Influence of assisted reproductive technologies on imprinting centers and functional characterization of conserved elements in Beckwith Wiedemann Syndrome (BWS) region

Thesis
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by

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"My mother taught me very early to believe I could achieve any accomplishment I wanted to. The first to walk without braces."

Wilma Rudolph

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History

Ordinarily it is expected that parents contribute equally to the offspring owing to the Mendel's laws of inheritance. However there are evidences in support to the deviations from the laws of inheritance such as Alexander Blink's study on R locus in maize, (early 1950), Barbara McClintock's (1958) study on suppressor mutator (*Spm*), a transposable element in maize and the inactivation of X chromosome in mammals (initially identified as Barr body). The X inactivation is a phenomenon where one of the X chromosome is excluded from gene expression after fertilization for the rest of the lifespan of an organism (Panning and Jaenisch 1998); Lyon M F Nature 1961).

Unequal contribution of parent of origin specific alleles to the offspring became more evident when McGrath and Solter (McGrath and Solter 1984) and Surani (Surani, Barton et al. 1984) tried pioneering experiments on generating gynogenetic (fusion of two female pronuclei) or androgenetic (fusion of two male pronuclei) embryos. These embryos were not normal and the simplest explanation was that the male and female gametes were unequal in contribution to the genetic programming during early development. The developing embryo expresses a set of genes from the allele contributed from the father and another set contributed from the mother during development and as a consequence gynogenetic and androgenetic embryos find themselves inappropriately diploid for imprinted loci.

The first evidence of parent of origin effects was observed in a transgene experiment. In this experiment the transmission of the transgene from a autosomal chromosome showed parent of origin specific gene expression (Swain, Stewart et al. 1987). Soon *Igf2* in mice and later many other endogenous genes were also identified in mammals that showed parent of origin specific transcription (also referred as imprinting) (DeChiara, Robertson et al. 1991). In no time it was realized that this parent of origin effect is regulated by epigenetic modifications, such as DNA methylation and modifications on histone tails.

Several theories were also put forth to explain how and why such a complicated regulatory mechanism has evolved and its pivotal role in development. These theories were prevention of female reproductive diseases (also called as “ovarian time bomb”), theory of evolvability (i.e. imprinting has evolved as a mechanism enhancing the adaptability of a species to a changing environment), defence against parasitic diseases by silencing and the theory of conflict between parental alleles. (Moore and Haig 1991; Barlow 1993; Varmuza and Mann 1994; Beaudet and Jiang 2002) Parental conflict is the most widely accepted theory of imprinting. It states that imprinting only occurs in organisms in which nutrients pass directly from the mother to the foetus and eventually leads to unequal investment of parental alleles to the growth of the foetus. Another theory proposed earlier in 1995, has also gained much attention in recent years. This theory describes the importance of imprinting during development and shows a link between recombination hot spots and imprinted regions. It states that differential chromatin remodelling during male and female meiosis is associated with epigenetic reprogramming at imprinted chromosomal regions. This as a result leads to different recombination rates between the two sexes (Paldi, Gyapay et al. 1995; Sandovici, Kassovska-Bratinova et al. 2006).

In conclusion genomic (or parental) imprinting is observed in mammals and is a phenomenon in which allele of a gene is expressed depending on the parent of origin. This differential gene expression is regulated by epigenetic marks present on different alleles.

I Epigenetic and development

I.1 Definition of imprints and imprinting

The term **imprinting** throughout this work relates to the ability of cells to maintain a different epigenetic memory on parental chromosomes. The heriTable, non-mutational modifications on chromosomal regions define the **epigenetic** memory. Epigenetic marks can be attained either by modifying the DNA nucleotides or modifying the histone proteins. The differential marking on the chromosome influences the transcription of the genes and the replication timing of the two parental alleles.

Genes showing differences in transcriptional activity based on parent of origin are termed as **imprinted genes**. To this date, 40 and 80 imprinted genes are identified in human and mouse respectively (documented for human and mouse at http://www.mgu.har.mrc.ac.uk/research/imprinting; http://igc.otago.ac.nz) (Morison, Ramsay et al. 2005). The extent of imprinting in mouse or human is not fully known, but has been estimated in mouse to range between 100 and 600 genes (Luedi, Hartemink et al. 2005). Likewise other mammalian species also show the presence of imprinted genes (Young, Fernandes et al. 2001; Young, Schnieke et al. 2003; Dindot, Farin et al. 2004; Arnold, Lefebvre et al. 2006; Lucifero, Suzuki et al. 2006). The evidence that imprinting of a gene is conserved in different mammalian species was first demonstrated by Killian J K on *Igf2r* gene. In this study authors observed that *Igf2r* is imprinted in most mammals but not in human (Killian, Nolan et al. 2001).

