glycerol, 3% 2-Mercaptoethanol, and bromophenol blue) was added and the samples boiled for 5 minutes before loading on a SDS-PAGE gel.

4.2.6 Chromatin Immunoprecipitation Assay (ChIP)

Cells were crosslinked with 1% formaldehyde and quenched with 125mM glycine. Cells were washed with PBS+PMSF and then lysed in SDS lysis buffer (1% SDS, 10mM EDTA, and 50 mM Tris-Cl pH 8.1). The samples were sonicated using the Bioruptor (Diagenode) at medium speed for 20 minutes total, diluted 1:5 with dilution buffer (0.1% SDS, 10% Triton-X, 0.5M EDTA, 1M Tris pH 8.1, 5M NaCl), and pre-cleared with Protein G Agarose beads at 4°C > 4 hours. Samples were then incubated with 10µg of α-Bright rabbit polyclonal antibody overnight at 4°C. Protein G Agarose beads were incubated with the protein-antibody complex at 4°C > 4 hours and then washed twice in a low salt buffer (1% Deoxycholate, 1% Triton-X, 1mM EDTA, 50mM

HEPES pH 7.5, 150mM NaCl), once in a high salt buffer (1% Deoxycholate, 1% Triton-X, 1mM EDTA, 50mM HEPES pH 7.5, 500mM NaCl), once in a LiCl buffer (250mM LiCl, 0.5% NP40, 0.5% Deoxycholate, 1mM EDTA, 10mM Tris pH 8.1), and twice in TE. Sample was eluted at 65°C in elution buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.1). Crosslinking was reversed by incubating overnight at 70°C. Sample was incubated in RNaseA (15µg) at 37°C for 30 minutes, then in 40µg of glycogen and 120µg of proteaseK at 37°C for 2 hours. DNA was Phenol/Chloroform extracted, ethanol precipitated, and resuspended in TE buffer. For ChIP primers used, see Appendix.

4.2.7 Electrophoretic Mobility Shift Assay (EMSA)

In vitro translated Bright was prepared per manufacturer's instructions (Pierce). Nuclear extracts were prepared by Iso-osmotic / NP-40 lysis; protein concentrations were quantified with Bradford reagents (Bio-Rad, Richmond, CA); and electrophoretic mobility shift assays (EMSAs) were performed in 4% nondenaturing acrylamide gels after incubation for 1 hour at 37 C with γ-32P-labeled probe, as previously described (Nixon et al., 2004). The prototypic Bright-binding site (a 150 bp *Bam*HI–*Fok*I fragment called bf150) from the S107 V1 5′ flanking sequence (Nixon et al., 2004) was used as a probe. The Nanog probe used was a 120bp AccI-SspI fragment approximately 350bp upstream from the Nanog start site. The Oct4 distal enhancer probe (DE, 289bp) and Bright binding probe (Bb, 353 bp) were PCR amplified from the Oct4 promoter region. In some instances antibodies were added after 20 minutes at RT and then incubated 30 minutes on ice. α-Bright rabbit polyclonal antibody used for supershifts. Cold

competition performed using protein+ rxn buffer + cold + hot, performed 1X, 100X 1000X based on scintillation counts. 70-80,000 counts per reaction

4.2.8 Semi-quantitative Real-Time PCR (qPCR)

All qPCR assays were run on Applied Biosystems ViiA7 machines using Syber green chemistry (Applied BioSystem SyberGreen or Quantus SyberGreen) with either ChIP materials or reverse transcribed cDNA. Relative occupancy fold enrichment of targets to input and relative expression fold change over control were calculated from three replicates of ChIP samples and cDNAs, respectively. For qPCR primers used, see Appendix.

4.2.9 Luciferase Assay

The indicated 2µg luciferase vector, 0.7 µg Renilla vector, and 3µg Bright or empty vector were electroporated into ~500,000 single cell suspension of sub-confluent mouse embryonic stem cells (Lonza, VPH-1001) and plated in 6 well plates either in standard ES conditions (undifferentiated) or in the absence of either feeder cells or LIF (differentiated). Luciferase vectors used were: Oct4-luciferase (Addgene plasmid 17221) (Takahashi et al., 2007), Nanog-luciferase (Addgene plasmid 16337), and Sox2 luciferase, which was a kind gift from Dr. Angel Martin (Fundación Inbiomed, San Sebastian, Spain).

4.3 RESULTS

4.3.1 Characterization of Bright Knockout Mouse Embryonic Fibroblasts

To determine whether Bright acts as a regulator of reprogramming, Bright knockout (Bri-/-), heterozygous (Bri+/-), and wildtype (Bri+/+) mouse embryonic fibroblasts (MEFs), were derived from E10.5 Bright embryos. Bri-/- MEFs, like other Bright null cell lines (An et al., 2010), do not undergo crisis under standard culture conditions and continued to proliferate > 6 months in contrast to Bri+/+ and Bri+/- MEFs, which senesced in culture as is typical for primary cell lines (Bandyopadhyay et al., 2001). Remarkably, early passage KO-MEFs spontaneously form ES-like colonies (**Figure 4.1 a**) that stained positive for alkaline phosphatase (AP) (**Figure 4.1 b**) within 2 weeks in standard culture conditions. Bri+/- MEFs at no point formed similar colonies. These colonies were difficult to expand and easily differentiated, suggesting they were only partially reprogrammed. This partial reprogramming indicated that under standard fibroblast culture conditions, Bright-deficient MEFs were "poised" for de-differentiation.

4.3.2 Bright Knockout Mouse Embryonic Fibroblasts are Spontaneously Pluripotent

We have shown that loss of Bright alone is sufficient to initiate reprogramming in somatic cells. We wanted to further explore the ability of Bri-/- MEFs to completely reprogram. If Bri-/- MEFs are truly 'poised,' we reasoned that they should undergo standard 4-factor (Oct4, Sox2, Klf4, and c-Myc) more efficiently then Bri+/+ MEFs. Bri-/- MEFs undergo standard 4-factor (KO+4F) reprogramming ~15-fold more efficiently than sibling Bri+/+ MEFs (WT+4F), with a ~23-fold enhancement of surviving clones

(**Table 4.1**)[1]. Colonies were visible 7-10 days earlier in Bri-/- MEF versus Bri+/+ MEF cultures. Unlike Bri+/+ MEFs, Bri-/- MEFs bypassed the requirements for Sox 2 (KO-S) or Klf4 (KO-K), although with lower efficiency and survival than KO+4F (**Table 4.1**)[1]. These data indicate that Bri-/- MEFs are more permissive to reprogramming.

Remarkably, Bri-/- MEFS grown under typical mES/iPS culture conditions containing LIF result in clones (termed BriPS) at higher efficiency and survival rates (~30-fold) than WT+4F colonies (**Table 4.1**)[1]. These clones are stable under standard ES culture conditions. BriPS and KO-S clones, but not KO-K, are indistinguishable from WT+4F in expression of alkaline phosphatase (AP), Oct4, Sox2, Nanog and SSEA1 (**Figure 4.2**)[2]. KO-4F and BriPS form teratomas in NSG mice indistinguishable in latency and morphology from WT+4F clones (**Figure 4.3**)[3]. Parental KO-MEFs were also injected into NSG mice, but no tumors grew, reinforcing the reprogramed nature of the BriPS.

Global gene expression analysis confirmed that BriPS and mES are comparable with respect to upregulation of conventional pluripotency genes, (**Figure 4.4 a, b**)[4]. Global analyses indicated that, as is commonly observed in standard reprogrammed iPS, some pathways (eg, cell cycle) were not comparable with mES (**Figure 4.5**)[4] (Chin et al., 2009). Gene ontogeny analysis of misregulated genes in the BriPS show no misregulation of pluripotency pathways, but, as seen in the Bri-/- ES microarray, misregulation of early

[1] Reprogramming experiments performed in Dr. Carol Webb's lab.
[2] Immunocytochemistry performed in Dr. Carol Webb's lab
[3] WT+4F and KO+4F teratoma experiments performed in Dr. Carol Webb's lab
[4] Reverse transcription of RNA, cDNA labeling, microarray hybridization and following analyses performed by Dr. Bum Kyu Lee in Dr. Vishy Iyer's lab

differentiation pathways (**Figure 4.5**). Epiblast stem cells (EpiSC) are pluripotent, and while they share many characteristics with mES, differential protein expression is consistently observed for a number of genes, including Klf4, Rex1, Fgf5, and Nodal (Maurer et al., 2008; De Miguel et al., 2010; Greber et al., 2010). Within this signature set, BriPS showed an intermediate expression pattern **(Figure 4.6 a, b**). Collectively the data indicate that BriPS share a highly similar gene expression signature with pluripotent cells. Thus, loss of Bright dramatically improves standard reprogramming efficiency and, alone, is sufficient to convert MEFs to fully pluripotent cells.

4.3.3 Bright Interacts with the Conventional Pluripotency Pathway through Oct4, Sox2, and Nanog

The efficiencies achieved for standard reprogramming in the absence of Bright suggested that it might be acting through the conventional Oct4, Sox2, and Nanog pathway. Core factors function as part of a larger transcriptional complex, providing maintenance of pluripotency by controlling gene expression of many target proteins while auto-regulating each other (Guenther, 2011; Ng and Surani, 2011; Young, 2011; Sterneckert et al., 2012). Mass spectrometry had previously identified Bright as a Nanog binding partner (Wang et al., 2006). We confirm that observation by co-immunoprecipitation (co-IP) in mES cells (**Figure 4.7 a**). Additionally, we found that endogenous levels of Oct4 and Sox2 were sufficient to co-IP with endogenous Bright in mES cells, whereas only the endogenous Oct4-Bright interaction was detected in the embryonic carcinoma (mEC) cell line P19 (**Figure 4.7 b**). We observed no interaction of

these factors when overexpressed in 293T cells, suggesting that additional ES-specific factors might be required as part of a larger protein complex (**Figure 4.7 c**).

There are multiple Bright consensus binding sites within the *Oct4, Sox2* and *Nanog* promoter regions (**Figure 4.8 a-c**), suggesting that Bright might act by directly repressing the transcription of these (and potentially other) core pluripotency factors. We found that Bright is enriched in chromatin corresponding to the proximal promoter regions of *Oct4,* and *Nanog* in 293Tcells—a transformed human epithelial line which we previously showed to undergo robust expression of pluripotency genes following repression of Bright (**Figure 4.9**)[5] (An et al., 2010). Binding within the *Nanog* promoter was mapped to a 120bp A/T-rich region ~350bp upstream of the start site, and within *Oct4*, to both the proximal enhancer and to a region directly downstream which contains several Bright consensus motifs (**Figure 4.10 a-c**). The proximal enhancer was previously suggested to act as a target for *Oct4* repression (Yeom et al., 1996; Favaedi et al., 2012).

These data suggested that, reciprocal to what is observed for loss of Bright in somatic cells, gain of Bright in mES or mEC cells, either via enforced over-expression or upon differentiation, would lead to repression of core pluripotency factor transcription. Accordingly, overexpression of Bright in undifferentiated mES cells (**Figure 4.11 a**) or in P19 mEC (**Figure 4.11 b**) leds to modest repression of the endogenous loci of Oct4, Sox2 and Nanog. As a more stringent test of the model, we employed Oct4, Sox2 and Nanog promoter/enhancer-driven luciferase constructs carrying the consensus Bright binding

[5] ChIP assay performed by Dr. Bum-Kyu Lee in Dr. Vishy Iyer's lab

sites noted above (**Figure 4.8 a-c**) and previously shown to be responsive to down-regulation of the corresponding ES factors during differentiation (Sato et al., 2006; Takahashi et al., 2007; Leis et al., 2012). Bright overexpression strongly represses each reporter activity except for the Sox2-core reporter (**Figure 4.12 a-d**). This repression occurred regardless of the mES differentiation state (**Figure 4.12 e-h**). Lack of complete repression of endogenous or reporter transcription, regardless of the extent or duration of Bright overexpression, is consistent with Bright acting as part of a larger protein complex and perhaps an essential co-repressor(s) component may be the limiting factor in ES cells. We conclude that singular elimination of Bright is itself sufficient to initiate Oct4, Sox2, and Nanog de-repression adequate to induce cellular reprogramming.

4.4 DISCUSSION

We show here for the first time, somatic cellular reprogramming to a pluripotent state through loss of a single gene, *Bright/ARID3a*. Bright -/- mouse embryonic fibroblasts were employed as a model system to test the ability of loss of Bright to mediate reprogramming. Typically, reprogramming is accomplished through cell-cell fusion, nuclear transfer, or forced overexpression of pluripotency factors. What is common between these methods is the introduction of pluripotency related proteins that initiate the reprogramming process. We show here that loss of Bright mediates cellular reprogramming and identify possible mechanisms by which it does so.

Bri-/- MEFs, like other Bri-/- cell lines (An et al., 2010), do not undergo senescence in culture. This remarkable phenotype, while not fully understood, may

contribute to reprogramming, as overcoming the senescence barrier is an important step to cellular reprogramming (Utikal et al., 2009). In low passage cultures, Bri-/- MEFs showed a propensity for forming alkaline phosphatase positive colonies. Alkaline phosphatase is a well-established marker of stem cells. These colonies are not stable and were difficult to expand, leading us to suggest they may have been partially reprogrammed. Microarray analysis confirmed that the parental Bri-/- MEFs (the cell line before any morphological changes have begun) has increased expression of the pluripotency factors Sox2 and Sall1. These observations indicate that loss of Bright creates a permissive environment for reprogramming.

Bri-/- MEFs cultured under confluent conditions in the presence of LIF form spontaneous iPS-like colonies that we termed Bright repression induced pluripotent stem cells (BriPS). What further factors exist, or what pathways are activated, under cell culture conditions that promote reprogramming remains to be determined. BriPS express pluripotency markers at similar levels to wildtype mES cells as shown by immunocytochemistry and microarray analysis. As a stringent test of pluripotency, we show that BriPS were capable of forming teratomas *in vivo*, confirming their pluripotency. Microarray analysis confirmed expression of pluripotency factors, but also showed multiple up- and down-regulated pathways. Comparing these results with the microarray results in Chapter 5 analyzing Bri-/- ES gene expression, we see many commonalities in misregulated pathways. These commonly misregulated pathways indicate Bright's normal function in stem cells may be regulating early gene expression pathways.

We found that Bri-/- MEFs reprogram more efficiently under standard conditions (Oct4/Sox2/Klf4/c-Myc) compared to wildtype MEFs. Interestingly, they could reprogram without Sox2 or Klf4. Considering the parental Bri-/- MEFs show increased Sox2 expression, independence of Sox2 is perhaps not surprising. Independence from Klf4 is unexpected, although these colonies did not persist. There has been evidence that Sox2 and Klf4 have redundancies in MEF reprogramming, which may account for this observation (Nemajerova et al., 2012). Further work is required to determine if under more careful conditions, such as defined, inhibitor based media (Silva et al., 2008), improvements can be made in Bri-/- MEF 4-factor reprogramming.

All current reprogramming methods involve the activity of pluripotency factors or their downstream targets mediating the reprogramming process (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Anokye-Danso et al., 2011). This led us to investigate the role Bright plays in directly regulating the core pluripotency factors Oct4, Sox2, and Nanog. We found that Bright interacts with these proteins in mES cells. Oct4, Sox2, and Nanog are transcription factors well known to bind to themselves and positively regulate their own expression (Young, 2011). We have found Bright consensus sequences in all three promoters and show binding of Bright to the Oct4 and Nanog promoters. Investigations into the ability of Bright to bind to the Sox2 promoter are underway. We show that, as the previous data would indicate, Bright is capable of transcriptionally repressing all three genes. This ability fits with the increase of Bright in the nuclear matrix with differentiation (see Chapter 5). It seems likely that, while there are certainly redundancies in the system, loss of Bright relieves some of the repression on core pluripotency factors, notably Sox2. This action of Bright, together with a more

permissive reprogramming environment, as suggested by the partially reprogrammed cells and the increase in standard reprogramming efficiency suffices to induce reprogramming under specific cell culture conditions. Further work will help to determine other factors with which Bright interacts to initiate reprogramming and the signaling pathways that are activated during the reprogramming process, specifically, what additional signaling events that occur in cell culture that promotes reprogramming. Bri-/- animals are developmentally delayed but do not develop any cancers or teratomas as one might expect if reprogramming were occurring *in vivo*. Elucidating these mechanisms will allow us to create a faster, more efficient alternative reprogramming method.

4.5 FIGURES AND TABLES

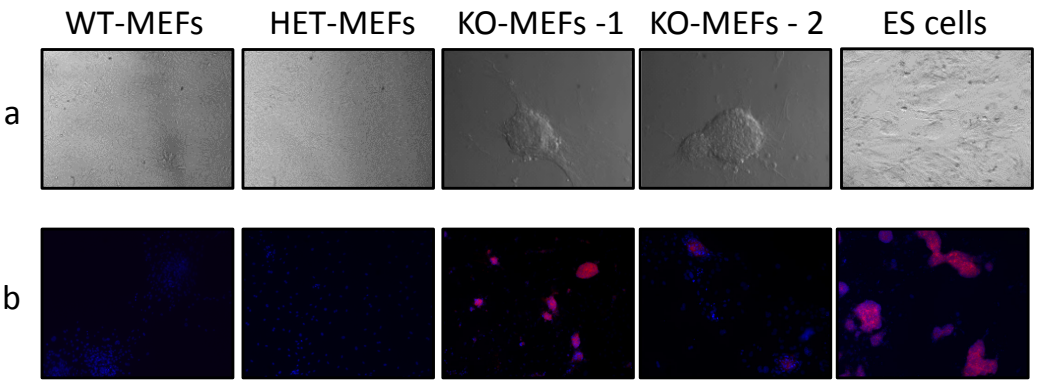

FIGURE 4.1 BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLASTS SPONTANEOUSLY FORM STEM CELL-LIKE COLONIES. (a) As soon as two weeks after initial culturing, multiple Bright KO MEF cell lines (KO-MEFs-1 and KO-MEFs-2) began forming distinct colonies. These colonies morphologically resemble ES cells. Brightfield image, 20x magnification. **(b)** Spontaneous Bright KO MEF express low levels of alkaline phosphatase (AP). AP is a marker of ES cells. AP: red, nuclei: blue. Abbreviations: AP: alkaline phosphatase; MEFs: mouse embryonic fibroblasts; WT: wildtype; Het: heterozygous for Bright; KO: knockout Bright; ES: embryonic stem cells

Cell Type	No. of Initial Clones	Efficiency	Stable surviving clones
WT+4F[a]	12	0.025%	2%
KO+4F[a]	82	0.37%	45%
KO-S[b]	50	0.13%	4%
KO-O[b]	10	0.027%	0%
KO-K[b]	18	0.045%	5.6%
KO-M[b]	28	0.07%	0%
BriPS[c]	5	0.05%	60%

[a] Average of 5 independent experiments

[b] Average of 2 independent experiments

[c] One experiment, 35 days

TABLE 4.1 **BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLASTS REPROGRAM MORE EFFICIENTLY.** Wildtype (Bri+/+) and Bright knockout (Bri-/-) mouse embryonic fibroblasts (MEFs) were infected using a retrovirus that overexpressed Oct4, Sox2, Klf4, and c-Myc to produce induced pluripotent cells (iPS). Bri-/- MEFs reprogrammed more efficiently than Bri+/+ MEFs and could form stable clones in the absence of Sox2 (KO-S) or Klf4 (KO-K). Bri-/-MEFs spontaneously formed stable reprogrammed cell lines (BriPS) in the presence of LIF. Abbreviations: WT: Bri+/- MEFs; KO: Bri-/- MEFs; 4F: MEFs infected with overexpression vectors for Oct4, Sox2, Klf4, and c-Myc; -S, -O, -K, -M: MEFs infected with overexpression vectors except for Sox2, Oct4, Klf4, or c-Myc respectively.

