ESTABLISHMENT OF A PHENOTYPICAL MODEL OF ADVERSE OUTCOMES ASSOCIATED WITH ASSISTED REPRODUCTIVE TECHNOLOGIES

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By

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

ESTABLISHMENT OF A PHENOTYPICAL MODEL OF ADVERSE OUTCOMES ASSOCIATED WITH ASSISTED REPRODUCTIVE TECHNOLOGIES

Presented by Katherine Marie Robbins

A candidate for the degree of Master of Science

And hereby certify that, in their opinion, it is worthy of acceptance.

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NOMENCLATURE

(-) RT	**Minus Reverse Transcriptase**
ac	**Acetylation**
ApE	**A plasmid Editor**
ART	**Assisted Reproductive Technology**
AS	**Angelman Syndrome**
BLAST	**Basic local alignment search tool**
BLAT	**Blast-like alignment tool**
BORIS	**Brother of Regulator of Imprinted Sites**
bp	**Base Pairs**
BWS	**Beckwith-Wiedemann Syndrome**
CDKN1C	**Cyclin-Dependent Kinase Inhibitor 1**
cDNA	**Complementary Deoxyribonucleic Acid**
COBRA	**Combined Bisulfite Restriction Analysis**
CTCF	**CCCTC-Binding Factor**
CTCFL	**CCCTC-Binding Factor-Like (CTCF-like)**
DMR	**Differentially Methylated Region**
DMSO	**Dimethyl Sulfoxide**
DNA	**Deoxyribonucleic Acid**
DNMT1o	**DNA Methyltransferase 1 oocyte**
DNMT1s	**DNA Methyltransferase 1 somatic**
DNMTs	**DNA Methyltransferase**
dNTPs	**Deoxynucleoside Triphosphates**

DTT	**Dithiothreitol**
ED	**Embryonic Day**
EST	**Expressed Sequence Tag**
H3K27me3	**Histone 3 Lysine 27 methyl 3**
H3K9	**Histone 3 Lysine 9**
H3K9me	**Histone 3 Lysine 9 methylation**
HIV	**Human Immunodeficiency Virus**
HP1	**Heterochromatin Protein 1**
HYMAI	**Hydatidiform Mole Associated and Imprinted Gene**
IC1	**Imprinting Control 1 (*H19/IGF2*)**
IC2	**Imprinting Control 2 (KvDMR1)**
ICM	**Inner Cell Mass**
ICR	**Imprinting Control Region**
ICSI	**Intra-Cytoplasmic Sperm Injection**
IGF2	**Insulin Growth Factor II**
In/del	**Insertion/deletion**
IVD	***In vivo* Derived**
IVF	***In vitro* Fertilization**
IVP	***In vitro* Production**
kb	**Kilobases**
KCNQ1	**Potassium Voltage Gated Channel 1**
KCNQ1OT1	**Potassium Voltage Gated Channel 1 Overlapping Transcript 1**
LOI	**Loss-of-Imprinting**

LOM	**Loss of Methylation**
LOS	**Large Offspring Syndrome**
M. Sss1	**CpG Methyltransferase**
me1, me2, me3	**Methyl group 1, 2, 3**
Mb	**Megabases**
MBD	**Methyl-CpG Binding Domain**
me	**Methylation**
MEST	**Mesoderm Specific Transcript Homolog**
NCBI	**National Center for Biotechnology Information**
ncRNA	**noncoding RNA**
PAGE	**Polyacrylamide gel electrophoresis**
PcGs	**Polycomb Group Protein**
PCR	**Polymerase Chain Reaction**
PGC	**Primordial Germ Cells**
PHLDA2	**Pleckstrin Homology-like Domain, Family A**
PLAGL1	**Pleomorphic Adenoma Gene like-1**
PRC1 / PRC2	**Polycomb Repressive Complex 1 or 2**
PRMT7	**Protein Arginine Methyltransferase Seven**
PTM	**Post-Translational Modifications**
PWS	**Prader Willi Syndrome**
RFLP	**Restriction Fragment length polymorphim**
RNA	**Ribonucleic Acid**
RT	**Reverse Transcriptase**

SAM	**S-adenosyl-methionine**
SCNT	**Somatic Nuclear Transfer**
SNP	**Single nucleotide polymorphism**
SRS	**Silver Russell Syndrome**
SSCP	**Single Stranded Conformation Polymorphism**
UCSC	**University of California Santa Crus Genome Browser**
UMD 3.1	**University of Maryland 3.1 Build**

CHAPTER I

LITERATURE REVIEW

General Introduction

Infertility is the inability to be able to produce a child. The advancement of assisted reproductive technologies (ART) has allowed infertile couples an increased likelihood of conceiving a child. ART procedures commonly used to help circumvent infertility include: ovarian hyperstimulation, *in vitro* fertilization, intra-cytoplasmic sperm injection, embryo culture, and embryo transfer. In the United States, one percent of all children born alive (*i.e.* 46,326) in 2006 were conceived by the use of ART (CDC, 2010).

Several retrospective studies have shown a greater likelihood of adverse outcomes to ART-conceived children when compared to naturally-conceived children (Bergh *et al.,* 1999; Hansen *et al.,* 2002). For example, an increased incidence of the loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann (BWS) in children conceived by the use of ART has been reported (DeBaun *et al.,* 2003; Gicquel *et al.,* 2003; Halliday *et al.,* 2004; Sutcliffe *et al.,* 2006). Genomic imprinting is an epigenetic modification that results in the monoallelic expression of approximately 100 genes in mammals (Nakabayashi *et al.,* 2011). Imprinted genes are involved in regulating the growth and development of the conceptus (fetus and placenta).

BWS is thought to occur because of the dysregulation of several imprinted genes found primarily on chromosome 11p15 (Rump *et al.,* 2005; Manipalviratn *et al.,* 2009; Weksberg *et al.,* 2010). The ART-induced overgrowth phenotype of offspring not only occurs in humans but has also been documented in ovids and bovids. In ruminants, the overgrowth phenotype is referred to as large offspring syndrome (LOS). LOS is known to be caused by a variety of *in vitro* culture conditions (Young *et al.,* 1998). Several phenotypical similarities exist between BWS and LOS. These include somatic overgrowth, birth defects, abdominal wall defects, hypoglycemia, and enlarged organs (Sangild *et al.,* 2000; McEvoy *et al.,* 2001; Hansen *et al.,* 2002; Weksberg *et al.,* 2003; Hiendleder *et al.,* 2004; Cohen, 2005; Li *et al.,* 2005; Manipalviratn *et al.,* 2009; Weksberg *et al.,* 2010).

Currently there are no animal models that recapitulate BWS. Given that ART induces an overgrowth phenotype in human and cattle, we speculate that BWS and LOS are epigenetically similar. The work presented in this thesis will delineate the initial steps taken to determine if bovids are an appropriate model to study how loss-of-imprinting results in BWS and specifically, how ART may induce this phenotype.

This literature review (Chapter I) will describe in detail two epigenetic modifications, namely DNA methylation and genomic imprinting. In addition, a short section on histone modifications will be included to enhance the understanding of the literature review. Unless otherwise specified, the descriptions given in the literature review on the various epigenetic modifications and epigenetic modifiers are those described using the mouse as a model. Chapter I will also discuss the loss-of-imprinting

syndrome BWS, ART, and the association between BWS and ART. Chapter I will conclude by describing LOS and the advantages of using bovids as a model to study BWS.

Chapter II will include definitions of frequently used terms as well as detailed descriptions of the procedures used to ascertain DNA methylation and gene expression at imprinted loci. In Chapter III, I will discuss the identification of DNA sequence polymorphisms between the *Bos taurus indicus (B. t. indicus*) and *Bos taurus taurus* (*B. t. taurus*) in regions known to be misregulated in BWS. Chapter III will also describe the steps taken to develop one DNA methylation assay and one imprinted gene expression assay. Lastly, Chapter IV will discuss the generation of *B. t. indicus x B. t. taurus* F1 day 65 naturally-conceived fetuses used to establish imprinting baseline in cattle.

Note: The gene symbol nomenclature varies between the human, cow, and mouse as to whether they are capitalized (*i.e. KCNQ1OT1*) or whether only the first letter is capitalized followed by lower case letters (*i.e. Kcnq1ot1*). For this thesis I have chosen to use the human nomenclature (*i.e. KCNQ1OT1*) for all species included.

EPIGENETICS

Histone modifications

The basic repeating unit of eukaryotic chromatin is called the nucleosome. Nucleosomes are composed of an octamer of core histone proteins that are wrapped twice by 146 base pairs (bp) of DNA (Jenuwein and Allis, 2001). The interaction between deoxyribonucleic acid (DNA) and histone proteins is the result of their net

charge. Histone proteins are positively charged due to their high content of the amino group containing amino acids arginine and lysine while DNA has an overall negative charge as a result of the presence of phosphate groups.

The histone octamer consists of two units of each of the following core histones: 2A (H2A), H2B, H3, and H4. Further, the entry/exit site of DNA interacts with the linker histone, H1 (Zhang and Reinberg, 2001). The core histone tails protrude from the nucleosome and undergo the following covalent post-translational modifications (PTM): methylation (me), acetylation (ac), phosphorylation (p), and ubquitination (ub). The most studied PTMs include methylation (me) and acetylation (ac). Each lysine residue is able to accept one to three methyl groups (me1, me2, me3) while arginine can accept one or two methyl groups (Peterson and Laniel, 2004).

Histone acetylation results in transcriptional gene activation examples include: H3K9ac and H4K12ac (Hebbes *et al.,* 1988). Histone methylation can lead to both repressive and active states of transcription. An example of an activating histone methylation mark would be methylation on lysine (K) 4 of H3 (H3K4me; Bernstein *et al.,* 2002) whereas transcriptional repressive marks for methylation would be H3K27me3, H3K9me2, and H4K12me (Umlauf *et al.,* 2004). The acetylation and methylation of histone proteins is catalyzed by histone acetyltransferases and histone methyltransferases, respectively (Zhang and Reinberg, 2001).

Chromatin is found in one of two states, euchromatin (accessible for transcription) or heterochromatin (inaccessible for transcription) and depends on the histone PTM as well as histone variants. Euchromatin is characterized by having

histones that are highly acetylated (Zhang and Reinberg, 2001) while the histones in heterochromatin typically have repressive methyl marks. The repressive histone marks allow for chromatin modifiers to bind which will act to induce a repressive chromatin conformation (Bartova *et al.,* 2008). For example, the histone methyltransferase SUV39H transfers a methyl mark to H3K9 which in turn allows heterochromatin protein 1 (HP1) to bind through its methyl binding domain (*i.e.* chromodomain; Latchner *et al.,* 2001) thus condensing the chromatin into an inactive state (D'Alessio and Szyf, 2006). HP1 can recruit DNA methyltransferases (see next section) leading to DNA methylation which ensures an enhanced state of transcriptional repression (Bartova *et al.,* 2008).

DNA Methylation

DNA methylation is an epigenetic modification that is inherited through cell division and alters gene expression usually in a repressive manner. DNA methylation is critical for development and reprogramming of the embryo (Okano *et al.,* 1999), gene expression (Nagae *et al.,* 2011), genomic imprinting (DeBaun *et al.,* 2003), retrotransposons repression (Walsh *et al.,* 1998), and X chromosome inactivation (reviewed in Dean *et al.,* 2005; Cotton *et al.,* 2011). This modification occurs when a methyl group (CH_3) is added to the fifth carbon position of a cytosine when next to a guanine base. Methylated cytosines promote a silent state of genes by attracting enzymes that have chromatin modifying capabilities which interfere with the binding of transcriptional regulators.

DNA methylation together with methyl-CpG binding domain proteins (MBD) act to form transcriptionally inactive chromatin (Fujita *et al.,* 2003). The four mammalian MBDs that recognize and bind to methylated DNA are MeCP2, MBD1, MBD2, and MBD4 (Fujita *et al.,* 2003). Mammalian MBD3 has a mutation in the MBD domain and is unable to bind methylated DNA (Clouaire and Stancheva, 2008). MeCP2 binds to methylated DNA and then recruits histone deacetylases to further repress transcription (Jones *et al.,* 1998). MBD1 acts as a transcriptional repressor binding to methylated DNA. The repression is enhanced by the interaction of MBD1 with SUV39H1 and HP1, a histone 3 lysine-9 methylase and methyl lysine binding protein, respectively (Fujita *et al.,* 2003). MBD2 and MBD3 associate with the NuRD complex which is composed of chromatin remodeling ATPases and histone deactelyases (Clouaire and Stancheva, 2008). The activities of these enzymes ultimately result in the spreading of a transcriptionally repressive state to a locus (Clouaire and Stancheva, 2008).

In the mammalian genome up to 70% of CpGs are methylated (Strichman-Almashanu *et al.,* 2002). CpG islands are regions of DNA that are larger than 500 bp in size with an observed over expected CpG ratio >0.6, and C + G frequency >0.5 (Ponger *et al.,* 2001). CpG islands are mainly associated with the promoter region of genes however, they are also found in exons, introns, and repetitive elements (reviewed in Robertson, 2005). Ninety percent of housekeeping gene promoters contain a CpG island whereas 41.6% of tissue-specific genes promoters contain a CpG Island (Ponger *et al.,* 2001). Promoter regions of ubiquitously-expressed housekeeping genes are generally

hypomethylated (Beatty *et al.,* 2006) while tissue specific/developmental stage specific are hypermethylated in tissues that do not express the gene.

DNA methylation is established and maintained by the DNA methyltransferase (DNMTs) enzymes DNMT3A, DNMT3B, DNMT3L, and DNMT1 (Dean *et al.,* 2005). The DNMTs attack the sixth carbon of cytosine and catalyze the covalent addition of the methyl group from S-adenosyl-L-methionine (SAM) to the fifth carbon position of cystosine (Jurkowska *et al.,* 2011).

DNMT3A and DNMT3B are commonly known as *de novo* DNA methyltransferases. The *de novo* methyltransferases are responsible for establishing DNA methylation during early germ cell development (Okano *et al.,* 1999). DNMT3A is highly expressed in the oocyte during oocyte growth (Lucifero *et al.,* 2004) and it is the main DNMT involved in remethylating the maternal genome. The protein level of DNMT3A is almost undetectable during preimplantation development. DNMT3A is moderately expressed in the embryonic ectoderm on embryonic day (ED) 8.5 – 9.5 and is ubiquitously expressed in the ventral part of the embryo (Okano *et al.,* 1999; Watanabe *et al.,* 2002). When DNMT3A is knocked out in mice, fetuses are born at term although they are underdeveloped and die within four weeks of birth (Okano *et al.,* 1999).

DNMT3B is first immunolocalized in the inner cell mass (ICM) around the time of implantation (*i.e.* ED 4.5; Watanabe *et al.,* 2002). DNMT3B is highly expressed in the embryonic neural and chorionic ectoderm at ED 7.5 while in the later stages of development it is present only in the forebrain of the embryo (Okano *et al.,* 1999).

DNMT3B knockout mice have severe developmental defects that impairs neural tube formation and prohibits survival past ED 9.5 (Okano *et al.,* 1999).

DNMT3L is catalytically inactive, however, this co-factor increases the catalytic activity of DNMT3A and DNMT3B by 2-33 fold based on the sequence flanking the CpG site (Wienholz *et al.,* 2010). DNMT3L is present in oocytes and seminiferous tubules (Hata *et al.,* 2002). The importance of DNMT3L has been demonstrated with knockout studies. For example, the testes of DNMT3L knockout mice are reduced in weight by eight weeks of age and the spermatogonia are not able to differentiate into spermatocytes resulting in infertility (Hata *et al.,* 2002). On the other hand, DNMT3L null females are fertile but the transmitted null allele to the pups results in neural tube defects and death by ED 10.5 (Hata *et al.,* 2002).

DNMT1 mainly acts on hemimethylated DNA (Takeshita *et al.,* 2011). This enzyme localizes to the replication fork by associating with proliferating cell nuclear antigen (PCNA) and copies the pattern of methylation from the parent strand unto the newly synthesized daughter stand (Jurkowska *et al.,* 2011). The maintenance DNMT is crucial for mammalian embryonic development (Hirasawa *et al.,* 2008). To that effect, mice deficient for DNMT1 have a three-fold reduction in global methylation along with adverse phenotypical features consisting of stunted growth, neural tube, and abdominal defects that lead to embryonic loss by ED 10.5 (Li *et al.,* 1992). Mice with a hypomorphic DNMT1 allele (10% protein expression) are phenotypically runts and by four to eight months of age develop aggressive thymic tumors (Gaudet *et al.,* 2003).

These investigators concluded that genomic hypomethylation due to a decrease in DNMT1 expression increases the likelihood of tumorigenesis (Gaudet *et al.,* 2003).

There are two oocyte-specific splice variants of DNMT1: somatic DNMT1 (DNMT1s) and oocyte-specific DNMT1 (DNMT1o). The most abundant DNMT1 in the oocyte, DNMT1o, is excluded from the nucleus of blastomeres during most stages of preimplantation development. The exception is at the 8-cell stage when DNMT1o traffics back into the nucleus. The movement of DNMT1o to the cytoplasm is thought to be responsible for the global demethylation observed during preimplantation development. Trafficking of DNMT1o to the nucleus at the 8-cell stage is hypothesized to act in maintaining methylation at imprinted loci (Howell *et al.,* 2001). When DNMT1o is deleted from oocytes, homozygous animals develop normally; however the majority of heterozygous embryos derived from homozygous females die by ED 14 because of misregulation of imprinted gene expression (Howell *et al.,* 2001). DNMT1s, on the other hand, is present in the nucleus throughout preimplantation development (Cirio *et al.,* 2008) and appears to be involved in maintaining the methylation imprints during preimplantation development at stages other than the eight cell stage (Cirio *et al.,* 2008).

The mammalian genome undergoes two rounds of global demethylation. The first occurs in primordial germ cells (PGC) and the second takes place during preimplantation development (Hajkova *et al.,* 2010). The PGCs are derived from the epiblast and first appear at ED 7.25 in mice (Ginsburg *et al.,* 1990; Hajkova *et al.,* 2010). The PGCs then migrate along the hindgut to the genital ridge where they divide

mitotically until approximately ED 10.5 (Anderson *et al.,* 2000). A study from Hajkova *et al.* (2002) demonstrated that in mouse male and female feti the PGCs are highly methylated at ED 10.5-11.5 but are completely unmethylated by ED 13.5. It should be noted that genome-wide demethylation in PGCs occurs at both single copy and imprinted genes while demethylation of repetitive elements is incomplete (Hajkova *et al.,* 2002).

The second phase of genome-wide demethylation occurs after fertilization and during preimplantation development. Before replication begins and within six hours after fertilization, the DNA in the paternal pronucleus is actively demethylated (Mayer *et al.,* 2000). On the other hand, maternally-inherited genome demethylates passively with each cell division and by day five of development demethylation of the maternal genome is complete (Howlett and Reik, 1991). Imprinted genes escape the second round of global demethylation. The observed demethylation pattern in preimplantation mouse embryos is similar for cow, pig, and rat embryos (Dean *et al.,* 2001). After implantation the *de novo* DNA methyltransferases remethylate the parental genomes.

The parental genomes remethylate independently of each other. In males, the gonocytes are arrested in the G1-phase of mitosis during ED 13.5-15.5 (Davis *et al.,* 2000; reviewed in Sasaki and Matsui, 2008) after which DNA methylation is acquired and completed by ED 18.5. In contrast, in the female, remethylation of the oocyte's genome does not occur until after birth and is initiated every time a crop of follicles is recruited (Davis *et al.,* 2000).

Genomic Imprinting

Genomic imprinting is an epigenetic modification that directs parent-specific gene expression. Imprinted genes are responsible for regulating the growth and development of the conceptus (Biliya and Bulla, 2010). Studies done in the 1980's first demonstrated the requirement for both parental genomes for proper embryonic and fetal development (Barton *et al.,* 1984; McGrath and Solter, 1984; Surani *et al.,* 1984). Those investigators generated embryos which had two maternal genomes (parthenotes and gynogenotes) or two paternal genomes (androgenotes) and recorded their developmental progress at mid-gestation. Their results showed that in gynogenotes and parthenotes the fetuses had developed normally, although they were smaller in size when compared to controls. However there was significant reduction in the size and development of the extraembryonic tissues when compared to fertilized controls (Surani *et al.,* 1984). The opposite was observed in androgenetic embryos which developed apparently normal extraembryonic tissues but had extremely underdeveloped embryonic tissues (McGrath and Solter, 1984; Surani *et al.,* 1984; Surani *et al.,* 1987).

We now know that the observations made in the previously mentioned studies showed differences in parental genome contribution during embryonic development as the result of genomic imprinting. The term "imprinting" was coined by Helen Crouse in 1960 when explaining that the male germline inherits an "imprint" or mark that is expressed in the opposite manner in the female germline during gametogenesis. By 1987, the differences observed between the male and female germlines during

gametogenesis were referred to as “genomic imprinting” (Reik *et al.,* 1987; Sapienza *et al.,* 1987).

Knockout studies in mice have uncovered a theme for the function of imprinted genes (Baker *et al.,* 1993; Lefebvre *et al.,* 1998; Li *et al.,* 1999; Takahashi *et al.,* 2000; Frank *et al.,* 2002). Generally, paternally-expressed genes drive growth and maternally-expressed genes control growth of the fetus and the placenta. This tug of war between the parental genomes was coined the “kinship theory of genomic imprinting” by Moore and Haig (2000). This hypothesis suggests that the products of paternally-expressed imprinted genes are used to exploit maternal resources to benefit and stimulate development of the fetus. On the other hand, the maternally-expressed imprinted genes conserve nutrients to restrict fetal overgrowth among offspring (Haig, 2000). Currently, there are over 200 predicted imprinted genes in the human genome of which parent-specific expression has been determined for 53 genes (Zhang *et al.,* 2009).

Imprinting control regions

Approximately 80% of imprinted genes are found in clusters (reviewed in Reik and Walter, 2001). These clusters are regulated by discrete regions of allele specific differentially methylated DNA known as the imprinting control region (ICR). In addition to differential DNA methylation, the ICRs have specific signatures of histones modifications on each one of the parental alleles (Ideraabdullah *et al.,* 2008). The ICR directs the correct allelic expression of the clustered imprinted genes (Zhang *et al.,*

2009). The epigenetic control afforded by the ICR can span megabases in a bidirectional manner (Pandey *et al.,* 2008).

ICRs are always differentially methylated regions (DMR) of DNA, however not every imprinted gene-associated DMR acts as an ICR. ICRs have several different functions and the misregulation of these regions result in loss-of-imprinting of the clustered genes. The two described mechanisms that allow ICRs to regulate imprinted gene expression are; 1) insulator activity and 2) expression of a noncoding ribonucleic acid (ncRNA; Jelinic *et al.,* 2006; Ideraabdullah *et al.,* 2008). Following is a description of the aforementioned mechanisms.

Insulator Activity

The insulator mechanism has been best studied in the *H19/IGF2* imprinted gene cluster (**Figure 1**). *IGF2* is a paternally-expressed fetal mitogen (Jelinic and Shaw, 2007) that is located 90 kilobases (kb) upstream of the maternally-expressed gene *H19* (Kaffer *et al.,* 2000). *H19* is a long ncRNA whose function is not yet determined but it may act as a tumor suppressor (Hao *et al.,* 1993). There are two types of shared enhancers that control the expression of *H19* and *IGF2*. Tissues of the endodermal lineage (*e.g.* liver, gut, and yolk sac) use the enhancers located 8 kb downstream of *H19* while tissues of mesodermal origin (*e.g.* skeletal and cardiac muscle) use the enhancers located 25 kb downstream of *H19* (reviewed in Phillips and Corces, 2009).

The *H19/IGF2* ICR is located 2-4 kb upstream from the start of transcription of *H19* and is paternally methylated and maternally unmethylated (reviewed in Reese and Bartolomei, 2006). There are four CTCF binding sites located within the 2 kb *H19/IGF2*

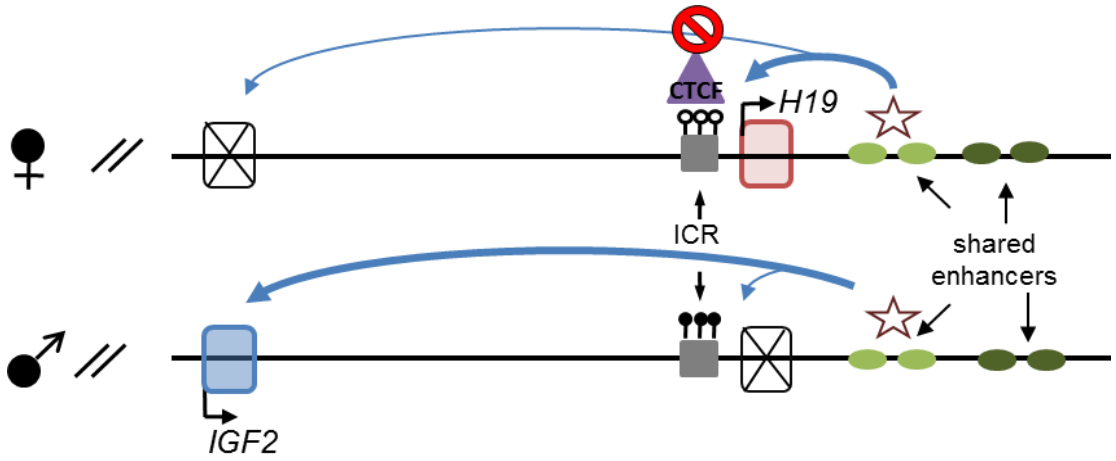

Figure 1. *H19/IGF2* imprinted gene cluster. *H19/IGF2* ICR is a differentially methylated region located on chromosome 7, 11, and 29 in the mouse, human, and bovine, respectively. This ICR is 2 kb in the mouse, 5 kb in humans, and at least 3 kb in bovine. The ICR acts to regulate parent-specific gene expression of *H19* and *IGF2*. There are two sets of downstream enhancers: endodermal and mesodermal located 8 kb and 25 kb, respectively. CTCF binding to the maternal ICR creates a chromatin conformation that hampers *IGF2*'s promoter-enhancer interaction thus allowing *H19*'s promoter to interact with the shared enhancers. Lack of CTCF binding on the methylated paternal allele results in activation of *IGF2* and repression of *H19*.