Imprinted genes are often observed in clusters, referred as **imprinted domains**. These clusters are under the regulation of one or more imprinting centers (ICs). These regulatory imprinting centers are enriched in CpG dinucleotides and often constitute CpG islands. Often array of tandem repeats (i.e. two or more approximate copies of a pattern of nucleotides) or presence of non coding transcript is observed near or within the imprinting center (Neumann, Kubicka et al. 1995; Hutter, Helms et al. 2006). Functionality of the imprinting center differs between parental alleles and depends on the epigenetic modifications present on the alleles.

I.2 Imprinting in bovine

Imprinted genes have thus far been identified in marsupial (metatherian), placental (eutherian) mammals (Scott, Spielman et al. 1998; Killian, Byrd et al. 2000; O'Neill, Ingram et al. 2000) and in flowering plants (Scott, Spielman et al. 1998). However, no indication of imprinting was found in monotreme (prototherian) mammals and other vertebrates and invertebrates. Since most of the presented work in this thesis relates to the imprinting in bovine (placental mammal) therefore I would like to present the current knowledge of imprinting in this species.

Imprinted genes are functionally involved in growth and development of the mammalian foetus and placenta. Recently, lesions in imprinted domains have also been shown in association to the failure in animal cloning. Bovine and ovine represent their importance in both farm animal industries as well as in cloning technology. Even though of their importance, these both species are far behind in the study of imprinted genes and related regulatory mechanisms. One of the reasons accounts for the lack of sequence information in the past, however many sequencing centers are presently in process of sequencing not only cow (bovine) and sheep (ovine) but also many other animal species. Studies on imprinted genes indicate that imprinting mechanisms are conserved in different mammalian species including bovine. However, minor differences can be visualized among species such as *Ascl2*, which is not imprinted in bovine and human, while it is imprinted in mouse. Similarly *Igf2*r is imprinted in most of the mammals but not in humans. The Table below summarizes the imprinted genes in bovine and their imprinting status in human, mouse and ovine.

	Imprinting status				Reference for bovine genes
Imprinted genes	Bovine	Human	Mouse	Sheep	
IgF2	Yes	Yes	Yes	Yes	(Dindot, Farin et al. 2004)
Xist	Yes	Yes	Yes	Yes	(Dindot, Kent et al. 2004)
Gtl2	Yes	Yes	Yes	Yes	(Dindot, Kent et al. 2004)
Peg3/Zim2	Yes	Yes	Yes	Yes	(Kim, Bergmann et al. 2004)
H19	Yes	Yes	Yes	Yes	(Zhang, Kubota et al. 2004)
IgF2r	Yes	No	Yes	Yes	(Killian, Nolan et al. 2001)
Ascl2	No	No	Yes	nd	(Arnold, Lefebvre et al. 2006)
Snrpn	Yes	Yes	Yes	nd	(Lucifero D et al 2006)

Table 1: Known Imprinted genes in bovine compared to human, mouse and sheep

I.3 **Dynamics of epigenetic modifications**

To gain more knowledge on the regulatory mechanisms involved at imprinting domains, it essential to know how the chromosomal DNA is packed in a cell. In a eukaryotic cell, the chromosomal DNA is condensed and compacted with histone proteins. Histone proteins form an octamer, which is comprised of two molecules of each highly conserved H2A, H2B, H3, and H4 histones, around which the chromosomal DNA is wrapped. This nucleoprotein complex is called a nucleosome core. Nucleosomes are connected with a linker DNA, and are stabilized through the binding of histone H1 protein ("*Beads on String*") (Figure 1). This combination of DNA and histone proteins is called as chromatin. There are several levels of chromatin condensation before higher level of compacted metaphase chromosomal structure is achieved. Such condensed chromatin structures at a locus are dynamic and are capable of undergoing folding and unfolding transitions. These transitions are critical for the gene regulation since they determine the accessibility of regulatory factors to the underlying DNA. Different modifications on DNA or on histone tails modulate the folding and unfolding transitions. These non mutagenic changes on a chromosome are termed **epigenetic modifications**. When these epigenetic modifications depend on parent of origin, they are termed as **imprints**.

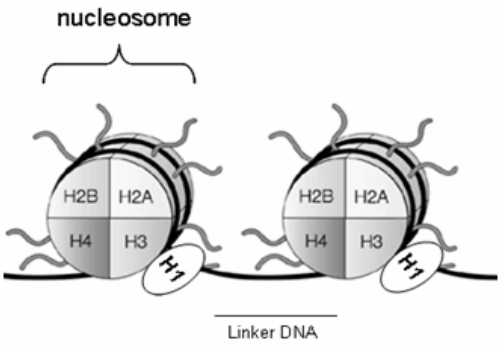


Figure 1: Structural organisation of nucleosomes in chromatin

In chromatin, the extruding tails of the histone proteins are the preferential sites for post translational modifications. These histone 'tails' do not contribute significantly to the structure of individual nucleosomes nor to their stability, but they do play an essential role in controlling the folding of nucleosomal arrays into higher order

structures. Enormous numbers of posttranslational modifications are known on these histone tails such as acetylation and methylation of lysine (K) and arginine (R), phosphorylation of serine (S) and threonines (T), ubiquitylation and sumoylation of lysine, as well as ribosylation. Adding to the complexity is the fact that each lysine residue can accept one, two or even three methyl groups, and an arginine can be mono or di-methylated. Studies on histone modifications show site-specific combinations of histone modifications correlating with a particular biological function. For example, a transcriptional inactive state is characterized by histone deacetylation at Lys-14, which precedes methylation at Lys-9 (Noma, Allis et al. 2001). In contrast, the active transcriptional state has a combination of H3 K14 acetylation and H3 S10 phosphorylation (Lo, Duggan et al. 2001).