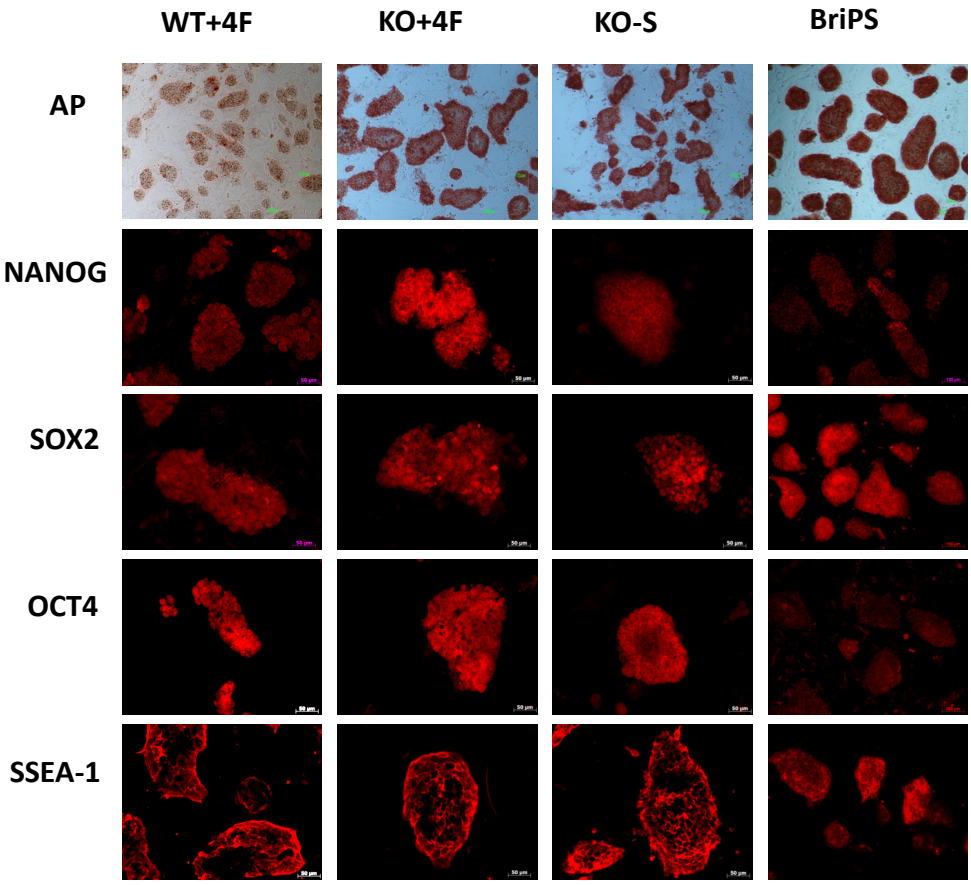

FIGURE 4.2 SPONTANEOUS BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLAST CLONES AND REPROGRAMMED BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLASTS EXPRESS PLURIPOTENCY MARKERS. Clonal BriPS, KO+4F and KO-S all express pluripotency markers AP, Nanog, Sox2, Oct4, and SSEA-1 at similar levels to standard 4-factor reprogrammed wildtype MEFs (WT+4F). For further detail, see Table 4.1 Abbreviations: WT+4F: standard 4 factor reprogrammed wildtype MEFs; KO+4F: Bri-/-MEFs reprogrammed using standard 4 factor reprogramming; KO-S: Bri-/- MEFs reprogrammed using standard factors without Sox2; BriPS: cell lines derived from spontaneous Bri-/- MEFs colonies; AP: alkaline phosphatase.

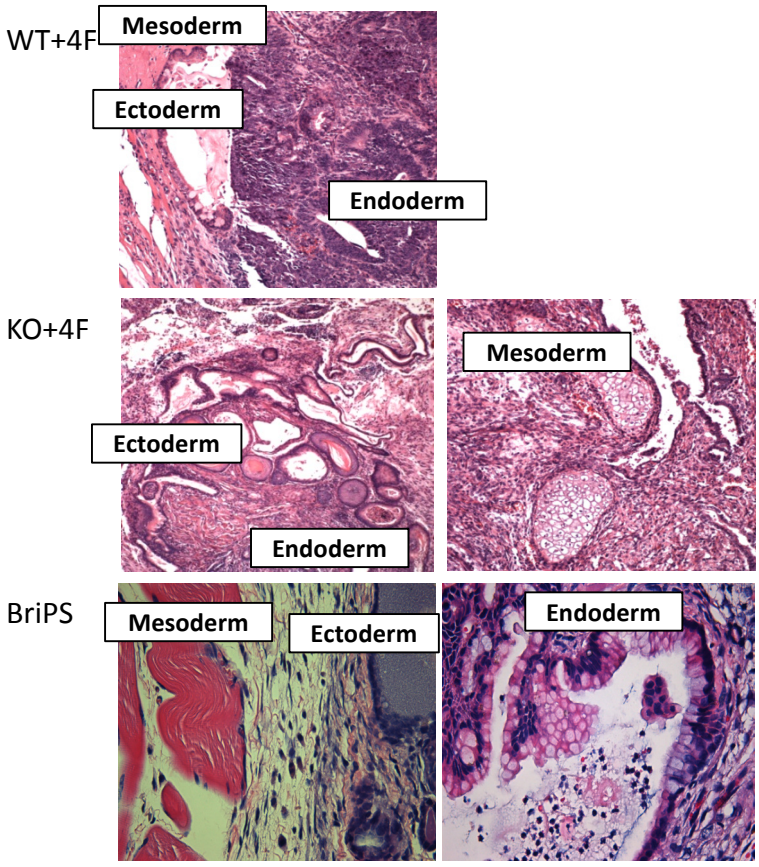

FIGURE 4.3 SPONTANEOUS BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLAST CLONES AND REPROGRAMMED BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLASTS FORM TERATOMAS. 350,000 feeder-depleted clonal cell lines derived from BriPS, KO+4F and WT+4F were injected into the flanks of immune compromised NSG mice. All tumors showed the same latency and were typically palpable after 3-4 weeks. Representative images with cells from all three germ layers are labeled. WT+4F and KO+4F 20x, BriPS 40x magnification. Abbreviations: WT+4F: standard 4 factor reprogrammed wildtype MEFs; KO+4F: Bri-/-MEFs reprogrammed using standard 4 factor reprogramming; BriPS: cell lines derived from spontaneous Bri-/- MEFs colonies;.

Figure 4.4

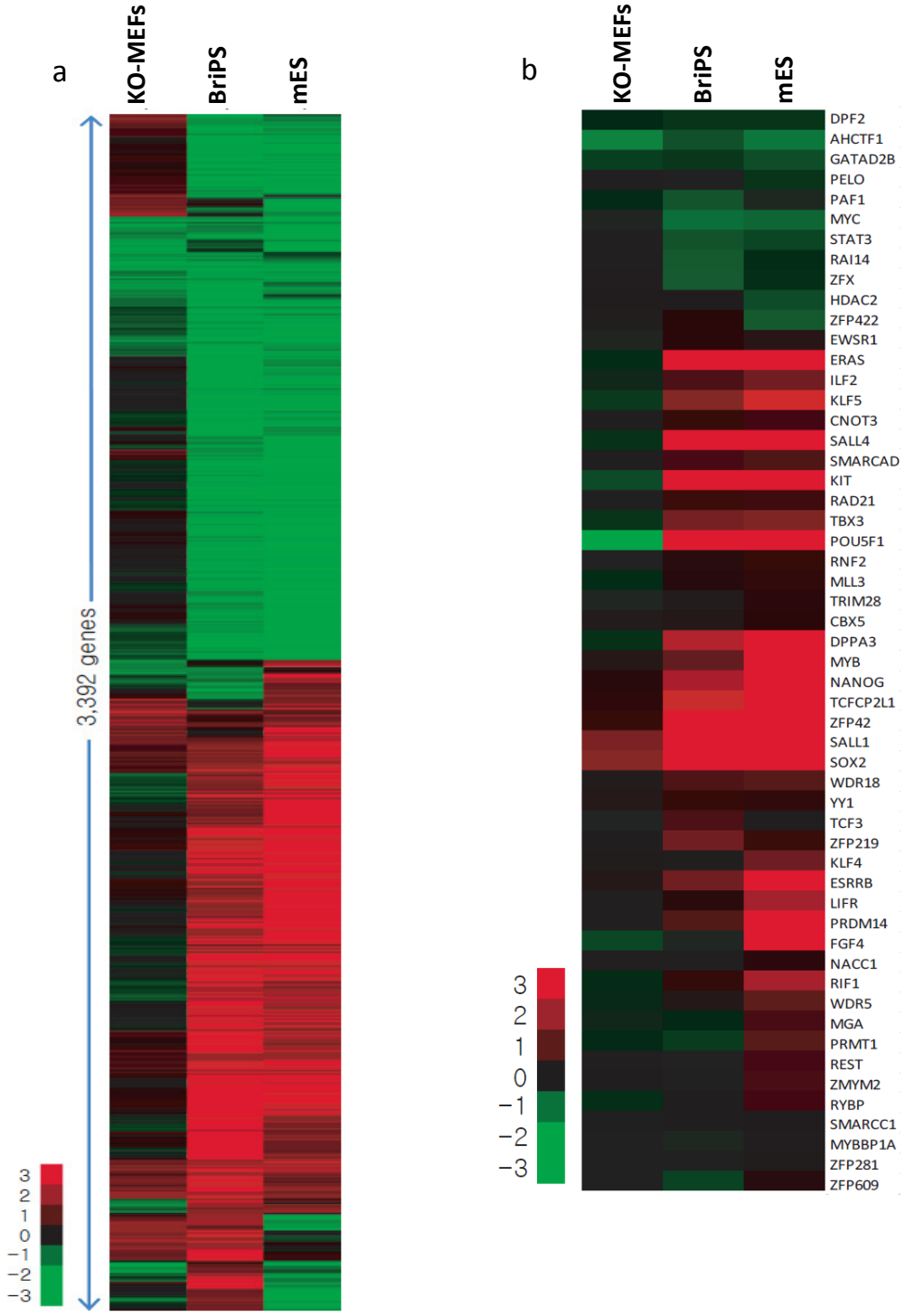
a
KO-MEFs
BriPS
mES
3,392 genes
3
2
1
0
−1
−2
−3
b
KO-MEFs
BriPS
mES
DPF2
AHCTF1
GATAD2B
PELO
PAF1
MYC
STAT3
RAI14
ZFX
HDAC2
ZFP422
EWSR1
ERAS
ILF2
KLF5
CNOT3
SALL4
SMARCAD
KIT
RAD21
TBX3
POU5F1
RNF2
MLL3
TRIM28
CBX5
DPPA3
MYB
NANOG
TCFCP2L1
ZFP42
SALL1
SOX2
WDR18
YY1
TCF3
ZFP219
KLF4
ESRRB
LIFR
PRDM14
FGF4
NACC1
RIF1
WDR5
MGA
PRMT1
REST
ZMYM2
RYBP
SMARCC1
MYBBP1A
ZFP281
ZFP609
3
2
1
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FIGURE 4.4 GLOBAL GENE EXPRESSION IS SIMILAR BETWEEN SPONTANEOUS BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLAST CLONES AND MOUSE EMBRYONIC STEM CELLS. **(a)** Global gene expression analysis performed by microarray demonstrates that BriPS are reprogramed. The overall gene expression pattern is similar to mES. The BriPS gene expression pattern has sharply diverged from the parental KO-MEFs. The heatmap represents total genes >1-fold different from WT-MEFs. **(b)** Pluripotency gene expression is similar between BriPS and mES. The heatmap represents fold changes seen in KO-MEFs, BriPS and mES compared to WT-MEFs of key pluripotency genes. Abbreviations: BriPS: cell lines derived from spontaneous Bri-/- MEF colonies; mES: mouse embryonic stem cells; KO-MEFs: Bright knockout mouse embryonic fibroblasts; WT-MEFs: wildtype mouse embryonic fibroblasts.

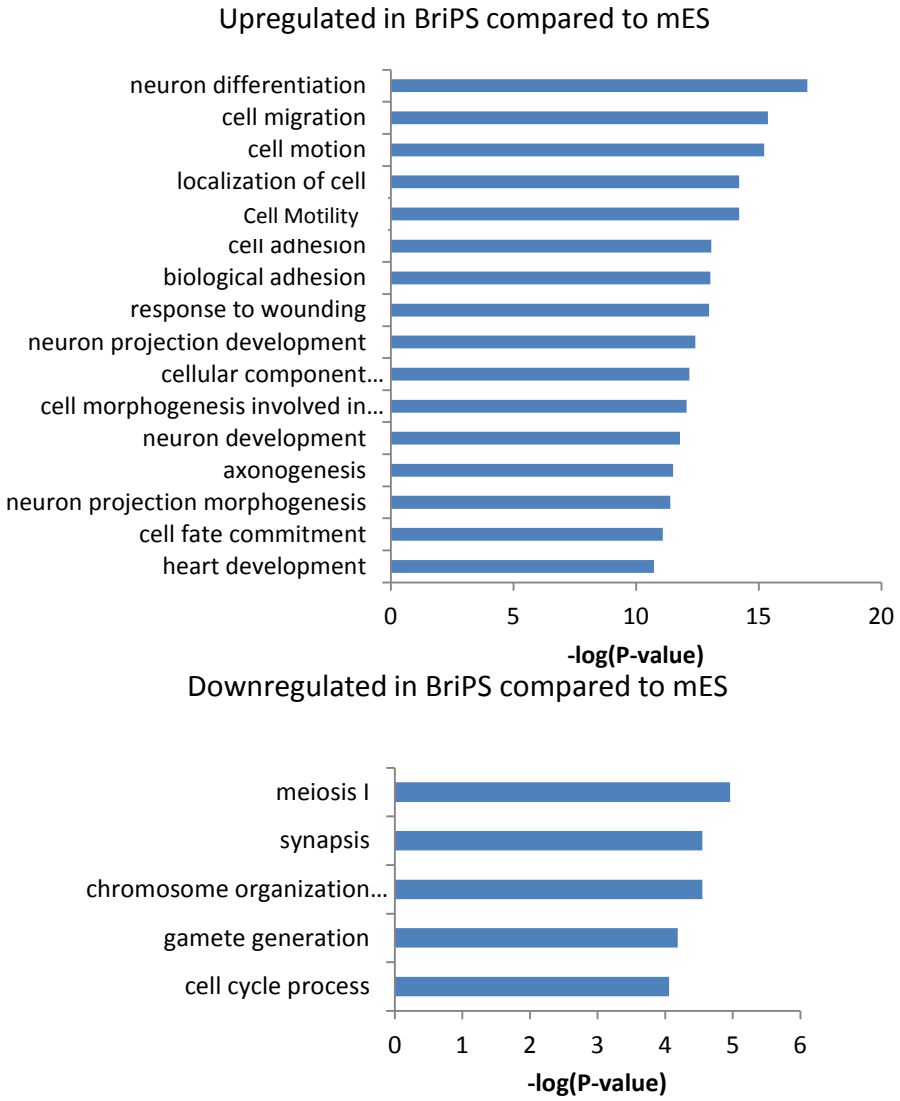


FIGURE 4.5 GENE ONTOLOGY ANALYSIS OF MICROARRAY BETWEEN SPONTANEOUS BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLAST CLONES AND MOUSE EMBRYONIC STEM CELLS. This analysis indicates pathway differences between BriPS and mES. Some of these pathways are known to be misregulated between mES and standard iPS, but of particular note, we see early developmental pathways but no misregulation is seen in any pluripotency pathways. Abbreviations: BriPS: cell lines derived from spontaneous Bri-/- mouse embryonic fibroblast colonies; mES: mouse embryonic stem cells; iPS: induced pluripotent stem cells;

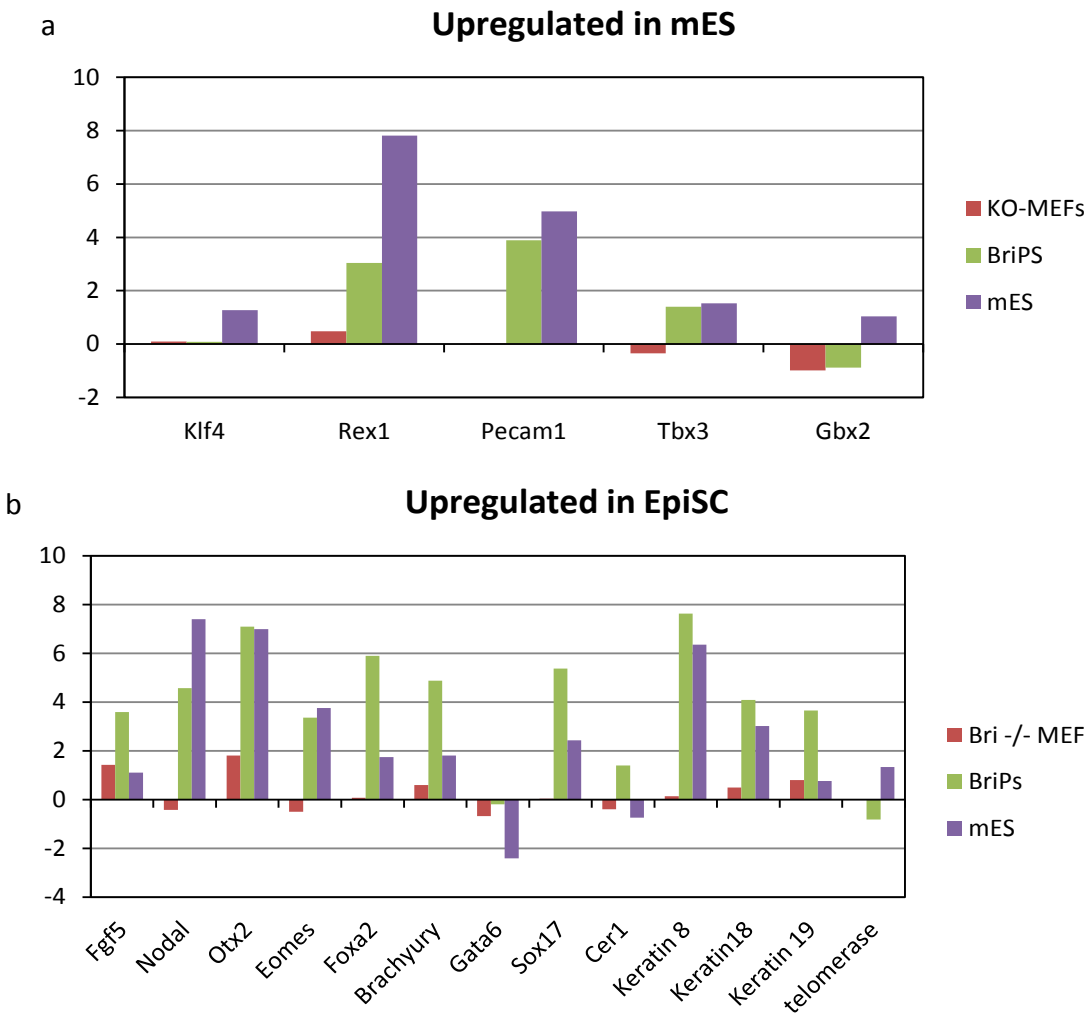


FIGURE 4.6 EXPRESSION ANALYSIS OF A SUBSET OF EPIBLAST STEM CELL GENES SHOWS AN INTERMEDIATE PATTERN FOR SPONTANEOUS BRIGHT KNOCKOUT MOUSE EMBRYONIC FIBROBLAST CLONES. **(a)** Graphical representation of gene expression upregulated in mES compared to EpiSC from microarray analysis of Bri-/- MEFs, BriPS, and mES (Figure 4.4). **(b)** Graphical representation of gene expression upregulated in mES compared to EpiSC from microarray analysis of Bri-/- MEFs, BriPS, and mES (Figure 4.4). Abbreviations: mES: mouse embryonic stem cells; EpiSC: epiblast stem cells BriPS: cell lines derived from spontaneous Bri-/- MEF colonies; Bri-/- MEFs: Bright knockout mouse embryonic fibroblasts;

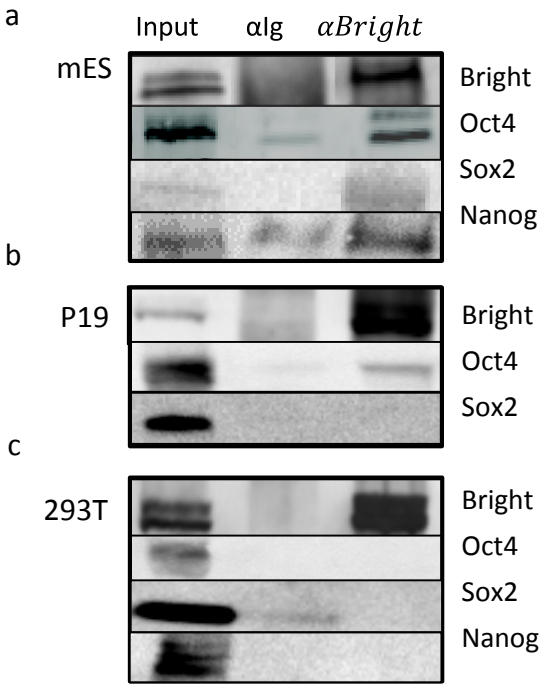


FIGURE 4.7 BRIGHT INTERACTS WITH PLURIPOTENCY FACTORS IN MOUSE EMBRYONIC STEM CELLS. **(a)** Bright binds to Oct4, Sox2, and Nanog as shown by co-immunoprecipitation in mouse embryonic stem cells (mES). **(b)** Bright binds to Oct4 but not Sox2 in P19 embryonic carcinoma cell lines. **(c)** Bright did not bind to Oct4, Sox2, or Nanog as shown by co-immunoprecipitation when overexpressed in 293T cells.