The female and male and symbols represent the maternal and paternal alleles, respectively. The blue box represents the paternally expressed gene *IGF2*. The red box represents the maternally-expressed noncoding RNA, *H19*. The white boxes with X through them represent repressed imprinted genes. The grey box is the ICR which is differentially methylated. The black lollipops represent methylated CpGs (paternal allele) while the unfilled lollipops represent unmethylated CpGs (maternal allele). The ▲ represents CTCF protein bound to the unmethylated maternal allele. The ovals represent the downstream endodermal (light green) and mesodermal (dark green) shared enhancers. The figure is not drawn to scale and non-imprinted genes are not shown. The orientation of the arrows shows the direction in which the genes are expressed.

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The female and male and symbols represent the maternal and paternal alleles, respectively. The blue box represents the paternally expressed gene *IGF2*. The red box represents the maternally-expressed noncoding RNA, *H19*. The white boxes with X through them represent repressed imprinted genes. The grey box is the ICR which is differentially methylated. The black lollipops represent methylated CpGs (paternal allele) while the unfilled lollipops represent unmethylated CpGs (maternal allele). The ▲ represents CTCF protein bound to the unmethylated maternal allele. The ovals represent the downstream endodermal (light green) and mesodermal (dark green) shared enhancers. The figure is not drawn to scale and non-imprinted genes are not shown. The orientation of the arrows shows the direction in which the genes are expressed.

DMR (Engel *et al.,* 2006). CTCF is a highly conserved eleven zinc finger DNA-binding protein with diverse functions including transcriptional activation and repression, insulation, imprinting, and X chromosome inactivation (reviewed in Phillips and Corces, 2009). In addition, CTCF act as a boundary element by blocking the spreading of heterochromatin (Cho *et al.,* 2005; reviewed in Robertson, 2005).

The binding of CTCF to the CTCF sites can only occur on unmethylated DNA (Engel *et al.,* 2006). The *H19/IGF2* ICR is unmethylated on the maternal allele, therefore CTCF is able to bind and by using its enhancer blocking ability prevents *IGF2* from utilizing the downstream enhancers (reviewed in Robertson, 2005; Engel *et al.,* 2008). Using chromosome conformation capture (3C) assays, Kurukuti *et al.* (2006) demonstrated that CTCF achieves enhancer usage control by causing the *H19/IGF2* locus to undergo looping. This looping creates a chromatin conformation refractory for the association of the *IGF2* promoters to the downstream enhancers (Kurukuti *et al.,* 2006; reviewed in Phillips and Corces, 2009). Without CTCF's insulator function on the maternal allele the enhancers downstream of *H19* can activate *IGF2*, ultimately leading to biallelic expression of *IGF2* (Jelinic *et al.,* 2006; Engel *et al.,* 2008).

BORIS (brother of regulator of imprinted sites) also known as CTCF-like (CTCFL) is responsible for establishing the paternal imprint at the *H19/IGF2* ICR. BORIS is 74% homologous to CTCF (Jelinic and Shaw, 2007) and has the potential to bind to CTCF binding sites on both methylated and unmethylated sequences (Nguyen *et al.,* 2008). BORIS is normally expressed only in the testis; however aberrant expression has been

documented in certain cancers such as Wilms' tumors and retinoblastoma (Jelinic *et al.,* 2006; Nguyen *et al.,* 2008).

The DNA methylating capability of BORIS appears to be mediated by its association with PRMT7, a protein arginine methyltransferase (Jelinic *et al.,* 2006). PRMT7 methylates arginines on histones H2A and H4 (Jelinic *et al.,* 2006). These histone modifications in turn recruit the *de novo* DNMTs (Jelinic *et al.,* 2006). This mechanism of DNA methylation at the *H19/IGF2* locus was demonstrated using oocytes from an organism that does not exhibit genomic imprinting, namely *Xenopus laevis*. In their study Jelinic *et al.* (2006) co-injected complementary DNA (cDNA) expression plasmids for BORIS, PRMT7, DNMT3A, 3B, and 3L together with a plasmid which contained the *H19* ICR into Xenopus oocytes and found that the ICR was *de novo* methylated. Significant *de novo* methylation of the ICR was not observed when the cDNA expression vectors were injected individually.

ncRNA

In the second mechanism, the ICR functions as the promoter for a ncRNA; a well-documented case being the KvDMR1 (**Figure 2**). In the mouse, the ~ 1 megabase (Mb) KvDMR1-directed imprinted locus consists of one gene encoding a paternally-expressed ncRNA (*KCNQ1OT1*) and eight maternally-expressed protein coding genes (*CDKN1C, PHLDA2, KCNQ1,* TSSC4, CD81, ASCL2, *SLC22A1L, OSBPL5*; Ideraabdullah *et al.,* 2008; Pandey *et al.,* 2008). *KCNQ1OT1*'s promoter, KvDMR1, is located within the tenth intron of the maternally-expressed gene *KCNQ1*. Transcription of this intronless ~90 kb ncRNA

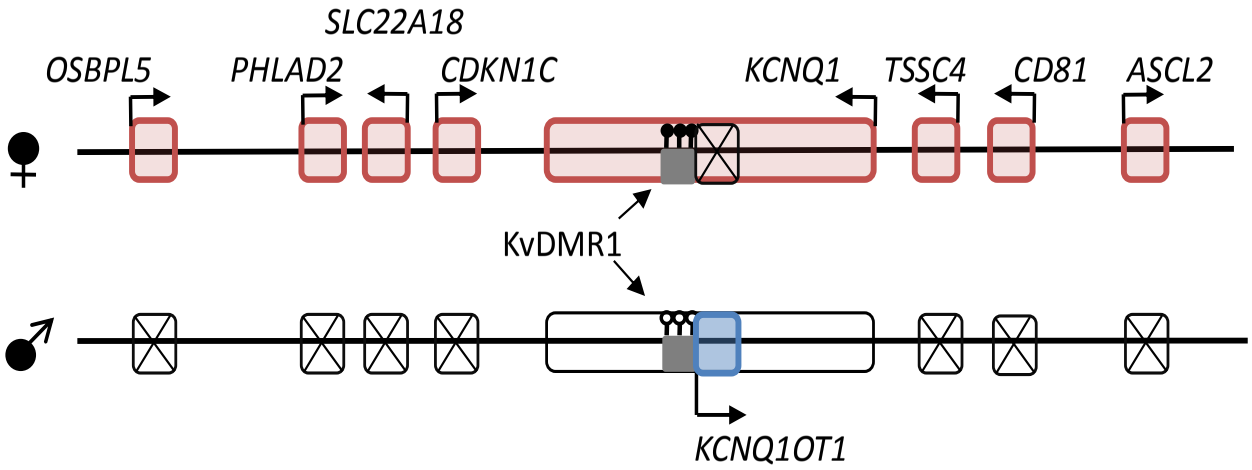

Figure 2. KvDMR1 imprinted gene cluster. KvDMR1 directs expression of a 1 Mb cluster of imprinted genes. KvDMR1 is located on chromosome 7, 11, and 29 in the mouse, human, and bovine, respectively. The KvDMR1 is a 2 kb differentially methylated region that acts as the promoter region for the noncoding RNA *KCNQ1OT1*. The paternal allele is unmethylated at the KvDMR1. When expressed, *KCNQ1OT1* bidirectionally silences the maternally-expressed flanking genes. *KCNQ1OT1* is transcribed in the antisense direction to the maternally-expressed *KCNQ1*. The maternal allele is hypermethylated at the KvDMR1, therefore *KCNQ1OT1* is not expressed and the maternally-expressed flanking genes are expressed. The female and male and symbols represent the maternal and paternal alleles, respectively. The blue box represents the paternally-expressed long noncoding RNA *KCNQ1OT1*. The red boxes represent the maternally-expressed genes. The white boxes with X through them represent repressed genes. The grey box is the ICR which is differentially methylated. The black lollipops represent methylated CpGs (maternal allele) while the unfilled lollipops represent unmethylated CpGs (paternal allele). The figure is not drawn to scale and non-imprinted genes are not depicted in the figure. The orientation of the arrows shows the direction in which the genes are expressed.

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occurs in the antisense direction relative to *KCNQ1* (Beatty *et al.,* 2005; Pandey *et al.,* 2008).

KvDMR1 is unmethylated on the paternal allele resulting in the expression of the ncRNA *KCNQ1OT1 (*Ideraabdullah *et al.,* 2008). *KCNQ1OT1* is responsible for the bidirectional silencing of the maternally-expressed imprinted flanking genes on the paternal allele (Ideraabdullah *et al.,* 2008). The maternal allele is hypermethylated at the KvDMR1, therefore *KCNQ1OT1* is not expressed resulting in transcription of the maternally-expressed imprinted genes. Loss of DNA methylation on the maternal allele results in the biallelic expression of *KCNQ1OT1* (Lee *et al.,* 1999a).

Besides DNA methylation, histone modifications have also been found to assist in the silencing of KvDMR1 flanking imprinted genes on the paternal allele. Recently, Terranova and coworkers (2008) described an association of the KvDMR1 imprinted locus with members of the Polycomb group protein (PcGs) complexes. PcGs are histone tail modifiers that are essential during early development and act by remodeling chromatin. There are two sets of PcG complexes: Polycomb repressive complex 1 and 2 (PRC1 and PRC2, respectively). The PRC2 complex has several components namely EZH1/2, SUZ12, EED, and RdAp46/48 (Margueron and Reinberg, 2011). EZH2 is the histone methyltransferase of the complex and catalyzes the trimethylation of H3K27 while EED can interact with histone deacetylases to remove the active acetyl marks from the neighboring histones thus creating a repressive transcriptional state (Lin *et al.,* 2011).

The PRC1's chromodomain member CBX2 recognizes the trimethylated state of H3K27 and promotes the ubiquitilation of H2AK119 by RNF2. These histone modifications contract the chromatin bidirectionally creating a compartment devoid of RNA polymerase (Terranova *et al.,* 2008; Magueron and Reinberg, 2011). RNA immunoprecipitation studies place *KCNQ1OT1* as the main player in attracting the PcG to the locus (Pandey *et al.,* 2008). Along with H3K27me3 another repressive mark found on the chromatin of the paternal allele is H3K9me2, an activity of the histone methyltransferases G9A and KMT1C (Lewis *et al.,* 2006; Malecová and Morris, 2010).

Methylation Imprints

During mammalian development imprinted loci undergo genome wide DNA methylation reprogramming during primordial germ cell development. Demethylation of PGCs followed by remethylation is crucial to remove the previously existing parental-specific methyl marks and to establish methylation imprints in a sex-specific manner (Biliya and Bulla, 2010).

In mice, there are 21 gametic/primary imprints of which 17 are methylated on the maternal genome and four are methylated on the paternal genome (Tomizawa *et al.,* 2011). The maternally methylated gametic imprints are: KvDMR1*, ZAC, MEST, GRB10, GNAS, NAP1L5, SNRPN, MCTS2, INPP5FV2, L3MBTL, DIRAS3, DIRAS1, NNAT, PEG3, PEG10, RB1,* and *IGF2R* region 2 (Koerner *et al.,* 2009; Tomizawa *et al.,* 2011; Woodfine *et al.,* 2011). The four paternal methylation imprints are: *H19/IGF2*, *RASGRF1, DLK1/GLT2*, and *ZDBF2* (Kato *et al.,* 2007; Kobayashi *et al.,* 2009; reviewed in

Bartolomei and Ferguson-Smith, 2011). Imprints are established within the germline during gametogenesis by the *de novo* DNMTs (Lucifero *et al.,* 2004; Hirua *et al.,* 2006).

Methylation imprints in oocytes are established asynchronously in a gene specific manner (Lucifero *et al.,* 2004; Hiura *et al.,* 2006). Imprinting marks are acquired postnatally during oocyte growth and require the methylating activity of DNMT3A and DNMT3L (Lucifero *et al.,* 2004). Establishment of methylation imprints in the male germ cell starts between ED 14.5-15.5 (Davis *et al.,* 2000; Kato *et al.,* 2007; Kobayashi *et al.,* 2009; reviewed in Bartolomei and Ferguson-Smith, 2011) and is completed by ED 18.5. Germline specific knockout studies have demonstrated that only DNMT3A and 3L are responsible for *de novo* methylation at the *H19/IGF2* and *DLK1/GLT2* loci (Kato *et al.,* 2007), while methylation at the *RASGRF1* locus requires all three *de novo* DNMTs (Kato *et al.,* 2007). It is not fully understood how the methylation imprints escape the second round of global demethylation that occurs after fertilization but DNMT1s and *PGC7/STELLA* appear to be involved (Lucifero *et al.,* 2004; reviewed in Robertson, 2005; Nakamura *et al.,* 2007). In somatic cells, DNMT1 is responsible for faithfully maintaining the methylation imprints (reviewed in John and Lefebvre, 2011).

Loss-of-imprinting syndromes in humans

Imprinted genes are functionally haploid and therefore more vulnerable to epigenetic mutations (epimutations; Fowden *et al.,* 2010). Loss-of-imprinting (LOI) refers to the misregulation of imprinted gene expression which results in biallelic expression or loss of expression of these genes. Epigenetically, LOI is often caused by

inappropriate DNA methylation of imprinted loci. There are several known loss-of-imprinting syndromes in humans. Some of these syndromes will be briefly discussed.

Angelman syndrome (AS) occurs in one in 15,000 births and is characterized by mental retardation, speech and behavioral delays (reviewed by Feinberg *et al.,* 2007). The loss of methylation (LOM) on the maternal allele at the *SNRPN* locus (chromosome 15) causes loss of expression of the maternally-expressed gene *UBE3A* in the brain. *UBE3A* encodes a ubiquitin protein ligase which is part of the ubiquitin-proteasome protein degradation pathway (Buiting *et al.,* 1999; reviewed in Robertson, 2005). Loss of function of this gene in AS results in protein build-up in the brain.

Prader Willi syndrome (PWS) is another LOI syndrome associated with the *SNRPN* locus. The frequency of PWS is one in 20,000 live births and is characterized by hyperphagia, obesity, and behavioral problems in early childhood (reviewed in Goldstone, 2004; reviewed in Robertson, 2005). PWS is the result of gain of methylation of the promoter region for the paternally-expressed *SNRPN* gene which leads to loss of expression of small nucleolar RNAs (Ozcelik *et al.,* 1992; Reed and Leef, 1994; reviewed in Horsthemke and Wagstaff, 2008; reviewed in Peters, 2008).

Silver Russell syndrome (SRS) is an intrauterine and postnatal growth restriction disorder that results in body asymmetry, broad forehead, and triangular face (Bandara and Idirisinghe, 2004; Gicquel *et al.,* 2005). The incidence of SRS is one in 100,000 live births (Falkert *et al.,* 2005). The growth retardation observed in these patients is the result of the loss of methylation at the *H19/IGF2* ICR on the paternal allele (Gicquel *et*

al., 2005). The LOM at *H19/IGF2* ICR results in biallelic expression of *H19* and decreased expression of the fetal growth factor *IGF2* (Gicquel *et al.,* 2005).

Transient neonatal diabetes mellitus is a rare (one in 400,000 births) form of diabetes during infancy that is associated with epimutations at the ICR that controls *PLAGL1* and *HYMAI*; two paternally-expressed imprinted genes (Kamiya *et al.,* 2000; Mackay and Temple, 2010). *PLAGL1* is involved in control of insulin secretion in pancreatic islet cells (Kamiya *et al.,* 2000). The loss of maternal methylation on the *HYMAI/PLAGL1* ICR results in biallelic expression of *PLAGL1* leading to hyperglycemia in infants (Diatloff-Zitto *et al.,* 2007).

BWS is an overgrowth syndrome that is associated with abdominal wall defects and embryonic tumors (reviewed in Feinberg, 2007; Choufani *et al.,* 2010). Two ICRs located on chromosome 11 are most often misregulated in this syndrome (Weksberg *et al.,* 2003; Choufani *et al.,* 2010). These are the maternally methylated KvDMR1 and paternally methylated *H19/IGF2*. This syndrome will be explained in detail in the following section.

Beckwith-Wiedemann Syndrome

Background of BWS

BWS is the most common pediatric overgrowth syndrome (reviewed in Cohen, 2005; Weksberg *et al.,* 2010). The incidence of BWS is one in 13,700 live births (reviewed in Cohen, 2005; Weksberg *et al.,* 2010). The most prevalent features found in BWS patients are macroglossia, macrosomia, and exomphalos (Choufani *et al.,* 2010).

There is equal incidence between males and females except in the case of monozygotic twins where there is an increased likelihood in females (Bliek *et al.,* 2009a; Weksberg *et al.,* 2010). In addition, monozygotic twins show discordance (*i.e.* one individual is afflicted by the syndrome while the other twin is unaffected) for the syndrome which points at the epigenetic origin of this disorder (Weksberg *et al.,* 2002; Bliek *et al.,* 2009a).

Clinical features associated with BWS

BWS has been classified as a disorder involving somatic overgrowth, predisposition to embryonic tumors, and congenital malformations (Weksberg *et al.,* 2003; Weksberg *et al.,* 2010). The overgrowth parameters for height and weight in children with BWS are in the ~97th percentile (Weksberg *et al.,* 2010).

The primary features of BWS patients are macroglossia (enlarged tongue), macrosomia (gigantism), and abdominal wall defects which include exomphalos and diastasis recti (reviewed in Elliott and Maher, 1994; Cooper *et al.,* 2005). Surgery is commonly performed for macroglossia to help offset the problems that will arise such as feeding difficulties and speech impairment (reviewed in Elliott and Maher, 1994). Secondary features include: ear creases and helical pits, renal abnormalities, facial nevus flammeus, hypoglycemia, and hemihyperplasia (reviewed in Elliott and Maher, 1994; Weksberg *et al.,* 2010). There is no consensus for diagnosing BWS, but typically, if a child presents with all three of the primary features or at least two primary features

and three secondary features are considered to have BWS (reviewed in Elliott and Maher, 1994).

Other clinical features are polyhydramnios and visceromegaly of the spleen, liver, pancreas, kidneys, and adrenals (Weksberg *et al.,* 2003; reviewed in Cohen, 2005; Cooper *et al.,* 2005). Children with BWS are also prone to congenital cardiac malformations (reviewed in Elliott and Maher, 1994; Weksberg *et al.,* 2010). Lastly, developmental delays are common along with mild to moderate mental retardation (reviewed in Elliott and Maher, 1994; reviewed in Cohen, 2005).

Loss-of-imprinting associated with BWS

The phenotypes associated with BWS were first correlated with paternally derived duplications of chromosome 11p15.5 (reviewed in Cohen, 2005; Cooper *et al.,* 2005; Choufani *et al.,* 2010; Weksberg *et al.,* 2010). Chromosome 11p15.5 in humans harbors the *H19/IGF2* and the KvDMR1 imprinted clusters. In humans these clusters of genes are referred to as imprinting control region 1 (IC1) and imprinting control region 2 (IC2), respectively. Paternal duplication of the locus results in disruption to imprinting given that both of these clusters have maternally and paternally-expressed genes. In this case, a genetic mutation leads to an epigenetic syndrome. Of the sporadic cases observed in BWS, 20% are the result of paternal 11p15 uniparental disomy (Weksberg *et al.,* 2003; Cooper *et al.,* 2005). Other sporadic molecular defects include duplications, translocations, and inversions of 11p15.5 with each accounting for ~1% of all cases (Weksberg *et al.,* 2003; Cooper *et al.,* 2005; Weksberg *et al.,* 2010).

The majority (50-60%) of BWS individuals do not present with genetic mutations but rather with epimutations at the KvDMR1 and/or *H19/IGF2* ICR making this syndrome mainly epigenetic in nature (Weksberg *et al.,* 2003; Weksberg *et al.,* 2010). The LOM at the KvDMR1 on the maternal allele is the most common (50%) epigenetic defect found in patients with BWS (Weksberg *et al.,* 2003; Sparago *et al.,* 2007; Weksberg *et al.,* 2010). This LOM results in aberrant expression of *KCNQ1OT1* from the maternal allele resulting in bidirectional silencing of the maternally-expressed flanking imprinted genes. An imprinted gene that is often silenced as a result of *KCNQ1OT1*'s biallelic expression is the maternally-expressed gene *CDKN1C* (Choufani *et al.,* 2010). *CDKN1C* is a cyclin-dependent kinase inhibitor that acts by negatively regulating the cell cycle. The most prevalent phenotypes for LOM at the KvDMR1 are exomphalos and hemihyperplasia (DeBaun *et al.,* 2003; Weksberg *et al.,* 2003; Choufani *et al.,* 2010; Weksberg *et al.,* 2010).

The gain of methylation on the maternal allele at the *H19/IGF2* domain results in *H19* repression from the maternal allele and biallelic expression of *IGF2.* This epigenetic misregulation is found in 2-10% of BWS patients (Sparago *et al.,* 2007; Weksberg *et al.,* 2010). This epimutation results in an estimated tumor risk of 35-45% as well as hemihyperplasia (DeBaun *et al.,* 2003; Rump *et al.,* 2005; Weksberg *et al.,* 2010).

Recently, BWS has been correlated with LOM at other imprinted loci (*i.e. MEST, PLAGL1/HYMAI, GRB10;* Rossignol *et al.,* 2006; Bliek *et al.,* 2009b; Lim *et al.,* 2009). The clinical features depend on the magnitude of LOM and how many imprinted loci are affected. Patients that had LOM *at HYMAI/PLAGL1, MEST, GRB10*, and KvDMR1,

generally had smaller birthweight, decreased frequency for hemihyperplasia and nevus flammeus but instead were premature and had hearing problems, feeding difficulties, apnea, and speech problems (Bliek *et al.,* 2009b). Continued investigation into these loci will be required to determine how LOI at these loci may affect BWS phenotype.

BWS-associated imprinted genes

As mentioned before, BWS is an epigenetic disorder that is associated primarily with the misregulation of imprinted genes found in the IC1 and IC2 of chromosome 11p15.5 (reviewed in Cohen, 2005; Cooper *et al.,* 2005; Choufani *et al.,* 2010; Weksberg *et al.,* 2010). However, there are two other ICRs that have been found to be misregulated in a few BWS patients, namely the *HYMAI/PLAGL1* ICR and *MEST* ICR which are located on chromosome 6q24.2 and 7q32, respectively (Bliek *et al.,* 2009b; Lim *et al.,* 2009). Simultaneous LOM at the KvDMR1 on the maternal allele was observed when *HYMAI/PLAGL1* and *MEST* were hypomethylated (Rossignol *et al.,* 2006; Bliek *et al.,* 2009b; Lim *et al.,* 2009).

Imprinting Control Region 2

The centromeric imprinted cluster directed by the KvDMR1 is the most common (50%) misregulated loci in BWS patients (Lim *et al.,* 2009). In humans, the KvDMR1 is a ~2 kb CpG island containing 178 CpGs that is located within the tenth intron of the maternally-expressed gene *KCNQ1* (Beatty *et al.,* 2006; Geuns *et al.,* 2007). The KvDMR1 acts as the ICR for a 1 Mb imprinted cluster of genes that contains six

maternally-expressed genes (*KCNQ1, CDKN1C, PHLDA2, SLC22A18, CD81, ASCL2*) and one paternally-expressed gene (*KCNQ1OT1*; reviewed in Maher and Reik, 2000; Weksberg *et al.,* 2003; Geuns *et al.,* 2007). The KvDMR1 is also the promoter for the paternally-expressed antisense ncRNA *KCNQ1OT1* (Mancini-DiNardo *et al.,* 2003). The genes that have been reported to be associated with BWS within this cluster are *KCNQ1, CDKN1C, PHLDA2, and KCNQ1OT1* (Weksberg *et al.,* 2003).

KCNQ1OT1- Potassium voltage gated channel 1 overlapping transcript 1, is a paternally-expressed ncRNA that is transcribed antisense to *KCNQ1* (Mitsuya *et al.,* 1999). Studies in the mouse have shown that *KCNQ1OT1*'s expression begins at the two cell stage and continues throughout the blastocyst stage (Lewis *et al.,* 2006). *KCNQ1OT1* is expressed in a lineage specific manner and has higher expression in the placenta than in the liver (Pandey *et al.,* 2008). Monoallelic paternal expression of *KCNQ1OT1* at ED 14.5 in mice was observed in the following tissues: lung, gut, heart, and kidney (Mancini-DiNardo *et al.,* 2003). Paternal allelic expression was observed in the following adult mouse tissues: brain, skeletal muscle, spleen, liver, heart, kidney, placenta, and lung (Yatuski *et al.,* 2002). In human, paternal expression was detected in the fetal liver, heart, spleen, cerebrum, muscle, lung, and thymus (Mancini-DiNardo *et al.,* 2003).

KCNQ1- Potassium voltage gated channel 1 is a six transmembrane potassium voltage gated channel (Lee *et al.,* 1997). It functions as a slow activating cardiac potassium channel in cardiac myocytes and loss of function of this gene leads to a

reduction in the repolarization of action potentials (Yamagata *et al.,* 2011). Mutations associated with *KCNQ1* are associated with cardiac arrhythmia syndromes (Weksberg *et al.,* 2005). *KCNQ1* is monoallelically expressed in mice in ED 15 fetal tissues: tongue, heart, lung, liver, kidney, placenta but biallelically expressed in fetal brain along with most neonatal tissues (Paulsen *et al.,* 1998). In contrast to mice, humans show monoallelic expression in lung, kidney, liver, gut, trachea, limb, but not the heart which was biallelically expressed in fetal tissue (Lee *et al.,* 1997). It is possible that there are two isoforms of *KCNQ1* in which isoform 2 is biallelically expressed only in cardiac tissue while isoform 1 is monoallelically expressed in all other fetal tissues (Lee *et al.,* 1997).