Another known epigenetic modification that can alter the chromatin, is on chromosomal DNA. Genomic DNA can be modified on nucleotides as methylated cytosines or methylated adenines (e.g. plant, bacteria). Methylation of cytosine occurs as a covalent modification in which methyl group is added to a cytosine base, mostly at CpG dinucleotides. This modification on cytosine is the major known DNA modification in mammals. Mammalian gene promoters are often associated with CpG rich (CpG island) regions and are unmethylated at all stages of development and tissue types (Antequera and Bird 1993). However, if the CpG island is methylated then this modification prevents the recruitment of transcriptional factors to the promoter and as a result the associated promoter is stably silent. *De novo* DNA methylation is rare in adult somatic tissues and is mostly observed during differentiation, ageing and in cancer cells. Even so, occasionally methylation marks can also disappear from the DNA segment during cell differentiation. This is often observed on tissue specific enhancers or promoters of tissue specific genes (Lucarelli, Fuso et al. 2001; Tagoh, Melnik et al. 2004).

I.4 Establishment and propagation of DNA methylation

Imprinting mechanisms define the differential epigenetic memory on parent of origin specific alleles. In order to preserve this phenomenon from one generation to another, imprints should be:

1) Faithfully transmitted from one cell division to another.
2) Erased during germ cell differentiation.
3) Established thereafter according to the sex of the individual and maintained for the rest of the life.

Different DNA methyl transferase (Dnmts) enzymes plays crucial role in maintaining the phenomenon of imprinting in mammalian species. The DNA methyltransferase family involves two types of enzyme activity: one with de novo methylation activity, while the other having the ability to maintain the methylation on the genome. The d*e novo* methylation activity is required for targeting specific unmethylated CpGs for the proper establishment of imprints in the germ cells and during differentiation (discussed later). DNA methylation maintance activity on the other hand is required for the faithful propagation of the imprint marks from one cell division to another. In mammals 5 genes have been identified as members of the DNA (5 cytosine) methyltransferases family. These are broadly classified as Dnmt1, Dnmt2 and Dnmt3 families (Dnmt3a & Dnmt3b and Dnmt3L). The function of these genes solely or in combination with other factors or within the family is not yet clear.

The DNA methylation marks are faithful transmitted from one cell to another during mitosis. This activity is called DNA methylation maintance activity and is mediated by Dnmt1 enzyme. Dnmt1 preferentially methylates at hemi methylated sites of double stranded DNA. It is recruited at the replication fork machinery during DNA replication and methylates the newly formed daughter strands depending on the epigenetic state of the parent template (Leonhardt, Page et al. 1992).

The Dnmt3 family mainly constitutes the de novo methyltransferase enzymes. Among Dnmt3 family proteins, Dnmt3a and Dnmt3b are the active de novo methyltransferases and are expressed in male and female germ cells (Okano, Bell et al. 1999; Lucifero, Mann et al. 2004) These enzymes have equal preferences for unmethylated and hemi-methylated DNA in *in-vitro* experiments (Okano, Xie et al. 1998) The Dnmt3b enzyme functions to methylate centromeric satellite repetitive elements and is also involved in transcriptional repression (Okano, Bell et al. 1999; Bachman, Rountree et al. 2001). Mutations in human DNMT3b enzyme are often associated with syndromes such as immunodeficiency, chromosomal instabilities,

and facial abnormalities (ICF) syndrome (Hansen, Wijmenga et al. 1999; Okano, Bell et al. 1999; Xu, Bestor et al. 1999) On the other hand, Dnmt3a along with truncated Dnmt3L (lacking conserved catalytic motifs, Figure 2) enzyme are involved in establishing germ line specific DNA methylation imprints. Knockout mice for Dnmt3L gene are normal but show improper germ cell differentiation. Male mice show sterility and females show the failure of establishing methylation at imprinted loci during oogenesis (Bourc'his, Xu et al. 2001; Hata, Okano et al. 2002).