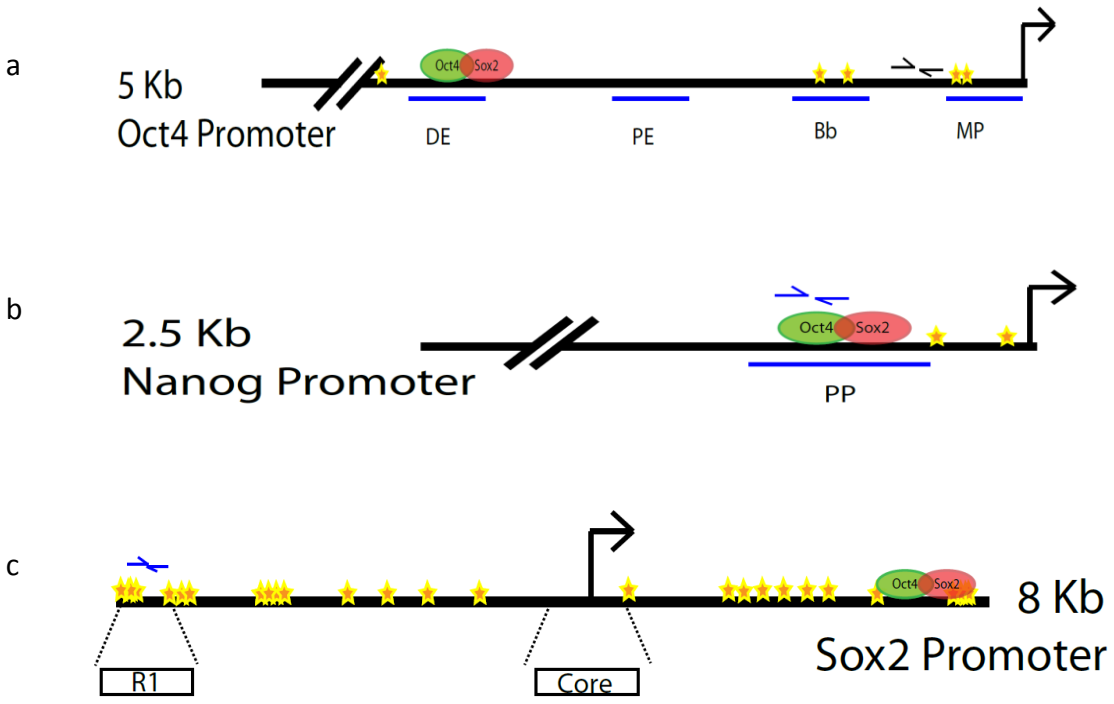


FIGURE 4.8 OCT4, NANOG, AND SOX2 PROMOTER CONTAIN BRIGHT CONSENSUS BINDING SITES. **(a)** Diagram of the Oct4 promoter. **(b)** Diagram of the Nanog promoter. **(c)** Diagram of the Sox2 promoter and enhancer sites. Promoters or partial promoter sequences as indicated by boxes were used in Figure 4.9 luciferase vectors. Oct4 and Sox2 binding sites are indicated by green and red balloons, respectively. ChIP primers used in Figure 4.8 are indicated by a pair of blue arrows. Probes tested for direct binding in Figure 4.10 are indicated by blue lines. Start of transcription is indicated by right-facing arrows. Bright consensus sequences are indicated by orange/gold stars.

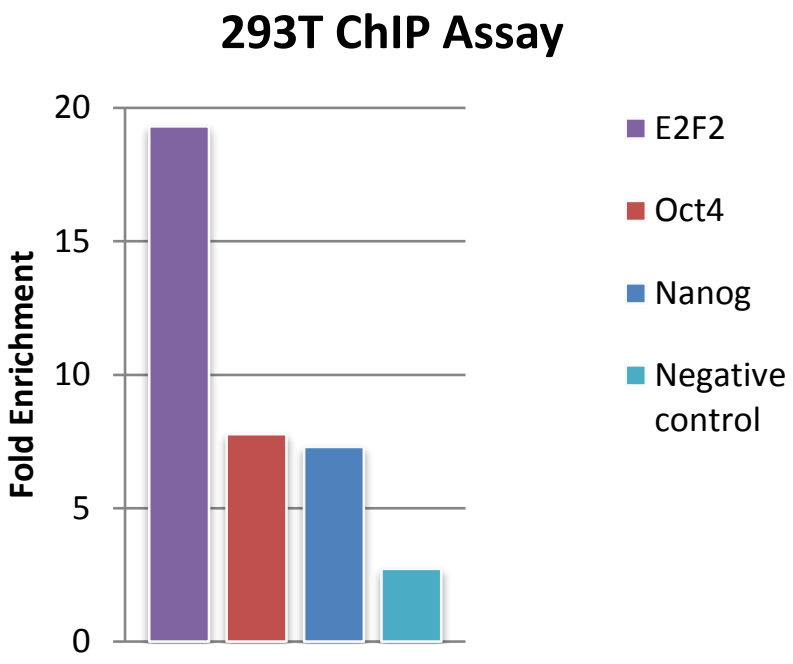


FIGURE 4.9 BRIGHT BINDS THE OCT4 AND NANOG PROMOTERS. Chromatin immunoprecipitation (ChIP) assay was performed using crosslinked human 293T cell lines and a Bright specific polyclonal antibody. Semi-quantitative Real-Time PCR was used to determine fold enrichment over input samples.

Figure 4.10

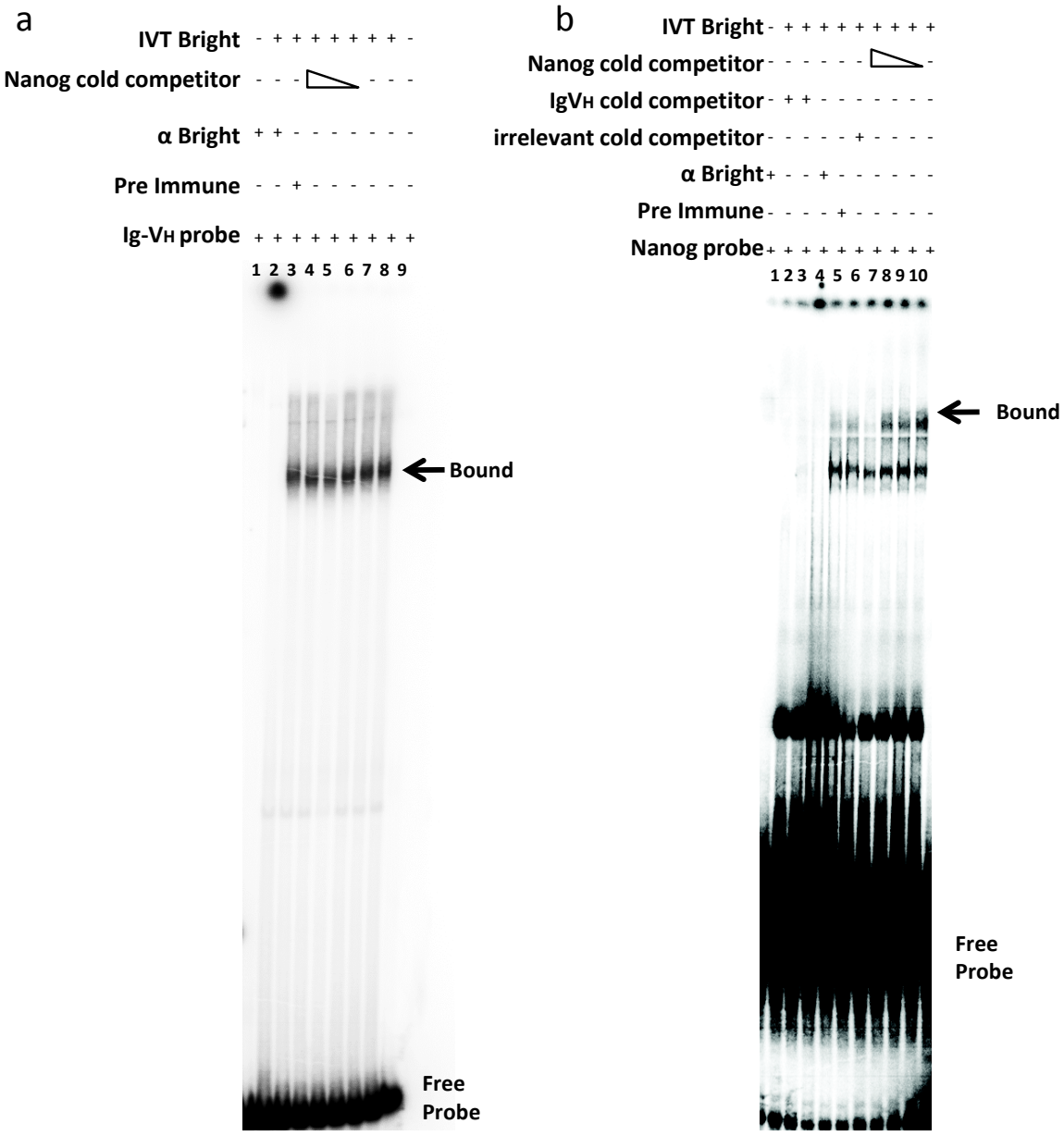

a
IVT Bright - + + + + + + + -
Nanog cold competitor - - -
- - -
α Bright + + - - - - - - -
Pre Immune - - + - - - - - -
Ig-VH probe + + + + + + + + +
1 2 3 4 5 6 7 8 9
Bound
Free
Probe
b
IVT Bright - + + + + + + + + +
Nanog cold competitor - - - - - -
-
IgVH cold competitor - + + - - - - - - -
irrelevant cold competitor - - - - - + - - - -
α Bright + - - + - - - - - -
Pre Immune - - - - + - - - - -
Nanog probe + + + + + + + + + +
1 2 3 4 5 6 7 8 9 10
Bound
Free
Probe

Figure 4.10

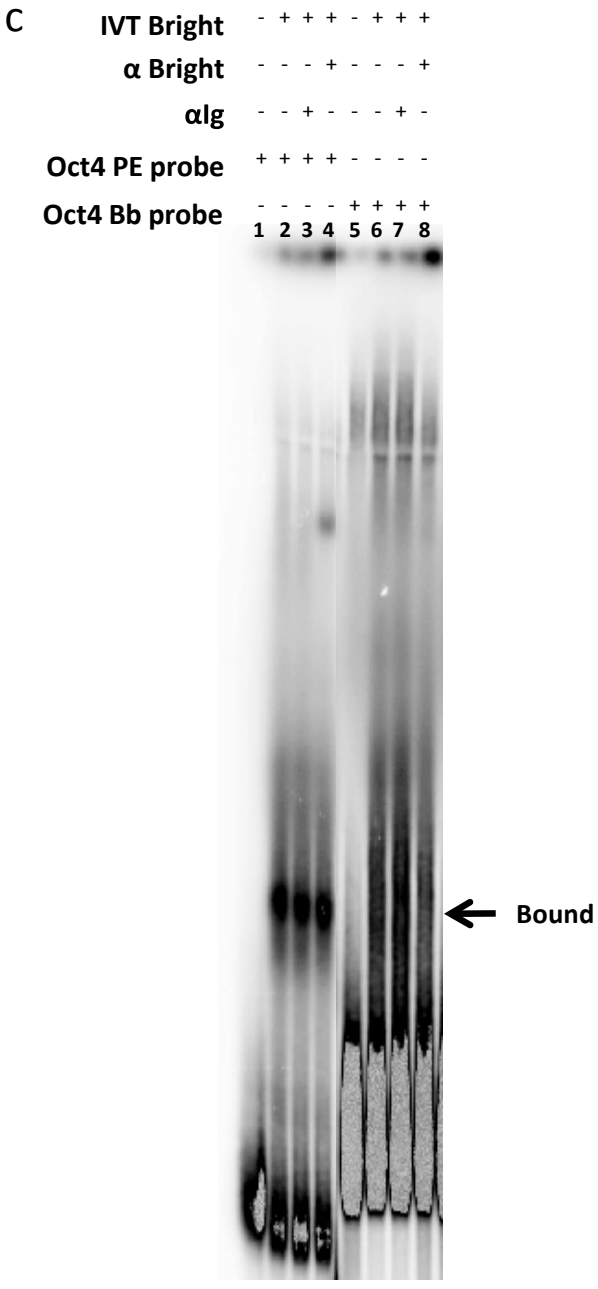

C
IVT Bright - + + + - + + +
α Bright - - - + - - - +
αIg - - + - - - + -
Oct4 PE probe + + + + - - - -
Oct4 Bb probe - - - - + + + +
1 2 3 4 5 6 7 8
Bound

FIGURE 4.10 BRIGHT DIRECTLY BINDS THE NANOG PROMOTER. **(a)** Electrophoretic mobility shift assay (EMSA) was performed using in vitro translated Bright protein and a prototypic Bright-binding site, Ig-Vh. Nanog probe could slightly cold compete Bright from the Ig-Vh fragment (lanes 4-6). **(b)** EMSA was performed using *in vitro* translated Bright protein and a 120 base pair fragment from the Nanog promoter. Bright could bind to the Nanog fragment (lane 10). This interaction was shown to be specific through supershifting the band using an anti-Bright polyclonal antibody (lane 4). **(c)** EMSA was performed using *in vitro* translated Bright protein. Probes used were a 289 base pair fragment (DE) that corresponds to the distal enhancer region and a 353 base pair fragment (Bb) that contains Bright consensus binding sequences from the Oct4 promoter. Bright could bind to these Oct4 fragments (lanes 2 & 6). This interaction was shown to be specific through supershifting the band using anti-Bright polyclonal antibody (lane 4 & 8).

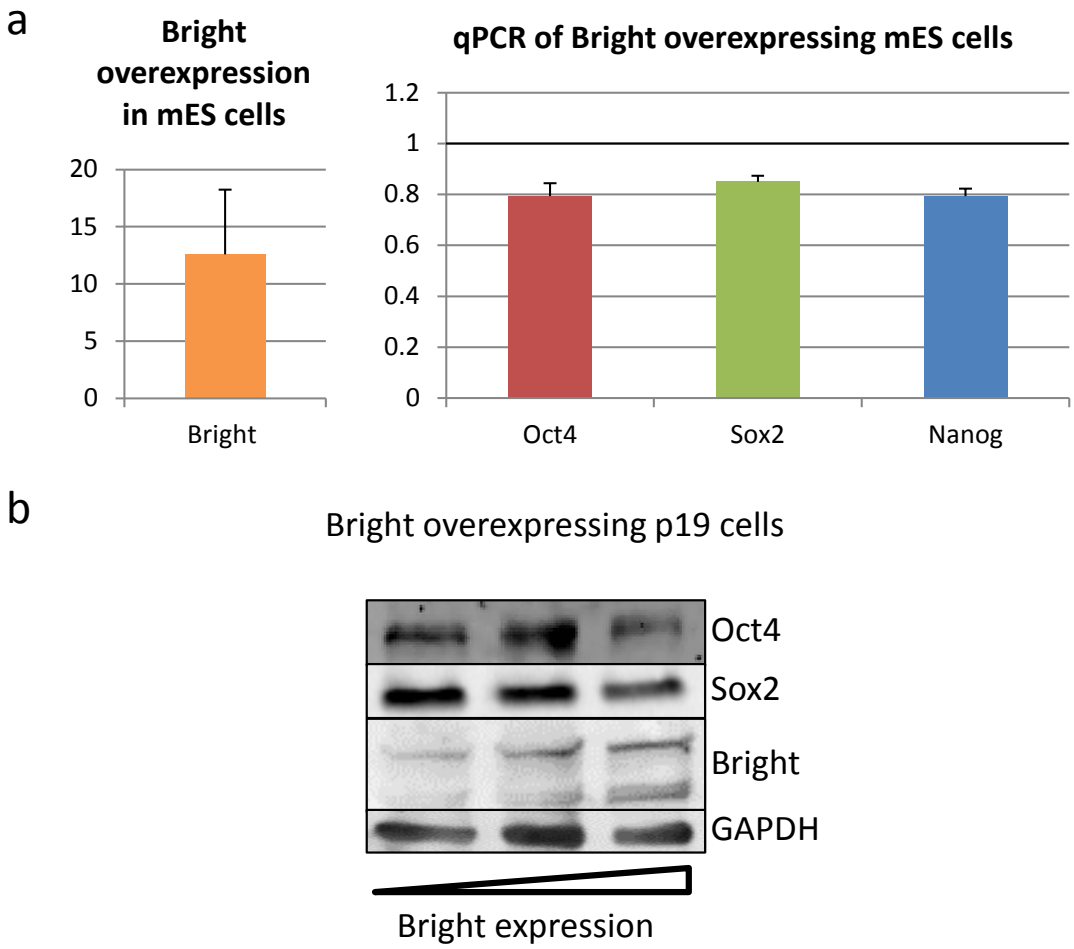
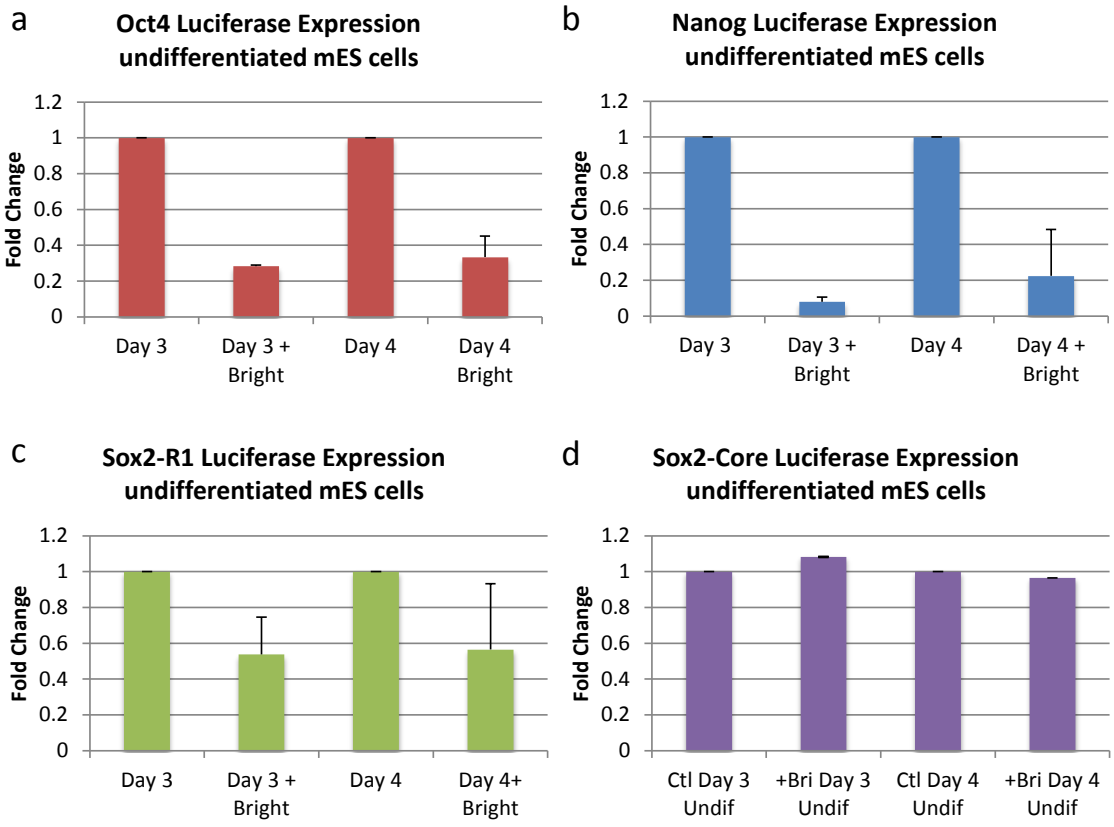


FIGURE 4.11 BRIGHT REPRESSES ENDOGENOUS LEVELS OF OCT4, SOX2, AND NANOG. **(a)** Mouse embryonic stem cells (mES) were transfected with either empty vector or Bright expression vector. Bright, Oct4, Nanog, and Sox2 levels were measured using semi-quantitative Real-Time PCR, and levels were normalized to empty vector control. Graphs represent the average of three separate experiments. Error bars correspond to the standard error of the mean. **(b)** Bright was overexpressed by transfecting a Bright expression vector in increasing concentrations into p19 embryonic carcinoma cell lines. Cells were harvested two days after transfection. Protein expression of Oct4, Sox2, Bright, and GAPDH was determined by western blot analysis.