CDKN1C- Cyclin-dependent kinase inhibitor is a maternally-expressed cell cycle suppressor gene (reviewed in Maher and Reik, 2000). It functions as a putative tumor suppressor gene (Higashimoto *et al.,* 2005; Larson *et al.,* 2008). The *CDKN1C* DMR is located approximately 500 kb from the start of transcription and extends 1.5 kb through exon two (Bhogal *et al.,* 2004; Cerrato *et al.,* 2005). Contrary to what has been reported for mice, no differential methylation is observed for *CDKN1C* in humans (Chung *et al.,* 1996). A reason for the differences in methylation between mice and humans at the *CDKN1C* gene could be because of the abundance of H3K9me2 found on the paternally methylated allele in mice (Higashimoto *et al.,* 2006). The presence of the repressive histone modification, H3K9me2, on the paternal allele in mice could possibly signal the DNMTs to *de novo* methylate the locus resulting in the difference observed between species.

The expression of *CDKN1C* is found to be imprinted in the kidney, brain, lung, heart, skeletal muscle, eye, and liver in the fetal, neonatal, and adult mouse (Matsuoka *et al.,* 1995; Mancini-DiNardo *et al.,* 2003). Expression has also been detected in humans in the liver and kidney (Algar *et al.,* 1999). In addition, a study by Monk *et al.* (2006) found human placenta to have monoallelic expression of this gene. *CDKN1C* deficient mouse embryos show placental overgrowth as compared to wild type controls suggesting that the product of this gene also functions to control placental development (Takahashi *et al.,* 2000).

CDKN1C is biallelically silenced in 50% of BWS patients as a result of the biallelic expression of *KCNQ1OT1* (Weksberg *et al.,* 2003; Diaz-Meyer *et al.,* 2005). The misregulation of *CDKN1C* has been observed in pediatric cancers such as rhabdomyosarcoma and hepatoblastoma as well as in breast cancer (Weksberg *et al.,* 2003; Larson *et al.,* 2008). Lastly, *CDKN1C* misregulation is also implicated in the omphalocele phenotype exhibited in many BWS patients (Diaz-Meyer *et al.,* 2005; Weksberg *et al.,* 2010).

PHLDA2- Pleckstrin homology-like domain family A member 2 (also known as imprinted in liver and placenta*; IPL*) is a maternally-expressed imprinted gene. *PHLDA2* functions to regulate placental growth (Salas *et al.,* 2004). The PH-domain of *PHLDA2* is able to bind to phosphoinositides and function in multiple cell signaling pathways necessary for cytoskeletal regulation and intracellular vesicular transport (Salas *et al.,* 2004). A study by Yatsuki *et al.* (2002) showed that in the adult mouse kidney and

embryo both parental alleles remained hypomethylated in *PHLDA2* showing that at least for the mouse there is no DMR for this gene.

Monoallelic expression of *PHLDA2* is highly detectable in human placenta although the chorioamnionic membrane moderately expresses this gene (Qian *et al.,* 1997; Monk *et al.,* 2006). Northern blot analysis detected *PHLDA2* expression in human fetal kidney, lung, and liver as well as in the adult kidney, liver, and pancreas (Qian *et al.,* 1997). In the mouse, expression was highest in the yolk sac membranes followed by the placenta (Qian *et al.,* 1997). Variable tissues expression for *PHLDA2* has been observed in murine fetal lung, liver, kidney, and limb, as well as in the adult kidney (Qian *et al.,* 1997). *PHLDA2* knockout mice exhibit placental overgrowth (Frank *et al.,* 2002).

Imprinting Control Region 1

The *H19/IGF2* ICR domain on chromosome 11p15.5 is found to be misregulated in 2-10% of BWS patients (Sparago *et al.,* 2007; Weksberg *et al.,* 2010). This misregulation is the result of gain of methylation of the *H19/IGF2* ICR on the maternal allele (Cerrato *et al.,* 2008). Epimutation at this ICR is associated with an estimated tumor risk of 35-45% in BWS individuals (DeBaun *et al.,* 2003; Rump *et al.,* 2005; Weksberg *et al.,* 2010).

H19- is a maternally-expressed ncRNA with elusive function, however studies show that it may act as a tumor suppressor (Hao *et al.,* 1993). *H19* is monoallelically expressed from endodermal and mesodermal tissues during fetal mouse development

(Gabory *et al.,* 2006). In humans, *H19* is monoallelically expressed in fetal kidney, cardiac and skeletal muscle, lung, liver, adrenal gland, spleen, thymus, tongue, placenta and conflicting studies show differences of expression in the fetal brain where both biallelic and monoallelic expression has been detected (Zhang *et al.,* 1992; Kalscheuer *et al.,* 1993; Ulaner *et al.,* 2003). *H19's* expression in most tissues stops shortly after birth however it is detected in human adult kidney and liver (Kalscheuer *et al.,* 1993; Ekstrom *et al.,* 1995) as well as in skeletal and cardiac muscle in adult mice (Pachnis *et al.,* 1984).

IGF2- Insulin like-growth factor 2 is a paternally-expressed gene. It encodes a fetal growth factor and acts as a regulator of metabolism in adults (Jelinic and Shaw, 2007). In mice the following fetal and postnatal tissues are monoallelically expressed: liver, heart, kidney, muscle, and tongue (Weber *et al.,* 2001) while being biallelically expressed in the leptomeninges and choroid plexus of the brain (Dechiara *et al.,* 1991). In humans, monoallelic paternal expression of *IGF2* has been observed in human fetal liver, tongue, skin, lung, brain, kidney, muscle, and placenta as well as in adult kidney (Kalscheuer *et al.,* 1993; Weksberg *et al.,* 1993; Zhang *et al.,* 1994). In addition, a study by Esktrom *et al.* (1995) showed that *IGF2* is biallelically expressed in the human liver from 18 months of age throughout adulthood. In BWS patients biallelic expression of *IGF2* was observed in the tongue and in fibroblasts (Weksberg *et al.,* 1993).

Other BWS-Associated Imprinting Control Regions - *HYMAI/PLAGL1*

HYMAI/PLAGL1 ICR is located on chromosome 6q24 (Bliek *et al.,* 2009b; Lim *et al.,* 2009). The *HYMAI/PLAGL1* ICR is a CpG island that starts 500 bp upstream from the start of transcription of *HYMAI* and extends through exon 1 of *HYMAI*; it also overlaps with exon one of *PLAGL1* (Arima *et al.,* 2005; Arima and Wake, 2006; Valleley *et al.,* 2007). Nine BWS naturally and ART-conceived individuals have been reported to have LOM at the *HYMAI/PLAGL1* ICR and KvDMR1 (Bliek *et al.,* 2009b; Lim *et al.,* 2009).

***HYMAI*-** Hydatidiform mole associated and imprinted gene is a ncRNA whose function is unknown, however it is associated with transient neonatal diabetes mellitus (Docherty *et al.,* 2010). *HYMAI* is paternally-expressed and found on 6q24 in humans. *HYMAI* is expressed in mouse liver, sclerotome, telencephalon, and placenta (Arima *et al.,* 2001). Transgenic mouse embryos generated with the human *HYMAI* gene expressed in the brain, pancreas, pituitary, adrenal gland, with less detection in liver (Ma *et al.,* 2004).

PLAGL1- Pleomorphic adenoma gene like-1 is a zinc finger protein that can function as a tumor suppressor and induce apoptosis (Arima *et al.,* 2005; Valleley *et al.,* 2007). In a study by Arima *et al.* (2005) it was determined that *PLAGL1* is expressed similarly to *CDKN1C* in many tissues. This study also showed that *PLAGL1* binds to the unmethylated promoter region of *KCNQ1OT1* (*i.e.* KvDMR1) and acts as a transcriptional

activator increasing *KCNQ1OT1*'s expression. *KCNQ1OT1* can silence *CDKN1C* and this suggest that *PLAGL1* may indirectly regulate *CDKN1C* (Arima *et al.,* 2005).

This paternally imprinted gene is expressed in the following human fetal tissues; placenta, kidney, muscle, lung, heart, adrenal gland, and spinal cord (Valleley *et al.,* 2007). In the mouse *PLAGL1* expression is seen in the: heart, lung, brain, ovary, and pituitary but biallelic expression in the liver as well as kidney and skeletal muscle (Valleley *et al.,* 2007).

Other BWS-Associated Imprinting Control Regions - *MEST* Domain

The *MEST* ICR is located on human chromosome 7q32 (Rossignol *et al.,* 2006). The CpG island acting as the ICR overlaps the promoter region and exon one of *MEST* (Imamura *et al.,* 2005). Thirteen BWS patients that have been conceived both naturally and by ART had maternal LOM at this ICR (Rossignol *et al.,* 2006; Bliek *et al.,* 2009b; Lim *et al.,* 2009).

MEST- Mesoderm specific transcript homolog is paternally-expressed. *The MEST* DMR is maternally methylated in oocytes and unmethylated in spermatogonia (Imamura *et al.,* 2005). Differential methylation was observed in two-cell embryos, blastocyts, and somatic cells (Imamura *et al.,* 2005). In mice, LOM on the maternal allele leads to the biallelic expression of *MEST* and results in increased fetal growth and visceromegaly (Shi *et al.,* 2004).

MEST is expressed in an isoform-specific manner in which isoform 1a is biallelically expressed (except in the placenta). Six kilobases upstream of 1a is isoform (1c) which is paternally-expressed (maternally imprinted) in both humans and mice (Nakabayashi *et al.,* 2002; McMinn *et al.,* 2006). In humans there is also a rare isoform, 1b, which has a single expressed sequence tag (EST) in Genbank that is located ~100 bp upstream from 1c (McMinn *et al.,* 2006). The 1c isoform of *MEST* is monoallelically expressed in human fetal brain, eye, liver, intestine, lung, skin, spinal cord, stomach, skeletal muscle, kidney, tongue, adrenal gland, heart, and placenta (Nakabayashi *et al.,* 2002; McMinn *et al.,* 2006).

Embryonic tumor susceptibility

Children with BWS have an increased susceptibility (4-21%) of having either malignant or benign embryonic tumors develop within the first five years of age (Weksberg *et al.,* 2003; Rump *et al.,* 2005; Choufani *et al.,* 2010). The most common embryonic malignancies that occur in BWS patients are Wilms' tumor of the kidney (67% of tumor cases), hepatoblastoma, rhabdomyosarcoma, neuroblastoma, and adrenocortical carcinoma (Weksberg *et al.,* 2003; Cohen, 2005; Rump *et al.,* 2005). There are several factors that increase the risk of developing embryonic tumors such as hemihyperplasia, nephromegaly, and nephrogenic rests (Weksberg *et al.,* 2005).

A retrospective meta-analysis study by Rump *et al.* (2005) determined the molecular defects associated with specific tumor development in patients with BWS. Their study showed that gain of methylation at the *H19/IGF2* ICR leads to a 43%

increased risk of embryonic tumors. Wilms' tumors in particular, were only detected in patients with LOI at the *H19/IGF2* locus or a combination of LOI at both the *H19/IGF2* and KvDMR1 domains (Rump *et al.,* 2005).

There is an overall higher incidence of embryonic tumor development associated with gain of methylation on the maternal allele at *H19/IGF2* than at the KvDMR1 locus (Bliek *et al.,* 2001). If patients have LOI at both the *H19/IGF2* and KvDMR1 domains they have a 28% susceptibility to developing embryonic tumors (Rump *et al.,* 2005). Rump *et al.* (2005) also concluded that patients with LOM only at the KvDMR1 had a three percent risk of developing embryonic tumors (Rump *et al.,* 2005). The specific types of embryonic tumors associated with this locus are hepatoblastoma and rhabdomyosarcoma (Rump *et al.,* 2005; Choufani *et al.,* 2010).

ASSISTED REPRODUCTIVE TECHNOLOGIES (ART)

Background on ART

Assisted reproductive technologies (ART) are used primarily to improve a woman's ability to conceive a child. The United States' Centers for Disease Control and Prevention 2008 report (CDC, 2010) showed that in the United States 10% of women of reproductive age had sought the help of infertility clinics in 2002. In developed countries, 0.1-5% of all children born each year are conceived with the use of ART procedures (Waldman, 2006; Andersen *et al.,* 2009; Manipalviratn *et al.,* 2009). Since

Louise Brown was born in 1978, estimates place the number of ART-conceived children worldwide at four million (Dondorp and Wert *et al.,* 2011).

Commonly used ART procedures include: ovarian hyperstimulation, *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI), embryo culture, and embryo transfer. Ovarian hyperstimulation is defined as pharmacological treatment used to stimulate the ovaries to obtain multiple oocytes (Zegers-Hochschild *et al.,* 2009). The CDC defines IVF as retrieving a woman's eggs, fertilizing the eggs in a laboratory, and transferring the resulting embryos back into the woman's uterus (CDC, 2010). ICSI is a specialized form of IVF where a single sperm is directly injected into the woman's egg (CDC, 2010). Embryo culture is the growing of potential embryos for two or more days in culture medium (CDC, 2010). Embryo transfer is the last procedure in which one or more embryos are placed into the woman's uterus or fallopian tubes.

It should be noted that in our lab we consider ovarian hyperstimulation to be an ART procedure even though this procedure is not considered an ART procedure by the Centers for Disease Control and Prevention (2010).

Adverse Outcomes of ART

Several retrospective studies have cautioned of an increased likelihood of developmental abnormalities in children conceived by the use of ART (Bergh *et al.,* 1999; Hansen *et al.,* 2002; Hvidtjorn *et al.,* 2006). For example, Bergh *et al.* (1999) compared the incidence of congenital malformations in infants conceived by IVF between 1982-95 and compared that to the frequency in the naturally-conceived

population. That study found that children conceived by IVF had an increased incidence (2.4-12.9%) of anencephaly, hydrocephalus, esophageal atresia, and spina bifida (Bergh *et al.,* 1999).

In another study, Hansen *et al.* (2002) compared naturally-conceived infants to those conceived by IVF and ICSI during 1993-1997 in Western Australia. Their results showed that infants conceived by ART were twice as likely to have a major birth defect (*i.e.* renal, musculoskeletal, and chromosomal defects) detected by one year of age than naturally-conceived infants (Hansen *et al.,* 2002). That study also determined that infants conceived by IVF and not ICSI had a higher risk of developing cardiovascular and urogenital defects (Hansen *et al.,* 2002). Lastly, a study performed at the University of Iowa in which 1,462 children conceived by IVF between 1989-2002 were compared to 8,422 naturally-conceived children found that the incidence of cardiovascular and musculoskeletal defects and syndromes were higher among IVF children than the control group (Olson *et al.,* 2005).

In addition, there have been several studies that suggest that ART may adversely affect the epigenetic regulation of genes (Cox *et al.,* 2002; Halliday *et al.,* 2004). Two LOI syndromes that have been documented to be associated with ART are BWS and AS. Loss of methylation at the *SNRPN* locus on the maternal allele accounts for only five percent of all AS cases (*i.e.* the incidence of LOM is 1 in 300,000 births; Cox *et al.,* 2002). However, Cox *et al.* (2002) found two children conceived by ICSI that were diagnosed with AS and both had loss of methylation on the maternal *SNRPN* locus. Another ART-conceived child was added to this report by Orstavik *et al.* (2003). Therefore, it has

been proposed that ICSI may perturb methylation of the maternal allele at the *SNRPN* locus (Cox *et al.,* 2002). The most often observed ART-induced LOI syndrome in humans is BWS. The association between ART and BWS will be addressed in the following section.

However, not all studies have found adverse outcomes associated with ART. A Danish study, concluded that the increased likelihood of cerebral palsy observed in IVF-conceived children between 1995-2000 may have been due to the fact that IVF children have a higher risk of being born prematurely (Hvidtjorn *et al.,* 2006). Furthermore, Bowdin *et al.* (2007) failed to find an increased risk of BWS or AS in a cohort of ART-conceived children. The study included 47 children that had been conceived by ART in the United Kingdom between 1989 and 2003.

BWS and ART associations

Children that are conceived by the use of ART have a higher incidence (3-9 times) of having the loss-of-imprinting overgrowth syndrome BWS (DeBaun *et al.,* 2003; Gicquel *et al.,* 2003; Maher *et al.,* 2003; Halliday *et al.,* 2004; Sutcliffe *et al.,* 2006; Lim *et al.,* 2009). A retrospective study of ART-conceived children born from 1995-2000 in the United Kingdom found that four percent of all the BWS cases included in the study were conceived by IVF or ICSI (Maher *et al.,* 2003). A study in the United States by DeBaun *et al.* (2003) found a six fold increased likelihood of BWS in children conceived by ART when compared to non-ART counterparts. In their study, five of the six ART-related

BWS patients had aberrant methylation at either KvDMR1 or both KvDMR1 and *H19/IGF2* (DeBaun *et al.,* 2003).

In the Gicquel study (2003) in France, they determined the epigenetic status of the KvDMR1 in 149 BWS patients. Six of these patients (4%) were born from various ART procedures and all had loss of methylation on the maternal allele at the KvDMR1. In that study, the prevalence of BWS in ART-conceived children was threefold higher than in non-ART-conceived infants (Gicquel *et al.,* 2003). The fourth study (Halliday *et al.,* 2004) compared 37 BWS cases in Australia that were born from 1983-2003. Four of the 37 children were conceived by ART. In that study, the likelihood of having BWS if conceived by ART was nine times greater than in naturally-conceived infants. Sutcliffe *et al.* (2006) found that there was an approximate threefold increase in BWS in children conceived by ART. That study also showed that all of the eight ART-conceived BWS children had LOM at the KvDMR1.

A recent study from the United Kingdom by Lim *et al.* (2009) compared 25 BWS children conceived by IVF or ICSI to 87 non-ART BWS conceived children that had LOM at the KvDMR1. Of the ART-conceived BWS cases 24 out the 25 had LOM at the KvDMR1 (Lim *et al.,* 2009). The ART-conceived group was more prone to facial nevus flammeus and less prone to having exomphalos when compare to the non-ART group (Lim *et al.,* 2009). None of the non-ART children had development of embryonic tumors compared to two of children in the ART-conceived group who developed hepatoblastoma or rhabdomyosarcoma by one year of age (Lim *et al.,* 2009). Lim *et al.* (2009) also analyzed other imprinted loci for possible LOM and found that three children

from the BWS ART group had LOM at two other loci (*i.e. MEST* and *SNRPN,* or *PLAGL1* and *MEST*) whereas three non-ART BWS children had LOM only at single loci (*i.e. MEST* or *PLAGL1*).

As explained in the loss-of-imprinting syndrome in humans section, LOM at the KvDMR1 on the maternal allele accounts for 50% of cases in the naturally-conceived BWS population. However, in the aforementioned studies, the majority (83-100%) of BWS ART-conceived patients presented this epimutation. There are many unanswered questions that still need to be resolved in order to conclude that ART is the culprit in the increased incidence of LOI syndromes in humans. Perhaps the most pressing is whether or not the infertility problem of the parents is the reason for the epimutation that results in the discussed syndromes.

Though it is possible that infertility may add to the incidence of BWS, a very recent case report (Kuentz *et al.,* 2011) still points at ART as responsible for the previously reported adverse outcome. In that case a human immunodeficiency virus (HIV) serodiscordant couple needed to use IVF to help conceive a child without the risk of HIV. The mother had previously conceived and was not infertile and the father had correct sperm morphology, indicating that no previous infertility problems existed (Kuentz *et al.,* 2011). Both hormonal stimulation and embryo manipulation under IVF protocols were used and during ultrasound exams exomphalos, macrosomia, and macroglossia were discovered indicating BWS phenotype. BWS was confirmed after birth as the child has visceromegaly, hypoglycemia, bilateral inguinal hernia, and DNA testing revealed LOM at the KvDMR1.

Ruminant fetal overgrowth syndrome

Large offspring syndrome (LOS) is an overgrowth phenotype that has been observed in bovine and ovine fetuses and offspring that had been cultured during their preimplantation development (Young *et al.,* 1998). The phenotypical features of LOS are: significant increase in birth weight, visceromegaly (specifically heart and liver), breathing difficulties, skeletal defects, hypoglycemia, polyhydramnios, difficulty suckling and perinatal death (Farin and Farin , 1995; Young *et al.,* 1998; Sangild *et al.,* 2000; Bertolini and Anderson, 2001; Hiendleder *et al.,* 2004; Farin *et al.,* 2006).

Ruminant blastocysts that have been cultured with serum or somatic cells are darker in appearance due to excess lipid accumulation in the cytoplasm which is increased in the presence of serum (Sinclair *et al.,* 2000). These cultured embryos typically have fewer cells distributed to the inner cell mass and undergo cytoplasmic fragmentation (Sinclair *et al.,* 2000).

Many studies have reported the adverse outcome as a result of ruminant embryo culture and have concluded that serum supplementation and/or co-culture with somatic cells is responsible for the overgrowth phenotype (Young *et al.,* 1998). One example is a study by Hiendleder *et al.* (2004) in which Day 80 fetuses produced from somatic cell nuclear transfer (SCNT) or IVF had significantly increased fetal weight, liver weight, and thorax circumference than their control counterparts (fetuses produced from artificial insemination).

Smith *et al.* (2009) did a microarray study to determine if global bovine gene expression is altered by *in vitro* production (IVP; *i.e. in vitro* maturation, IVF, and embryo culture) or if it is the culture of embryos (*i.e. in vivo* derived and cultured; IVD) that causes misregulation of genes that could potentially lead to LOS. Their results show that both the IVF and IVD groups had significant differences in gene expression when compared to control embryos (*in vivo*-produced after artificial insemination). They concluded that *in vitro* culture alone can affect gene expression levels (Smith *et al.,* 2009). Further, in that study the imprinted genes *CDKN1C* and *CD81* were upregulated in IVD embryos. These genes are located on the BWS locus in humans. However, DNMT3A mRNA levels were lower in IVP embryos than in the IVD and control embryos (Smith *et al.,* 2009). It could be speculated that the downregulation of DNMT3A could result in misregulation of genes by causing loss of *de novo* DNA methylation.

Also, an association between embryo culture and increased levels of *IGF2* have been reported in cattle (Blondin *et al.,* 2000). Blondin *et al.* (2000) analyzed *IGF2* messenger RNA (mRNA) levels of liver and skeletal muscle from Day 70 *in vitro*-produced (IVP) embryos cultured in the presence of serum, IVP serum restricted to *in vivo*-produced embryos from superovulated cows. In both IVP groups the male fetuses expressed *IGF2* 1.8-2.4 fold higher than the *in vivo*-produced male fetuses (Blondin *et al.,* 2000). The opposite was true in skeletal muscle in which the *in vivo*-produced group had higher levels of *IGF2* than the IVP serum restricted group but neither group was significantly different from the IVP serum group (Blondin *et al.,* 2000).

ADVANTAGES OF USING THE BOVINE AS AN ANIMAL MODEL TO STUDY BWS

Currently, there are no animal models that recapitulate the overgrowth phenotype of BWS. Murine knockout models for BWS have been unable to display all of the primary features observed in BWS patients (Caspary *et al.,* 1999).

There are several reasons to believe that bovids can serve as a good model to study the human overgrowth syndrome BWS. First, the bovine LOS exhibits many of the same features seen in BWS patients such as macrosomia, increased birthweight, hypoglycemia, visceromegaly, polyhydramnios, and difficulty breathing as well as misregulation of *IGF2* (Farin and Farin, 1995; Young *et al.,* 1998; Sangild *et al.,* 2000; Bertolini and Anderson, 2001; reviewed in McEvoy *et al.,* 2001; Hiendleder *et al.,* 2004; Farin *et al.,* 2006). Second, both infants conceived through ART and *in vitro*-produced bovine offspring display similar overgrowth phenotypes as the result of minimal ART procedures (Young *et al.,* 1998; reviewed in McEvoy *et al.,* 2001; Lazzari *et al.,* 2002, DeBaun *et al.,* 2003; Gicquel *et al.,* 2003; Halliday *et al.,* 2004; Farin *et al.,* 2006). Third, the bovine (unlike mice) is a non-litter bearing species and shares a similar gestation length of nine months with humans. Finally, comparative analysis of the murine, bovine and human genomes have revealed a more similar association between human and bovine (73.8%) than that of human to mouse (66.8%; Miziara *et al.,* 2004; Khatib *et al.,* 2007; Miller *et al.,* 2007).

RATIONALE FOR THESIS

Assisted reproductive technologies (ART) account for 0.1-5% of live births in developed countries throughout the world. Several studies have suggested that minimal manipulation of gametes (*i.e.* hormonal stimulation, embryo culture, embryo transfer) can alter DNA methylation and transcription of imprinted and non-imprinted genes.

Genomic imprinting is an epigenetic modification that directs parent-specific gene expression. Studies have shown that imprinted genes regulate the growth and development of the conceptus. Beckwith-Wiedemann syndrome (BWS) is a loss-of-imprinting overgrowth condition in humans that occurs primarily as a result of misregulation of two clusters of imprinted genes on locus 11p15.

Several retrospective studies have analyzed the likelihood of adverse outcomes associated with ART. ART-conceived infants appear to have an increased risk for developing major birth defects and syndromes. In particular these studies have revealed that there is a 3-9% increase incidence of BWS in ART-conceived infants. In addition, BWS infants conceived by ART have a higher incidence of loss of methylation at the KvDMR1 than naturally-conceived individuals. This suggests that the ART procedures are interfering with the epigenetic program of embryos.

Currently there are no animal models that can recapitulate the human overgrowth phenotype that is associated with BWS. Ruminants have an ART-related overgrowth syndrome (*i.e.* LOS) that phenocopies BWS. We hypothesize that LOS is

epigenetically similar to BWS and that it results from misregulation of one or more clusters of imprinted genes, specifically the cluster of imprinted genes regulated by the KvDMR1.