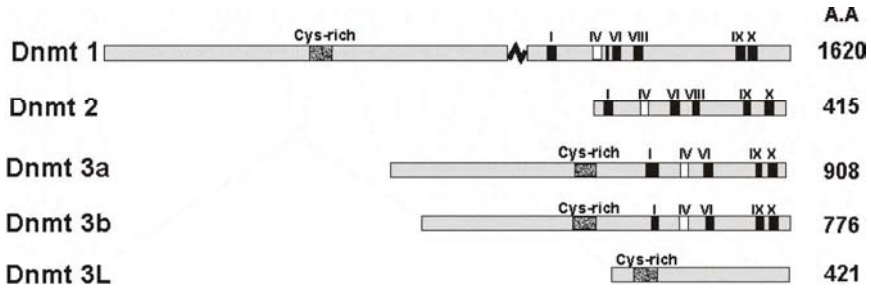


Figure 2: The DNA methyltransferases in mammals

The Dnmt family proteins consist of cysteine rich motif in the N terminal and the catalytic activity resides (I to X conserved motifs) in the C terminal. Here a.a means length of amino acid of the protein (adopted from Bestor 2000).

Another member of Dnmt enzymes is Dnmt2. Dnmt2 protein only consists of conserved catalytic motifs, is highly conserved and recently been shown to be involved in methylating ^{asp}tRNA (Goll, Kirpekar et al. 2006). Its involvement in methylating chromosomal DNA is not clear.

Epigenetic marks should also be reprogrammed in order to ensure that every generation receives the appropriate sex specific imprint. There are two well known events in the life cycle of an individual when the DNA methylation marks are actively erased. These events occur at the time of early embryo development and during germ cell (PGC) differentiation. (Monk, Boubelik et al. 1987; Kafri, Ariel et al. 1992; Tada, Tada et al. 1998; Oswald, Engemann et al. 2000). Both active and passive demethylation (through replication) activities are required for the erasure of DNA methylation, although the enzyme performing active de-methylation on cytosine residues is still unknown.

The first major demethylation event is observed after fertilization. The paternally derived genome in mice (also in other mammalian species with some differences) undergoes active demethylation and most of the methylation marks established during spermatogenesis are eliminated. (Mayer, Niveleau et al. 2000; Oswald, Engemann et al. 2000; Santos, Hendrich et al. 2002). This demethylation event occurs before the first cell division and is independent of DNA replication. In contrast, maternally derived genome retains DNA methylation during this process, but subsequently also undergoes passive demethylation during genome replication. This may be due to the fact that Dnmt1o (splice variant of DNA methylation maintance enzyme) resides in the cytoplasm during early stages of embryo development (Doherty, Bartolomei et al. 2002). During this demethylation event, most of the imprinting centers and some repetitive elements faithfully maintain the DNA methylation state (Olek and Walter 1997; Reik, Dean et al. 2001). Later during implantation the de novo DNA methylation activity (Dnmt3a and Dnmt3b) restores the normal levels of methylation on the genome. In mouse and in other mammals, the cycle of early embryonic demethylation followed by de novo methylation is critical in determining somatic DNA methylation patterns.

A second wave of genome wide reduction in DNA methylation is observed in primordial germ cells (PGCs), in proliferating oogonial and spermatogonial stages. Here inherited imprinting patterns are also erased (Tada, Tada et al. 1997; Reik and Walter 2001; Hajkova, el-Maarri et al. 2002). The DNA demethylation of germ cells is crucial if the correct sex specific epigenetic information has to be laid down during sperm and oocyte maturation. Demethylation in PGCs starts at around embryo day 11.5 (E11.5) and is completed by day E13-E14 (Hajkova, Erhardt et al. 2002). Once the supposedly ground state of DNA methylation is archived, the male and female PGCs begin to enter mitotic and meiotic arrest respectively. Over the period of oocyte growth, the maternal imprints as well as non imprinted sequences become methylated. Dnmt 3a, Dnmt3b and Dnmt3L all are expressed during postnatal oocyte growth. Different maternally methylated imprinting centers show appearance of DNA methylation at different stages of oocyte maturation (Lucifero, Mertineit et al. 2002).