Figure 4.12


a
Oct4 Luciferase Expression
undifferentiated mES cells
Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Day 3
Day 3 + Bright
Day 4
Day 4 Bright
b
Nanog Luciferase Expression
undifferentiated mES cells
Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Day 3
Day 3 + Bright
Day 4
Day 4 + Bright
c
Sox2-R1 Luciferase Expression
undifferentiated mES cells
Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Day 3
Day 3 + Bright
Day 4
Day 4+ Bright
d
Sox2-Core Luciferase Expression
undifferentiated mES cells
Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Ctl Day 3 Undif
+Bri Day 3 Undif
Ctl Day 4 Undif
+Bri Day 4 Undif

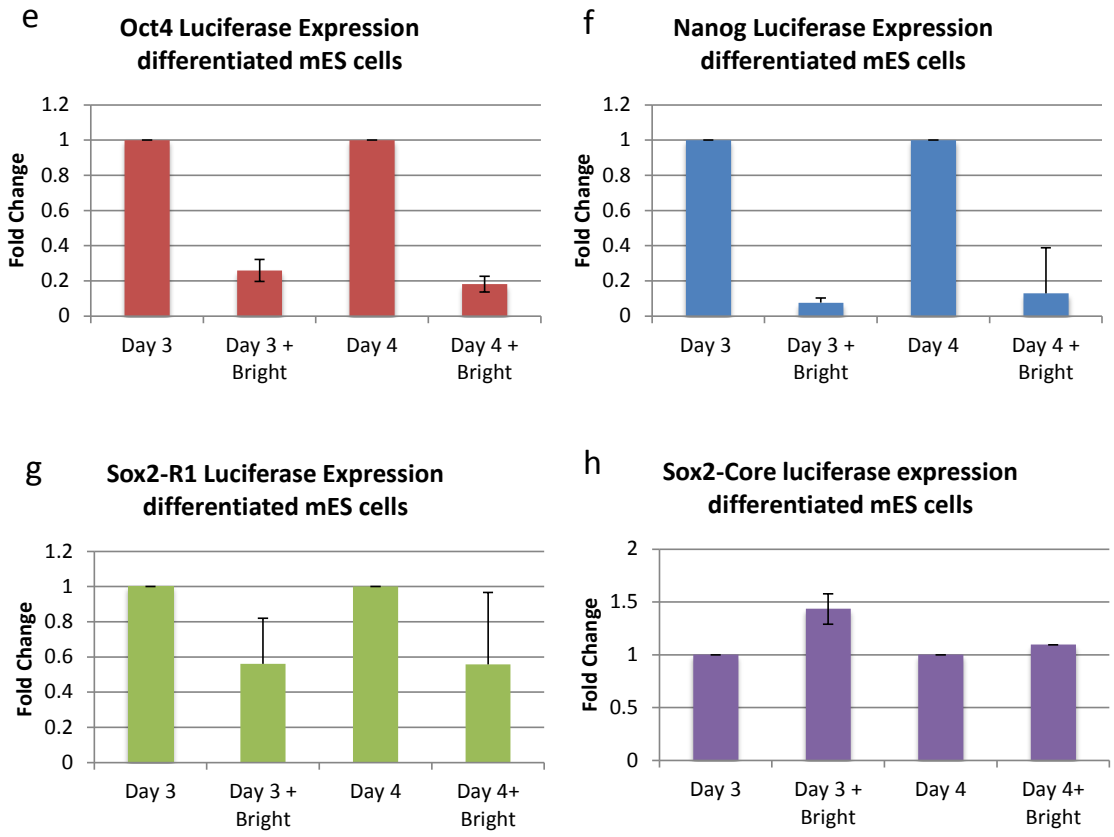


FIGURE 4.12 BRIGHT REPRESSES THE OCT4, NANOG, AND SOX2 PROMOTERS. Indicated promoter driven luciferase vectors (see Figure 4.7) were transfected into undifferentiated mouse embryonic stem cells (mES). Cells were allowed to grow either under non-differentiating conditions **(a-d)** or under differentiating conditions **(e-h)**. Cells were harvested at day 3 and 4 and luciferase expression determined. Graphs represent at least two biological replicates each done in triplicate. All samples were normalized to the internal control renilla. Fold change is expressed over empty vector controls.

Chapter 5

Bright Regulates Timely Differentiation of Embryonic Stem Cells

5.1 INTRODUCTION

Mouse embryonic stem (mES) cells are invaluable research tools in the lab. They have allowed researchers to unravel the early stages of cell differentiation and lineage commitment. In normal development, mES cells are only a transient state. These cells quickly lose their pluripotency and become committed to specific cell lineages. Fluid interactions between lineage-specifying transcription factors, an open chromatin state, and epigenetic modifiers prevents cells from becoming terminally differentiated until the entire organism is correctly prepared. Cell-cell interactions and timing of differentiation are critical to the correct formation of the embryo (Hemberger et al., 2009).

We have previously established that Bright/ARID3A is broadly expressed in the early developing embryo. Expression becomes restricted primarily to the fetal liver around embryonic development day 12.5 (E12.5) (Webb et al., 2011). It is possible for Bright null mice to fully develop, indicating that Bright is not necessary for differentiation. We have noted, however, that Bright null (Bri-/-) embryos and young

pups are typically smaller than either their wildtype (Bri+/+) or heterozygous (Bri+/-) littermates. We showed in Chapter 3 that this size difference does not appear to be a proliferative issue. We have also shown that Bright represses key pluripotency factors, indicating that it has a role in early differentiation. These observations lead us to investigate how Bright influences early embryonic development.

5.2 MATERIALS AND METHODS

5.2.1 Derivation of Bright Null and Heterozygous Embryonic Stem Cells

To obtain Bright-/- mES lines, blastocysts were flushed out of the horns of 3.5 day pregnant Bright+/- females which had been mated with Bright+/- males (Nagy, 2002). Blastocysts were transferred onto STO feeder layers in mES media (DMEM supplemented with 20% FBS, penicillin/streptomycin, nucleosides, non-essential amino acids, and β-mercaptoethanol) and cultured at 37°C in 5% CO2 in humidified air for 6–7 days without media changes. The inner cell masses were identified, treated with trypsin, disrupted, and then transferred individually and subcultured in 24-well STO feeder plates. Four days later, single cell clones of compact mES colonies were passaged onto 6-well plates and then split after 2–3 generations for confirmation of null genotype by PCR.

5.2.2 Embryoid Body Formation Assay

Embryonic stem cell colonies were lightly trypsinized, feeder cells were removed, and cells were resuspended in mES media that contained no LIF at a concentration of 20,000 cells/ml. Approximately 80-20ul droplets (each containing ~400 cells) were placed on the lid of a petri dish. The lid was then inverted and placed over the petri dish filled with PBS to maintain proper humidity. Hanging drops were kept in standard cell culture conditions for three days. On the third day hanging drops were collected into 10ml mES media that contained no LIF on bacterial culture plates to prevent attachment. Media was changed every other day, and EBs were collected by centrifugation at the timepoints indicated. For adherent experiments, EBs were collected at day 8 and plated onto gelatin coated 10 cm cell culture dishes. Protocol modified from David Stewart lab protocol, personal communication.

5.3 RESULTS

5.3.1 Bright Expression Increases with Embryonic Stem Cell Differentiation

We have shown Bright is expressed in embryonic development (E5.5-E8.5) but not at earlier timepoints. To determine Bright expression levels, we analyzed publically available microarray data of Bright expression across normal human and mouse tissues. Bright is expressed at low levels in all tissues tested but high expression in many B cell lineages (**Figure 5.1 a, b**) (Su et al., 2004). Notably, both in human and mouse, Bright is highly expressed in the placenta. Additionally, published microarray data of retinoic acid

induced differentiation of mES cells showed that Bright was highly upregulated upon differentiation od mES cells (see **Figure 5.9 b**) (Wang et al., 2006). This upregulation demonstrates that Bright likely plays a role in multiple tissue types both during development and in the adult.

mES cells must be maintained under strict cell culture conditions in order to maintain pluripotency, or they will spontaneously differentiate. mES cells kept in suspension and allowed to differentiate will form embryoid bodies (EBs). An EB is a round mass of differentiated cells that represent all three germ layers (Itskovitz-Eldor et al., 2000; Kurosawa, 2007). Consistent with the observations of Wang et al (2006), undifferentiated mES cells express Bright. We also find that upon differentiation, either by withdrawal of LIF or formation of EBs, Bright expression increases steadily to a maximum by day 6 (**Figure 5.2 a, b**).

Bright has a number of cellular functions other than as a transcription factor. For instance, Bright shuttles between the nucleus and the cytoplasm as well as localizes to lipid rafts (Kim and Tucker, 2006; Schmidt et al., 2009). Bright transactivation activity is maximal when it localizes to the nuclear matrix (Zong and Tucker, 2000) to enhance chromatin accessibility (Lin et al., 2007). We determined by four-way fractionation of undifferentiated and differentiated mES cells that the increased levels of Bright seen upon differentiation preferentially accumulate within the nuclear matrix (**Figure 5.2 c**). These data suggest that Bright may act as a transcriptional regulator in early differentiating mES cells. If Bright is acting as a regulator of differentiation, then pluripotent cells may maintain low levels of sequestered Bright to respond quickly to developmental signals.

5.3.2 Bright Knockout Embryonic Stem Cells are Pluripotent

Standard reprogramming techniques utilize overexpression of pluripotency factors to induce expression of endogenous factors that lead to restructuring of chromatin to the 'open' state found in pluripotent cells. Understanding the role Bright plays in mES cells and differentiation may help to unravel the role it plays in reprogramming.

To understand the role Bright plays in normal mES cells, we utilized Bright null embryonic stem cells (Bri-/- mES) (Webb et al., 2011). These cells were derived from E3.5 blastocysts produced from Bright heterozygous matings. These cells maintain mES morphology (**Figure 5.3 a**) and pluripotency gene expression pattern (**Figure 5.6**). They grow at equivalent rates compared to Bri+/+ and Bri+/- mES cells and showed no abnormal phenotype. As a more stringent test of pluripotency, teratoma assays were performed using Bri-/- mES cells[6]. Bri-/- mES cells form teratomas with a similar latency compared to Bri+/+ mES cells, indicating that they maintained pluripotency in culture (**Figure 5.3 b**).

5.3.3 Bright Knockout Embryonic Stem Cells Do Not Resemble Wildtype Embryonic Stem Cells Upon Differentiation

Further in vitro differentiation experiments were performed to determine more precisely whether the loss of Bright affected the mES cell's ability to differentiate. Briefly, cells were removed from feeder cells and plated in hanging drop cultures for 2

[6] Histological analysis of tumors was performed by Dr. Kusewitt at MD Anderson Science Park.

days. On the third day, EBs were collected and grown in suspension conditions for up to 20 days (**Figure 5.4 a**). The Bri-/- EBs grew much faster than the Bri+/+ controls, and the majority formed very large structures termed cystic embryoid bodies (CEB) (Wang et al., 1992). CEBs are models for early extraembryonic tissues development, as they contain yolk-sac-like structures (Doetschman et al., 1985; Yasuda et al., 2009) and early vasculature (Wang et al., 1992; Ng et al., 2004). The Bri-/- CEBs were on the order of 10 times larger than any Bri+/+ EBs. On the eighth day, Bri-/- and Bri+/+ EBs were plated on gelatin coated plates to encourage cardiomyocyte differentiation (**Figure 5.4 b**)[7] (Fuegemann et al., 2007; Kurosawa, 2007). The Bri+/+ EBs plated down and formed regular round colonies with differentiated cells radiating out from the center of less differentiated cells and were able to form beating cardiomyocytes (**Figure 5.4 c**). Of particular note, while Bri-/- EBs could form beating cardiomyocytes (**Figure 5.4 c**), they did not spread out on the plate or form regular differentiating colonies. These morphological differences show that Bri-/- mES cells are clearly deficient in their ability to undergo differentiation correctly.

5.3.4 Timing of Differentiation is Disrupted in Bright Knockout Embryonic Stem Cells

We have shown that loss of Bright does not prevent self-renewal or maintenance of pluripotency in mES cells, but it does affect differentiation. To investigate the cellular processes that may be involved in the differentiation defect, we performed an initial

[7] Additional methodology and advice provided by Dr. David Stewart, University of Houston.

microarray analysis on Bri-/- and Bri+/+ undifferentiated mES cells[8]. Arrays were performed using two different Bri-/- mES cell lines (Bri-/- mES-1 and Bri-/- mES-2) with two biological replicates (**Figure 5.5 a, b**). The first array consisted of Bri-/- and Bri+/+ mES cells that were removed from their feeder cells. The second array consisted of cells that were FACS sorted based on size (mES cells being smaller than the feeder cells). Gene ontogeny analyses of the microarrays show several common downregulated pathways between the two microarrays, including neurological system processes, regulation of neurotransmitter levels, and cell-cell signaling. Only one upregulated pathway was common between the two arrays: embryonic organ development. Within the upregulated pathways for each array, however, there was more similiarity. The first replicate showed upregulation in early development pathways such as positive regulation of cell fate, cell fate commitment, embryonic morphogenesis, organ development, and organ morphogenesis (**Figure 5.5 a**, ***right***). The second biological replicate showed upregulated pathways associated with heart and vasculature development such as heart, blood vessel, vasculature development and blood vessel morphogenesis (**Figure 5.5 b**, ***right***). These data support the increase of CEB formation seen in the Bri-/- ES. Notably, placenta development is also upregulated. We found this to be a particularly interesting deregulated pathway considering the high levels of Bright expression in the normal placenta (**Figure 5.1**).

To study the pathways in which Bright functions during differentiation, we performed gene expression analysis on undifferentiated Bri-/- and Bri+/+ mES cells and

[8] Reverse transcription of RNA, cDNA labeling, microarray hybridization, and following analyses performed by Dr. Bum Kyu Lee in Dr. Vishy Iyer's lab

EBs harvested at day 6 and 15. We used Bri-/- mES-1, Bri-/- mES-2, and Bri+/+ mES cells with two biological replicates for each timepoint. The heat map was generated by averaging all Bri-/- replicates for each timepoint and normalizing them to the Bri+/+ control. Genes that were >2-fold different in expression for any given timepoint were plotted and clustered according to expression pattern over the full time course (**Figure 5.6**). The heat map shows that over the course of differentiation, there are few differences at day 0 but an increasing number at day 6, with the largest gene expression difference observed at day 15. Gene ontogeny analysis of select clusters (numbered in red) showed a range of misregulated pathways (**Figure 5.7 a-e**). Cluster 6 included downregulation of multiple pathways involved in pluripotency, such as stem cell maintenance, stem cell development, and gastrulation. Conversely, there is also downregulation of some differentiation pathways, including stem cell differentiation, formation of the primary germ layer, pattern specification process, and anterior/posterior pattern formation in cluster 6. The same pathways seen in undifferentiated mES cell microarrays (**Figure 5.5 a-b**) are again present in the upregulated clusters 2, 5, 8, and 10 (**Figure 5.7 a-d**). These can roughly be broken into two groups: the first relates to vasculature (heart, blood vessel, vasculature development, and blood vessel morphogenesis) and the second to cell movement (cell motion, cell adhesion, biological adhesion, cell-cell adhesion, and regulation of cell migration). The upregulation of vasculature related pathways correlates with the increased formation of CEBs in Bri-/- mES cells. We noted previously that the Bri-/- EBs did not properly adhere to the cell culture plate or spread out when plated out during hanging drop experiments. The increase in adhesion pathways seen from the GO analysis may explain this observation.

Analysis of the gene expression data comparing lineage specific genes indicated that Bri-/- mES cells differentiate more quickly down certain lineages (**Figure 5.8 a-d**). This increase differentiation may account for the greater change in gene expression seen at D15 compared to D0. The Bri-/- mES cells are further differentiated down certain cell lineages compared to Bri+/+ ES. Bri-/- mES appear to differentiate down the endoderm lineage at the same pace as the Bri+/+ mES (**Figure 5.8 a**). They also appear to follow a similar but not identical timing pattern for the ectoderm lineage (**Figure 5.8 b**). However, Bri-/- mES differentiated into neuroectoderm and mesoderm lineage pathways more efficiently than did Bri+/+ mES (**Figure 5.8 c, d**). Correlation analysis indicated that Bri-/- day 6 EBs more closely resemble Bri+/+ day 15 EBs gene expression patterns for the neuroectoderm and mesoderm (**Figure 5.8 c, d, *bottom***).

These data suggest that Bright is required for timely differentiation down the neuroectoderm and mesoderm pathways. It may be informative in this regard that cluster 6 revealed downregulation of mesoderm formation and morphogenesis, suggesting that Bright has additional functions in mesoderm differentiation.

5.3.5 Further Analysis of Bright-interacting Proteins and Pathways

Bright was originally discovered as an activator of immunoglobulin gene transcription via binding to specific ATC-rich sites within promoter and enhancer associated MARs (Herrscher et al. 1995). Several additional Bright consensus binding sequences have been reported in publically available protein binding microarrays (UniProbe database; **Figure 5.9 a**). The transcription start sites of previously determined

core pluripotency factors were probed for Bright consensus binding sites[9] (**Table 5.1**). Of the total genes examined, 13 are highly differentially expressed during RA-induced mES cell differentiation (**Figure 5.9 b**). Of these potential Bright pluripotency targets, we noted includes proteins both upregulated and downregulated with differentiation.

In light of the multiple pathways and apparent context dependence of Bright action, we reasoned that determination of Bright interacting partners would be informative. We performed mass spectrometry peptide sequencing analysis[10] of Bright immunoprecipitates to search for potential candidates under conditions of endogenous Bright expression levels (**Table 5.2**). Initially, we investigated the binding partners in the somatic cell line 293T, a human embryonic kidney epithelial carcinoma, transformed by adeno- and SV40 viruses. Previous work had shown that these cells are capable of undergoing partial reprograming in response to Bright shRNA knockdown (An et al., 2010). Mass spectrometry analysis was performed in triplicate. Of these potential targets, we further validated Bright interactions with SATB2, PELP1, and RelA recombinant proteins by tagged overexpression/Co-IP (**Figure 5.10**).

Each of these proteins may be implicated in mES maintenance or reprogramming. SATB2 is a transcription factor that binds AT-rich DNA and acts as a docking protein for chromatin remodeling enzymes (Gyorgy et al., 2008). As with Bright, SATB2 binds to MAR regions and undergoes SUMO modifications by PIAS1 to modulate immunoglobulin gene expression (Dobreva et al., 2003). PIAS1 is the same E3 ligase that

[9] Promoter analysis performed by Dr. Edward Marcotte
[10] Protein digestion and mass spectrometry performed by Dr. Daniel Boutz in Dr. Edward Marcotte's lab

sumoylates Bright (Schmidt et al., 2009). SATB2 regulates specific neuronal fates in the developing central nervous system (Britanova et al., 2005; Alcamo et al., 2008), craniofacial development (Dobreva et al., 2006), and osteoblast formation (Hassan et al., 2010). Of particular interest, SATB2 regulates Nanog expression and reprogramming in mES cells. Overexpression of SATB2 increases Nanog expression and reprogramming capabilities in mES cells and B lymphocytes, respectively (Savarese et al., 2009). Bright and SATB2 interact in somatic cells, so it is possible that in Bri-/- MEFs, increased expression of SATB2 could aid in reprogramming.

PELP1 is a non-DNA binding scaffolding protein which connects signaling pathways of nuclear hormone receptors to enhance transcription (Vadlamudi and Kumar, 2007). PELP1 is also a nuclear matrix protein which appears to remodel chromatin through interactions with histone 1 and 3 (Choi, 2004; Nair et al., 2004). It plays a permissive role in E2-mediated cell cycle progression via pRb phosphorylation (Balasenthil and Vadlamudi, 2003). Bright is a known E2F binding protein (Suzuki et al., 1998). In fibroblasts, overexpression of Bright overcomes RAS (V12) induced senescence, and depletion induces premature senescence through the $p16^{ink4A}$ and pRb pathways. It is possible that Bright and PELP1 interact in the E2-mediated cell cycle pathway to promote cell division. Of note is our observation that multiple Bri-/- cell lines do not undergo senescence (An et al., 2010), implying that Bright plays a role in regulating cell cycle and senescence in a context-specific manner. Further work needs to be done to determine in which context Bright is interacting with PELP1.