The research conducted for this thesis determined baseline methylation and allele-specific expression in day 65 bovine fetuses of imprinted loci known to be misregulated in BWS as a result of minimal ART procedures. The works completed for this thesis are the initial steps taken to determine if the bovine will serve as a good model to study BWS.

CHAPTER II

Procedures and Techniques used to develop imprinted gene expression and DNA methylation assays.

INTRODUCTION

This chapter is designed to provide the reader a quick reference for terms frequently used in this thesis. In addition, detailed explanations of the techniques and procedures used to determine gene expression and DNA methylation at BWS-associated imprinted loci will be provided.

DEFINITIONS

1) **Accession Number-** is a specific code given to a sequence once it has been submitted to a database (*i.e*. NW_001494547.3; NCBI Handbook, 2002).
2) **ApE-** A plasmid Editor is a freely available program that is used to work with DNA sequences and find restriction enzyme sites within the sequence (Davis, 2011).
3) **Bisulfite Mutagenesis –** During the bisulfite mutagenesis procedure unmethylated cytosines will get converted to uracil while the methylated cytosines will remain unchanged. During PCR, the uracils will be substituted by thymine.

4) **BLAST-** Basic local alignment search tool is an NCBI (NCBI Handbook, 2002) nucleotide query database that allows to search for similar nucleotide sequences among a given genome (*e.g.* bovine) based on specific set of algorithms.
5) **BLAT**- Blast-like alignment tool is the UCSC DNA database query that allows searching for similar sequences of DNA that are 25 nucleotides or longer within the entire genome of an organism (Kent *et al.,* 2002).
6) **COBRA-** Combined bisulfite restriction analysis involves the conversion of genomic DNA into bisulfite-mutagenized DNA. After the bisulfite-converted DNA is amplified by PCR one of the parental ICRs will contain cytosines within its sequence (*i.e.* the methylated allele) while the unmethylated allele will lack cytosines making the two parental DNA sequences different. The PCR product of the bisulfite amplicon can be digested with a restriction enzyme that has a specific recognition site for the methylated allele due to the presence of cytosines within the sequence. Polyacrylamide gel electrophoresis (PAGE) is then performed to determine the methylation status of a region.
7) **Ensembl -** genome database of various vertebrate species, by the joint partnership between the European Bioinformatics Institute, the European Molecular Biology Laboratory (EMBL), and the Wellcome Trust Sanger Institute (http://www.ensembl.org/index.html).
8) **ESTs-** Expressed sequence tags are short sequence reads of cDNA. The NCBI BLAST database (2002) can be used to compare the transcript of interest against their EST database to see if similar regions have been previously sequenced.

9) **Informative Polymorphism -** for the purpose of our research is defined as a difference in DNA sequence that remains unchanged after bisulfite conversion (*e.g.* any nucleotide variation other than a T/C mutation). For example,

Informative Individual 1: **A**AT

Individual 2: **G**AT

Non-informative Individual 1: **T**AT

Individual 2: **C**AT

10) **In/del-** Insertion/deletion is a DNA sequence variation among individuals in which either a single or group of nucleotides are either added or removed from the sequence of an individual/breed/subspecies. For example,

Individual 1: GAG**CCC**TG

Individual 2: GAGTG

11) **MacVector-** is an application that can be used for multiple sequence alignments and sequence assembly to a reference genome.

12) **NCBI-** National Center for Biotechnology Information is a genome browser that contains the annotated collection of all publically available nucleotide sequences of various organisms (NCBI handbook, 2002).

13) **RepeatMasker-** will compare the DNA sequence of interest against a library of known repetitive elements and then return the DNA sequence back with the repeated sequences masked (Smit *et al.,* 2011).

14) **Restriction enzymes-** are bacterial endonucleases that recognize and cleave a specific sequence of nucleotides (called the restriction site).

15) **SNP-** Single nucleotide polymorphism is a single nucleotide sequence variation among individuals. For example,

Individual 1: **A**AT

Individual 2: **C**AT

16) **UCSC-** University of California Santa Cruz Genome Browser contains all reference and working draft sequencing assemblies of various organisms (Kent *et al.,* 2002).

17) **UMD 3.1-** University of Maryland 3.1 Build is a genome assembly for Bos taurus sequences (http://www.cbcb.umd.edu/research/bos_taurus_assembly.shtml).

PROCEDURES AND TECHNIQUES USED FOR THE IDENTIFICATION OF DNA SEQUENCE POLYMORPHISMS

Data Mining: Several public genome databases were used to help retrieve sequence information for annotated and non-annotated genes associated with BWS in the bovine. The databases used to generate genomic sequencing for genes commonly misregulated in BWS were the National Center for Biotechnology Information (NCBI Btau 4.2; NCBI handbook, 2002), University of California Santa Cruz Genome Browser (UCSC; Kent *et al.,* 2002), Ensembl Genome Browser

(http://www.ensembl.org/index.html), and University of Maryland 3.1 Build (UMD 3.1; http://www.cbcb.umd.edu/research/bos_taurus_assembly.shtml).

All misregulated genes associated with BWS in humans are annotated in NCBI (NCBI handbook, 2002), UCSC (Kent *et al.,* 2002), and Ensembl databases (http://www.ensembl.org/index.html). The following genes are annotated in the bovine genome in at least one of the four previously mentioned genome browsers: *H19, IGF2, KCNQ1, CDKN1C, PHLDA2, MEST,* and *PLAGL1*. Annotated bovine genes were compared for similarity to the human genome to make sure they associated to the correct imprinted region. The following regions were not annotated in the bovine KvDMR1*,* *KCNQ1OT1*, and *HYMAI*. For these genes and regions the annotated human reference genome was used to align to the bovine genome browser databases to find similar regions within the bovine genome.

After the sequences were retrieved from these freely available databases they were put into A plasmid Editor (ApE; Davis, 2011). Then, RepeatMasker (Smit *et al.,* 2011) was used to determine the portion of the DNA sequence that was composed of repetitive elements. Once the repetitive elements were identified and masked, polymerase chain reaction and sequencing primers were designed. Two primer programs were used to generate sequencing primers for the BWS associated genes; these were Primer3 (Rozen and Skaletsky, 2000) and Integrated DNA technologies (IDT) PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/). Primer parameters were selected based on annealing temperature (55-65 °C), temperature less

than (1.0-0.5 °C) of the annealing temperature between primer pairs, the length of the primers (18-30 bp), and product size (~200-1200 bp).

Gel Electrophoresis: In order to separate the different size fragments of template (DNA and cDNA) gel electrophoresis was used. This method uses an electric current to separate different sizes of template. Negatively charged DNA migrates to the positively charged electrode. The two types of gel electrophoresis matrices used were agarose and polyacrylamide.

Agarose gel electrophoresis was used when bands needed to be excised from the gel in order for DNA to be extracted. Typically one percent agarose gels (*e.g.* 0.5 grams of agarose mixed with 50ml of 1X Tris/Borate/EDTA; TBE) were used. Visualization of the nucleic acid was possible by the addition of ethidium bromide to the agarose gel. The PCR product was mixed (1:10 ratio) with 10X loading buffer (0.3% Xylene Cyanol and 0.3% Bromophenol Blue in a 65% sucrose solution containing 10mM Tris and 10mM EDTA) before loading into the gel. The gel was then analyzed by the use of a UV *wavelength* transilluminator.

Polyacrylamide gel electrophoresis (PAGE) was used when more precise resolving power was necessary (*e.g.* when determining size band and amount after PCR product restriction). Different percentages of PAGE gels were used based on the range of separation that was needed between base pairs (bp). For example, to separate between 35 bp a 20% PAGE was made whereas to separate between 400 bp a 7% PAGE was used. An example of the components used to make 30ml of a 18% polyacrylamide

gel cast are 13.5ml of a 40% acrylamide solution (acrylamide:bis-acrylamide, 29:1; Fischer BioReagents), 6ml of 5X TBE, 300µl of 10% Ammonium persulfate (Sigma), 30 µl Temed (Fischer BioReagents), with 10.17ml of water. The gel polymerized within an hour and the samples were loaded with loading dye as previously mentioned. After the gel ran it was put into a vessel containing approximately 0.1µg ethidium bromide solution. Then the gel was analyzed by the transilluminator and PCR amplicon sizes were determined.

DNA Isolation: A small piece of tissue (4-5mm) was cut and put into a 1.7ml microcentrifuge tube containing lysis buffer (50mM Tris-HCl, pH 8.0, 100mM EDTA, 0.5% sodium dodecyl sulfate supplemented with 20mg/ml proteinase K) and incubated overnight at 55°C.

DNA extraction was done using a standard phenol-chloroform extraction protocol. Briefly, after adding 500µl phenol-chloroform to the microcentrifuge tube containing the lysed tissue the tube was gently vortexed and centrifuged for five minutes. The aqueous layer was carefully removed and transferred to a newly labeled tube and the previous step was repeated again. Then, 500µl of 100% ethanol was added to the aqueous layer and vortexed until a precipitate was seen and then centrifuged again for five minutes. The ethanol was then aspirated and the pellet was washed with 70% ethanol (500µl) and centrifuged for one minute. Then, the ethanol was aspirated and the pellet air dried for ten minutes. The DNA was resuspended in Buffer PEB (10mM Tris-Cl, pH 8.5; PerfectPrep; 5’ Prime) and incubated at 65°C for two

hours or until the DNA was completely in solution. The concentration of DNA (in ng/µl) was determined by spectrophotometry (NanoDrop). The quality of the genomic DNA preparation was determined by running 1-2µg in an 80cm 0.7% agarose gel for ~5 hours. Once it was determined that the DNA was not degraded then it became template for sequencing and assay development.

DNA extraction of spermatozoa was performed with a commercially available kit (QIAamp DNA Micro Kit; Qiagen). The procedure was as follows; semen straws were thawed and the content was transferred to a 1.7ml microcentrifuge tube containing 500µl of PBS, proteinase K (20 mg/ml), and 1M Dithiothreitol (DTT). The tube was then centrifuged at low speed so that a spermatozoa pellet would form. The supernatant was discarded and 900µl of ATL buffer (from the kit) added to resuspend the pellet and the final resuspension was divided separately into three new microcentrifuge tubes. The tubes were placed on a 56°C hot block and vortexed every ten minutes for two hours.

After the two hour incubation period 300µl of Buffer AL was added to the samples and placed on 70°C hot block for ten minutes and the samples were vortexed every three minutes. Then the samples were centrifuged (20,000 x g) for one minute. The supernatant was then transferred to a QIAamp column followed by centrifugation (6,000 x g) for one minute. The column was then transferred to a new collection tube and 500µl Buffer AW1 was added and centrifuged (6,000 x g) for one minute. This step was repeated except that 500µl of Buffer AW2 was used. Then the membrane was dried after centrifuging (20,000 x g) for three minutes. The DNA was eluted in 20µl of

Buffer AE after a five minute incubation at room temperature. DNA was stored at -20°C or used immediately for assay testing.

Bisulfite Mutagenesis: The sodium bisulfite conversion of single stranded DNA involves several steps. The first step is to denature the double stranded DNA into single stranded DNA by heating. The next step of bisulfite mutagenesis is the sulfonation of unmethylated cytosines at the sixth carbon position (Eads and Laird, 2002). Then the fourth carbon position will be deaminated and become uracil sulfonate (Eads and Laird, 2002). The final step is desulfonation into uracil. It is not until amplification by PCR that the uracil will be replaced by a thymine. The methylated cytosines will not be converted during bisulfite mutagenesis and during PCR they will remain cytosines.

Bisulfite conversion of genomic DNA can be done using commercially available kits (*i.e.* Imprint Modification Kit; Sigma-Aldrich). I will briefly describe the method used to bisulfite mutagenize DNA. Five hundred nanograms of genomic DNA diluted in 10µl of water were converted for each sample at any given time. DNA modification solution (1.1ml) was added to the DNA modification powder and vortexed for two minutes until the solution was clear. Next, 40µl of balance solution was added to the DNA modification solution and briefly vortexed. For the rest of the procedure the tubes containing the DNA were covered in foil to protect from direct light. Then, 110µl of DNA modification solution was added to each of the 500ng DNA samples and were incubated at 99°C for six minutes followed by 65°C for 90 minutes using a thermal cycler.

The post modification DNA clean up protocol was followed and the DNA was purified through a spin column system. Three hundred microliters of DNA capture solution were added to the columns followed by the modified DNA solution containing the DNA and then centrifuged at 12,000 x g for one minute. Next, 200µl of ethanol-diluted cleaning solution were added to the spin columns and centrifuged at 12,000 x g for one minute. Fifty microliters of ethanol balance wash solution were added to the columns and allowed to incubate for eight minutes before performing a one minute centrifugation at 12,000 x g. Two 90% ethanol washes were performed followed by one minute centrifugation steps (12,000 x g). The columns were then transferred to new microcentrifuge tubes and eluted in 20µl of water.

RNA Isolation: In order to perform gene expression assays RNA must first be extracted from the cells/tissues. Tissue samples from which we intended to isolate RNA from were frozen immediately upon collection either in liquid nitrogen or at -80°C to prevent RNA degradation. Tissues were kept on dry ice while working at the bench. I used a commercially available kit (*i.e.* High Pure RNA Tissue Kit; Roche) in order to isolate total RNA from the tissues. First, a small piece (3-4mm) of tissue was added to a 1.7ml microcentrifuge tube that contained 450µl of the kit's lysis binding buffer. Then the tissue was homogenized manually using a plastic pestle before passing through a 22 and then a 26 gauge needle several times and then centrifuged at maximum speed. Absolute ethanol was added to the microcentrifuge tube and centrifuged before transferring the entire volume to the kit's RNA binding columns. DNA contamination

was eliminated by adding 100µl of Dnase (10µl of DNase I; 10kU and 90µl of DNase Incubation Buffer; 1M NaCl, 20mM Tris-HCl, 10mM MnCl2, at pH 7.0) to the column. The RNA was then eluted in water, the concentration determined by spectrophotometry, and immediately stored at -80°C until cDNA synthesis.

cDNA Synthesis: Complementary DNA is DNA that has been transcribed from mature RNA by reverse transcriptase. The final concentration of master mix components for cDNA synthesis included: DTT (10mM; Invitrogen), First Strand buffer (1X; Invitrogen), deoxynucleoside triphosphates (dNTPs- 1mM each; Fischer), random primers (0.5 µg; Promega), Superscript II (reverse transcriptase- 100 units; Invitrogen), and RNaseOUT Ribonuclease Inhibitor (20 units; Fischer). Equal volumes of RNA in solution and cDNA master mix were mixed and incubated for one hour at 42°C followed by 10 minutes at 95°C. cDNA was stored at -20°C until use. In order to make sure there was no DNA contamination a "minus" reverse transcriptase (-RT) reaction was performed in which all components mentioned above were added to RNA except the Superscript II. Presence of a product after PCR would have indicated DNA contamination in the cDNA. When this occurred the original RNA tube underwent another DNAse step to get rid of any contamination before repeating the –RT step.

Polymerase Chain Reaction: PCR allows the amplification of DNA/cDNA sequences. Primer pairs used to amplify the region of interests were selected with the assistance of Primer3 (Rozen and Skaletsky, 2000) and IDT PrimerQuest software

programs (http://www.idtdna.com/Scitools/Applications/Primerquest/). The typical final concentrations of reagents in a PCR reaction included 1X Colorless Buffer (Promega), 4mM MgCl2 (Promega), .2mM each dNTP (Fischer), forward and reverse primers at 0.3µM each, and 1.25units of Go Taq Polymerase (Promega).

The PCR reaction takes place in a thermal cycler and involves an initial denaturation step of approximately two minutes in length followed by 25-40 cycles of repeated temperature changes. The initial denaturation step (94°C) is used to separate the double stranded DNA and denature the polymerase bound antibody. The following denaturation steps (94°C) separate the double stranded DNA into single strands creating a template for the primers to bind. During the annealing steps (temperature depends on the primers) the primers will bind to the DNA/cDNA sense or antisense strand. During the extension step (72°C), the DNA polymerase synthesizes the complement strand of DNA (5' to 3' direction) by base pairing dNTPs. After each cycle the number of copies is expected to double. PCR products were resolved by agarose gel electrophoresis or PAGE.

PCR assay optimization was often necessary due to lack of amplification (no bands on the gel) or amplification of multiple products during PCR (*i.e.* bands of the incorrect size were present on the gel). For the former, we tried to relax the PCR conditions by adding 5% dimethyl sulfoxide (DMSO) or 1M betaine. This was especially useful when trying to amplify difficult regions of DNA such as CpG islands. In the event that too many bands (or bands of the incorrect size) were present we tried to make the PCR conditions more stringent by adjusting to the $MgCl_2$ and primer concentrations as

well as changing the annealing temperatures of the primers. In addition, touchdown PCR reactions were used in which the annealing temperature was set five degrees above the original temperature and decreased by one degree each cycle until the optimal primer temperature was reached. If none of these steps were successful, new primers were designed and the procedures repeated.

If a PCR amplicon of the predicted size was present in an agarose gel it was excised from the gel using a clean blade in order to send for sequencing to verify that the correct region of DNA had been amplified. I used a commercially available kit (*i.e.* Promega Wizard SV Gel and PCR Clean-Up System) to extract the DNA from the gel slice.

Sequencing: All samples were sent to the University of Missouri DNA Core Facility to be sequenced. The sequencing results were given in the form of chromatographs or text files. Once the results came back they were downloaded into the sequencing alignment program MacVector. Sequencing was used to detect sequence differences between *Bos taurus indicus* and *Bos taurus taurus* subspecies of cattle (Chapter III). Sequencing was also used when trying to determine the methylated state of cytosines. For the latter, the bisulfite converted sequence was read and all CpGs annotated as to whether they were Ts (implying that that C was unmethylated in the tissue) or Cs (implying that the C was methylated in the tissue).

Bacteria Cloning: The bisulfite-converted DNA amplicons generated from the bisulfite PCR reactions were cloned into a vector system using a commercially available

kit (*i.e.* Promega T Easy Vector and NEB 5-alpha F competent *E. coli*; Copy Control cDNA, Gene & PCR Cloning Kit, EPICENTRE Biotechnologies). This was done in order to separate the PCR amplicons into independent parental alleles. The ligation reaction is set up according to protocol and includes the addition of the PCR product of interest, ligation buffer, vector, and T4 DNA ligase. The PCR product has a 3' A overhang added by the Taq Polymerase during the final PCR extension step that will base pair to the T overhang of the vector. Then DNA ligase forms phosphodiester bonds to stabilize the amplicon to the vector. Two types of competent cells namely TransforMax EPI300 *E. coli* (which are electrocompetent) or NEB 5-alpha F'I^q *E. coli* (which are chemically competent) were used depending on the region that was amplified. The reaction was then plated on antibiotic resistant plates to grow overnight according to the antibiotic selection within the vector (*e.g.* ampicillin, chloramphenicol). If the *lacZ* gene was present in the vector then blue and white colony selection was done. When necessary, we performed colony PCR to determine if the colony that grew had the complete insert and in some instances we sequenced this product. Plasmid isolation was done by using a mini-prep kit (*i.e.* PerfectPrep Spin Mini Kit, 5 PRIME). Plasmids were then sequenced, and the sequences aligned to a reference sequence (using MacVector) to determine methylation status of the amplicon.

Restriction Fragment Length Polymorphism: The ability of restriction enzymes to recognize specific sequences of DNA was used to differentiate parental contribution or methylation status of the amplicons after PCR. It is known that the methylated and

unmethylated alleles do not always amplify equally during PCR. Therefore, I used COBRA as a diagnostic test to determine if the PCR amplicons generated after bisulfite mutagenesis of DNA indeed contained both alleles. Then PAGE was performed to resolve the restricted products and determine if both alleles were being amplified at equal rates during PCR. Restriction enzymes were also used to differentiate between the parental alleles by using enzymes that would recognize either the *B. t. indicus* or *B. t. taurus'* sequence at the polymorphic site.

Single-Strand Conformation Polymorphism - SSCP is a technique used to separate by PAGE small PCR amplicons that contain difference/s in their sequence. SSCP is based on the principle that the mobility of single-stranded DNA in a non-denaturing gel depends on the primary DNA sequence. This procedure can be used to determine parental expression of imprinted genes between *B. t. indicus* and *B. t. taurus* when no restriction enzymes exists that recognize the polymorphism of interest. I have used the literature on SSCP analysis to generate PCR primers for expression assays for the two genes (*i.e. PHLDA2* and *IGF2*; Chapter III) where a restriction digestion will not be possible. The non-denaturing gel used for SSCP is highly dependent on size and SSCP is not able to distinguish between single mutations for amplicon sizes more than 300 bp (Fujita and Silver, 1994). All primers ordered for future SSCP expression assays were less than 300 bases and the genome browsers were used to compare with expressed sequence tags (ESTs) of the bovine to verify that these amplicons had been sequenced before.

CHAPTER III

Identification of DNA sequence polymorphisms between *Bos taurus indicus* and *Bos taurus taurus* and development of DNA methylation and gene expression assays for imprinted loci known to be misregulated in Beckwith-Wiedemann Syndrome

INTRODUCTION

In order to perform genomic imprinting studies we must be able to distinguish between the maternal and paternal alleles. In mice identification of the parental alleles is typically done by breeding animals of two inbreed strains and then studying the F1 offspring generated by the breeding. Known DNA sequence polymorphisms for each strain are then used to assign the expressed and silenced alleles. We are going to use subspecies of *Bos taurus* (*i.e. B. t. taurus and B. t. indicus*) to serve the same purpose.

B. t. taurus and *B. t. indicus* are subspecies of cattle that diverged from each other 620,000 years ago (MacHugh *et al.,* 1997). Studies have shown that single nucleotide polymorphisms should be found every 172 base pairs (bp) within the exon regions of genes between *B. t. taurus* and *B. t. indicus* (Heaton *et al.,* 2001; Taylor *et al.,* 2006). Therefore, we hypothesized that there would be sequence diversity between *B. t. taurus* and *B. t. indicus* at imprinted regions that we could use to be able to differentiate between the parental alleles at the DNA methylation and gene expression level.

ANIMAL IDENTIFICATION

Bull

The first step was to determine which *B. t. indicus* bull to use for our studies. The criteria used to select the bull were 1) commercial availability from suppliers (ABS Global Inc. and Select Sires), 2) successful pregnancy after artificial insemination and, 3) the ability to fertilize and produce embryos *in vitro*. A total of eight *B. t. indicus* bulls (7 Brahman and 1 Nelore) were commercially available for testing. Frozen semen from each of the bulls were sent to Dr. Jeremy Block (OvaTech-University of Florida) who performed *in vitro* production procedures (2 IVP runs/bull) using Holstein oocytes. Results showed that the Nelore bull produced the highest blastocyst rates (data not shown).

Dams

We decided that the dams of the F1 fetuses that would be used for the imprinting studies should be *B. t. taurus*. The rationale behind this decision is the fact that Holstein cattle (a *B. t. taurus* breed) are easy to identify due to their skin color pattern. This, we expected, would give us some assurance that the ovary collector at the slaughterhouse would have certainty of the breed.

DNA SEQUENCE DETERMINATION

The next step was to sequence the Nelore bull's DNA to ensure that sequence differences existed at our loci of interest when compared to the DNA sequence of 5-12 *B. t. taurus* females (Holstein, Angus, and Hereford). We included other *B. t. taurus* breeds in our sequencing project to ascertain if the polymorphisms were fixed within the subspecies or were breed specific. Subspecies specific sequence differences would give us more options in the future.

EXAMPLE OF ASSAY DEVELOPMENT TO ASCERTAIN DNA METHYLATION AND GENE EXPRESSION AT IMPRINTED LOCI IN BOVIDS

The procedures and techniques used for identification of DNA sequence polymorphisms, for DNA methylation, and expression assays for genes associated with BWS will be summarized in this section. In order to avoid redundancy I will only explain in depth the steps taken to develop one DNA methylation (*i.e. H19/IGF2* ICR) and one gene expression assay (*i.e. KCNQ1OT1*). All other data generated for DNA methylation and imprinted gene expression analysis will be presented in several tables at the end of this chapter.

DNA methylation assay development

The *H19/IGF2* imprinted region has been identified in both the mouse (chromosome 7) and human (chromosome 11) and we know that in both species the ICR is located -2 to -4 kb upstream of the start of transcription of *H19* (Jinno *et al.,* 1996; Vu *et al.,* 1999; Engel *et al.,* 2006). However, unlike in the mouse, there are discrepancies among where the human ICR actually starts. Differential methylation is proposed to be present anywhere from the start of transcription to -500 bp from *H19* and span for ~ 5.2 kb upstream of *H19*. This region is estimated to be differentially methylated for 4.5-5 kb (Takai *et al.,* 2001; Cerrato *et al.,* 2008; Ideraabdullah *et al.,* 2008). In human, the *H19/IGF2* ICR is located on chromosome 11p15.5 [accession # ref NT_009237.18; GRCh37.p2 (reference bases 1964545-1958496)].

The location of both *H19* and *IGF2* are annotated in the bovine genome. The bovine *H19/IGF2* ICR is located on chromosome 29 [accession # NCBI Btau 4.2 ref NW_001494547.3 (bases 3718166 to 3725900)]. According to the UCSC genome browser, this region in bovine is 86% homologous to the *H19/IGF2* ICR in humans.