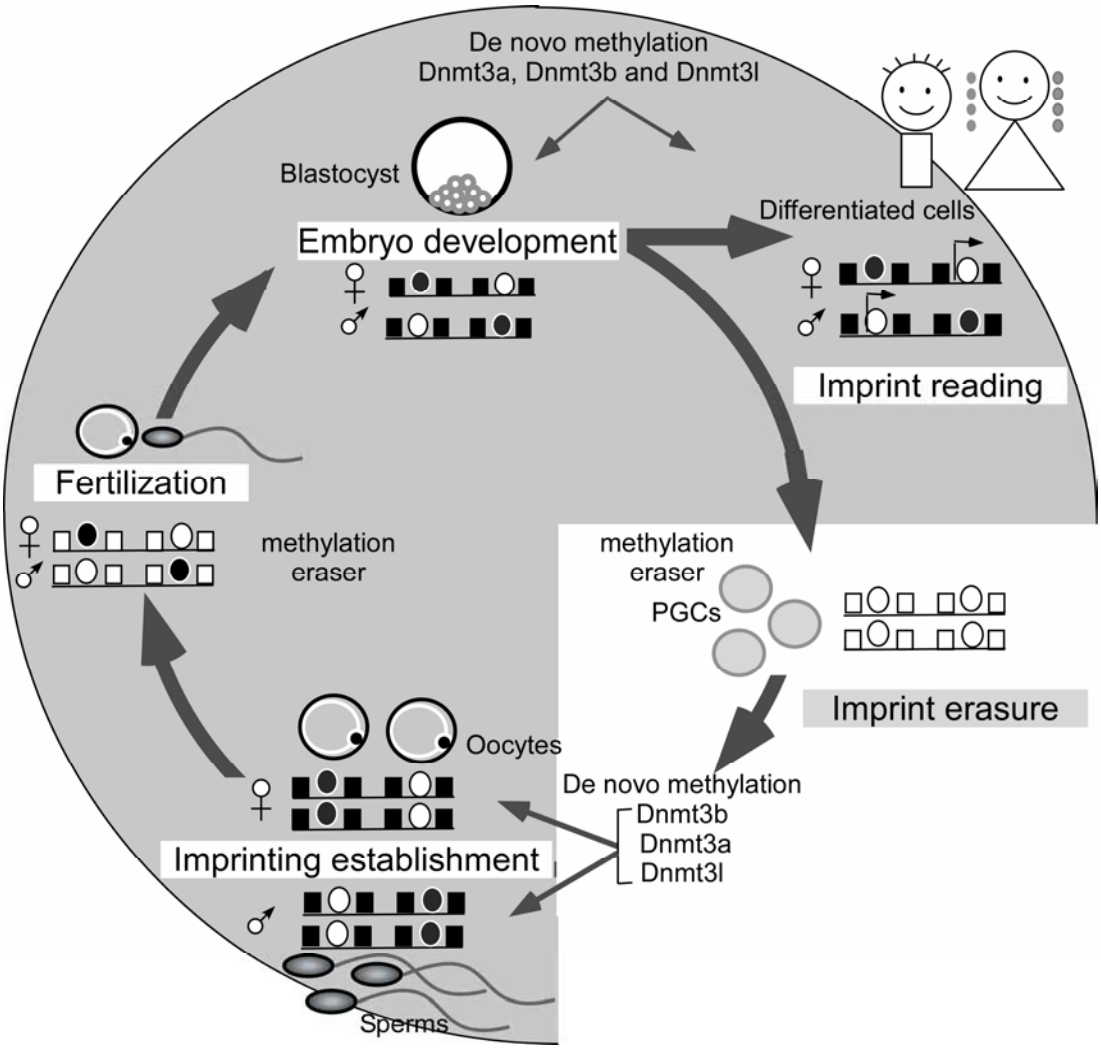


Figure 3: DNA methylation imprint establishment and propagation

The grey shaded region in the background demarcates the expression of Dnmt1 and the *de novo* DNA methyl transferases are indicated in respective stages. The imprinted regions on the DNA are marked as circle and these regions have parent of origin specific DNA methylation. The activity marks on imprinted regions are established during gametogenesis. The parental specific imprints are erased in primordial germ cells (PGCs) and the appropriate sex dependent imprint marks are re-established for the next generation. The maternal and paternal genomes (♂♀) have different imprint patterns in germ cell development. Both imprint marks and imprint reading are maintained after fertilization and during somatic cell division. DNA methylation at non imprinted regions (squares) behaves indifferently at both parental genomes. Filled circles or squares represent methylated DNA and open circles or squares represent unmethylated DNA regions. As with oocytes, the level of DNA methylation increases as sperm develops. This increase in DNA methylation content is attributed to both, paternal imprints as well as DNA methylation of non imprinted regions. All Dnmt enzymes are found during sperm maturation, although the expression level varies from stage to stage. For example Dnmt1 is found in high levels during meiosis except at pachytene stage. During spermatogenesis, de novo methyl transferases Dnmt3a and Dnmt3L are indispensable. Absence of any of them leads to DNA methylation errors in some of the paternally imprinted genes and also impairs spermatogenesis and gonad development (Okano, Bell et al. 1999; Hata, Okano et al. 2002).

I.5 Imprinting centers and their regulation

After gaining knowledge on epigenetic marks, their establishment and maintance during development and differentiation, we now focus to understand the regulation of imprinted regions. Imprinted regions (region where imprinted genes are clustered together) are regulated by one or more imprinting centers. Different epigenetic mark on parental alleles defines the differential regulation of the imprinting centers. Imprinting centers behave differently in their mechanisms of action; among them some are well understood while others are still under investigation. In the majority of cases these centers function either as silencers or as insulator/boundary elements. Presented below are the two well known imprinted regions, the Beckwith-Wiedemann syndrome (BWS) and Prader-Will (PWS) / Angelman syndrome (AS) regions, their regulation and the syndrome associated to them in humans.