RelA/p65 is a component of the most abundant form of the NF-κB complex (p65/p50 heterodimer). NF-κB is an abundant transcription factor that is involved in many cellular processes (Hayden and Ghosh, 2012). In mES cells, Nanog maintains pluripotency by inhibiting NFκ-B (Torres and Watt, 2008). It is possible that loss of Bright inhibits NFκ-B activity, creating a more permissive reprogramming environment. BTK is a known binding partner of Bright (Webb et al., 2000; Rajaiya et al., 2005) and is required for NFκ-B to activate B-cell growth (Petro et al., 2000). Potentially relevant in this regard, Bright cooperates with p53 (Ma et al., 2003; Lestari et al., 2012), a tumor suppressor which can repress the NFκ-B pathway (Rocha et al., 2003; Perkins, 2004). However, microarray analysis did not show significant downregulation of RelA (data not shown), indicating that Bright-RelA function acts at the posttranscriptional level. Further work will be required to determine the role Bright plays in regulating NFκ-B activity.

We further investigated Bright binding partners in mES using mass spectrometry peptide analysis. Mass spectrometry analysis was performed in triplicate (**Table 5.3**). ARID3B is a strong binding partner of Bright in both mES and 293T cells as well as HnRNP F and K, NPM, and HSP90. These protein collectively are involved in a wide range of cellular functions, reinforcing the idea that Bright has a broader role in the cell then previously appreciated. To determine if there was functional overlap between Bright interacting partners in 293T and mES cells, we performed clustering and gene ontogeny analysis using DAVID software (Huang et al., 2008). Comparison of the cluster analyses from 293T and mES shows several similar pathway groupings (**Table 5.4**). While some of these pathways are rather general, of interest is group 2, which contains pathways involved in translation. This indicates that Bright may have a role in

RNA binding and/or protein translation. Further work is needed to determine if Bright does play a role in translation and if that role differs between somatic cells and pluripotent cells.

Interestingly, Oct4, Nanog, and Sox2 did not appear as binding partners. The mES cells used for the mass spectrometry were grown under an inhibitor-based ES cell culture conditions whereas the mES cells used in the immunoprecipitation experiments were grown under standard ES cell conditions. The inhibitor-based culture conditions have been shown to repress spontaneous differentiation, commonly seen in mES cultures. Additionally, it promotes the establishment of the ground state of mES cells and prevents expression of lineage-specific genes (Ying et al., 2008; Marks et al., 2012). It is possible that the endogenous interaction we showed between Bright and Oct4, Sox2, and Nanog occurs in mES does not occur in the ground state of mES. Investigating Bright interacting partners during early differentiation of mES will further clarify Bright containing protein complexes in the different stages of pluripotency.

5.4 DISCUSSION

Evidence presented in previous chapters showed that Bright is essential for embryonic hematopoiesis in the fetal liver as well as normal kidney development in the adult. Bright is also required for maintaining the differentiation state of various somatic cell types, as its sole deficiency leads to cellular reprogramming. To further understand Bright's role in early differentiation as well as to unravel the mechanisms by which

Bright loss facilitates reprogramming, we generated Bright null mES lines and employed them in an *in vitro* differentiation system.

Bright is expressed at low levels in undifferentiated mES cells. During early differentiation, Bright expression increases gradually with a majority of the protein localized to the nuclear matrix. This gradual increase of Bright during differentiation suggests that Bright acts as a transcriptional function during early differentiation. In support of this hypothesis, we identified a cohort of genes whose promoters carry putative Bright binding sites that undergo strong expression modulation during RA-induced differentiation. Of note, potential Bright targets both increase and decrease with differentiation. Efforts are underway to determine directly by ChIPseq the global array of direct Bright targets in both undifferentiated and differentiating cells.

We observed that Bright knockout and wildtype mES cells were comparable in their abilities to self-renew and maintain pluripotency. However, during differentiation, Bright knockout mES cells showed earlier expression of genes involved in certain lineages (mesoderm and neuroectoderm), but not others (endoderm and ectoderm), indicating that Bright may regulate genes critical to lineage-specific pathways. Microarray analysis of undifferentiated Bright knockout mES cells showed upregulation of early differentiating and vasculature pathways. Proper mesoderm differentiation's apparent requirement for Bright is consistent with the need for Bright in hematopoietic stem cell and kidney development, both being mesoderm derived tissues. Additionally, MEF cell lines, the first fully reprogrammed Bright null cell line, are also derived from

the mesoderm. It is therefore possible that Bright is an important regulator of the mesoderm cell lineage.

These data lead us to speculate that Bright is specifically required for controlled differentiation through its role as a transcription repressor. Key regulatory mES cell transcription factors exist in a very balanced state; too little or too much of Oct4, Sox2, or Nanog can induce differentiation (Bosnali et al., 2009). Loss of Nanog commits cells to the endoderm lineage, while loss of Oct4 commits cells to the trophoectoderm. Raising the levels of Oct4 by just 50% commits cells to the endoderm and mesoderm lineage (Chambers, 2004). Overexpression of Sox2 by as little as twofold induces lineage differentiation except down the endoderm lineage (Kopp et al., 2008). We have shown that loss of Bright induces reprogramming in somatic cells and alters the differentiation pattern of mES cells. Taking into consideration both these observations and the transcriptional balance required for differentiation, we believe Bright may act not as a switch but as a knob, repressing levels of key pluripotency factors in a timely and controlled manner during differentiation. Therefore, Bright is necessary for proper repression of pluripotency, both in mES and somatic cells.

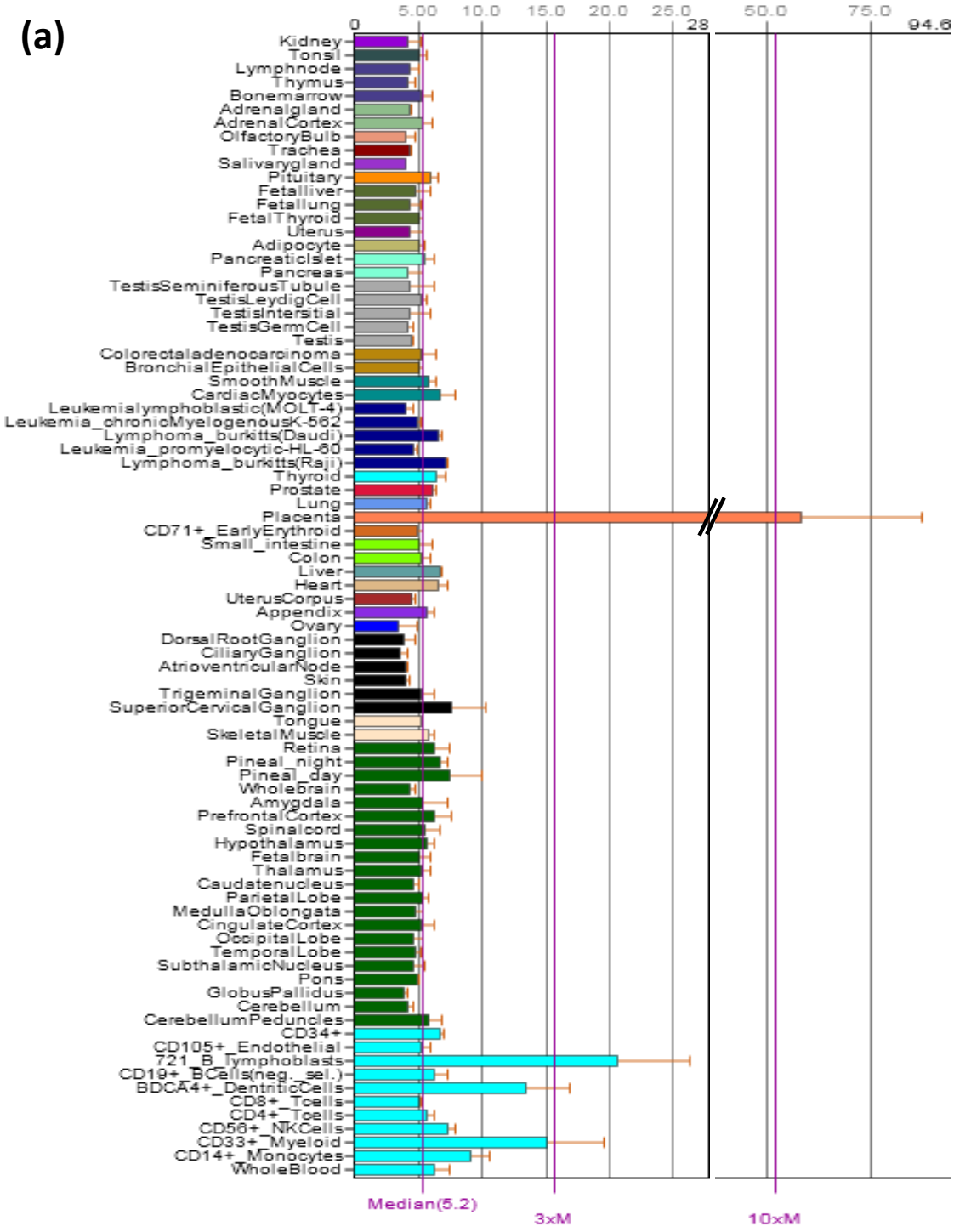
We have identified several previously unknown Bright interacting partners. Features associated with these interacting proteins indicate that Bright may be involved in previously unappreciated pathways and provide additional insights into mechanisms by which Bright loss stimulates reprogramming of MEFs. In the somatic cell line 293T, mass spectrometry and co-IP identified two MAR binding partners, SATB2 and PELP1, which modify chromatin in response to a range of signals. It is possible that Bright

normally interacts with these proteins within the nuclear matrix, and loss of Bright inhibits their normal functions. We also have confirmed Bright interaction with RelA, a component of the NF-κB complex. Additionally, TRIM21, another potential Bright binding partner, which we have yet to further validate, is a downstream effector of the NF-κB pathway (Yoshimi et al., 2009). Both the RelA and TRIM21 interaction strongly implicate Bright as a component of the KF-κB signaling pathway. Cell cycle entry and senescence bypass are necessary for reprogramming (Sullivan et al., 2006; Han et al., 2008). We have shown that Bright null cell lines maintain proliferative status to avoid natural senescence, potentially relieving a critical barrier to reprogramming somatic cells. How Bright interacts with KF-κB, p53, $p16^{ink4a}$, and other proliferative pathways remains to be seen. Comparisons between Bright interacting proteins in somatic cells and embryonic stem cells indicate that Bright may also play a role in translation, although there are few commonly bound proteins. Thus far, Bright has not been shown to have a role in translation, indicating another potential function of Bright in the cell.

The mES *in vitro* differentiation system has been informative in expanding the function of Bright beyond its role in hematopoietic stem cell development. Our data indicate that Bright is a critical component for mesoderm and potentially neuroectoderm differentiation. We have shown that Bright is potentially involved in multiple signaling pathways heretofore unknown. Further work will clarify Bright's role in these pathways, its transcriptional targets, and its other interacting partners. These clarifications will illuminate how reprograming occurs in Bright null cells.

5.5 FIGURES AND TABLES

Figure 5.1



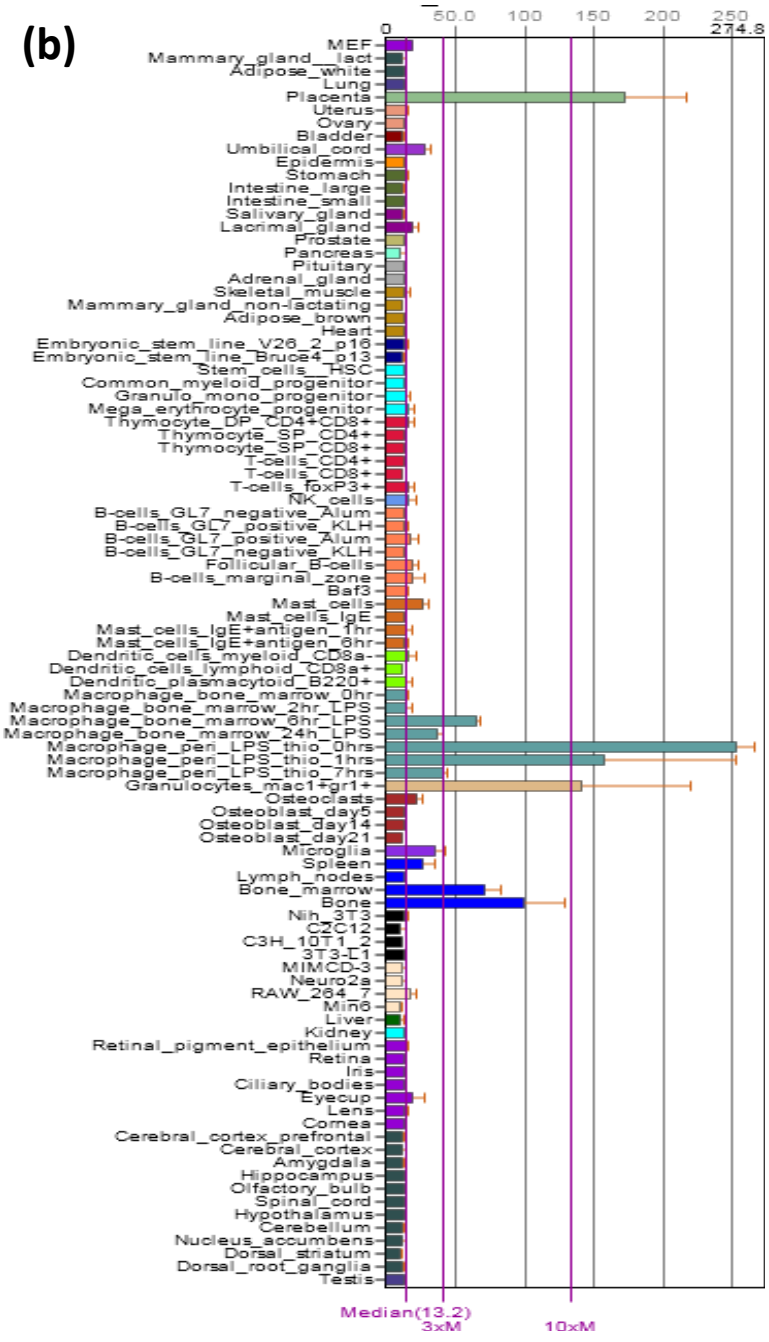


FIGURE 5.1 BRIGHT IS EXPRESSED HIGHLY IN HEMATOPOIETIC LINEAGES AND THE PLACENTA. Publicly available datasets of human **(a)** and mouse **(b)** tissues in duplicate, hybridized against affymatrix gene arrays (Su et al., 2004). Bright expression is low amongst most tissues except for many hematopoietic lineages and the placenta.

FIGURE 5.2

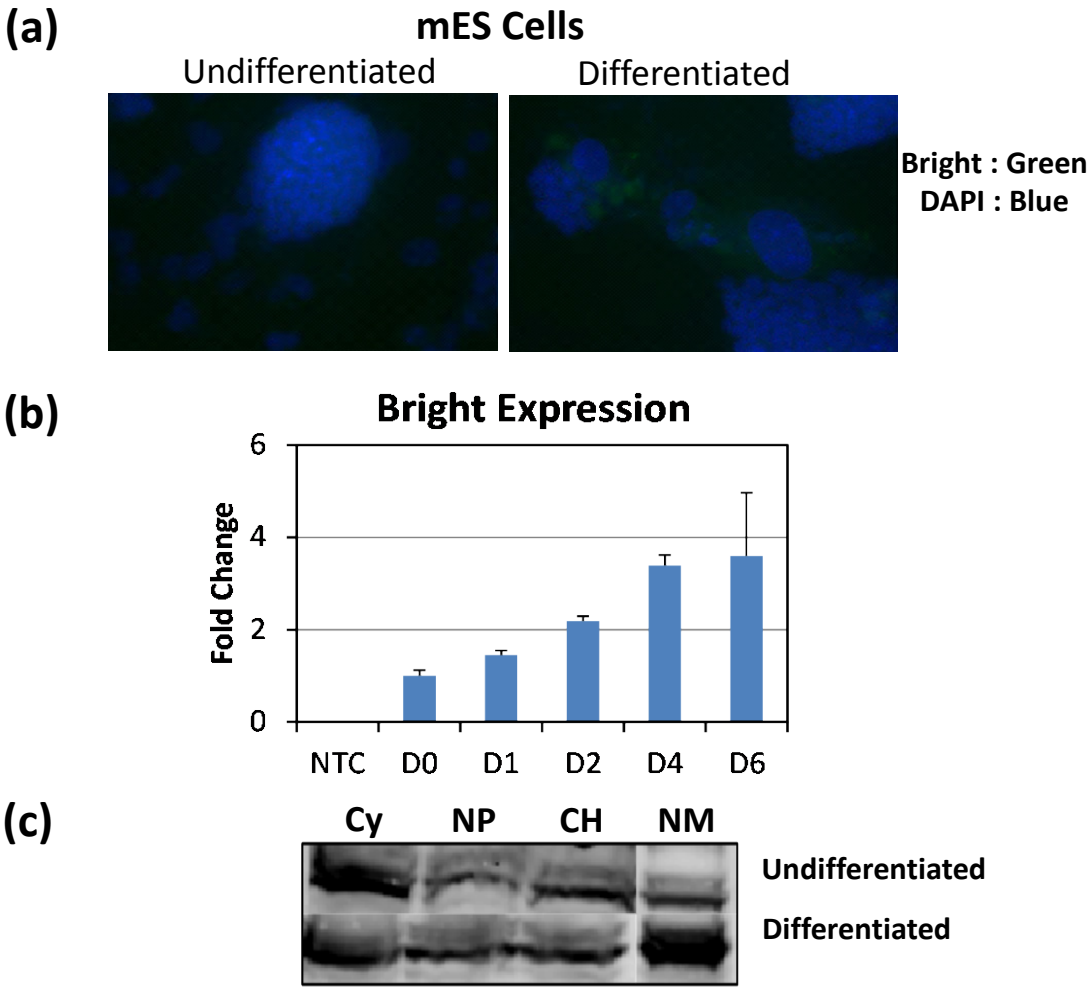

(a)
mES Cells
Undifferentiated
Differentiated
Bright : Green
DAPI : Blue
(b)
Bright Expression
Fold Change
6
4
2
0
NTC
D0
D1
D2
D4
D6
(c)
Cy
NP
CH
NM
Undifferentiated
Differentiated

FIGURE 5.2 BRIGHT INCREASES EXPRESSION WITH DIFFERENTIATION. **(a)** Mouse embryonic stem (mES) cells show an increase in Bright protein levels upon differentiation. mES cells were grown in chamber slides in the presence (undifferentiated) or absence (differentiated) of LIF for 3 days. Cells were stained for Bright expression (green), and nuclei were counterstained with DAPI (blue). **(b)** mES cells increase RNA levels of Bright upon differentiation. mES cells were grown in the absence of LIF in adherent cultures. Cells were harvested at the times indicated; total RNA isolated; reverse transcriptase PCR was performed; and fold change in Bright RNA levels was determined using Bright specific primers by semi-quantitative PCR. **(c)** Bright localizes to the nuclear matrix upon differentiation. mES cells were grown in the presence (undifferentiated) or absence (differentiated) of LIF for 3 days. Cells were chemically separated into cytoplasmic (Cy), soluble nuclear protein (NP), chromatin (CH), and nuclear matrix (NM) fractions. Fractions were separated on an SDS-page gel and probed using anti-Bright polyclonal antibody. Abbreviations: mES: mouse embryonic stem cells.

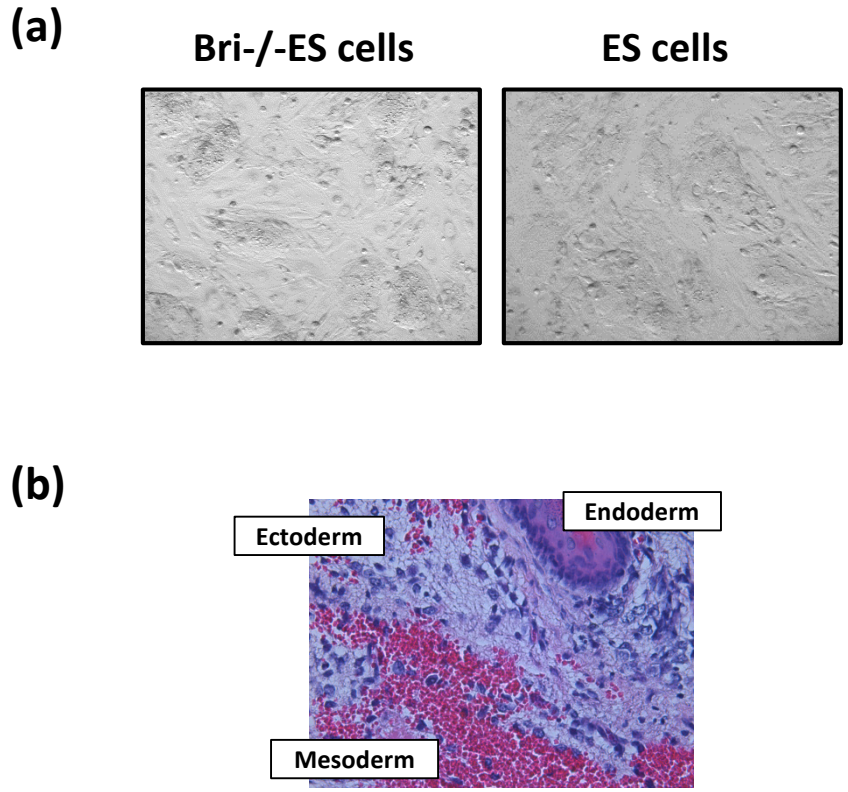


FIGURE 5.3 **BRIGHT KNOCKOUT EMBRYONIC STEM CELLS ARE PLURIPOTENT.** **(a)** Bri-/- ES morphologically resemble mES. Bri-/- ES cells grown on a feeder cell layer, in the presence of LIF, maintain their ES morphology. **(b)** Bright knockout embryonic stem cells form teratomas. ~350,000 Bri-/-ES cells were injected into the flanks of immune compromised mice. Tumors were harvested when ~ 1cm across. Sections were stain by H&E, and cells representing all three germ layers were found to be present. Abbreviations: Bri-/- ES: Bright knockout embryonic stem cells; mES: mouse embryonic stem cells.