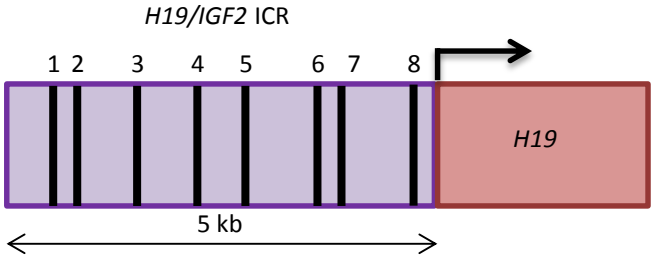
Previously published work on the bovine *H19/IGF2* ICR proved to be inaccurate with respect to the primers used to amplify the region (Curchoe *et al.,* 2009). We were unable to align the region they were studying in either the NCBI or UCSC genome browser. Therefore, since we did not know where the differentially methylated region was in bovine we decided to work backwards. Instead of finding DNA sequence polymorphisms with genomic sequencing primers and then making bisulfite assays, I

designed bisulfite primers first. This allowed us to determine where differential methylation was present in the *H19/IGF2* region and then, once that was determined, I sequenced the region to find sequence polymorphisms.

We began by using the UCSC genome browser to pull sequence data for the region upstream of *H19.* There was gap of unknown size in base pairs in the sequence just upstream from the start of transcription of *H19.* Therefore, primers were designed that amplified the region immediately upstream of the gap. *H19/IGF2* ICR uses CTCF proteins to form a chromatin barrier on the maternal allele to block maternal *IGF2* expression and allow for maternal *H19* expression (Engel *et al.,* 2006; Ideraabdullah *et al.,* 2008). This DMR in mice contains four CTCF sites while humans have seven (Ulaner *et al.,* 2003). Using the University of Essex CTCF searching database (http://www.essex.ac.uk/bs/molonc/binfo/ctcfbind.htm) I was able to determine putative CTCF binding sites in a 5kb region of the bovine's putative ICR. The software identified eight putative CTCF binding sites at this locus (**Figure 1**).

The next step was to mask the repeated sequences in the genomic region of *H19/IGF2* that was obtained from the UCSC genome browser. For this, I used RepeatMasker (Smit *et al.,* 2011). In the output of this program all repetitive sequences will be shown as "N". No primers were designed over repeated sequences. After the sequence information was pulled with the unknown gap, then the region was bisulfite converted *in silico* using Microsoft Word. First, I pasted the sequence information into Word and replaced all CGs with "XX" and then the find and replace feature was used to convert all Cs (cytosines) to Ts (thymine). The last step was to convert the XX back to

Figure 1. Putative CTCF binding sites at the *H19/IGF2* ICR in bovine



CTCF binding sequence	Location of CTCF sites
Site 1) cgttaagggg	-4739 to -4749 bp
Site 2) ccgcgaggcggcag	-4311 to -4325 bp
Site 3) ccgcggggcggcgg	-3882 to -3896 bp
Site 4) cgttaagggg	-3372 to -3382 bp
Site 5) ccgcgaggcggcag	-2944 to -2958 bp
Site 6) tggacagggg	-1739 to -1749 bp
Site 7) ccgcgaggcggcgg	-1492 to -1506 bp
Site 8) tgttgagggg	-251 to -261 bp

Location of putative CTCF binding sites relative to *H19*'s start of transcription in the bovine. The figure is drawn to scale.

CGs. This is how the methylated genome (the paternal allele in this example) will look once it has been bisulfite converted (*i.e.* the methylated CGs will remain unchanged). The unmethylated maternal allele will have all C's converted to T's since it should have only unmethylated cytosines.

I designed the bisulfite assay primers (Primer3; Rozen and Skaletsky, 2000 and IDT PrimerQuest; http://www.idtdna.com/Scitools/Applications/Primerquest/) to include one CTCF site within the amplicon. When designing bisulfite primers, areas with low CpG content were selected so that the primers would be able to bind to both the unmethylated and methylated alleles. At least three primer pairs were tested per region.

The next step was to perform bisulfite mutagenesis of the genomic DNA using the *B. t. indicus* (Nelore) bull sperm cells' DNA as well as the *B. t. taurus* females (Holstein, Angus, Hereford) liver and blood DNA samples to determine the methylation status of the region. The primers were tested in several PCR reactions using a gradient of temperatures to determine optimal annealing temperature. Standard PCR conditions were used: 2.5mM $MgCl_2$, .3µM of each primer, 2.5µM of dNTPs, Taq Polymerase, and roughly 5ng of bisulfite mutagenized DNA. The PCR reaction was loaded onto a 1% agarose to check if amplification of the sequence had occurred.

The first time I ran the primers (1F-1R) for this assay I saw no bands which meant one of two things; my bisulfite conversion was unsuccessful or the PCR conditions were not correct for the primers. After several different attempts adjusting $MgCl_2$ concentrations and changing the annealing temperature for the primers new bisulfite DNA was made. I also tried adding 5% DMSO and 1M betaine. Both DMSO and betaine inhibit secondary structures from forming in GC rich regions so that the polymerase will be able to amplify the sequence. After electrophoresis was performed I was able to get bands using 1M betaine for a region 485 bp in length which contained 43 CpGs and encompassed the 8^{th} CTCF site (**Figure 2**).

The successful amplification of the region gave us the opportunity to determine if differential methylation was present by the use of COBRA. The restriction enzyme DpnII (recognition sequence GATC) was used to digest the methylated allele. I expected to see even band intensity (50/50) for both the cut methylated allele and the uncut unmethylated allele. However, I noticed skewing of the digested product towards the

putative unmethylated maternal allele (**Figure 3**). This suggested to us that the unmethylated allele was preferentially amplified during PCR. As a result of the skewing, new bisulfite primers were generated closer to *H19*'s start of transcription. Several of the primers worked but I chose the one that amplified the region the best. The selected primer set (4F – 3R) amplifies a 493 bp region located at -687 to -194 from *H19* and contains 45 CpGs (**Figure 4**) as well as the 8th CTCF site. COBRA was performed again using DpnII and this time we were able to amplify both alleles equally (*i.e.* 50/50 methylation was observed; **Figure 5**).

Since the differentially methylated region had been located the next step was to sequence the genomic DNA encompassing that region in search of polymorphisms between *B. t. indicus* and *B. t. taurus*. Primer3 (Rozen and Skaletsky, 2000) was used to design sequencing primers (2F-2R) over the DMR using genomic DNA from Nelore, Angus, Hereford, and Holstein animals. The region was successfully amplified (**Figure 6**) and the PCR product was sent to the DNA Core to be sequenced. Alignment of the sequences revealed one polymorphism between the Nelore and the *B t. taurus* dams (G: to A, respectively; **Figure 7**).

Once the SNP had been determined between the *B. t. indicus* and *B. t. taurus,* I used this difference in DNA sequence to determine baseline methylation of the region using the chorioallantois from two independent F1 *B. t. indicus x B. t. taurus* concepti. Briefly, the bisulfite-converted DNA PCR product was ligated to a vector (*i.e.* Promega T Easy Vector) and introduced into NEB 5-alpha F competent *E. coli*. Fifteen individual clones from each sample were sent for sequencing. During alignment I first assessed

Figure 2. *H19/IGF2* ICR bisulfite assay 1F-1R. Assay position -770 bp to -285 bp from *H19's* start of transcription. Number of CpGs = 43.

Figure 3. *H19/IGF2* ICR bisulfite assay 1F-1R restriction enzyme analysis. COBRA was performed using the restriction enzyme DpnII which cuts the methylated allele at 323 bp (amplicon size 485 bp). The digest shows skewing towards the unmethylated allele. U = unmethylated. M = methylated.

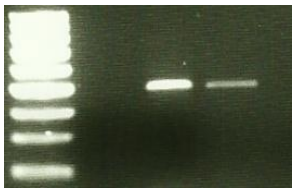


Figure 4. *H19/IGF2* ICR bisulfite assay 4F-3R. Assay position -687 bp to -194 bp from *H19's* start of transcription. Number of CpGs = 45.

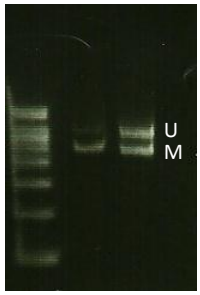


Figure 5. *H19/IGF2* ICR bisulfite assay 4F-3R restriction enzyme analysis. COBRA was performed using the restriction enzyme DpnII which cuts the methylated allele at 414 bp (amplicon size 493 bp). U = unmethylated. M = methylated.

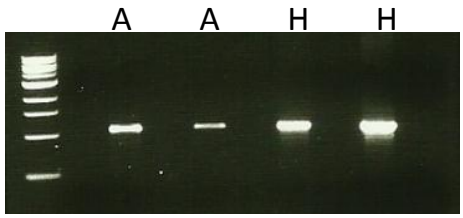


Figure 6. *H19/IGF2 ICR g*enomic sequencing primers. Sequencing primers (2F-2R) were generated to amplify the region encompassing the 4F-3R amplicon in order to determine if DNA sequence polymorphisms were present between *B. t. taurus* and *B. t. indicus*. Amplicon size = 1028 bp. A= Angus. H= Holstein.

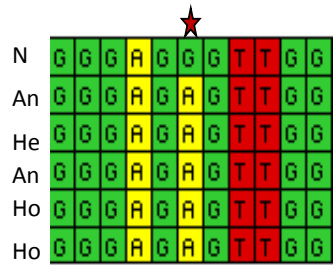


Figure 7. *H19/IGF2* ICR informative SNP between *B. t. indicus* and *B. t. taurus*. The red star denotes the SNP. N = Nelore, An = Angus, He = Hereford, Ho = Holstein.

parent of origin of each allele amplified by searching for the polymorphism described (**Figure 7**). The combined results from both samples' bisulfite sequencing data revealed that there was skewing toward the paternally methylated allele (75%; 6 maternally unmethylated and 20 paternally methylated strands) even though there was 50/50 methylation in the bisulfite PCR analysis (DpnII digest). From this we concluded that the bacteria favor the amplification of the methylated allele over the unmethylated allele. We were not totally surprised as this is a common problem with the product of bisulfite converted unmethylated DNA due to its high content of Ts. Given that this assay would not be useful and that in the meantime the gap upstream of *H19* had been filled we decided to generate another bisulfite assay.

A new bisulfite assay was generated approximately 2.5 kb upstream of *H19* (-3074 to -2574; primer pair 3F-3R) which coincides with the conserved DMR in mouse and human. The 3F-3R primer set amplify a region containing the 5^{th} CTCF site. I repeated all the steps described above except this time we were fortunate and found a polymorphism (G/A = *B. t. indicus/B.t taurus*) within the product of the bisulfite converted DNA amplicon (**Figures 8, 9**). Then, COBRA was performed and the digest showed a bias towards the unmethylated allele (70/30; **Figure 10**). Many unsuccessful steps were taken to try to resolve the skewing in allele amplification. As a result, another set of bisulfite primers (-3065 to -2747; primer pair AF-AR) were generated that amplified a 318 bp region (**Figure 11**) that included the same polymorphisms described in the previous assay. Then COBRA analysis was performed using a methylation specific enzyme PflfI which showed 50/50 methylation (**Figure 12**).

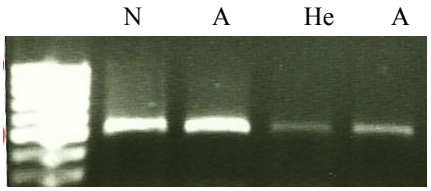


Figure 8. *H19/IGF2* ICR bisulfite assay 3F-3R. Assay position -3074 bp to - 2574 bp from *H19's* start of transcription. Number of CpGs = 25. N= Nelore, A= Angus, He= Hereford.

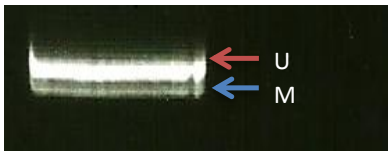


Figure 10. *H19/IGF2* ICR bisulfite assay 3F-3R restriction enzyme analysis. COBRA was performed using the restriction enzyme Taqα1 which cuts the methylated allele at 469 bp (amplicon size 500 bp). U = unmethylated. M = methylated. The bisulfite converted PCR product is 70% unmethylated and 30% methylated.

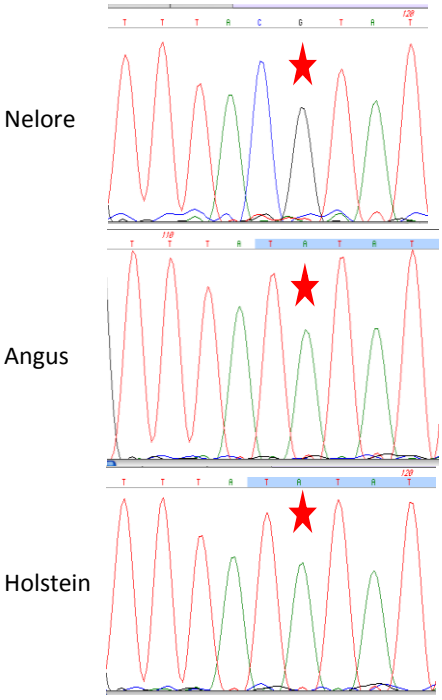


Figure 9. Sequencing chromatographs of the *H19/IGF2* ICR bisulfite amplicon 3F-3R. The red star denotes the informative SNP between the *B. t. indicus* (G) and the *B. t. taurus* (A).

Bisulfite DNA was cloned and sequenced as described above. The same problem occurred after cloning with preferential amplification of the methylated allele. From our results, we know that both alleles amplified at equal rates during PCR (**Figure 12**); however, our results from sequencing revealed that the majority of clones amplified were from the putative paternally methylated allele. This led us to speculate that the bacteria were the culprit of the problem.

We decided to test whether the skewing was indeed the result of cloning by preparing plasmids that contained products amplified from either fully methylated or fully unmethylated DNA. I generated genomic DNA primers (2F-2R) over the region

(spanning an extra ~200bp in either direction) where the bisulfite assay AF-AR was located. After PCR amplification, half of the product was methylated with the methyltransferase M. SssI. This enzyme methylates all CpG's, therefore creating a facsimile of the methylated paternal allele. The other half of the PCR product represented the unmethylated allele because genomic DNA methylation is lost during PCR amplification.

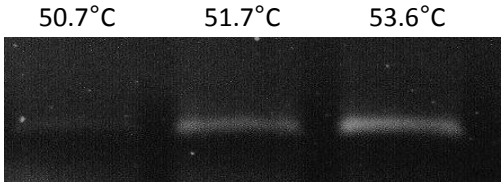


Figure 11. *H19/IGF2* ICR bisulfite assay AF-AR. A gradient of temperatures were tested to identify the optimal temperature for amplification. Assay position -3065 bp to -2747 bp from *H19's* start of transcription. Number of CpGs = 20. The picture's contrast was adjusted to see the bands more clearly.

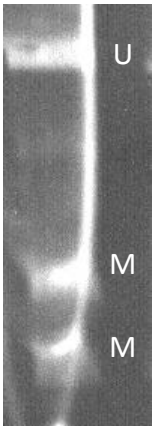


Figure 12. *H19/IGF2* ICR bisulfite assay AF-AR restriction enzyme analysis. COBRA was performed using the restriction enzyme PflfI which cuts the methylated allele at 214 bp (amplicon size 318 bp). U = unmethylated. M = methylated.

Both the methylated and unmethylated amplicons were bisulfite converted, PCR amplified (**Figure 13**), and cloned. This gave me the ability to distinguish between colonies that contained a methylated allele and those that contained an unmethylated allele. After colony PCR the samples were run on an agarose gel and it was clear that the bacteria were not amplifying both alleles at equal rate (3/5 and 1/5 for the methylated and the unmethylated colonies, respectively; **Figure 14**).

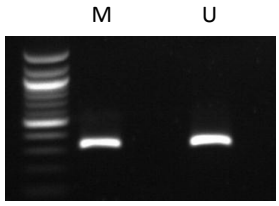


Figure 13. Methylated and unmethylated allele amplification for *H19/IGF2* ICR bisulfite assay AF-AR. The genomic region encompassing AF-AR was PCR amplified with primer set 2F-2R. To test the DNA polymerase ability to amplify equally methylated and unmethylated products, half of the 2F-2R product was methylated (M) with the methylase Sss1. The other half of the 2F-2R product remained unmethylated (U). The product then underwent bisulfite mutagenesis and amplification with primer pair AF-AR.

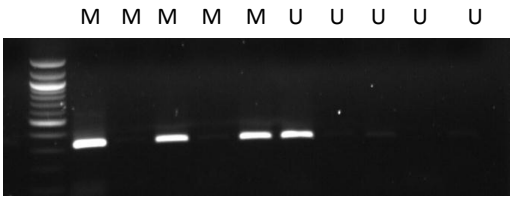


Figure 14. Colony PCR of *H19/IGF2* ICR bisulfite assay AF-AR. *E. coli* colonies containing methylated (M; M.Sss1 treated) and unmethylated (U) PCR amplicons were used for PCR amplification with bisulfite DNA specific primer pair AF-AR.

In summary, differential methylation was determined by COBRA for the *H19/IGF2* ICR for the bovine in the conserved region 2-4 kb upstream of *H19*. However, after cloning there was skewing towards the methylated paternal allele. Therefore, it is necessary to test other types of bacteria that will be able to equally replicate plasmids containing methylated and unmethylated alleles.

Methylation assays were generated for other BWS-associated ICRs and DMRs (KvDMR1, *MEST* DMR, *CDKN1C* DMR) using the same methods as previously described above refer to **Tables 1.1-2.2** at the end of this chapter for further information on these assays.

Gene Expression assay development

KCNQ1OT1 is a paternally-expressed long ncRNA that has been identified in both the mouse (chromosome 7) and human (chromosome 11) and we know in both species

it is located downstream of the KvDMR1, which also serves as its promoter region (Mancini-DiNardo *et al.,* 2003; Beatty *et al.,* 2006). Studies have shown that the KvDMR1 is located in the tenth intron of *KCNQ1* (Mancini-DiNardo *et al.,* 2003; Beatty *et al.,* 2006). According to the NCBI (2002) and the UCSC genome browsers (Kent *et al.,* 2002) the human *KCNQ1OT1* spans from intron 10 to intron 9 of *KCNQ1* and is transcribed in the antisense direction of *KCNQ1*. In human, *KCNQ1OT1*'s transcript is 59.46 kb. Since this gene is intronless, the genomic DNA sequence is the same as the transcript sequence (NCBI human Reference Sequence: NC_000011.9 region from base 2661768 to 2721228; 2002).

KCNQ1OT1 is not annotated in the bovine genome. Therefore, we had to align the human *KCNQ1OT1* sequence to the bovine genome browser databases. The BLAST feature in NCBI (2002) and BLAT feature in UCSC (Kent, 2002) were used to compare the human *KCNQ1OT1* sequence to similar regions within the bovine database. The databases revealed 74-85% homology between the human and bovine genomes with respect to *KCNQ1OT1*. The alignment from both NCBI (2002) and UCSC (Kent, 2002) revealed that *KCNQ1OT1* is located on chromosome 29 between the 10^{th} and 11^{th} intron of the *KCNQ1* gene. We were able to retrieve ~28 kb from the genome browsers that could be used to generate sequencing assays for the bovine *KCNQ1OT1* gene (NCBI accession # ref NW_001494547.3 bases 3138866 – 3152100; 2002).

Genomic sequencing primers were generated for *KCNQ1OT1* in order to determine where DNA sequence polymorphisms existed between *B. t. indicus* and *B. t. taurus.* The first step was to mask the repetitive elements in the sequence obtained

from the NCBI database. The RepeatMasker program was used to mask the repetitive sequences (Smit *et al.,* 2011). Genomic sequencing primers were not generated over the repetitive elements. The primer programs used to generate genomic sequencing primers were Primer3 (Rozen and Skaletsky, 2000) and IDT PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/). When designing genomic sequencing primers, the annealing temperatures were selected to be between 55-65°C and the primer length was 18-30 nucleotides long. The sizes of the amplicons generated were typically between 600-1200 bp. At least two primer pairs were made and tested for each region.

Next, DNA was extracted from *B. t. indicus* (Nelore) bull's sperm cell as well as from the *B t. taurus* females (Holstein, Angus, Hereford) liver and blood to be used as template for testing the genomic sequencing primers. In order to determine optimal annealing temperature for the primers a temperature gradient was used. In certain cases when multiple bands were observed for an amplicon the MgCl2 concentration was adjusted from 4mM to 2.5mM to make the reaction more stringent. In the opposite case, when no bands were observed 5% DMSO was used to try and relax the PCR reaction by allowing the polymerase to bind. Once the optimal PCR conditions were determined then Nelore, Holstein, Angus, Hereford samples were used as template to be sent for sequencing at the University of Missouri's DNA Core.

The first two regions to be amplified and sequenced were A1F-A2R (27073 to 28270 bp) and B1F-BR1 (13983 to 14644 bp) located furthest from the start of transcription of *KCNQ1OT1*. The samples were then sent to the University of Missouri's

DNA Core for sequencing according to the guidelines provided by the core. Alignment of the *B. t. indicus and B. t. taurus* sequences in MacVector revealed no differences in DNA sequence between the *B. t. indicus and B. t. taurus* in either A1F-A2R or B1F-B1R assays.

A new set of primers were designed that amplify flanking sequence of the previous regions. Those primers pairs amplified KCNQ1OT1 bases 2535-3546 (C1F-C2R) and 12311-13082 (B2F-B2R). During alignment, it was determined that a polymorphism existed between the *B. t. indicus* and *B. t. taurus* for both regions; C1F-C2R (C/T = *B. t. indicus*/*B. t. taurus*) and B2F-B2R (A/C = *B. t. indicus*/*B. t. taurus*). However, no commercially available restriction enzyme was found that would restrict either site.

In order to increase our chances to find a polymorphism four new regions were sequenced. I successfully located a SNP (G/A = *B. t. indicus/B. t. taurus;* **Figure 15**) using primer pair F2F-F2R which encompassed bases 8182-9100 bp of the *KCNQ1OT1* transcript. The restriction enzyme Hinfl was used to digest the Nelore allele.

Expression assays were generated for *KCNQ1OT1* over the region containing the SNP using the primer programs described above. Primers were designed based on the restriction fragment sizes in order to easily distinguish between the *B. t. indicus* and *B. t. taurus* alleles by PAGE.

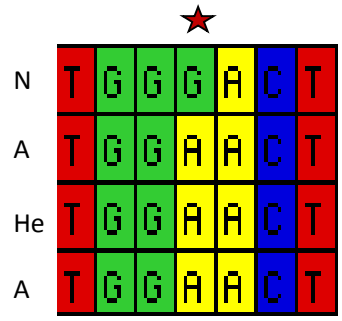


Figure 15. *KCNQ1OT1* SNP between *B. t. indicus* and *B. t. taurus. Primer pair = F2F-F2R.* The red star denotes the SNP. The SNP is recognized by the restriction enzyme HinfI (GANTC). N = Nelore, He = Hereford, A = Angus.

The template used for expression assay analysis was cDNA. Briefly, the RNA was extracted from tissue using the commercially available kit (*i.e.* High Pure RNA Tissue Kit; Roche). After RNA extraction, cDNA was synthesized using reverse transcriptase (RT; *i.e.* Superscript II; Invitrogen). Since intron-spanning primer design was not possible it was necessary to also run minus RT reactions to make sure that the samples were not contaminated with genomic DNA.

B. t. taurus and *B.t indicus* cDNA was amplified using expression assay primers F1.5F-F1.5R which amplified bases 8690 to 9191. After PCR amplification, the samples were digested with Hinfl and resolved by PAGE. As expected, Hinfl digestion of the *B. t. indicus* (Nelore) 502 bp amplicon was cut three times (at position 32, 300, and 489) and the *B. t. taurus* was cut twice (at position 32, and 489; **Figure 16**).

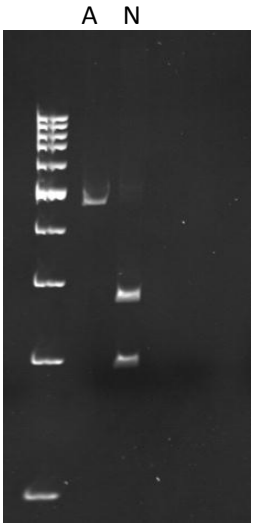


Figure 16. Expression assay analysis for *KCNQ1OT1.* The *KCNQ1OT1* expression assay F1.5F-F1.5R (502 bp) was used to differentiate between the *B. t. taurus* and the *B. t. indicus* transcripts. The restriction enzyme HinfI restricts the two alleles differently (*i.e*. *B. t.* taurus at position 32 and 489 and the *B. t. indicus* at position 32, 300, and 489). The smaller products of digestion (13 and 32 bp) are not visible in this gel. A= Angus, N= Nelore.

SSCP expression assay primers were designed (although not tested) when no commercially available restriction enzymes were available that restricted an identified polymorphism. Examples of polymorphisms found between the *B. t. indicus* and *B. t.* for other imprinted genes can be found in **Figure 17**. Lastly, expression assays were generated for other BWS-associated imprinted genes using the same methods as described above and the information is shown in **Tables 1.1-1.6 and 3.1-3.2**.