I.5.1 Imprinting centers in the Beckwith-Wiedemann syndrome (BWS) region

The human chromosome 11p15.5 and its homologous region on the mouse distal chromosome 7 harbour a cluster of imprinted genes. This cluster is regulated by two well studied imprinted centers.

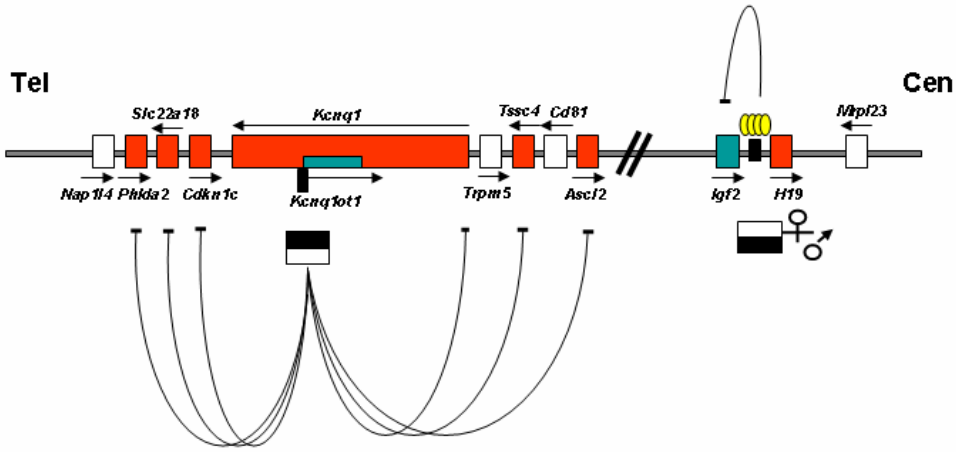


Figure 4: Beckwith-Wiedemann syndrome region in mouse

BWS region harbours two imprinting domains regulated mainly by imprinting centers *H19* DMR and KvDMR1 respectively. Both imprinting centers are reciprocally methylated i.e. *H19* DMR is methylated on paternal allele (♂) while KvDMR1 is methylated on maternal allele (♀). Methylated alleles at the imprinted centers lack their functionality. *H19* DMR functions as an insulator to which CTCF protein binds (yellow circles) and inhibits the access of endodermal enhancers to the *Igf2* promoter. KvDMR1 functions as a promoter and silencer and it silences genes on its both sides. Maternally expressed genes are indicated with red colour, paternally genes with blue and genes with biallelic expression with white.

The imprinting center located upstream of *H19* gene is known as *H19* DMR, and it regulates the *Igf2, Ins* and *H19* genes. The second cluster of imprinted genes is located on the telomeric end in mouse chromosome 7, and is mainly regulated by KvDMR1 imprinting center. Imprinted genes such as *Cdkn1c*, *Kcnq1, Tssc4, Phlda 2,* and the *Ascl2* are regulated by KvDMR1 in mouse (Figure 4)

I.5.1.1 Imprinting center 1 (*H19* DMR)

In mouse the centromeric imprinting sub-domain of BWS region, contains two well characterized imprinted genes, *Igf2* and *H19*. These are expressed widely during embryonic development in identical tissues and are down-regulated shortly after birth. One exception to this are some parts of brain, such as choroids plexus, where *Igf2* is expressed but *H19* is not (Charalambous, Menheniott et al. 2004). These two genes are reciprocally imprinted i.e. *Igf2* is expressed from the paternal allele while *H19* is expressed from the maternal allele. Both genes are mainly regulated by DNA methylation at a chromatin boundary element located 5' of *H19*, termed as differentially methylated region or imprinting center 1 (*H19* DMR) (Tremblay, Duran et al. 1997; Ishihara and Sasaki 2002).

The *H19* DMR is unmethylated on maternal allele and is methylated on paternal allele. Several endo-dermal and meso-dermal enhancers were also identified downstream of *H19* which functions on *H19* or *Igf2* promoters (Leighton, Ingram et al. 1995; Ishihara, Hatano et al. 2000) (Figure 5).

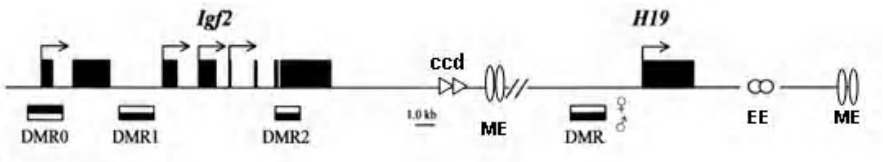


Figure 5: Differentially Methylated Regions at *H19* DMR imprinting domain

All exons of the *Igf2* gene and promoters of both genes in mouse are presented. DMRs are shown underneath together with their allele-specific DNA methylation status (filled, hypermethylated). DMR0 methylation pattern refers only to placenta. *H19* DMR and known enhancers to this domain are placed on the map (not to the scale), where ME (meso dermal enhancer), EE (endodermal enhancer) and CCD (Choroid plexus specific enhancer) (adopted and modified from Lopes S et al, 2003).