Figure 5.4

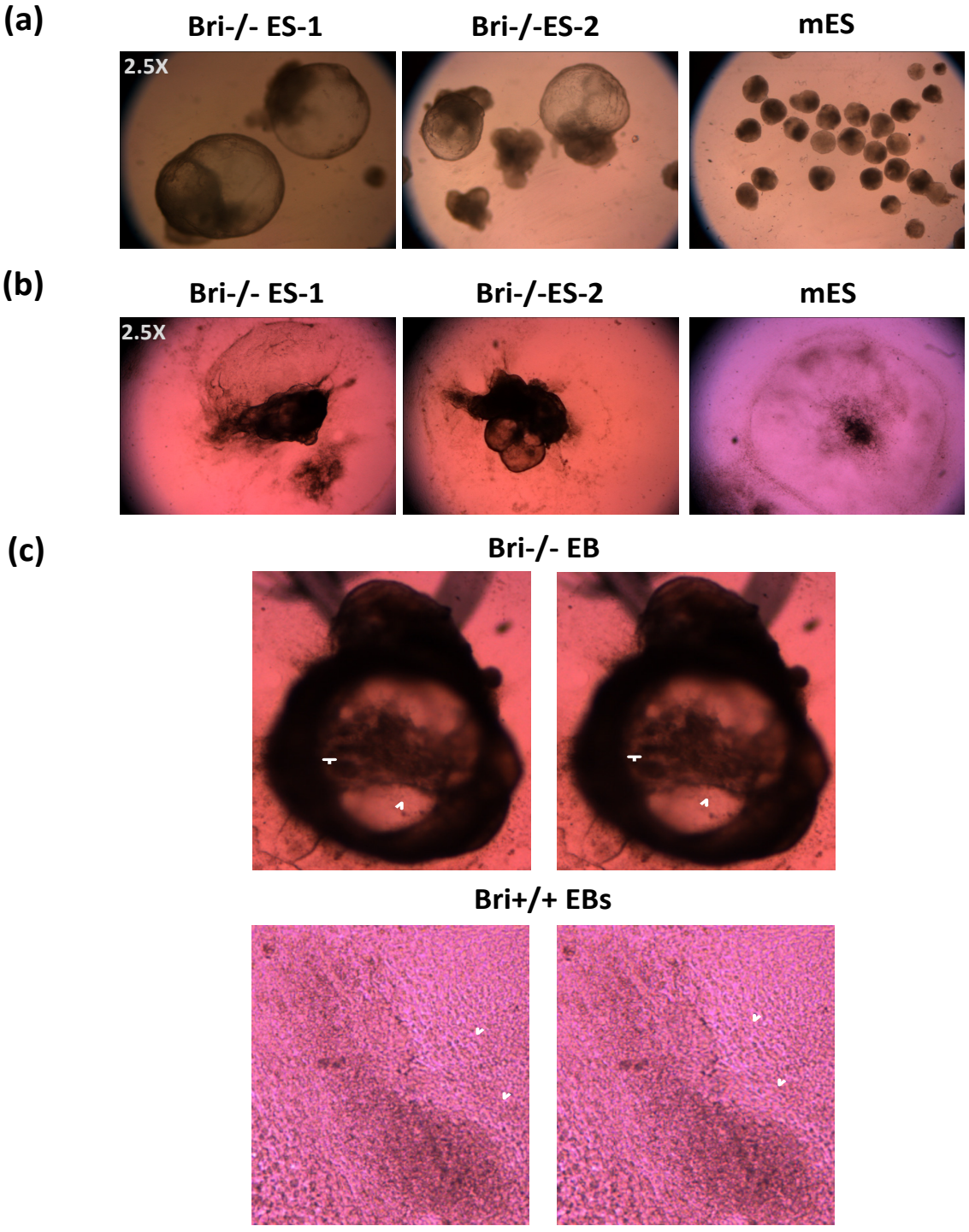
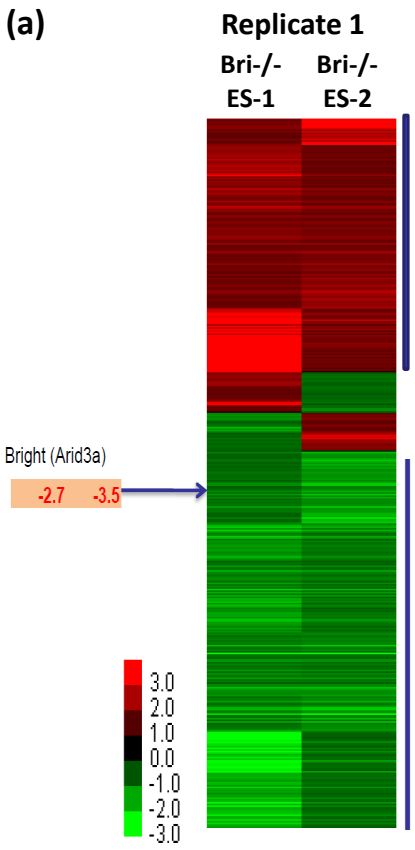

(a)
Bri-/- ES-1
Bri-/-ES-2
mES
2.5X
(b)
Bri-/- ES-1
Bri-/-ES-2
mES
2.5X
(c)
Bri-/- EB
Bri+/+ EBs

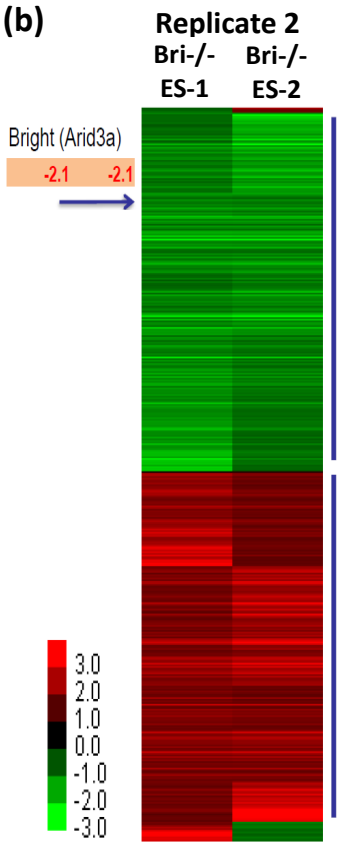
FIGURE 5.4 BRIGHT KNOCKOUT EMBRYONIC STEM CELLS DIFFERENTIATE INTO EMBRYOID BODIES MORE RAPIDLY COMPARED TO WILDTYPE. **(a)** Bri-/- ES CEBs. Two different Bri-/- ES cell lines (Bri-/- ES-1 and Bri-/- ES-2) and Bri+/+ ES cells were grown in suspension culture without LIF to promote differentiation. Bri-/- ES formed CEBs more quickly, more often, and much larger then Bri+/+ ES. Images are at day 10 differentiation. 2.5x magnification. **(b)** Bright knockout embryonic stem cells do not differentiate correctly in adherent cultures. Two different Bri-/- cell lines and Bri+/+ mES cells were grown in suspension culture without LIF to promote formation of EBs for eight days. Ebs were allowed to adhere to gelatin coated cell culture plates. Bri-/- mES cells did not appropriately adhere to the cell culture plate and appeared to incompletely differentiate. Images are at day 13 differentiation (2.5x magnification). **(c)** Bright knockout embryonic stem cells form larger areas of cardiomyocytes in culture. In differentiating culture, formation of cardiomyocytes is easily observed through the 'twitching' of the colonies. Arrows point to area of movement that can be seen between the two images presented. Images are taken at differentiation day 13 and are 2.5x for Bri-/- and 10x for Bri+/+. Abbreviations: Bri-/- ES: Bright knockout embryonic stem cells; Bri+/+ ES: wildtype mouse embryonic stem cells; EB: embryoid body; CEB: cystic embryoid bodies.

Figure 5.5



Term	Count	%	P-Value	Fold Enrichment	Bonferroni
Cell Fate Commitment	14	0.4	7.60E-07	5.9	1.20E-03
Embryonic Morphogenesis	22	0.7	3.80E-07	3.8	5.80E-04
Embryonic Organ Development	16	0.5	9.00E-06	4.1	1.40E-02
Embryonic Organ Morphogenesis	13	0.4	1.20E-05	5	1.80E-02
Skeletal System Development	17	0.5	1.60E-05	3.7	2.50E-02
Positive Regulation of cell differentiation	13	0.4	2.80E-05	4.6	4.20E-02

Term	Count	%	P-Value	Fold Enrichment	Bonferroni
Neurological System Process	70	10.8	1.70E-06	1.8	2.40E-03
Regulation of Neurotransmitter Levels	10	1.5	1.30E-05	6.9	1.80E-02
Cell-Cell Signaling	19	2.9	1.70E-04	2.8	2.10E-01
Cognition	57	8.8	1.90E-04	1.6	2.30E-01



Term	Count	%	P-Value	Fold Enrichment	Bonferroni	FDR
Neurological System Process	70	10.8	1.70E-06	1.8	2.40E-03	2.80E-03
Regulation of Neurotransmitter Levels	10	1.5	1.30E-05	6.9	1.80E-02	2.10E-02
Cell-Cell Signaling	19	2.9	1.70E-04	2.8	2.10E-01	2.80E-01

Term	Count	%	P-Value	Fold Enrichment	Bonferroni	FDR
Heart Development	22	3.8	1.80E-07	3.9	3.20E-04	3.10E-04
Blood Vessel Development	21	3.6	3.20E-06	3.4	5.70E-03	5.50E-03
Vasculature Development	21	3.6	4.70E-06	3.4	8.20E-03	7.90E-03
Blood Vessel Morphogenesis	19	3.3	2.30E-06	3.8	4.10E-03	4.00E-03
Embryonic Organ Development	19	3.3	3.50E-05	3.2	6.00E-02	6.00E-02
Placenta Development	11	1.9	6.00E-05	5.1	9.90E-02	1.00E-01

FIGURE 5.5 GENE EXPRESSION OF BRIGHT KNOCKOUT EMBRYONIC STEM CELLS IS SIMILAR TO MOUSE EMBRYONIC STEM CELLS. (a, b) Global gene expression analysis was performed on biological replicates (replicate 1 and replicate 2) of two independently derived Bright knockout embryonic stem cell lines (Bri-/- ES-1 &-2). Bri-/- ES were compared to Bri+/+ ES cells. Heatmaps represent the differentially expressed genes between Bri-/- ES lines and Bri+/+ ES greater than 1-fold difference. Gene ontogeny analyses of differentially expressed genes are displayed to the right of the heatmap. Abbreviations: Bri-/- ES: Bright knockout embryonic stem cells; Bri+/+ ES: wildtype mouse embryonic stem cells.

Figure 5.6

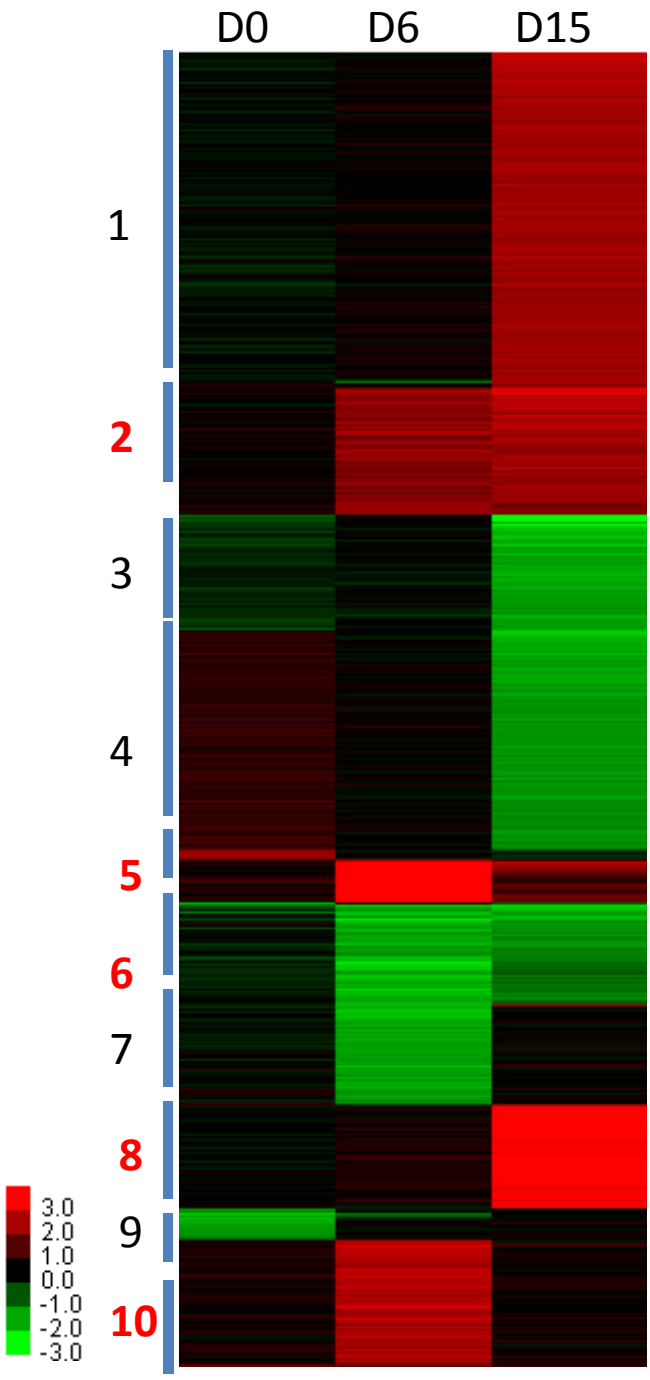


FIGURE 5.6 GENE EXPRESSION OF DIFFERENTIATED BRIGHT KNOCKOUT EMBRYONIC STEM CELLS IS DISSIMILAR TO WILDTYPE EMBRYONIC STEM CELLS. Global gene expression analysis was performed on biological replicates of two independently derived Bright knockout embryonic stem cell lines (Bri-/- ES-1 & 2) and Bri+/+ ES. Cells were cultured in hanging drops to allow differentiation into embryoid bodies and harvested at days 0, 6, and 15 (D0, D6, and D15, respectively). All Bri-/- ES replicates were compared together to Bri+/+ ES cells. The heatmap represents the differentially expressed genes (greater the 1.5-fold difference and a P value less than 0.05) clustered based on expressed patterns over time. Data are a compilation between replicates of both Bri-/- ES lines and Bri+/+ ES at each timepoint. Abbreviations: Bri-/- ES: Bright knockout embryonic stem cells; Bri+/+ ES: wildtype mouse embryonic stem cells.

Figure 5.7

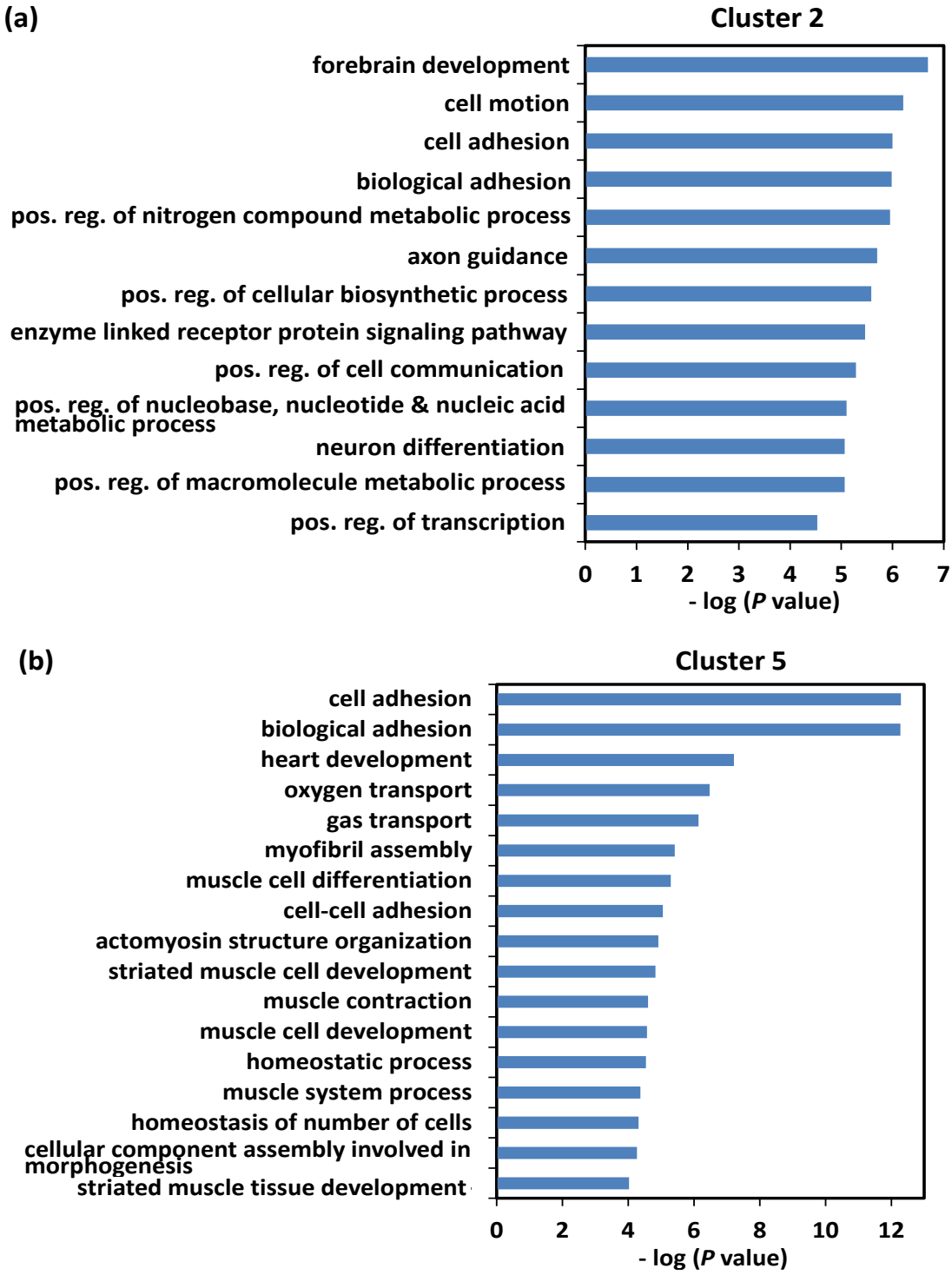
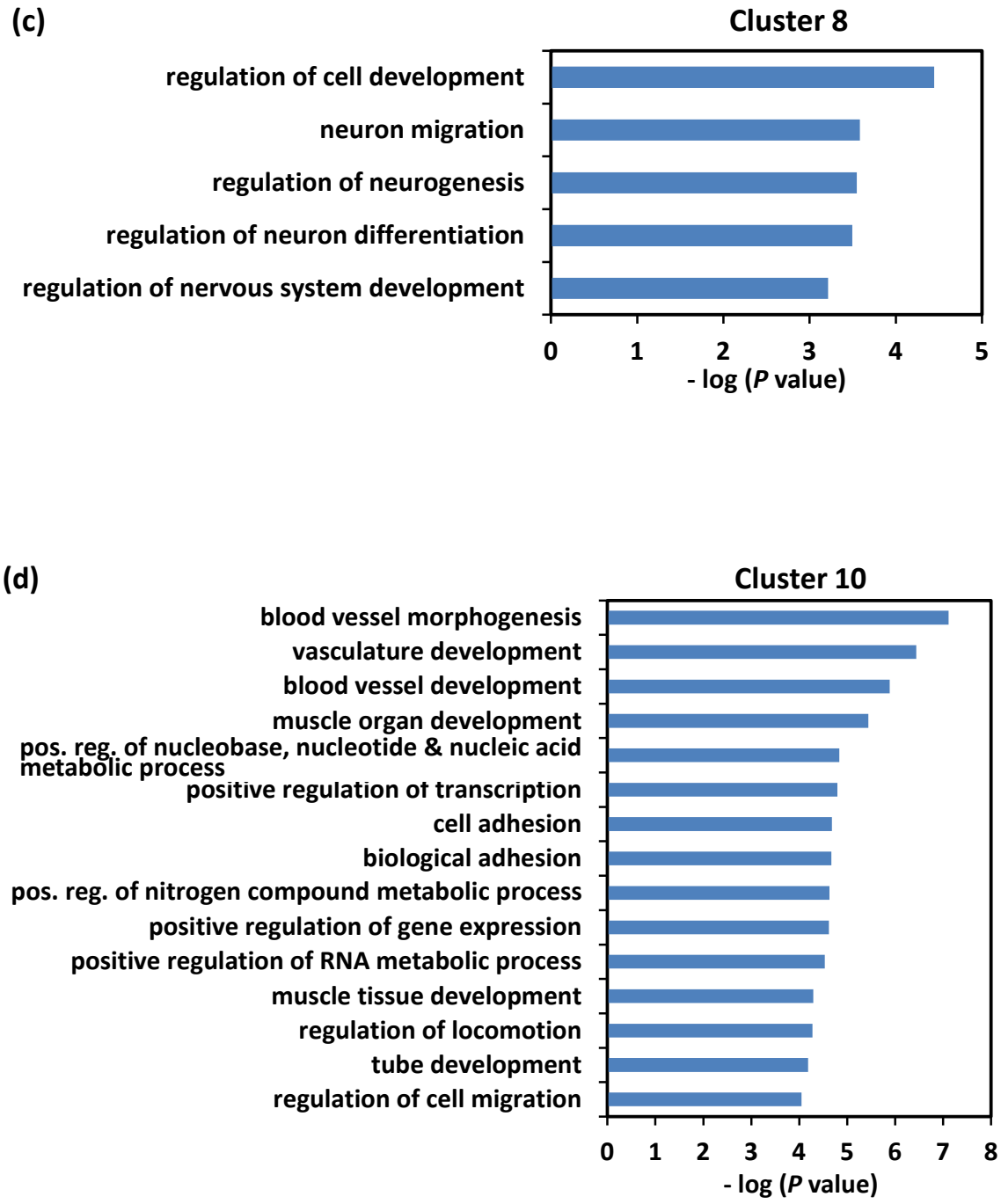