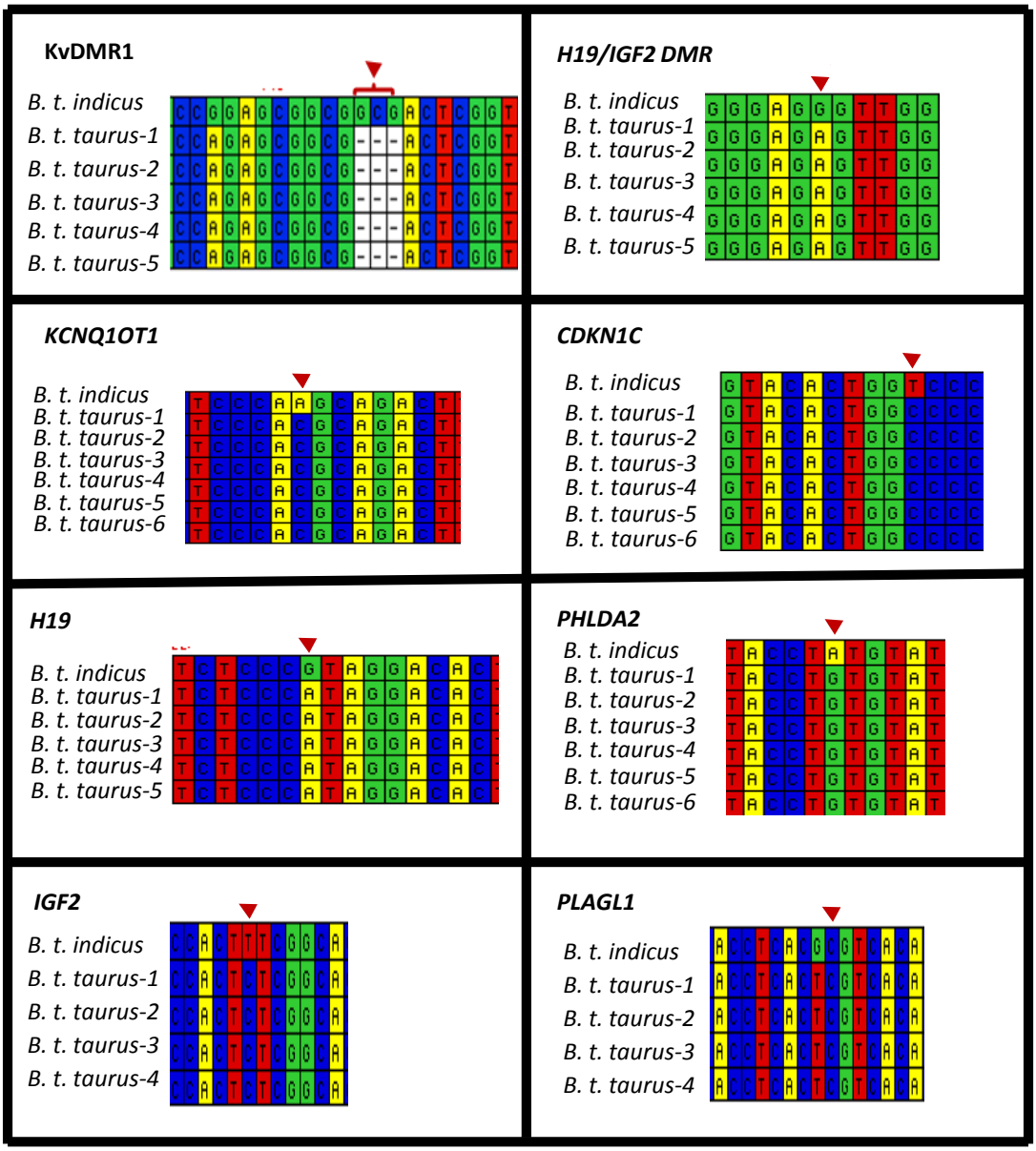


Figure 17. Examples of polymorphisms found between the *B. t. indicus* and *B. t. taurus* in genomic regions associated with BWS.

Table 1.1 Genomic Sequencing Primers

Gene Symbol		Genomic Primers (5'-3')	Maternal Bos taurus	Paternal Bos indicus	Exon	NCBI accession # (based on Btau_4.2)	PM location in reference Btau 4.2
H19	Forward	AGGTTGACCTAAAGGAACGGACGA	C	T	5	ref|NW_001494547.3	3727326
	Reverse	ATGGACACACGTTCATGCTTGGTG			5	3725159 - 3728163	
H19	Forward	CGGGCCTCTGCGGGCGATGACG	A	G	2-4 (PM in 3)	ref|NW_001494547.3	3726600
	Reverse	TCCGGTGGTGTGGGTCGTCCGT			2-4 (PM in 3)	3725159 - 3728163	
IGF2	Forward	TCCGCCATTTATCGCCCTGTTT	C	T	10	ref|NW_001494547.3	3636104
	Reverse	TGCTTTATTGGGATTGCGAGCG			10	3634659 – 3636601	
IGF2	Forward	TCTTCCAAGGCATCCAGCGATT	A(13)	A(14)	10	ref|NW_001494547.3	3634025
	Reverse	AGCAGGTTTGGGTCTTTGGTGT			10	3633168-3634394	
IGF2	Forward	AGCCAGGGAGTTCTTTACACCT	T	C	10	ref|NW_001494547.3	3634199
	Reverse	ATGGAAACTTGGAGGAGGAGCA			10	3633168-3634394	
H19/IGF2 ICR	Forward	TCACCCATGGTTAAAGCTAGAGT	A	G	~300-700bp upstream from H19 TSS	ref|NW_001494547.3	3724402
	Reverse	CCCGGGCTTTTTCTAACTGGA				3718166 -3725900	
H19/IGF2 ICR	Forward	AAGACCTTGGAGGCGTTTCCCAAT	A	G	~2.5-3 kb upstream from H19 TSS	ref|NW_001494547.3|	3721970
	Reverse	ATGGGTCTATTTGGTCAGCCCAAGAC				3718166 -3725900	

Genomic sequencing primers used to find polymorphisms (PM) between *B. t indicus* and *B. t taurus*

Table 1.2 Genomic Sequencing Primers

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Restriction Enzyme	Primer [] µM	$MgCl_2$ [] (mM)	5% DMSO	#Cycles	Taq Polymerase
H19	60	(DF-DR/BR) 505 bp	BsiHKAI	0.3 0.3	4	YES	35	Go Taq Promega
H19	60	(2F-1R) 782 bp	MSP1	0.3 0.3	4		40	Go Taq Promega
IGF2	59	(5F-5R) 1081	none	0.3 0.3	4		35	Go Taq Promega
IGF2	59.2	(3F-3R) 678	none	0.3 0.3	4		35	Go Taq Promega
IGF2	58.1	(2F-2R) 1079	none	0.3 0.3	4		35	Go Taq Promega
H19/IGF2 ICR	58	(2F-2R) 1038		0.3	4		35	Go Taq Promega
H19/IGF2 ICR	60.6	(2F-2R) 731		0.3	4		35	Go Taq Promega

Tm (°C) = annealing temperature, bp= base pairs, []= concentration, DMSO= dimethyl sulfoxide.

Table 1.3 Genomic Sequencing Primers

Gene Symbol		Genomic Primers (5'-3')	Maternal Bos taurus	Paternal Bos indicus	Exon	NCBI accession # (based on Btau_4.2)	PM location in reference Btau 4.2
KvDMR1	Forward Reverse	TCAACCTCCATCGCCACAACTTCT AGGATCGAGAATGGGAGGAGAGTCA	A,C, in/del of C between AG	C,G, in/del C between AG	closer to *KCNQ1OT1* TSS	ref|NW_001494547.3 3135438 -3133503	3135290, 3134901, 3134894
KvDMR1	Forward Reverse	TTCCGATCTTAAGGCGCGAACAGA CCCTGCAGGCTATCCAATCAGC	A, G, G, C,G, A, in/del between GA, C,G	G, A,A, G,A,G in/del between GA "GCG",G,C	further from *KCNQ1OT1* TSS	ref|NW_001494547.3 3135438 -3133503	3134377, 3134305, 3134284, 3134150, 3134131, 3134095, 3134086 - 3134084, 3134072, 3133883

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Restriction Enzyme	Primer [] µM	$MgCl_2$ [] (mM)	5% DMSO	#Cycles	Taq Polymerase
KvDMR1	62	(1F-1R) 697		0.3	2.5		35	Go Taq Promega
KvDMR1	58	(2F-3R) 1182		0.3				Go Taq Promega

Genomic sequencing primers used to find polymorphisms (PM) between *B. t. indicus* and *B. t. taurus*
Tm (°C) = annealing temperature, bp= base pairs, []= concentration, DMSO= dimethyl sulfoxide, TSS= transcription start site

Table 1.4 Genomic Sequencing Primers

Gene Symbol		Genomic Primers (5'-3')	Maternal Bos taurus	Paternal Bos indicus	Exon	NCBI accession # (based on Btau_4.2)	PM location in reference Btau 4.2
KCNQ1OT1	Forward Reverse	TGCTGTGTAGGTGGCAGAACACTT TTGCAATAAGCCCTGTGAGGAGGA	T	C	closest to TSS	ref NW_001494547.3 3140877 - 3139866	3139984
KCNQ1OT1	Forward Reverse	ATCTGCTTTCTCGGTGCCTCTGAA TCTGGTGTAAGGCATGCTGTGTCT	C	A	furthest from TSS	refNW_001494547.3 3150413 - 3149642	3150194
KCNQ1OT1	Forward Reverse	AGAAGGCTTTGGTCAGCAGATAC AAATGGCAGCAACTACTGAAGTCT	A	G	Close to C1F	refNW_001494547.3 3146431 -3145513	3146321
KCNQ1OT1	Forward Reverse	GGTCACACCTACACATTCTAAGGTC CATGCTAACCAATTTCTGAGTCTTT	T, G	C,C	downstream of G3F	refNW_001494547.3 3145151- 3144175	3144895, 3141830
KCNQ1OT1	Forward Reverse	TTTGTTTTCTCATCTCAGCCTATTC AGAGAAGCAGAACTCTTCCAAAGAT	A,A	T,G	downstream of C1F	refNW_001494547.3 3142393- 3141534	3142234, 3142201

Genomic sequencing primers used to find polymorphisms (PM) between *B. t indicus* and *B. t taurus*

Table 1.5 Genomic Sequencing Primers

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Restriction Enzyme	Primer [] µM	$MgCl_2$l [] (mM)	5% DMSO	#Cycles	Taq Polymerase
KCNQ1OT1	63	(C1F-C2R) 1012	none	0.4	GC Buffer 1		35	La Taq Takara
KCNQ1OT1	62	(B2F-B2R) 772	none	0.3 0.3	2.5		30	Go Taq Promega
KCNQ1OT1	60	(F2F-F2R) 919	HinfI	0.3 0.3	2.5		30	Go Taq Promega
KCNQ1OT1	60	(G1F-G1R) 977	HindIII (T/C)	0.3 0.3	2.5		30	Go Taq Promega
KCNQ1OT1	60	(G3F-G3R) 860	none	0.3 0.3	2.5		30	Go Taq Promega

Tm (°C) = annealing temperature, bp= base pairs, []= concentration, DMSO= dimethyl sulfoxide

Table 1.6 Genomic Sequencing Primers

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Restriction Enzyme	Primer [] µM	$MgCl_2$ [] (mM)	5% DMSO	#Cycles	Taq Polymerase
CDKN1C	(65-61) 60	(3F-1R) 857	AvaII	0.3 0.3	4		(2) 35*	Go Taq Promega
PHLDA2	62	(1F-2R) 733	none	0.3 0.3	4		35	Go Taq Promega
PLAGL1	60	(2F-2R) 889	MluI	0.3 0.3	4		35	Go Taq Promega

Gene Symbol		Genomic Primers (5'-3')	Maternal Bos taurus	Paternal Bos indicus	Exon	NCBI accession # (based on Btau_4.2)	PM location in reference Btau 4.2
CDKN1C	Forward Reverse	TCCTCGCGGCCCCTCTGTCGC GTCCCCGTTGTGCCCCGCCGT	C, (deletion A (26)	T, (Insertion A (27)	intron 3 intron 4	refNW_001494547.3 2955546-2956402	2955801, 2955909
PHLDA2	Forward Reverse	TCTCTGACGTCGGGTGGAGGT GTTGGTCGGCAAGCAGGGTC	G	A	intron 1 intron 2	refNW_001494547.3 2914497 -2917483	2915910
PLAGL1	Forward Reverse	ACCACTGCGACAGATGCTTCTACA TGGAAATGAGGCAGGATAGCCGAA	T	G	6 6	ref|NW_001495600.2 4087804 - 4086186	4087357

Genomic sequencing primers used to find polymorphisms (PM) between *B. t indicus* and *B. t taurus*

- * 2 cycles each for (65-61°C) followed by 35 cycles at 60°C
- Tm (°C) = annealing temperature, bp= base pairs, []= concentration, DMSO= dimethyl sulfoxide

Table 2.1 Bisulfite Assay Primers

Gene Symbol		Bisulfite Primers (5'-3')	Maternal Bos taurus	Paternal Bos indicus	Exon	NCBI accession # (based on Btau_4.2)	PM location in reference Btau 4.2
H19/IGF2 ICR	Forward	GGGGAGGTTGTCGGGTTTATGG	A	G	~200-700bp upstream from *H19* TSS	ref|NW_001494547.3 3719654-3724900	3724402
	Reverse	CCGCACCCCTCCTTTAACATC					
H19/IGF2 ICR	Forward	AGTGTGTAGAGGATATTG	A	G	~2.7-3 kb upstream from *H19* TSS	ref|NW_001494547.3 3719654-3724900	3721970
	Reverse	CTCCTCTCCTAACTTCAAC					
H19/IGF2 ICR	Forward	TGTTTTAGGGATGAGGTGGTTGG	A	G	~300-800 upstream from *H19* TSS	ref|NW_001494547.3 3719654-3724900	3724402
	Reverse	CCCAACCCCCAAAAACACAC					
H19/IGF2 ICR	Forward	ATATAGGGTAGTGTGTAGA	A	G	~2.5-3 kb upstream from *H19* TSS	ref|NW_001494547.3 3719654-3724900	3721970
	Reverse	AATCTTTAATCAACCCAAA					
KvDMR1	Forward	TTAGAGTATTGTGAGTTTAGA	A, G, G,	G, A,A,	Closer to *KCNQ1OT1* TSS	ref|NW_001494547.3	3134377, 3134305, 3134284,
	Reverse	TCTCTTCAAAATACTCCTCTAAC	C,G, A,	G,A,G		3134542- 3133908	3134150, 3134131, 3134095,
			in/del between GA, C	in/del between GA "GCG",G			3134086 - 3134084, 3134072
KvDMR1	Forward	GTGAGGAGTACGGTATTGAGG	G	C	further from *KCNQ1OT1* TSS	ref|NW_001494547.3	3133883
	Reverse	CCCGACCAATACGAACCTA				3134542- 3133908	
CDKN1C DMR*	Forward	GAGGACTGGGCGTTCCACAGGCCA			exon 1 intron 2	refNW_001494547.3	
	Reverse	GCCCTTTAACGGCCAGGAGGC				2952619 -2957867	
MEST ICR**		GCGTTGTCGCGGTTACGAGTATTT				ref|NW_001494939.3	
		TCCGACCGACAACGTCTTACCTACTA				3816040 - 3817019	

Bisulfite primers used to determine methylation status of the ICR in regions associated with BWS in the bovine.

- See *CDKN1C* DMR assay information in Appendix 1.
- ** The C will be converted to a T during bisulfite mutagenesis
- ***M*EST* differential methylation was determined for only the *B. t taurus* this region did not amplify for *B. t indicus*
- TSS= transcription start site, PM= polymorphism.

Table 2.2 Bisulfite Assay Primers

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Bisulfite Restriction Enzyme (50/50)	Primer [] µM	MgCl2 [] (mM)	5% DMSO	1M Betaine	# Cycles	Taq Polymerase
H19/IGF2 ICR	60	Bis (4F-3R) 493	DpnII	0.3	2.5		YES	40	Go Taq Promega
H19/IGF2 ICR	53.6	Bis (AF-AR) 318	PfifI	0.3	2.5		YES	40	Go Taq Promega
H19/IGF2 ICR	61	Bis (1F-1R) 485	DpnII	0.3	2.5		Yes	40	Go Taq Promega
H19/IGF2 ICR	45	Bis (3F-3R) 500	Taqα1	0.3	2.5		YES	50	Go Taq Promega
KvDMR1	51	Bis (4F-2R) 569	HPYCH4III	0.3	4			40	Go Taq Promega
KvDMR1	55	Bis (B23F-B23R) 417	Taqα1	0.3	2			40	Go Taq Promega
CDKN1C DMR*	59	(225L-1374R) 1090		0.4	GC Buffer II			35	LA Taq Takara
MEST ICR***	60.1	Bis (2F-2R) 376	Taqα1	0.3	4	YES		40	Go Taq Promega

- * See *CDKN1C* DMR assay information in Appendix 1.
- *** *MEST* differential methylation was determined for only the *B. t taurus* this region did not amplify for *B. t indicus*
- Tm (°C) = annealing temperature, bp= base pairs, []= concentration, DMSO= dimethyl sulfoxide

Table 3.1 Expression Assay Primers

Gene Symbol		Primers (5'-3')	Exon of primers	Maternal *B. t. taurus*	Paternal *B. t. indicus*	Exon of PM	NCBI accession # (based on Btau_4.2)	PM location in reference Btau 4.2
H19	Forward	GATATGGTCCGGTGTGATGGAGAGAGCA	2	C	T	5	NR_003958.2	1831
	Reverse	TTCGGAGCCTCCAGACTCGGTG	5			5	2069 bp	
H19	Forward	CGGGCCTCTGCGGGCGATGACG	1	A	G	3	NR_003958.2	1262
	Reverse	TCCGGTGGTGTGGGTCGTCCGT	5			3	2069 bp	
IGF2	Forward	TGCAGAGCAGAAGAGCTGCGTAGTTT	10	C	T	10	NM_174087.3	
	Reverse	GGTGTCGGGCCTCTAGCTCACTA	10			10	3824 bp	3296
KNCQ1OT1	Forward	TCGAGGGTACCGGATTCCCAGGC		A	G	Close to start of transcription of *KCNQ1OT1*	refNW_001494547.3	3146321
	Reverse	CGCAGGACACCCCAACTACAGCC					3146522-3146021	
CDKN1C	Forward	GGAGGCGCCGCGATCAAGAAG	2	C	T	4	refNW_001494547.3	2955801
	Reverse	GACAGCGAAAGCGCGAAGAGAC	4				2954777-2956157	
PHLDA2	Forward	GGCCAGCCCTAAGTCCCA	1	G	A	2	NM_001076521.1	694
	Reverse	GACAGATGGTTTGGATCAACAGGC	2				811 bp	
PLAGL1	Forward	TCAACCGGAAAGACCACCTGAAGA	6	T	G	6	NM_001103289.1	
	Reverse	GGTCAAAGCCTGCATTGAGCTTGT	6				2815 bp	867

Imprinted gene expression assay primers used to determine parental-specific expression using the polymorphism (PM) between *B. t. indicus* and *B. t. taurus* and restriction enzyme digestion

Table 3.2 Expression Assay Primers

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Expressed Allele	Restriction enzyme	Digested *B t. taurus* (bp)	Digested *B. t. indicus* (bp)	SSCP	Primer [] µM	$MgCl_2$ [] (mM)	#Cycles
H19	60	752	Maternal	BsiHKAI	609, 143	609, 35, 108		0.3 0.3	2.5	35
H19	60	457	Maternal	MSP1	245, 105, 64, 39, 4	309, 105, 39, 4		0.3 0.3	4	40
IGF2	61.9	210	Paternal	none	N/A	N/A	YES	0.3 0.3	4	35
KNCQ1OT1	64	502	Paternal	HinfI	457, 32, 13	268,189,32,13		0.3 0.3	2.5	35
CDKN1C	62	745	Maternal	AvaII	494, 251	361,251,133		0.3 0.3	4	35
PHLDA2	59.3	256	Maternal	none	N/A	N/A	YES	0.3 0.3	4	50
PLAGL1	60	834	Paternal	MluI	834	387, 447		0.3 0.3	4	35

Imprinted gene expression assay primers used to determine parental-specific expression using the polymorphism (PM) between *B. t indicus* and *B. t taurus* and restriction enzyme digestion

Tm (°C) = annealing temperature, bp= base pairs, SSCP= single stranded conformation polymorphism assay will be used, []= concentration

CHAPTER IV

Expression and methylation analysis of BWS-associated imprinted genes in F1 *B. t. indicus x B. t. taurus*

Abstract

Background: Beckwith-Wiedemann syndrome (BWS) is a loss-of-imprinting pediatric overgrowth syndrome. The primary features of BWS include macrosomia, macroglossia, and abdominal wall defects. Secondary features that are frequently observed in BWS patients are hypoglycemia, nevus flammeus, polyhyrdoamnios, visceromegaly, hemihyperplasia, cardiac malformations, and difficulty breathing. BWS is speculated to occur primarily as the result of the misregulation of imprinted genes associated with two clusters on chromosome 11p15.5, namely the KvDMR1 and *H19/IGF2.* There is a similar overgrowth phenotype that is observed in bovids and ovids as a result of embryo culture. In ruminants this syndrome is known as large offspring syndrome (LOS). The phenotypes associated with LOS are increased birth weight, visceromegaly, skeletal defects, hypoglycemia, polyhydramnios, and breathing difficulties. The genomic region/s associated with LOS have not yet been determined. The aim of the present study was to ascertain allelic expression in bovids of imprinted genes known to be misregulated in BWS. We also wanted to determine the methylation status in bovine of the imprinting control regions (ICRs) primarily affected in BWS patients.

Results: We found that imprinting is conserved between the bovine and human in imprinted genes known to be associated with BWS. *KCNQ1OT1* and *PLAGL1* were paternally-expressed in F1 *B. t. indicus* x *B. t. taurus* while *CDKN1C* and *H19* were maternally-expressed. We also show that in bovids, differential methylation exists at the KvDMR1 and *H19/IGF2* ICRs (the two ICRs primarily misregulated in BWS patients).

Conclusions: Based on these findings we conclude that the imprinted gene expression of *KCNQ1OT1*, *CDKN1C*, *H19*, and *PLAGL1* are conserved between the bovine and human. In addition, the KvDMR1 and *H19/IGF2* ICRs also have conserved DNA methylation patterns between humans and bovine.

Background

Genomic imprinting is an epigenetic modification that directs parent-specific gene expression. Imprinted genes are responsible for regulating growth and development of the conceptus (Biliya and Bulla, 2010). These genes are typically found in clusters containing both maternally- and paternally-expressed genes. The correct allelic expression of the clustered genes is regulated by a neighboring region of DNA which is differentially methylated and is known as the imprinting control region (ICR; Verona *et al.,* 2003; Zhang *et al.,* 2009). The effect of the ICR on a cluster of genes can span for megabases in a bidirectional manner (Pandey *et al.,* 2008).

Imprinted genes are functionally haploid and therefore are vulnerable to epigenetic mutations and loss-of-imprinting (Fowden *et al.,* 2010). Loss-of-imprinting (LOI) refers to the misregulation of imprinted gene expression which results in either loss of expression or biallelic expression of these genes.

There are several LOI disorders in humans including Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), Prader-Willi syndrome, and Silver Russell syndrome. BWS is the most frequent LOI syndrome observed in humans with an incidence of one in 13,700 live births (Choufani *et al.,* 2010; Weksberg *et al.,* 2010). BWS is also the most common pediatric overgrowth syndrome (Weksberg *et al.,* 2010). The overgrowth parameters for height and weight for BWS patients are among the 97th percentile (Weksberg *et al.,* 2010).

The primary features of BWS include macroglossia, macrosomia, and abdominal wall defects (reviewed in Elliott and Maher, 1994; Cooper *et al.,* 2005). The secondary features include visceromegaly, polyhydramnios, renal abnormalities, facial nevus flammeus, hypoglycemia, hemihyperplasia, ear creases and helical pits, and cardiac malformations (reviewed in Elliott and Maher, 1994; Weksberg *et al.,* 2003; Cooper *et al.,* 2005; Weksberg *et al.,* 2010). Children with this syndrome also have an increased susceptibility (4-21%) of developing embryonic tumors by the time they turn five years of age (Weksberg *et al.,* 2002; Rump *et al.,* 2005; Choufani *et al.,* 2010). Wilms' tumor of the kidney is the most common embryonic tumor (67% of cases) observed in BWS patients (Rump *et al.,* 2005).

BWS is thought to occur because of the dysregulation of several imprinted genes located primarily on chromosome 11p15.5 (Cooper *et al.,* 2005; Manipalviratn *et al.,* 2009; Weksberg *et al.,* 2010). The two main imprinted gene clusters associated with BWS are those directed by the *H19/IGF2* and KvDMR1 ICRs (Weksberg *et al.,* 2003; Sparago *et al.,* 2007). The BWS-associated imprinted genes regulated by the KvDMR1 include *KCNQ1OT1* (paternally-expressed) and *CDKN1C*, *KCNQ1*, and *PHLDA2* (all three maternally-expressed).

The KvDMR1 is methylated on the maternal allele and unmethylated on the paternal allele. Loss of methylation (LOM) at the KvDMR1 on the maternal allele is the most common epigenetic defect (50%) observed in BWS patients (Weksberg *et al.,* 2003; Sparago *et al.,* 2007; Weksberg *et al.,* 2010). This LOM results in the aberrant expression of the long noncoding RNA (ncRNA) *KCNQ1OT1* from the maternal allele which results in bidirectional silencing of the maternally-expressed flanking genes, in particular *CDKN1C* (Choufani *et al.,* 2010).

The *H19/IGF2* domain regulates the expression of the paternally-expressed gene *IGF2* and the maternally-expressed ncRNA *H19*. This ICR is unmethylated on the maternal allele and methylated on the paternal allele (Weksberg *et al.,* 2003). The gain of methylation on the maternal allele results in the repression of *H19* from both parental alleles and biallelic expression of *IGF2*. This epimutation occurs in 2-10% of BWS patients and is highly associated with tumor development (DeBaun *et al.,* 2003; Sparago *et al.,* 2007; Weksberg *et al.,* 2010). Recent studies have also found that some

BWS patients also have LOM at the following ICRs: *HYMAI/PLAGL1*, *MEST*, and *GRB10* (Rossignol *et al.,* 2006; Bliek *et al.,* 2009b; Lim *et al.,* 2009).