Besides *H19* DMR, this region also harbours differentially methylated regions in the *Igf2* gene, i.e. DMR0, DMR1 and DMR2 (Figure 5). The DMR0 located in Igf2 gene, is differentially methylated only in placenta tissue in mouse and acts as a placenta specific DMR (Moore, Constancia et al. 1997; Lopes, Lewis et al. 2003; Monk, Sanches et al. 2006).

When *H19* DMR is un-methylated, it acts as a boundary/insulator element and this function is mediated by the CTCF (zinc finger protein) protein. The CTCF protein complex at *H19* DMR interacts with DMR1 (located in intron of *Igf2* gene) and this interaction places *Igf2* gene in a silent chromatin state and inaccessible to endodermal enhancers. The enhancers downstream of *H19* are now in the vicinity of the *H19* gene and eventually enhance its gene transcription. On contrary to this, on the paternal allele, the *H19* DMR is methylated and CTCF protein is excluded from binding to the *H19* DMR. Unknown protein complex machinery binds at the methylated *H19* DMR and also interacts with the methylated DMR2 at *Igf2* gene. The interaction now places *H19* gene in a silent chromatin state and the endodermal enhancers in the vicinity of *Igf2* promoter. This results in *Igf2* gene transcription and hence *Igf2* is paternally expressed. This is a good example illustrating that epigenetic mark (DNA methylation) determining the different long range interactions between the DMR sites. These different combinations of interactions among DMRs also show the complex regulation underlying in regulating *Igf2* and *H19* imprinted gene expression. (Murrell, Heeson et al. 2004)

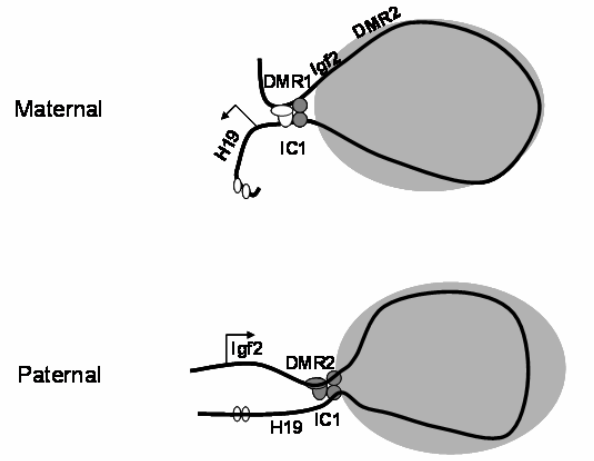


Figure 6: Parent specific interactions between the DMRs provide an epigenetic switch for *Igf2* Here *H19* DMR is presented as IC1 (reproduced from Murrell et al 2004)

The meso-dermal enhancers were also identified later between *H19* and *Igf2* gene (Drewell, Arney et al. 2002). Tissue specific enhancers for choroid plexus are mapped between the *H19* and *Igf2* genes and in this tissue *Igf2* is bi-allelic expressed (Figure 5). Since the enhancers for choroid plexus are between the *Igf2* promoter and *H19* DMR, hence the promoter is not under the influence of *H19* DMR insulator. This also explains why *Igf2* has biallelic expression in choroid plexus (Charalambous, Menheniott et al. 2004).

I.5.1.2 Imprinting center 2 (KvDMR1)

Since most of the work is done at the KvDMR1 imprinting center, therefore it is here discuss in detailed.

Imprinting center 2 (KvDMR1) was initially identified by studying chromosomal breakpoints and DNA methylation changes in BWS patients. It was mapped in intron 10 of *KCNQ1* gene and was shown to possess differential DNA methylation on parent of origin specific alleles (Smilinich, Day et al. 1999).

The imprinting center 2 (KvDMR1) is methylated on the active maternal allele of the *KCNQ1* gene while is unmethylated on the paternal allele. On the paternal allele it functions as promoter for an oppositely oriented and paternally expressed gene known as *KCNQ1OT1* or *Lit1* (Mitsuya, Meguro et al. 1999; Engemann, Strodicke et al. 2000). On analysis of the known DMRs in KvDMR1 sub domain, only KvDMR1 center maintains differential DNA methylation from the germ line (sperm and oocyte) till adult stages (Yatsuki, Joh et al. 2002). The targeted deletion of KvDMR1 on the human paternal chromosome 11 when propagated in a chicken DT40 cell line resulted in activation of the normally silent paternal alleles of *KCNQ1* and *CDKN1C* (Horike, Mitsuya et al. 2000). This removal of silencing by KvDMR1 on neighbouring imprinted genes was also observed after deletion of the KvDMR1 center in mice (Fitzpatrick, Soloway et al. 2002).