(a)
Cluster 2
forebrain development
cell motion
cell adhesion
biological adhesion
pos. reg. of nitrogen compound metabolic process
axon guidance
pos. reg. of cellular biosynthetic process
enzyme linked receptor protein signaling pathway
pos. reg. of cell communication
pos. reg. of nucleobase, nucleotide & nucleic acid metabolic process
neuron differentiation
pos. reg. of macromolecule metabolic process
pos. reg. of transcription
0 1 2 3 4 5 6 7
- log (P value)
(b)
Cluster 5
cell adhesion
biological adhesion
heart development
oxygen transport
gas transport
myofibril assembly
muscle cell differentiation
cell-cell adhesion
actomyosin structure organization
striated muscle cell development
muscle contraction
muscle cell development
homeostatic process
muscle system process
homeostasis of number of cells
cellular component assembly involved in morphogenesis
striated muscle tissue development
0 2 4 6 8 10 12
- log (P value)

Figure 5.7


(c)
Cluster 8
regulation of cell development
neuron migration
regulation of neurogenesis
regulation of neuron differentiation
regulation of nervous system development
0
1
2
3
4
5
- log (P value)
(d)
Cluster 10
blood vessel morphogenesis
vasculature development
blood vessel development
muscle organ development
pos. reg. of nucleobase, nucleotide & nucleic acid metabolic process
positive regulation of transcription
cell adhesion
biological adhesion
pos. reg. of nitrogen compound metabolic process
positive regulation of gene expression
positive regulation of RNA metabolic process
muscle tissue development
regulation of locomotion
tube development
regulation of cell migration
0
1
2
3
4
5
6
7
8
- log (P value)

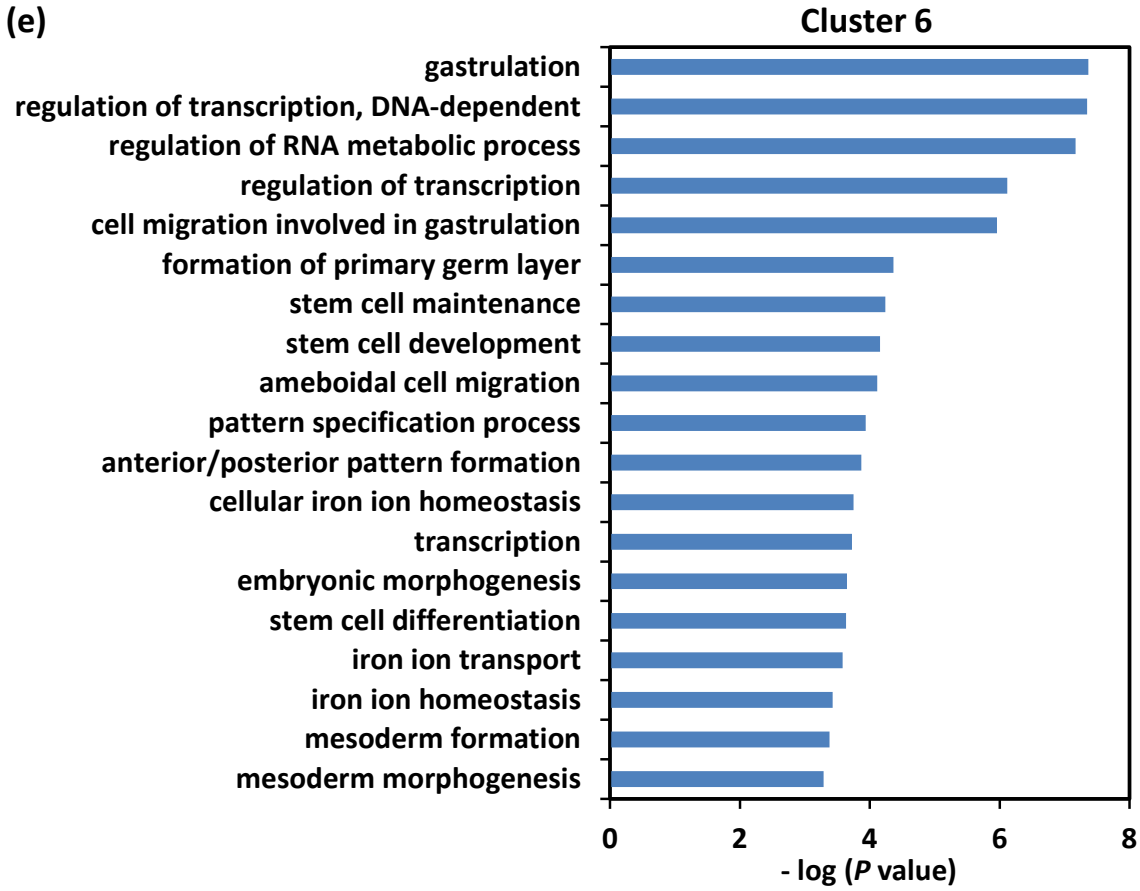
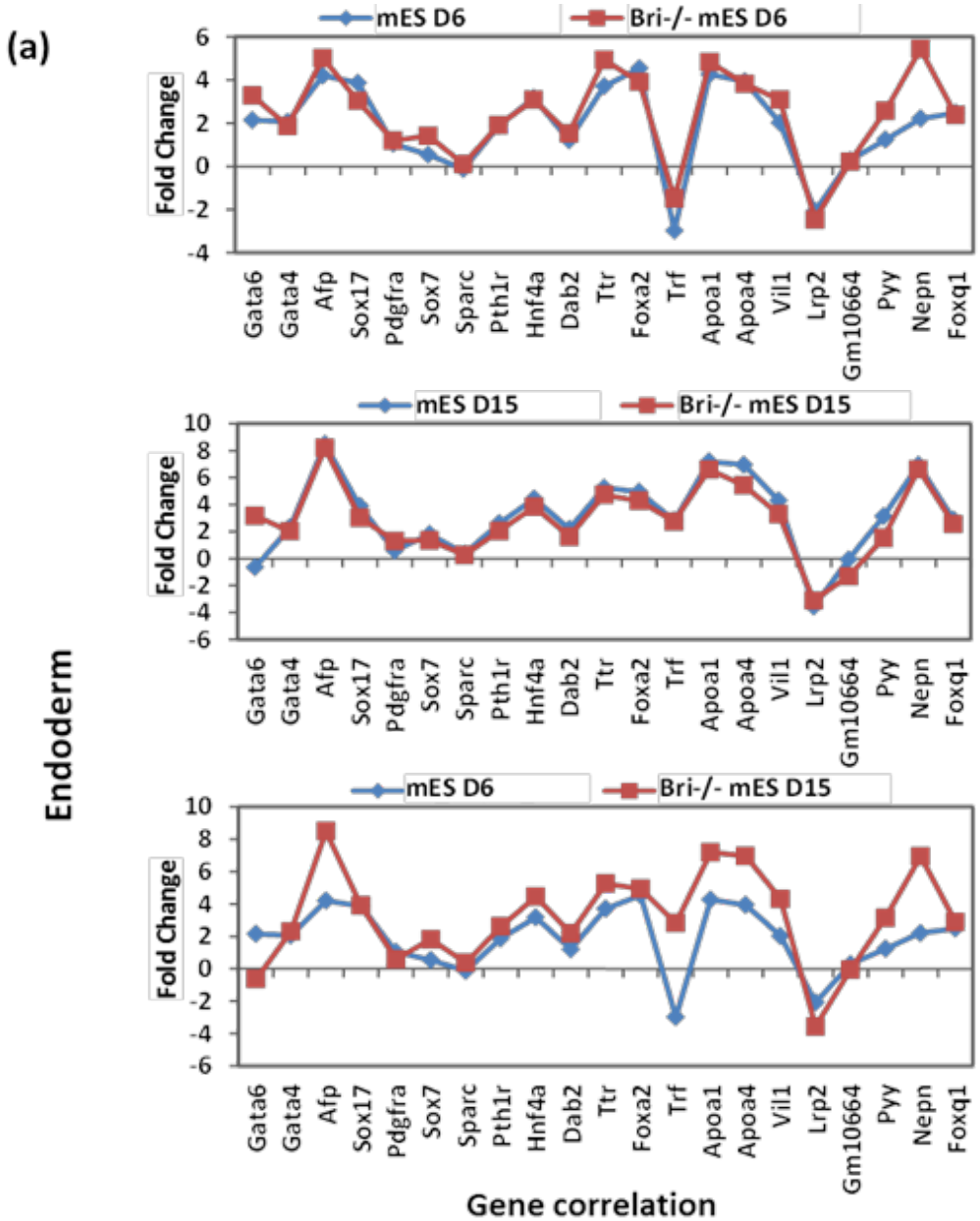


FIGURE 5.7 GENE ONTOGENY ANALYSES OF DIFFERENTIALLY EXPRESSED GENES IN DIFFERENTIATED BRIGHT KNOCKOUT EMBRYONIC STEM CELLS. Clusters indicated on Figure 5.6 in red numbers were analyzed using Gene ontogeny. Upregulated clusters **(a-d)** and the downregulated cluster **(e)** show a wide range of misregulated gene pathways. Generally, differentiation associated pathways are more commonly upregulated while stem cell related programs are more downregulated.

Figure 5.8



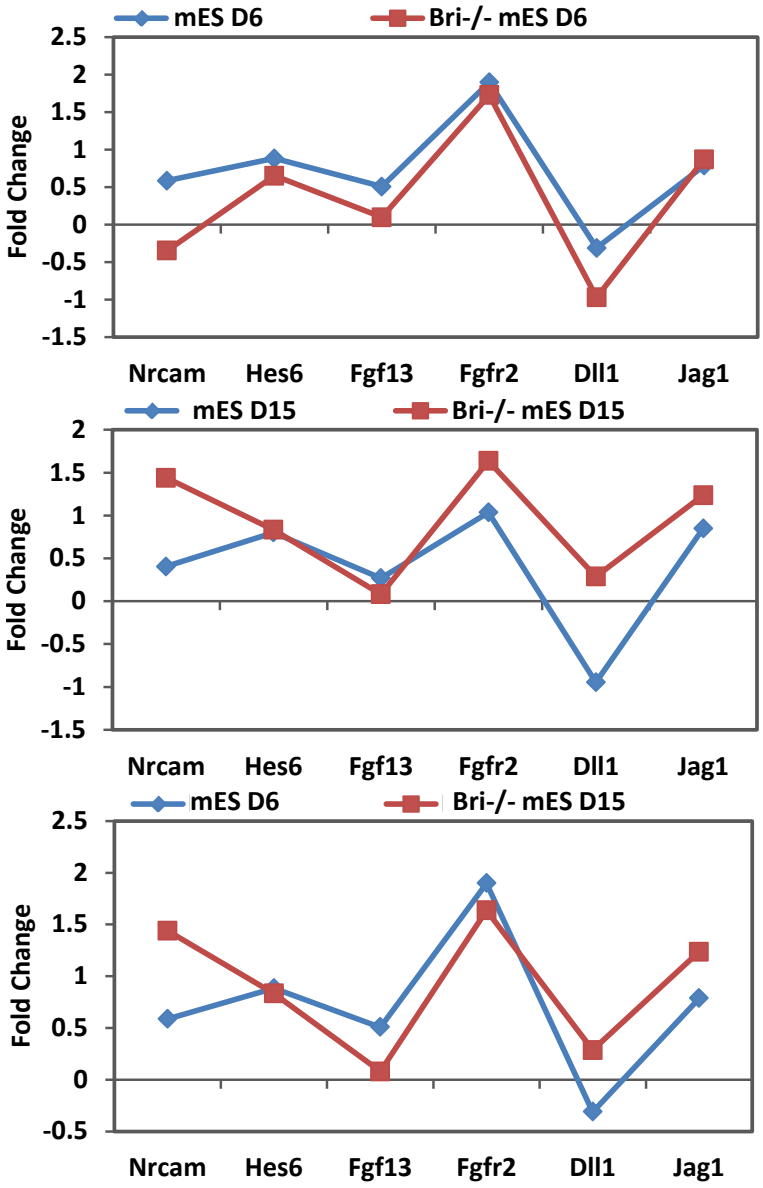
Gene correlation

	Bri-/- mES D0	Bri-/- mES D6	Bri-/- mES D15
mES D6	0.68	0.90	0.73
mES D15	0.35	0.82	0.93

Figure 5.8

(b)

Ectoderm

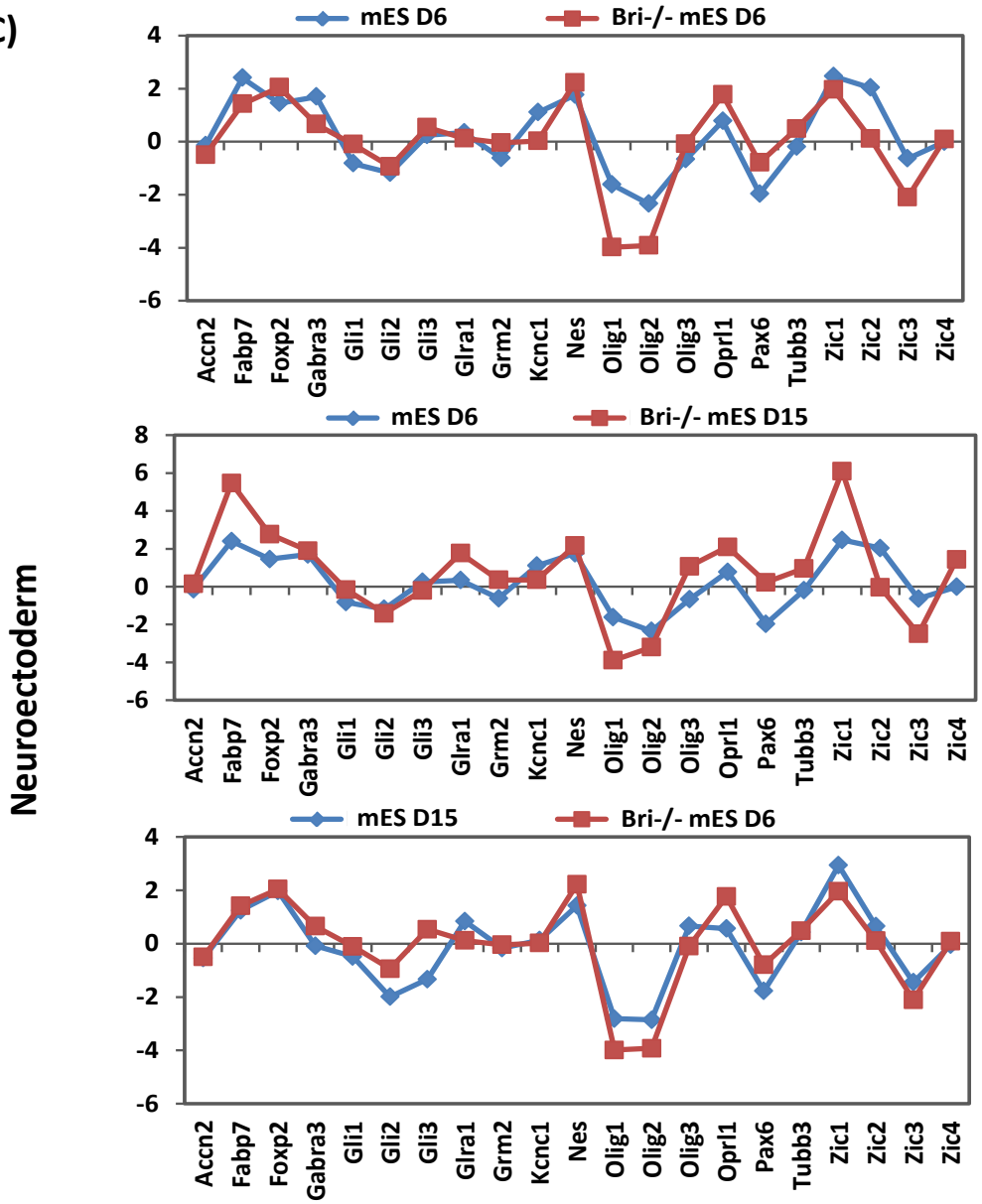


Gene correlation

	Bri-/- mES D0	Bri-/- mES D6	Bri-/- mES D15
mES D6	0.68	0.90	0.73
mES D15	0.35	0.82	0.93

Figure 5.8

(C)



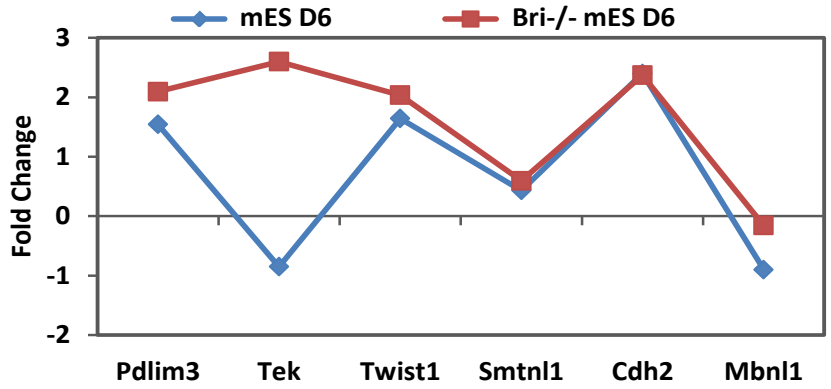
Gene correlation

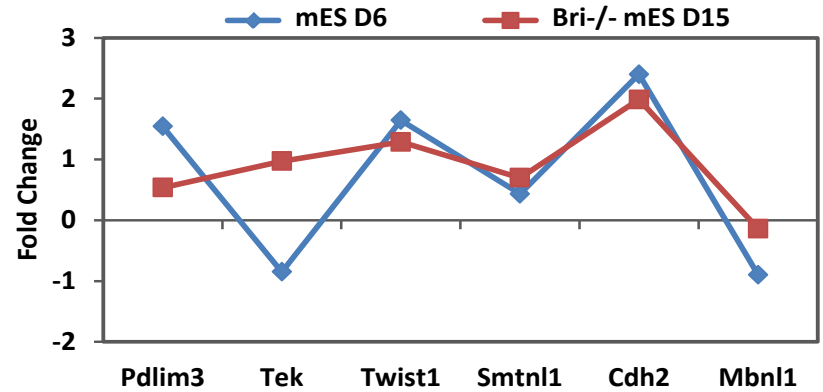
	Bri-/- mES D0	Bri-/- mES D6	Bri-/- mES D15
mES D6	0.57	0.79	0.79
mES D15	0.73	0.87	0.89

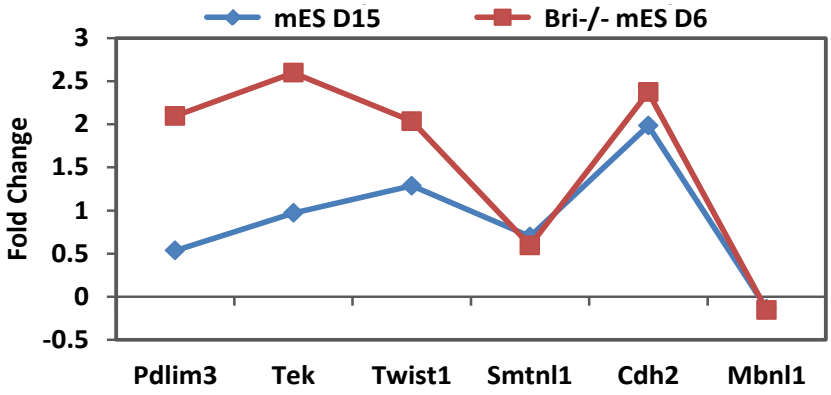
Figure 5.8

(d)

Mesoderm







Gene correlation

	Bri-/- mES D0	Bri-/- mES D6	Bri-/- mES D15
mES D6	0.27	0.48	0.53
mES D15	-0.02	0.74	0.70

FIGURE 5.8 BRIGHT KNOCKOUT EMBRYONIC STEM CELLS DIFFERENTIATE MORE RAPIDLY COMPARED TO WILDTYPE. **(a, b)** Bri-/- ES differentiate into endoderm and ectoderm lineages in a similar manner to Bri+/+ ES. **(c-d)** Bri-/- ES cells express neuroectoderm and mesoderm markers sooner than the Bri+/+ ES cells. Fold expression of all genes relative to Bri+/+ ES undifferentiated control (D0). Correlation between Bri+/+ and Bri-/- at day 0, 6, and 15 gene expression pattern of lineage specific genes from tables shown below each image. Abbreviations: Bri-/- ES: Bright knockout embryonic stem cells; Bri+/+ ES: wildtype mouse embryonic stem cells.