In ruminants there is an overgrowth syndrome that resembles BWS. The overgrowth syndrome in ruminants is known as large offspring syndrome (LOS; Young *et al.,* 1998). LOS has been documented to result from several embryo culture conditions (Farin and Farin, 1995; Blondin *et al.,* 2000; Bertolini and Anderson, 2002; Lazzari *et al.,* 2002) and high protein diet supplementation to the dam prior to conception and early pregnancy (McEvoy *et al.,* 1997). The phenotypical features of LOS include: increased birth weight, macrosomia, skeletal defects, hypoglycemia, polyhydramnios, visceromegaly, difficulty suckling, and perinatal death (Farin and Farin, 1995; Young *et al.,* 1998; Sangild *et al.,* 2000; Bertolini and Anderson, 2001; Hiendleder *et al.,* 2004; Farin *et al.,* 2006).

Currently, no animal models exist that recapitulate the overgrowth phenotype of BWS. To that effect, murine knockout models for BWS have been unable to display all the primary features observed in children with BWS (Leighton *et al.,* 1994). We are interested in determining if LOS could be used as an animal model to understand the etiology of BWS. The aim of the present study was to ascertain allelic expression in bovids of imprinted genes known to be misregulated in BWS. We show that as in humans *KCNQ1OT1*, *H19*, *CDKN1C* and *PLAGL1* are imprinted in the bovine. We also have determined that the KvDMR1 and *H19/IGF2* ICR are also differentially methylated in the bovine as it is in humans.

Methods

Production of *Bos taurus indicus x B. taurus taurus* day 65 F1 concepti

Seven *B. t. taurus* heifers (6 Angus, 1 Hereford) were synchronized using the 14-CIDR®-PG synchronization protocol. Briefly, CIDRs were inserted for 14 days to synchronize estrus by regulating progesterone levels. Sixteen days after the removal of the CIDRs, 25 mg of prostaglandin F_2 alpha (Lutalyse; dinoprost tromethamine; Pfizer Animal Health, New York, NY) was administered intramuscularly (i.m.). Three days later, 100mcg of gonadotropin releasing hormone Cystorelin was administered i.m. (gonadorelin diacetate tetrahydrate; Merial; Duluth, GA). Heifers were then artificially inseminated with semen from one *B. t. indicus* bull (Nelore breed; ABS CSS MR N OB 425/1 677344 29NE0001 97155). Three out of the seven heifers (2 Angus, 1 Hereford) were confirmed pregnant by ultrasonography examination on day 30 of gestation. Two males and one female *B. t. indicus* x *B. t. taurus* F1 concepti were collected on day 65 of gestation at the University of Missouri Veterinary School's abattoir.

The reason we collected day 65 concepti was because a study by Cezar *et al.* (2003) determined that DNA methylation levels were the same between day 60 of gestation and adult animals. The following tissues were collected; amnion, chorioallantois, brain, tongue, heart, kidney, liver, lung, intestines, and reproductive tract. Tissues were snap frozen in liquid nitrogen and stored at -80°C until use.

RNA extraction and cDNA synthesis for parental-allelic expression analysis

The chorioallantois, liver, brain, heart, and tongue of day 65 *B. t. indicus x B. t. taurus* F1 concepti were homogenized with a plastic disposable pestle (Fischer Scientific; Pittsburgh, PA) in 450µl of lysis binding buffer. The tissue lysates were then passed through a 22 and 26 gauge needle connected to a 1ml syringe. RNA was extracted from the tissues using a commercially available kit (High Pure RNA; Roche Applied Science; Mannheim, Germany) following manufacturer's specifications. cDNA was synthesized in a 20µl reaction using 10µl of RNA (130 ng Total RNA) and 10µl of a master mix containing: 10mM DTT (Invitrogen; Carlsbad, CA), 1X First Strand buffer (Invitrogen; Carlsbad, CA), 0.5 µg random primers (Promega; Madison, WI), 1mM dNTPs (each dNTP; Fischer Scientific; Pittsburgh, PA), 100 units Superscript II reverse transcriptase (Invitrogen; Carlsbad, CA), and 20 units of Optizyme RNase Inhibitor (Fischer Scientific; Pittsburgh, PA). Then the samples were incubated in a thermal cycler PCR machine for one hour at 42°C followed by ten minutes at 95°C. The samples were then stored in the -20°C until further analysis. To verify that there was not DNA contamination a minus Reverse Transcriptase control was prepared for each sample processed. RNA was also collected and cDNA prepared from several *B. t. taurus* and *B. t. indicus* tissues to serve as RFLP assay controls (data not shown).

Imprinted expression analysis of *B. t. indicus* x *B. t. taurus* concepti

B. t. indicus x *B. t. taurus* F1 tissues were used to determine gene expression of *KCNQ1OT1*, *CDKN1C*, *H19*, and *PLAGL1*. The PCRs primers generated for expression

analysis were intron-spanning for *CDKN1C* and *H19*. However, the primers used to amplify *KCNQ1OT1 and PLAGL1* were designed within a single exon. The possibility of DNA contamination in the cDNA was eliminated by the exclusion of the Reverse Transcriptase from the cDNA master mix. The RT-PCR program started with an initial denaturation step at 94°C for 2:15 min. This initial step inactivates the antibody bound to the polymerase and separates the double stranded DNA into single strands. The denaturation (94°C for 30 sec), annealing (**Table 1**), and extension (72°C for 1 min) steps were repeated for 35 cycles with a final extension at 72°C for five minutes.

Restriction fragment length polymorphism (RFLP) was used to identify allelic expression for each gene. The SNPs between *B. t. taurus* and *B. t. indicus* used as the restriction site are shown in **Table 2**. After restriction enzyme digestion the assays were resolved by polyacrylamide gel electrophoresis (PAGE; **Table 3**). In cases when the repressed allele was expressed the band intensity was measured by the UN-SCAN-IT gel 5.3 alias gel analysis software (Silk Scientific; Orem, UT) that functions as a gel band densitometer. To be considered biallelic a sample had to have 10% or higher expression from the repressed parental allele (Rivera *et al.,* 2008).

DNA extraction, bisulfite mutagenesis and COBRA procedures

DNA was extracted from day 65 *B. t. indicus x B. t. taurus* F1 tissues using a phenol-chloroform extraction procedure. Then, bisulfite mutagenesis was performed following the instructions for the Imprint DNA Modification Kit One-Step Modification kit (Sigma-Aldrich; St. Louis, MO). During the bisulfite mutagenesis procedure all unmethylated

Table 1. Imprinted gene expression PCR primers

Gene Symbol		Primers (5'-3')	PCR Annealing Tm (°C)	PCR size (bp)	Primer [] µM	$MgCl_2$ [] mM	# Cycles
H19	Forward Reverse	GATATGGTCCGGTGTGATGGAGAGAGCA TTCGGAGCCTCCAGACTCGGTG	62.8	752	0.3 0.3	2.5	35
KNCQ1OT1	Forward Reverse	TCGAGGGTACCGGATTCCCAGGC CGCAGGACACCCCAACTACAGCC	64	502	0.3 0.3	2.5	35
CDKN1C	Forward Reverse	GGAGGCGCCGCGATCAAGAAG GACAGCGAAAGCGCGAAGAGAC	62	745	0.3 0.3	4	35
PLAGL1	Forward Reverse	TCAACCGGAAAGACCACCTGAAGA GGTCAAAGCCTGCATTGAGCTTGT	60	834	0.3 0.3	4	35

Tm= temperature, [] concentration, bp= base pair

Table 2. Polymorphisms used for restriction analysis between *B. t. indicus* and *B. t. taurus*

Gene Symbol	Maternal *B. t. taurus*	Paternal *B. t. indicus*	Exon of PM	NCBI accession # (based on Btau_4.2)	PM location in reference NCBI: Btau 4.2
H19	C	T	5 5	NR_003958.2 2069 bp	1831
KNCQ1OT1	A	G	Closer to start of transcription	refNW_001494547.3 3146522-3146021	3146321
CDKN1C	C	T	4	refNW_001494547.3 2954777-2956157	2955801
PLAGL1	T	G	6	NM_001103289.1 2815 bp	867

PM= polymorphism

Table 3. Restriction enzyme digest analysis

Gene Symbol	Expressed Allele	Restriction enzyme	Digested *B. t. taurus* (bp)	Digested *B. t. indicus* (bp)	PAGE Details
H19	Maternal	BsiHKAI	609, 143	609, 35, 108	18%
KNCQ1OT1	Paternal	HinfI	457, 32, 13	268,189,32,13	7%
CDKN1C	Maternal	AvaII	494, 251	361,251,133	10%
PLAGL1	Paternal	MluI	834	387, 447	10%

PAGE= Polyacrylamide gel electrophoresis, bp= base pair

cytosines are converted to uracils while methylated cytosines remain as cytosines. During PCR the uracils will be replaced by thymines. Therefore, after undergoing bisulfite mutagenesis, differentially methylated alleles will have unequal sequences which are recognized differently by restriction enzymes. Primers for the bisulfite mutagenized DNA were designed for the *H19/IGF2* ICR and the KvDMR1. PCR was used to amplify a 493 bp region of the H19/IGF2 ICR and a 417 bp region of the KvDMR1.

Methylation status of the loci was determined by combined bisulfite restriction enzyme assay (COBRA; **Table 4**). The enzyme used to restrict the methylated *H19/IGF2* ICR was DpnII while Taqα1 restricted the methylated allele of the KvDMR1. The PCR amplicons and digested products were resolved by 7% polyacrylamide gel electrophoresis (PAGE).

Table 4. Polymorphisms used for differential methylation analysis between *B. t. indicus* and *B. t. taurus*

Gene Symbol		Bisulfite Primers (5'-3')	Maternal *B. t. taurus*	Paternal *B. t. indicus*	NCBI accession # (based on Btau_4.2)	PM location in reference Btau 4.2	Restriction enzyme
H19/IGF2 ICR	Forward Reverse	GGGGAGGTTGTCGGGTTTATGG CCGCACCCCTCCTTTAACATC	A	G	ref|NW_001494547.3 3724214 -3724706	3724402	DpnII
KvDMR1	Forward Reverse	GTTAGAGGAGTATTTTGAAGAGA CCCTCTCAACCAATAACAAAAC	G	C**	ref|NW_001494547.3 3133974- 3133558	3133883	Taqα1

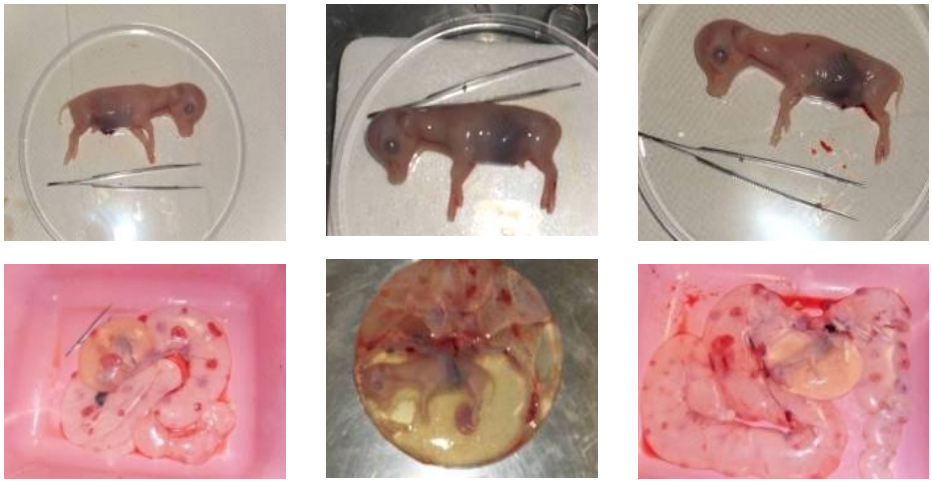
PM= polymorphism
** The C will be converted to a T during bisulfite mutagenesis

Results

F1 *B. t. indicus x B. t. taurus* day 65 concepti for baseline imprinted gene expression in BWS-associated genes

In order to determine if the bovine would be a good model to use to study BWS we must first determine baseline expression of imprinted genes known to be misregulated with BWS. Three F1 *B. t. indicus x B. t. taurus* concepti were collected on day 65 of gestation (**Figure 1**) and the following tissues were analyzed: brain, tongue, heart, liver, and chorioallantois. These tissues were analyzed for imprinted gene

Figure 1. F1 *B. t. indicus x B. t. taurus* day 65 concepti



A B C

Figure 1. Three F1 B. t indicus x B. t. taurus day 65 concepti collected to determine baseline imprinted gene expression in BWS-associated genes and differentially methylated regions.

expression of *KNCQ1OT1*, *CDKN1C*, *PLAGL1*, and *H19*. In the bovine, *KCNQ1OT1*, *CDKN1C*, and *H19* are located on chromosome 29 while *PLAGL1* is found on chromosome 9.

RFLP was the method used to determine allele-specific imprinted gene expression using SNPs identified by our lab (**Table 2 and 3**). *KNCQ1OT1*, *CDKN1C*, *PLAGL1*, and *H19* showed the correct monoallelic expression in all tissues analyzed (**Table 5**). However, gene expression was not detected for all tissues of each F1 concepti studied. For example, the RNA of the chorioallantois that belonged to *B. t. indicus x B. t. taurus* F1-C (**Table 5**) had degraded during collection and no expression was observed for any imprinted assay. Likewise, there was no expression detected for *KCNQ1OT1* in the heart of F1-A or for *PLAGL1* in the liver sample of conceptus F1-B.

Several of the tissues studied had low level expression from the repressed allele of *KNCQ1OT1*, *CDKN1C*, *PLAGL1*, however because this expression was not greater than 10% they were considered to be expressing those genes in a monoallelic manner (**Table 5 and Figures 2-4**). *H19* displayed no expression from the parentally-repressed allele from any F1 conceptus tissue sample (**Figure 5**).

Table 5. F1 *B. t. indicus x B. t. taurus* day 65 concepti for baseline imprinted gene expression in BWS-associated genes

	F1-A *B. t. indicus x B. t. taurus* day 65 conceptus			
	Genes Analyzed by Restriction Digest			
	KCNQ1OT1	***H19***	***CDKN1C***	***PLAGL1***
Tissue	(%) - expression from repressed allele			
Chorioallantois	Mono (2.65%)	Mono	Mono	Mono (3.75%)
Liver	Mono (6.90%)	Mono	Mono	Mono (4.73%)
Brain	Mono (6.01%)	Mono	Mono	Mono (1.66%)
Heart	N/A	Mono	Mono	Mono (2.17%)
Tongue	Mono (4.09%)	Mono	Mono (5.67%)	Mono (6.39%)

	F1-B *B. t. indicus x B. t. taurus* day 65 conceptus			
	Genes Analyzed by Restriction Digest			
	KCNQ1OT1	***H19***	***CDKN1C***	***PLAGL1***
Tissue	(%) - expression from repressed allele			
Chorioallantois	Mono (2.33%)	Mono	Mono	Mono (5.50%)
Liver	Mono (6.17%)	Mono	Mono	N/A
Brain	Mono (6.46%)	Mono	Mono	Mono (2.63%)
Heart	Mono (8.01%)	Mono	Mono	Mono (4.59%)
Tongue	Mono (7.08%)	Mono	Mono	Mono (9.58%)

	F1-C *B. t. indicus x B. t. taurus* day 65 conceptus			
	Genes Analyzed by Restriction Digest			
	KCNQ1OT1	***H19***	***CDKN1C***	***PLAGL1***
Tissue	(%) - expression from repressed allele			
Chorioallantois	N/A	N/A	N/A	N/A
Liver	Mono (4.01%)	Mono	Mono	Mono (2.40%)
Brain	Mono (5.60%)	Mono	Mono	Mono (4.77%)
Heart	Mono (9.74%)	Mono	Mono	Mono (5.74%)
Tongue	Mono (1.96%)	Mono	Mono	Mono

Table 5. Imprinted gene expression analysis was performed using restriction fragment length polymorphisms to differentiate between the parental alleles. F1 *B. t. indicus x B. t. taurus* day 65 embryonic and extraembryonic tissue samples were collected from three F1's (A,B,C). Monoallelic expression (Mono) was found in the following imprinted genes known to be misregulated in BWS: *KCNQ1OT1*, *CDKN1C*, *H19*, and *PLAGL1*. (%) is the percent expression from the repressed allele if less than 10% then the sample was considered Mono. N/A represents degradation of samples for those assays.

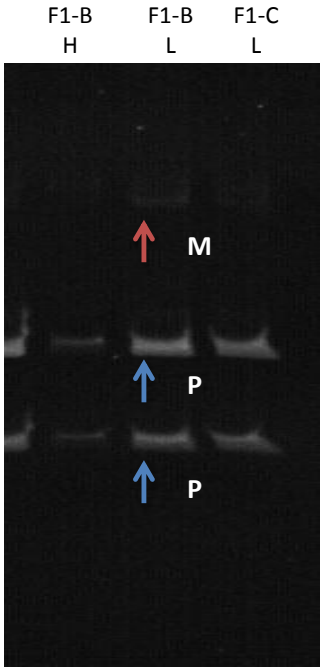


Figure 2. *KCNQ1OT1* paternal monoallelic expression. F1 (B,C) *B. t. indicus x B. t. taurus* naturally-conceived day 65 concepti determined by RFLP. H= heart, L= liver. Blue → (paternal allele; P), Red → (maternal allele; M)

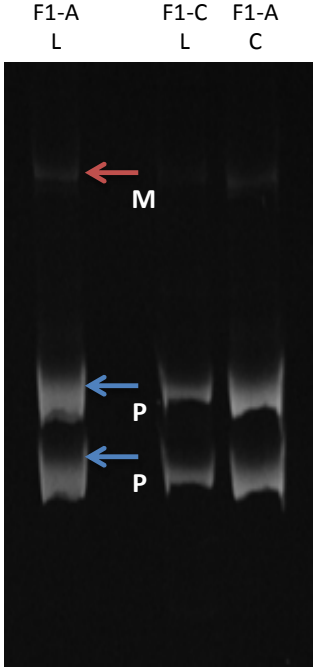


Figure 3. *PLALG1* paternal monoallelic expression. F1 (A,C) *B. t . indicus x B. t. taurus* naturally-conceived day 65 concepti determined by RFLP. L= liver, C= chorioallantois. Blue → (paternal allele; P), Red → (maternal allele; M)

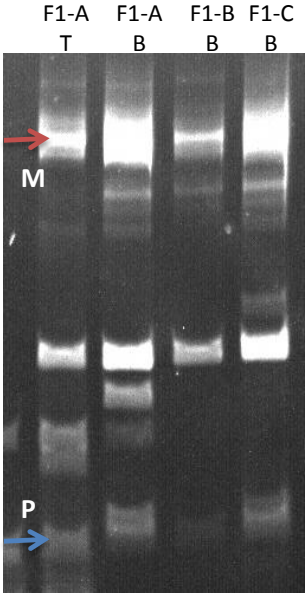


Figure 4. *CDKN1C* maternal monoallelic expression. F1 (A,B,C) *B. t. indicus x B. t. taurus* naturally-conceived day 65 concepti determined by RFLP. T= tongue, B= brain. Blue → (paternal allele; P), Red → (maternal allele; M).

Figure 5. *H19* maternal monoallelic expression. F1-A *B. t. indicus x B. t. taurus* naturally-conceived day 65 concepti determined by RFLP. A= muscle, N= Nelore fat, F1 –A ; h= heart). Blue → (paternal allele; P), Red → (maternal allele; M).

F1 *B. t. indicus x B. t. taurus* day 65 concepti for baseline methylation in BWS-associated imprinting control regions

Combined bisulfite restriction analysis was used to determine the methylation status of the *H19/IGF2* ICR and KvDMR1. These two ICRs are the two differentially methylated regions primarily misregulated in BWS patients (Weksberg *et al.,* 2010). From our study we were able to determine where differential methylation is observed within these ICRs associated with BWS in F1 *B. t. indicus x B. t. taurus* naturally-conceived concepti **(Figure 6-7**). Both the *H19/IGF2* and the KvDMR1 regions in the bovine showed 50/50 methylation which has also been observed in humans (Takai *et al.,* 2001; Beatty *et al.,* 2005; Cerrato *et al.,* 2008; Ideraabdullah *et al.,* 2008). This results

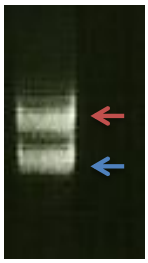


Figure 6. *H19/IGF2* ICR bisulfite assay restriction enzyme analysis. *H19/IGF2* ICR 4F-3R (493 bp; 45 CpGs) the methylation status was determined by RFLP using the restriction enzyme DpnII which cuts only the methylated allele at 414 bp. This digest shows 50/50 methylation of F1-B *B. t. indicus x B. t. taurus* naturally-conceived day 65 concepti. Blue → (methylated), Red → (unmethylated allele). C= chorioallantois.

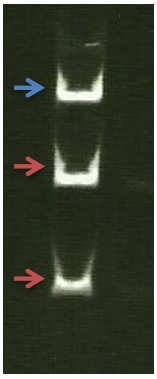


Figure 7. KvDMR1 bisulfite assay restriction enzyme analysis. KvDMR1 B23F-B23R (417 bp; 41 CpGs) the methylation status was determined by RFLP using the restriction enzyme Taqα1 which cuts only the methylated allele at 248 bp. This digest shows 50/50 methylation of F1-A *B. t. indicus x B. t. taurus* naturally-conceived day 65 concepti . Blue → (unmethylated allele), Red → (methylated allele). C= chorioallantois.

shows that the bovine is differentially methylated in the same manner as human and mouse in BWS-associated regions.

Discussion

In this study, we set to determine the pattern of expression of four imprinted genes associated with the human overgrowth syndrome Beckwith-Wiedemann in bovids. By using RT-PCR and RFLP analysis we were able to determine the imprinted gene expression for *KCNQ1OT1*, *PLAGL1*, *CDKN1C*, and *H19*. We analyzed gene expression and DNA methylation in embryonic and extraembryonic tissues of three F1 day 65 *B. t. indicus* x *B. t. taurus* concepti. Our results showed that similar to humans, *KCNQ1OT1* and *PLAGL1* are monoallelically expressed from the paternal allele while *CDKN1C* and *H19* are maternally-expressed genes in bovids. The imprinted gene expression was observed in all tissues analyzed which included brain, heart, liver, tongue, and chorioallantois. Another result from this study revealed that the KvDMR1 and the *H19/IGF2* ICRs are differentially methylated in the bovine which is also observed in the human and mouse.

BWS is the most common pediatric overgrowth syndrome with an incidence of one in 13,700 live births (reviewed in Cohen, 2005; Weksberg *et al.,* 2010). BWS has been classified as a disorder involving somatic overgrowth, predisposition to embryonic tumors, and congenital malformations (Weksberg *et al.,* 2003; Weksberg *et al.,* 2010). No current animal models are able to fully phenocopy BWS. The imprinted genes associated with BWS have been shown to be conserved between the human and mouse

(Qian *et al.,* 1997; Paulsen *et al.,* 1998; Weber *et al.,* 2000; Mancini-DiNardo *et al.,* 2003,; Gabory *et al.,* 2006; Lewis *et al.,* 2006). However, there been several mouse models which have not been able to recapitulate all the diagnostic clinical features associated with BWS (Leighton *et al.,* 1994; Caspary *et al.,* 1999).

There are many reasons to propose the use of bovids as a model to study BWS. First, LOS has several phenotypical similarities with BWS including somatic overgrowth, increased birth weight, visceromegaly, hypoglycemia and polyhydramnios (Farin and Farin, 1995; Young *et al.,* 1998; Bertoloini and Anderson, 2001; Hiendleder *et al.,* 2004; Farin *et al.,* 2006). Second, increased *IGF2* expression has been observed in day 70 LOS concepti (Blondin *et al.,* 2000). This is interesting since 2-10% of BWS patients' have biallelic expression of the paternally-expressed *IGF2* in tongue and in fibroblast (Weksberg *et al.,* 1993). In BWS, *IGF2*'s biallelic expression is due to gain of methylation at the *H19/IGF2* ICR. Third, both species have a nine month gestation period. This is particularly important because the sequence of events that result in a condition may occur at similar times during pregnancy. Fourth, comparative genome analysis (Miziara *et al.,* 2004; Khatib *et al.,* 2007) shows that the percent identity between the cow and human genomes is greater than between mouse and human (73.8 vs. 66.8%, respectively; Miller *et al.,* 2007) and the functional features within the human genome align better with cow than mouse (Miller *et al.,* 2007). Lastly, both the bovine and human gestations usually involve one offspring. The control of imprinted genes differs between human and mouse at the KvDMR1 domain and this has been speculated to be a result of the number of offspring normally carried during gestation (Monk *et al.,* 2006).

It is likely that there has been divergence for growth regulation between litter bearing and non-litter bearing species.

Another important similarity between humans and bovids is the adverse response of preimplantation embryos to *in vitro* manipulations. For instance, children that are conceived by the use of ART have a higher incidence (3-9 times) of having the loss-of-imprinting overgrowth syndrome BWS (DeBaun *et al.,* 2003; Gicquel *et al.,* 2003; Maher *et al.,* 2003; Halliday *et al.,* 2004; Sutcliffe *et al.,* 2006; Lim *et al.,* 2009). Likewise, a fetal overgrowth syndrome has also been documented in ruminants as a result of ART. In ruminants this syndrome is known as large offspring syndrome (LOS). Since the overgrowth phenotype has been observed in ruminants and humans as a result of assisted reproduction, we propose that both syndromes have similar epigenetic etiologies. In order to determine the plausibility of our hypothesis we need to ascertain if BWS-associated imprinted gene expression misregulation is recapitulated in LOS. However, until now no work has been done that shows if these imprinted regions are similarly regulated in bovids.

Our study shows that genomic imprinting is conserved between human and bovids in the imprinted regions known to be misregulated in BWS. Ongoing studies from our lab are determining if LOS and BWS are epigenetically similar.