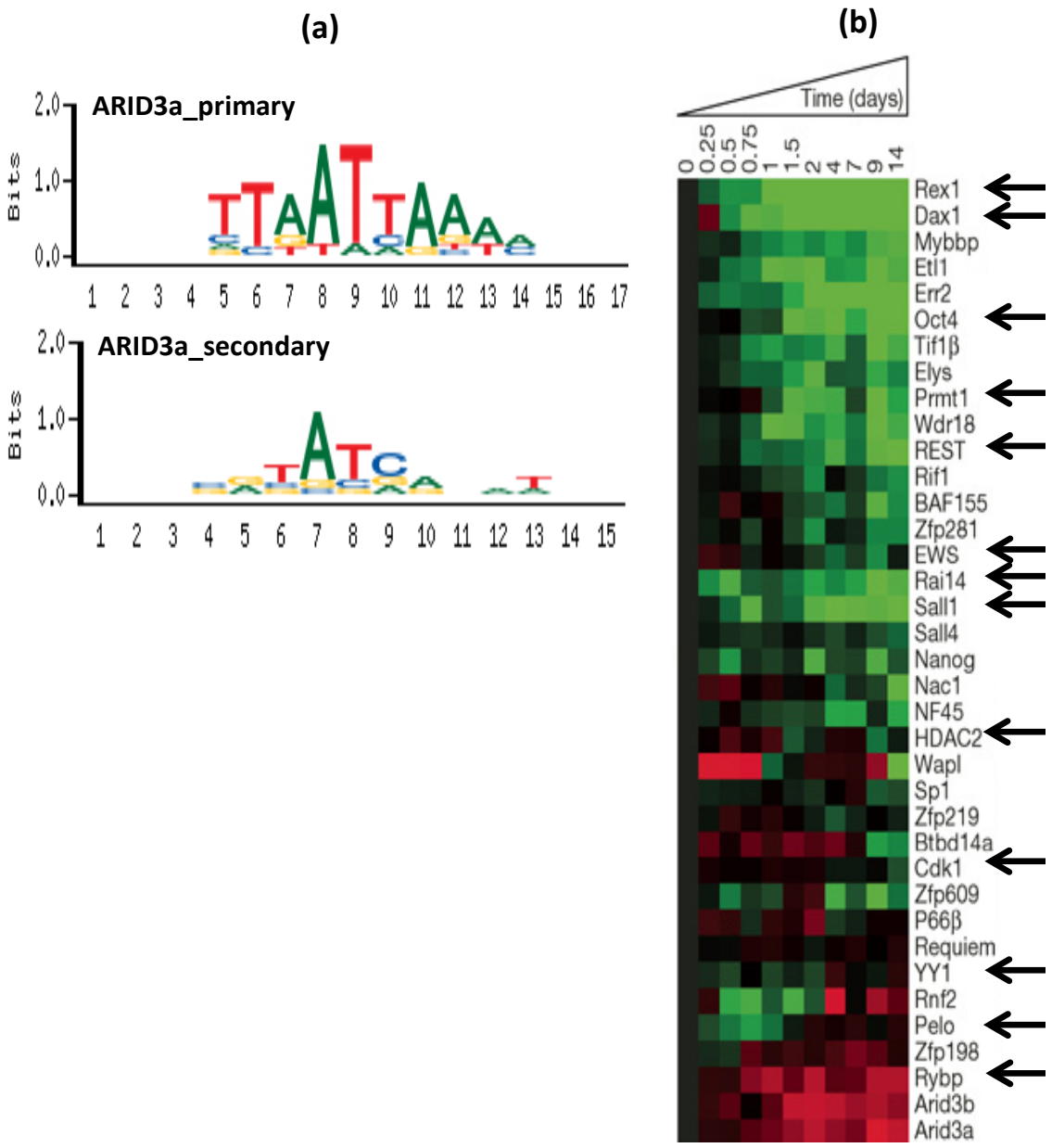


FIGURE 5.9 PROMOTERS OF EARLY DIFFERENTIATION GENES CONTAIN BRIGHT CONSENSUS SEQUENCES. **(a)** Publicly available data of protein binding array determined a primary and secondary Bright consensus sequence similar to previously published consensus sequences. **(b)** Meta-analysis of publicly available microarray data indicates that Bright expression increase with retinoic acid (RA) induced differentiation (Wang et al., 2006). Arrows indicate gene found in Table 5.1.

Both human and mouse
Protein arginine N-methyltransferase 1 (Prmt1)
POU class 5 homeobox 1 (POU5F1)
Sal-like 1 (Drosophila) (SALL1)
YY1 transcription factor (YY1)
Ewing sarcoma breakpoint region 1 (EWSR1)
AT hook containing transcription factor 1 (AHCTF1)
Human
Histone deacetylase 2 (HDAC2)
SWI/SNF related, matrix associated, actin dependent regulator of chromatin (SMARCC1)
Retinoic acid induced 14 (RAI14)
D4, zinc and double PHD fingers family 2 (DPF2)
AT rich interactive domain 3B (BRIGHT-like) (ARID3B)
RE1-silencing transcription factor (REST)
Pelota homolog (Drosophila) (PELO)
Cyclin-dependent kinase 1 (CDK1)
Nuclear receptor subfamily 0, group B, member 1 (NR0B1) (Dax1)
Tripartite motif-containing 28 (TRIM28)
Mouse
RING1 and YY1 binding protein (Rybp)

TABLE 5.1 PLURIPOTENCY RELATED GENES CONTAIN BRIGHT CONSENSUS SEQUENCES. Human and mouse genes that have Bright binding consensus sequences found using primary consensus sequence in (Figure 5.9 a).

Protein	Fold Change	Protein description
ARID3A	426.44	Bright
NDUFV1	197.07	NADH dehydrogenase
AMOT	166.92	angiomotin
MUT	148.61	methylmalonyl CoA
SATB2	94.77	SATB homeobox 2
AKAP8L	78.61	A kinase (PRKA) anchor protein 8-like
IGF2R	73.23	insulin-like growth factor 2 receptor
PELP1	65.69	proline, glutamate and leucine rich protein 1
ARID3B	47.38	BDP
RELA	30.15	Nuclear factor NF-kappa-B p65 subunit
CEP192	29.08	centrosomal protein 192kDa
ARID3C	29.08	Bright-like
SATB1	26.92	SATB homeobox 1
PDE1C	20.46	phosphodiesterase 1C, calmodulin-dependent 70kDa
ALDH16A1	20.46	aldehyde dehydrogenase 16 family, member A1
WDR18	19.38	WD repeat domain 18
RPIA	18.31	ribose 5-phosphate isomerase A
TRIM21	18.31	tripartite motif-containing 21
PPP1CB	17.23	protein phosphatase 1
DERA	15.08	deoxyribose-phosphate aldolase (putative)
PPP1CA	15.08	protein phosphatase 1
PPP1CC	15.08	protein phosphatase 1
PPP1R12A	14.00	protein phosphatase 1, regulatory (inhibitor) subunit 12A
DDX20	11.85	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20 -RNA helicase
SEC16A	10.77	SEC16 homolog A
TP53	2.15	p53

TABLE 5.2 **BRIGHT HAS A WIDE RANGE OF BINDING PARTNERS.** Mass spectrometry analysis of Bright immunoprecipitated from human 293T cells. Experiments performed in triplicate and results averaged. Proteins of particular interest are highlighted in blue.

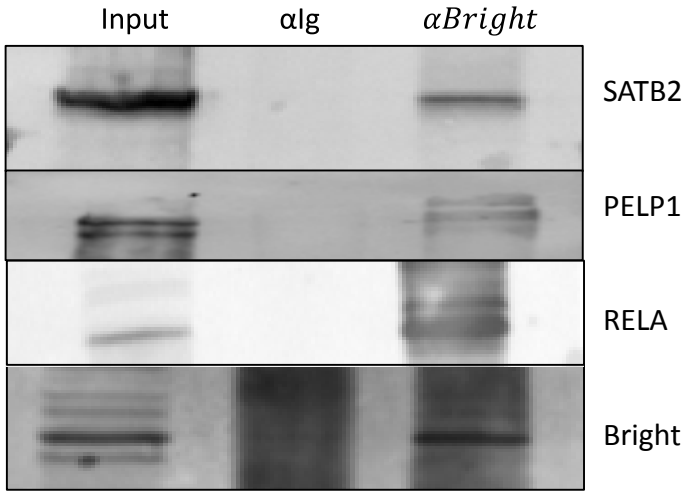


FIGURE 5.10 NOVEL BRIGHT PROTEIN INTERACTIONS. Bright and corresponding tagged gene expressing vectors were overexpressed in 293T cells. Immunoprecipitation was performed using anti-Bright antibody, and blots were probes for the tagged proteins.

mES Mass Spectrometry
Actin, cytoplasmic 2 (ACTG1)
Tubulin beta-5 chain (TUBB)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Alpha-enolase (ENO1)
AT-rich interactive domain-containing protein 3B (ARID3B)
Tubulin alpha-1B chain (TUBB1B)
Elongation factor 1-alpha 1 (EEF1A1)
Eukaryotic initiation factor 4A-I (EIF4A)
Isoform 2 of Heterogeneous nuclear ribonucleoprotein F (HnRNP F)
Nucleophosmin (NPM)
ATP synthase subunit alpha, mitochondrial (ATP5A)
Heat shock protein HSP 90-beta (HSP90B)
Fructose-bisphosphate aldolase A (ALDOA)
Stress-70 protein, mitochondrial (HSPA9)
ATP synthase subunit beta, mitochondrial (ATP5B)
Heat shock cognate 71 kDa protein (HSPA8)
Elongation factor 1-gamma (EEF1G)
Tubulin beta-4B chain (TUBB4B)
Heat shock protein HSP 90-alpha (HSP90A)
60S acidic ribosomal protein P0 (RPLP0)
Isoform 2 of Heterogeneous nuclear ribonucleoprotein K (HnRNP K)

TABLE 5.3 BRIGHT BINDING PARTNERS IN MOUSE EMBRYONIC STEM CELLS. Mass spectrometry analysis of Bright immunoprecipitated from mouse embryonic stem cells. Experiments performed in triplicate. Proteins common between mouse embryonic stem cell (mES) and 293T mass spectrometry experiments highlighted in light blue.

Group 1	Group 2	Group 3
intracellular	translation	intracellular non-membrane-bounded organelle
cell	ribonucleoprotein complex	non-membrane-bounded organelle
cytoplasm	translation factor activity, nucleic acid binding	cytoskeleton
organelle	translation initiation factor activity	intracellular organelle part
intracellular organelle		organelle part
intracellular part		protein complex
cell part		

TABLE 5.4 BRIGHT HAS COMMON PATHWAY INTERACTIONS. Mass spectrometry analysis of Bright immunoprecipitated from human 293T and mouse embryonic stem cells clustered using DAVID software and gene ontogeny performed. Pathways in the same cluster within each cell line and common between the two cell lines are shown. Mass spectrometry experiments performed in triplicate. All cluster analyses greater than 2.0 enrichment, all term p-value greater than .01.

Chapter 6

Conclusions and Future Directions

6.1 SUMMARY

This work has shown new roles for Bright/ARID3a. Bright is a well-established regulator of B-cell and hematopoietic stem cell development. By using a Bri-/- mouse model, we demonstrated that Bright plays a role in maintaining adult kidney structure and proliferation. Bright overexpression has previously been shown to overcome senescence in several cell models (Peeper et al., 2002; Fukuyo et al., 2004, 2011). That loss of Bright appears to increase proliferation in the kidney indicates a context dependent function for Bright regulation of proliferation.

A startling observation that loss of Bright induces developmental plasticity in somatic cells (An et al., 2010) led us to investigate the abilities of Bri-/- mouse embryonic fibroblasts (MEFs) to reprogram. We found that in the presence of LIF, Bri-/- MEFs were able to fully reprogram to a pluripotent state. Bright was shown to act as a repressor of key pluripotency factors in ES cells, which may account in part for Bri-/- MEFs ability to reprogram. Observations of Bri-/- ES cells confirm that Bright is necessary for properly timed differentiation. These data indicate that Bright's primary

role in early differentiation is the repression of key pluripotency factors, likely in complex with other proteins, to regulate the differentiation program temporally.

6.2 FUTURE DIRECTIONS FOR INVESTIGATING BRIGHT'S ROLE IN THE KIDNEY

Earlier observations of rare surviving Bri-/- mice suggested that there was no phenotype in fully grown mice. Analysis of adult animal organs showed no change compared to Bri+/+ and Bri+/- with the exception of the kidney. We found that in older animals, there was a distinct loss of tubular structure of the kidney. Interestingly, younger animals did not show a loss of organ morphology, indicating that initial development of the kidney is normal. We observed that in the Bri-/- younger animals, kidney proliferation was significantly increased compared to Bri+/+. Bright is a known binding partner of E2F, a cell cycle control transcription factor (Suzuki et al., 1998). Overexpression of Bright can overcome Ras-induced senescence (Peeper et al., 2002) and we have shown that loss of Bright can prevent senescence in multiple cell lines (An et al., 2010). These conflicting results indicate that Bright has a role in regulating cell cycle, but that role is most likely context specific. Determining kidney specific Bright interacting partners and transcriptional targets, either through mass spectrometery or microarray analysis, should shed light on how Bright regulates proliferation in the kidney. Further studies are needed to understand what factors may be regulating Bright in the kidney and if the observed increase in proliferation itself causes loss of kidney structure in older animals.

6.3 FUTURE DIRECTIONS FOR OPTIMIZING BRIGHT MEDIATED REPROGRAMMING

Loss of Bright has been shown to induce developmental plasticity as well as overcome senescence in somatic cells (An et al., 2010). We have shown here that loss of Bright alone is sufficient to induce reprogramming in mouse embryonic fibroblasts. Bri-/- MEFs are also more efficiently reprogrammed using standard 4-factor overexpression vectors. This novel observation led us to further investigate the boundaries of Bright-mediated reprogramming. Further studies investigating the loss of Bright by utilizing shRNA and siRNA technologies in different cell lines, both mouse and human, will expand on the limits of Bright-mediated reprogramming. Different combinations of inhibitors may be able to reduce the time it takes Bri-/- MEFs to reprogram; leading to a more efficient Bright mediated reprogramming method.

We further described previously unknown interactions between Bright and key pluripotency factors Oct4, Sox2, and Nanog. These proteins are key regulatory transcription factors in maintaining pluripotency. Bright interacts with these proteins and binds their promoters directly. Overexpression of Bright in ES cells represses Oct4, Sox2, and Nanog. These interactions are of great interest due to the pivotal role Oct4, Sox2, and Nanog play in pluripotency. Further studies determining all possible Bright binding sites on these promoters by ChIP-sequencing will help determine how Bright may be influencing gene expression as well as determine other genes under Bright transcriptional control. Mass spectrometry analysis of the protein complexes Bright is involved in both undifferentiated and differentiating ES cells will help determine Bright's role in development. This analysis is especially important in deciphering transcriptional roles versus any cytoplasmic signaling pathways in which Bright may be involved. Careful

Bright overexpression assays in ES cells and qPCR analysis may help to decipher the balance between Bright and other pluripotency related factors in mediating differentiation.

We have shown here that Bright plays an important role in reprogramming somatic cells to a pluripotent state. Additionally, we described novel interactions between Bright and key pluripotency factors. It is increasingly clear that Bright plays a significant role in the pluripotency pathway of ES cells, but more precise work is needed to fully describe what that role is.

6.4 FUTURE DIRECTIONS FOR DETERMINING BRIGHT'S ROLE IN DIFFERENTIATION

Bright null animals typically die by E12.5 due to failed hematopoiesis (Webb et al., 2011), but the mechanism behind this defect remains unknown. Bri-/- mES cells do not appear to have phenotypic changes regarding self-renewal or maintaining pluripotency, but they have a dramatic phenotype when they are allowed to undergo differentiation. These Bri-/- mES cells preferentially form cystic EBs and develop more quickly than Bri+/+ or Bri+/- ES cells. Microarray analysis confirmed misregulation of lineage specific pathways, suggesting a role for Bright in maintaining the correct timing of lineage differentiation. Most strikingly, mesoderm differentiation appears to be highly influenced by loss of Bright. Perhaps not coincidentally, hematopoietic stem cells develop from the mesoderm lineages. Further developmental studies are necessary to understand the pathways in which Bright is involved during differentiation. Analysis of

Bright binding, expression levels, and localization during directed differentiation of ES cells should lead to further understanding of how Bright regulates differentiation.

6.5 CONCLUDING REMARKS

Differentiation is not a static state. It is a dynamic state that requires modulation of chromatin structure, activation of lineage specific genes, and repression of alternate lineage programs. The process of reprogramming a fully differentiated cell to a pluripotent state most markedly demonstrates this. Our data suggests a critical role for the Bright/ARID3A transcription factor in maintaining the differentiation state through repression of key pluripotency genes *Oct4*, *Sox2*, and *Nanog*. We have also shown a role for Bright in the timely differentiation of ES cells as well as a role in maintaining kidney structure in the adult animal. Including the critical role we have previously shown Bright has in hematopoietic stem cell development, these data implicate Bright as a regulator of mesoderm differentiation and may explain why mesoderm descendants, including B lymphocytes and fibroblasts, undergo most efficient conversion. Collectively, this work indicates Bright plays a pivotal role in organizing and maintaining differentiation in somatic cells and suggests an alternative method to cellular reprogramming which may be more amenable to clinical use in the future.

Appendix

	Forward	Reverse
ChIP		
Oct4	AAAGTTTCTGTGGGGGACCT	AAAACCGGGAGACACAACTG
Nanog	GTTGGAAACGTGGTGAACCT	GAAAACCGAGCAACAGAACC
Neg control	GGAGTCCCCTAGGAAGGCATTAATAGTTT	GGATTCTCTCGGCTTCAGACAGACTTT
qPCR		
Bright	GAGGTTATCAACAAGAAACTGT	GATACTTCATGTACTGTGTCCG

Primers used in the chromatin immunoprecipitation assay (ChIP) and quantitative Real-Timee PCR (qPCR) assays.

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