Conclusion

In conclusion, our study established the imprinting status of *KCNQ1OT1*, *CDKN1C*, *PLAGL1*, and *H19* in bovine F1 day 65 *B. t. indicus* x *B. t. taurus* concepti and

found that imprinting was conserved with humans. These genes are associated with the human overgrowth and loss-of-imprinting syndrome BWS. We have also determined that the ICRs primarily affected in BWS, namely KVDMR1 and *H19/IGF2*, are differentially methylated as seen in humans. Currently no animal models are able to fully recapitulate BWS. Our results suggest that the bovine may be able to serve as an appropriate animal model for studying BWS.

General Summary and Conclusion

In our study, we determined DNA sequence polymorphisms between *B. t. indicus* and *B. t. taurus* subspecies of cattle in imprinted genes known to be misregulated in BWS. For our study we had to first determine the genomic sequence for BWS-associated genes that were not annotated in the bovine. Once this was accomplished we were able to find DNA sequence polymorphisms within the imprinting control regions and transcriptional units of the imprinted genes of interest.

In summary, 1) DNA sequence polymorphisms were found in the IC1 (*H19/IGF2* ICR) as well as within the transcripts of the maternally-expressed gene *H19* and the paternally-expressed gene *IGF2*; 2) DNA sequence polymorphisms were found for the IC2 (KvDMR1) as well as the maternally-expressed flanking genes, *CDKN1C* and *PHLDA2* and the paternally-expressed gene *KCNQ1OT1*; 3) Other loci (*i.e*. *PLAGL1/ HYMAI)* have been associated to have LOM in BWS. A polymorphism has been found at *PLAGL1* transcript. However, with 3 Kb left to sequence, no DNA sequence polymorphism for *HYMAI* has been identified thus far; 4) Sequencing of exons 2-12 of *MEST* revealed no DNA sequence polymorphisms between *B. t. indicus* and *B. t. taurus*.

Our data also shows where differential methylation occurs in the bovine at the KvDMR1 and *H19/IGF2* domains. We were unable to find the putative DMR for *HYMAI/PLAGL1*. Our bisulfite assays were never able to identify equal levels of methylation from both parental alleles. More work will need to be done in order to identify the *HYMAI/PLAGL1* DMR. The *MEST* DMR was determined in *B. t. taurus* but this

region was unable to be amplified in the *B. t. indicus* suggesting that there could be an insertion/deletion in this region that the primers are unable to amplify; further sequencing must be done before we can determine that this region is indeed the DMR.

The second part of this thesis was to generate parental-specific expression assays using restriction enzyme digestion to differentiate between the parental alleles. This was done for the following genes: *KCNQ1OT1*, *CDKN1C*, *H19*, and *PLAGL1*. Not all polymorphisms found in BWS-associated imprinted genes were useful for the use of restriction fragment length polymorphism assays to distinguish between the maternal and paternal alleles. Therefore, SSCP will need to be used as the method of choice to analyze expression for *PHLDA2* and *IGF2*. These assays will be tested in the future to determine the imprinting status of these genes.

The final part of our study was to determine baseline expression of *KCNQ1OT1*, *CDKN1C*, *H19*, and *PLAGL1* in the liver, brain, heart, tongue, and chorioallantois of F1 *B. t. indicus* x *B. t. taurus* day 65 concepti. Results from our study suggest that the bovine may serve as a good model to study BWS because imprinting is conserved in the regions primarily misregulated in BWS.

Now that baseline expression has been determined in F1 *B. t. indicus* x *B. t. taurus* naturally-conceived concepti, the next step is to use these assays to study day 100 concepti produced with culture conditions expected to induce LOS. Future studies will determine if LOS is epigenetically similar to BWS and if bovids will serve as an appropriate animal model to study this human overgrowth syndrome.

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APPENDICES

APPENDIX 1: Determination of the methylation status of *CDKN1C* in bovine

In the mouse, *CDKN1C's* DMR has been shown to extend from the promoter region through the second exon (Bhogal *et al.,* 2004; Cerrato *et al.,* 2005). However, the homologous region is not differentially methylated in humans (Chung *et al.,* 1996).

I successfully sequenced CDKN1C's exons 1 and 2 but no polymorphisms were found between *B. t. indicus* and *B. t. taurus*. Countless attempts were made to sequence the promoter region of the bovine *CDKN1C* gene. Over twenty sets of primer pairs were designed encompassing the promoter region of *CDKN1C* and extending through known sequenced region of *CDKN1C.* However, sequencing results never coincided with the expected region on chromosome 29 although, according to the databases, the primers aligned perfectly to the bovine *CDKN1C's* promoter.

Even though we failed in our attempt to amplify and sequence *CDKN1C*'s promoter region we decided to design bisulfite sequencing primers over the previously sequenced exons to at least determine if the region was hypomethylated as in the human or differentially methylated as in the mouse. Several sets of bisulfite primers were generated but no primer pair combination resulted in amplification of the bisulfite mutagenized DNA. One reason for this may be due to the fact that the region has 73% C + G content. Despite using GC specific buffers to help relax the PCR conditions no amplicon were ever obtained.

Therefore, in order to determine the methylation status of *CDKN1C* in bovine we decided to take a different approach by using restriction enzyme analysis. Isoschizomers are restriction enzymes that recognize and cleave the same recognition sequence. The two isoschizomers used to test the methylation status of *CDKN1C* were HpaII and MspI. These two restriction enzymes allowed us to differentiate between methylated and unmethylated CpGs. HpaII is methylation sensitive and is blocked by CpG methylation and therefore will not be able to restrict genomic DNA that is methylated at the CCGG recognition sites whereas, MspI is methylation insensitive and able to cleave both methylated and unmethylated DNA at the CCGG recognition sites.

First genomic DNA was isolated from the kidney of *B. t. taurus* (Holstein), *B. t. indicus* (Nelore), and F1 *B. t. indicus x B. t. taurus* day 65 conceptus (F1-C; female conceptus). The genomic DNA was divided into five groups and treated as follows: 1) untreated DNA, 2) DNA treated with the CpG methyltransferase M. Sss1 (methylates all CpGs), 3) DNA treated with M. Sss1 prior to digestion with HpaII, 4) DNA digested with HpaII, and 5) DNA treated with MspI. The primer pair 225L-1374R was used to amplify a 1090 bp region encompassing exon one through intron two which contained 19 HpaII/MspI sites.

As expected, MspI digestion cleaved the DNA thus fragmenting the template and preventing amplification of the region (**Figure A1.1**). The PCR primers were able to amplify a region of the correct size for the untreated genomic DNA, the M. Sss1 treated DNA, and the M. Sss1 + HpaII treated DNA groups. However, no amplicons were

detected for the genomic DNA treated with HpaII suggesting a hypomethylated state of the genomic region.

This preliminary result suggests that the methylation status of the *CDKN1C*'s DMR in bovine may be more similar to human than the mouse. However, further studies will need to be done to verify this conclusion.

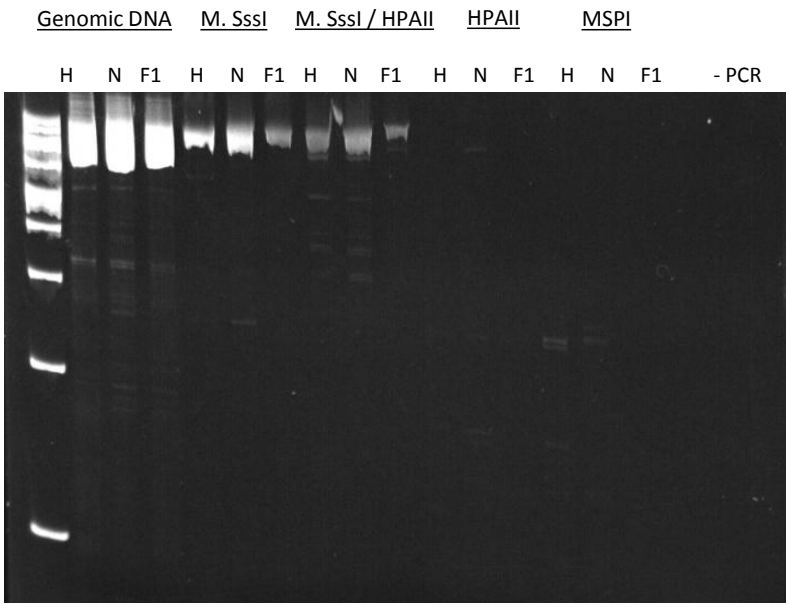

Figure A1.1. **Methylation analysis of *CDKN1C's* DMR in bovine**. Restriction enzyme analysis was used to determine the methylation status of *CDKN1C* DMR in the bovine. The restriction enzymes HPAII (blocked by CpG methylation) and MSPI (able to digest both methylated and unmethylated CpGs) were used to determine the methylation of *CDKN1C* exons 1 through intron 2. M. Sss1 (methylates all CpGs) was used as a positive control to show that HPAII is unable to cleave methylated CpGs. Our results show that at least one of the 19 CCGG recognition sites for HPAII was unmethylated because there was no PCR amplification of this region for the HPAII digested template. H = Holstein, N = Nelore, F1 = *B. t. indicus x B. t. taurus* F1-C conceptus. - PCR = water PCR control to show no DNA contamination.

APPENDIX 2: *In vitro* production (IVP) of *B. t. indicus* and *B. t. taurus* bovine embryos

The following steps were taken to generate potential large offspring syndrome day six blastocysts.

The IVP protocol was followed exactly as described by Rivera et al., (http://www.animal.ufl.edu/hansen/ivf/). All *In vitro* fertilization (IVF) media (Hepes-TL, IVF-TL, SP-TL) were purchased from Caisson Labs (Logan, UT). All chemicals used to prepare the media were purchased from Sigma (St. Louis, MO).

Holstein bovine cumulus-oocyte complexes (COCs) were shipped overnight in maturation medium from Trans Ova Genetics (Long Prairie, MN). The oocytes were rinsed in HEPES-TALP and immediately placed in IVF-TALP.

For sperm cell purification, we had to use glass wool columns because the sperm cells from the Nelore bull we used (ABS CSS MR N OB 425/1 677344 29NE000197155) were sensitive to the Percoll gradient. For this, the column was preequilibrated three hours before COCs arrived by rinsing with 4ml, 3ml, 2 ml of Hepes-TALP, SP-TALP, and IVF-TALP, respectively. For all experiments one frozen semen straw from Nelore (ABS CSS MR N OB 425/1 677344 29NE000197155) was used and thawed in the citothaw at 35.5°C. The semen straw contents were put into a 15 ml centrifuge tube containing 13ml of SP-TALP and spun for 15 minutes at 200 x g. Immediately after spinning, the pellet was resuspended in 300µl of IVF-TALP and then this volume was pipetted into the wool column filtered and collected into a sterile microcentrifuge tube. The optimum

Heparin concentration in the IVF-TALP for this bull is 2.5 µg/ml. The COCs and the sperm cells were incubated at 38.5°C in humidified air containing 5% CO_2 for 19 hours.

Practice IVP runs - Putative zygotes were grouped in 25-30 and placed in 50µl KSOM drops covered in mineral oil (Sigma, St. Louis, MO) and placed in a chamber injected with a gas mixture containing 90% N_2, 5% CO_2, and 5%O_2. Cleavage rate (**Table A2.1**) was assessed on day 3 post fertilization. On Day 5, the unfertilized zygotes and degenerated embryos were removed from the KSOM and 10% (5µl) estrus cow serum was added to half of the KSOM drops and the embryos were placed back into the chamber until day 8 when blastocyst rate was determined (**Table A2.1**).

Experimental concepti – The IVP procedures were followed as above except that cleavage rate was assessed on day 5 and the embryos were shipped overnight to Trans Ova Genetics (Sioux Center, IA) in a 38.5°C portable incubator. For that, 20-30 early blastocysts were split into two groups and placed in 2.0ml tubes (Nunc, Rochester, NY) with 1.8ml of KSOM supplemented with 180µl estrus cow and covered with mineral oil. Embryo transfer technicians at Trans Ova Genetics received the embryos on day 7 and prepared them for transfer into 30 synchronized Holstein cows (two blastocysts/surrogate). On day 40 pregnancies were verified via ultrasonography and 19 females were confirmed pregnant (60% pregnancy rate; **Figure A2.1**).

Table A2.1 Cleavage and blastocyst rate for IVP serum and serum restricted *B. t. indicus* x *B. t. taurus* embryos

(+) Serum

Date	Cleavage Rate %	Blastocyst Rate %
2/17/2011	n/a	n/a
3/3/2011	n/a	n/a
3/17/2011	58	46
4/5/2011	56	26
4/7/2011	60	14
4/8/2011	43	33
4/12/2011	57.6	28
4/14/2011	62.6	19
4/19/2011	64	20.3
4/21/2011	57	11
4/28/2011	61	18
5/18/2011	n/a	n/a
5/24/2011	n/a	n/a

(-) Serum

Date	Cleavage Rate %	Blastocyst Rate %
2/17/2011	78.8	12.4
3/3/2011	69.5	26.1
3/17/2011	58	20.5
4/5/2011	n/a	n/a
4/7/2011	n/a	n/a
4/8/2011	n/a	n/a
4/12/2011	n/a	n/a
4/14/2011	n/a	n/a
4/19/2011	n/a	n/a
4/21/2011	n/a	n/a
4/28/2011	n/a	n/a
5/18/2011	50	20.7
5/24/2011	59	11.5

(+)= 10% estrus cow serum added, (-)= no estrus cow serum added, %= percentage. Cleavage rate = total number of oocytes/number $\geq$ 2-cell embryos x 100. % blastocyst = number of blastocysts/numbers of cleaved embryos x 100. n/a = embryos were not generated for that treatment.

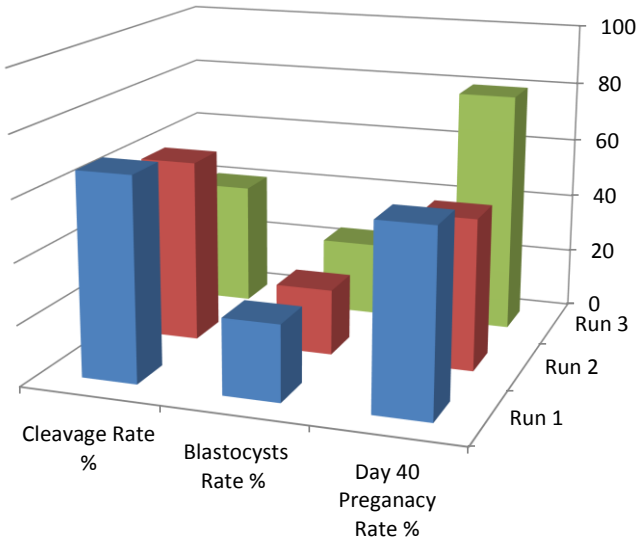

Figure A2.1 F1 *B. t. indicus* x *B. t. taurus in vitro*-produced and transferred embryos. These data corresponds to the embryos that were cultured with conditions expected to produce LOS offspring. Cleavage rate = total number of oocytes/number ≥ 2-cell embryos x 100. % blastocyst = number of blastocysts/numbers of cleaved embryos x 100. Pregnancy rate determined by ultrasonography on day 40 by Trans Ova Genetics. Ten cows received two embryos for each run.

APPENDIX 3: BWS-associated regions of exact DNA sequence between the *B. t. indicus* and *B. t. taurus* subspecies of cattle

Many regions which contained no polymorphisms between *B. t. indicus* and *B. t. taurus* in BWS-associated imprinted loci were sequenced. These primers, the genomic region amplified, and the NCBI bovine genome position number will be listed in a table format.

Table A3.1 - BWS-associated regions conserved between *B. t. indicus* and *B. t. taurus*

Gene Symbol		Genomic Primers (5'-3')	Exon of Primer	NCBI accession # Reference assembly (based on Btau_4.2)	Chromosome
IGF2	Forward	GCATCGTGGAAGAGTGTTGCTT	9	NC_007330.4	29
	Reverse	ACAGGACGGTACAGGGATTTCA	10	51369435..51388040	29
IGF2	Forward	GGCCAAATTTGCAGGTAGGCTTGT	10	NC_007330.4	29
	Reverse	AAACCGTTCCTCACTCTGCACGAT	10	51369435..51388040	29
KCNQ1OT1	Forward	ACAAGGATGCTGCCTTTCCCAAAC	1	refNW_001494547.3	29
	Reverse	CAAGCAGGAAGCTGTTCTTTGCCA	1	3163404-3166519	29
KCNQ1OT1	Forward	CCATGTTCACACGCTTTCCTTGCT	1	refNW_001494547.3	29
	Reverse	AGCAAAGACGGAGCAGGTTGGATA	1	3148624 -3152100	29
KCNQ1OT1	Forward	GTGACATTTGGTTCTGAGTAGCC	1	refNW_001494547.3	29
	Reverse	CTATAGCAATGGGATGGAAGCTG	1	3140520-3146187	29
CDKN1C	Forward	GAGGACTGGGCGTTCCACAGGCCA	exon 1	refNW_001494547.3	29
	Reverse	GCCCTTTAACGGCCAGGAGGC	intron 2	2952619 -2957867	29
PHLDA2	Forward	CCACTATAAAGGCGGCTCCCA	5' TSS	refNW_001494547.3	29
	Reverse	TACACCGAAGGTGAAAGCGACATC	intron 1	2914921 -2915632	29
PLAGL1	Forward	TCAACCGGAAAGACCACCTGAAGA	6	NC_007307.4	9
	Reverse	GGTCAAAGCCTGCATTGAGCTTGT	6	84129860 to 84185561	9

Table A3.1.1 - BWS-associated regions conserved between *B. t. indicus* and *B. t. taurus*

Gene Symbol	PCR Annealing Tm(°C)	PCR size (bp)	Primer [] µM	$MgCl_2$ [] (mM)	5% DMSO	#Cycles	Taq Polymerase
IGF2	58	(1F-1R) 762	0.3 0.3	4		35	Go Taq Promega
IGF2	60.5	(AF-AR) 802	0.3 0.3	4			Go Taq Promega
KCNQ1OT1	(65-58) 56	(A1F-A2R) 1116	0.3 0.3	2.5		(2) 35	Go Taq Promega
KCNQ1OT1	62	(B1F-B1R) 662	0.3 0.3	2.5		30	Go Taq Promega
KCNQ1OT1	60	(G2F-G2R) 907	0.3 0.3	2.5		30	Go Taq Promega
CDKN1C	59	(225L-1374R) 1090	0.4 0.4	GC Buffer II		35	LA Taq Takara
PHLDA2	59.2	(3F-3.2R) 712	0.3 0.3	4	yes	35	Go Taq Promega
PLAGL1	60	(1F-1R) 834	0.3 0.3	4	yes	35	Go Taq Promega

[]= concentration, Tm= temperature (°C) , DMSO= dimethyl sulfoxide.

Table A3.2 - BWS-associated regions conserved between *B. t. indicus* and *B. t. taurus*

Gene Symbol		Genomic Primers (5'-3')	Exon of Primer	NCBI accession # Reference assembly (based on Btau_4.2)	Chromosome
HYMAI	Forward	AGTCGTGTTCAATGCCTCCTCA	1	ref NW_001495600.2	9
	Reverse	AATGCTGGCCGGGAAATATACG	1	4134712 -4138967	9
HYMAI	Forward	TCACCCACATGCCTGTTACCTT	1	ref NW_001495600.2	9
	Reverse	TGGAATGTCACCTTCAAAGGGC	1	4134712 -4138967	9
HYMAI	Forward	ACGTGTCTCCGTGTGGACATTT	1	ref NW_001495600.2	9
	Reverse	ACCATCAATTCAGCTCCCTGGT	intron 1	4134712 -4138967	9
HYMAI	Forward	GCTTACAGCGTAGTCAGCAAATTAGGG	1	ref NW_001495600.2	9
	Reverse	TGGACTGAAGATCCAGTTAAAGGACAGAC	1	4134712 -4138967	9
MEST	Forward	ACCTCCATGCTGTTCTCTTCCACT	intron 11	NC_007302.4	4
	Reverse	CAAAGCCATTCGAGGCAAGGTGAA	12	97467668 to 97480398	4
MEST	Forward	TGACTCACACTGGTGAACAGCACA	12	NC_007302.4	4
	Reverse	ATACATTCAGCCCGGTGACTTGGA	12	97467668 to 97480398	4

Table A3.2.1 - BWS-associated regions conserved between *B. t. indicus* and *B. t. taurus*

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Primer [] µM	$MgCl_2$ [] (mM)	5% DMSO	#Cycles	Taq Polymerase
HYMAI	58.2	(1F-1R) 723	0.3 0.3	4		35	Go Taq Promega
HYMAI	58.2	(3F-3R) 736	0.3 0.3	4		35	Go Taq Promega
HYMAI	58.8	(4F-4R) 724	0.3 0.3	4		35	Go Taq Promega
HYMAI	59.6	(Af-AR) 608	0.3 0.3	4		35	Go Taq Promega
MEST	60	(3F-3R) 568	0.3 0.3	4		35	Go Taq Promega
MEST	60	(4F-4.5R) 750	0.3 0.3	4		35	Go Taq Promega

[]= concentration, Tm= temperature (°C) , DMSO= dimethyl sulfoxide.

Table A3.3 - BWS-associated regions conserved between *B. t. indicus* and *B. t. taurus*

Gene Symbol		Genomic Primers (5'-3')	Exon of Primer	NCBI accession # Reference assembly (based on Btau_4.2)	Chromosome
MEST	Forward	AGAAGCATTGTCCCTCCTGAGCTA	12	NC_007302.4	4
	Reverse	TGACCATGCAGGTGACTGTCAGAA	12	97467668 to 97480398	4
MEST	Forward	GACAGTAGCCTTTGTGCCAGAGAT	12	NC_007302.4	4
	Reverse	GCTCATAGGCCTTTCAGACTGTGT	12	97467668 to 97480398	4
MEST	Forward	TCCAGGGCAAAGAAGAGAGGAAGA	intron 10	NC_007302.4	4
	Reverse	CATCCAGTGAAGTCAGAGACAGGCTA	intron 11	97467668 to 97480400	4
MEST	Forward	AGTAAGTTAAGCGGGCCATTCCCA	Intron 9	NC_007302.4	4
	Reverse	AAACAGTCTGTCGTCCCAGCAGTT	Intron 10	97467668 to 97480402	4
MEST	Forward	TGGGTAAGCAAATCAAGAAACTGAAATCC	Intron 7	NC_007302.4	4
	Reverse	GTACCAAATGGCTTACGCTCAGTG	intron 9	97467668 to 97480404	4
MEST	Forward	TCCAGTGGGATAGAATCTGACCTG	intron 6	NC_007302.4	4
	Reverse	TTTAACTACAGCCTGTGGCAGC	intron 7	97467668 to 97480406	4
MEST	Forward	CTGGGAGGGATCATTGCCAAGTTA	intron 3	NC_007302.4	4
	Reverse	AGACGACCATTTAGACGTCACTGC	intron 6	97467668 to 97480408	4
MEST	Forward	GCCTGTTCTTACCTAAAGCAATAAGCTTGC	intron 2	NC_007302.4	4
	Reverse	CAATGATCCCTCCCAGATTGCCAAAG	intron 3	97467668 to 97480410	4
MEST	Forward	GAACGACGACCAAATACGGCAATGG	intron 1	NC_007302.4	4
	Reverse	TTCTGTGAGCTTCCCTGCTGTCAA	intron 2	97467668 to 97480412	4

Table A3.3.1 - BWS-associated regions conserved between *B. t. indicus* and *B. t. taurus*

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Primer [] µM	$MgCl_2$ [] (mM)	5% DMSO	#Cycles	Taq Polymerase
MEST	60	(3F-3R) 568	0.3 0.3	4		35	Go Taq Promega
MEST	60	(4F-4.5R) 750	0.3 0.3	4		35	Go Taq Promega
MEST	60	(5F-5R) 805	0.3 0.3	4		35	Go Taq Promega
MEST	58.7	(3' gap 12F-R) 294	0.3 0.3	4		35	Go Taq Promega
MEST	59.5	(Ex 11 1F-1R) 381	0.3 0.3	4	yes	35	Go Taq Promega
MEST	60.5	(Ex 10 1F-1R) 616	0.3 0.3	4		35	Go Taq Promega
MEST	58.1	(Ex 9, 8 1F-1R) 762	0.3 0.3	4		35	Go Taq Promega
MEST	57.7	(Ex 7 1F-1R) 352	0.3 0.3	4		35	Go Taq Promega
MEST	58.7	(Ex 456 1F-1R) 850	0.3 0.3	4	yes	35	Go Taq Promega
MEST	60.1	(Ex 3 1F-1R) 433	0.3 0.3	4	yes	35	Go Taq Promega
MEST	60.5	(Ex 2 1F-1R) 532	0.3 0.3	4		35	Go Taq Promega

[]= concentration, Tm= temperature (°C) , DMSO= dimethyl sulfoxide.

VITA

Katherine (Katie) Marie Robbins was born on May 15, 1987 in Newark, Delaware. She grew up in Woodstown, New Jersey with her parents, Mark and Diane, and sister and brother, Jackie and Jonathan. Katie is well known for her love of animals and in particular for her rabbits and dogs; Tucker and Hershey. Katie was active in 4-H and Girl Scouts during her Woodstown High School career. She raised and showed Holland Lop and Netherland Dwarf rabbits and was very involved in the American Rabbit Breeder's Association (ARBA). After graduating from Woodstown High School in 2005 she attended Delaware Valley College in Doylestown, Pennsylvania. During her time at Delaware Valley College Katie was an active member in the Biology and Chemistry clubs. Katie graduated magna cum laude with a Bachelor of Science degree in Biology with a specialization in Zoology in May 2009. In August 2009, Katie moved to Columbia, Missouri where she pursued her Master of Science degree in Animal Science; she studied developmental epigenetics in the laboratory of Dr. Rocío M. Rivera. After

defending her master's thesis in July 2011, Katie plans to move back to the East Coast to begin working on her Doctor of Philosophy degree in Biological Sciences at the University of Delaware in August 2011.