Studies on BWS patients showed that chromosomal breakpoints at 11p15.5 are often located in *KCNQ1* gene in intron 14, 9 and between exon1a and exon 1c (Reid, Davies et al. 1997). These breakpoints disrupt the imprinting domain but often leave

the imprinted *CDKN1C* gene and the regulatory center KvDMR1 intact on one chromosome. *CDKN1C* is the only gene known, where loss of protein function causes BWS syndrome in human (discussed later). These chromosomal breakpoint studies show that KvDMR1 in most cases is still functionally active to regulate the imprinting of *CDKN1C* and the BWS phenotype results from the misregulation by unknown *cis* acting regulators. Studies on transgenic lines in mice also highlight the essence of *cis* acting regulatory elements. These all transgene studies were directed on *Cdkn1c* gene expression.

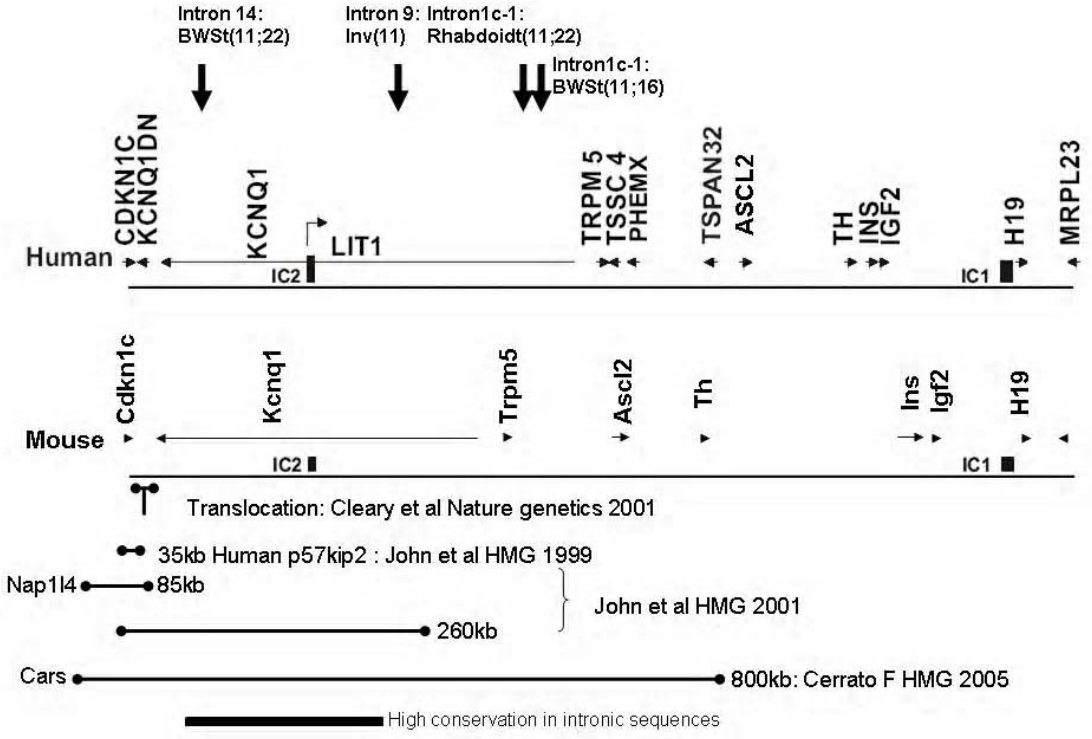


Figure 7: Mapping of human BWS breakpoints and mouse transgenes at KvDMR1 domain

BWS region in human and mouse illustrated with the gene order and transcript orientation. Vertical arrows above human BWS show various breakpoints known from BWS patients (Reid, Davies et al. 1997). Various transgene lines roughly mapped to mouse BWS region are shown as horizontal line below the mouse BWS region. Horizontal shaded bars represent various enhancers identified from the different transgene studies, (John, Hodges et al. 1999; John, Ainscough et al. 2001; Cerrato, Sparago et al. 2005) while the horizontal black bar below it represents the highly conserved intron 10 region (Paulsen, Khare et al. 2005).

Figure 7 shows different transgene studies in mice. Transgenes covering only *Cdkn1c* and its upstream promoter region (35 kb) (John, Hodges et al. 1999), or translocation of *Cdkn1c* and its upstream region to Chromosome 11 (Cleary, van Raamsdonk et al. 2001) showed that *Cdkn1c* gene is under the influence of the KvDMR1 center. In

both studies weak expression of *Cdkn1c* was observed and it was speculated that the lack of *cis* acting enhancers are responsible for this effect. Other transgene studies have only predicted tissue specific enhancers and none of the study showed the complete functionality of this domain in transgene constructs (John, Ainscough et al. 2001). Recently *Cerrato et al* took 800kb transgene extending from *Cars* till *Th* and showed the complete functionality of this sub domain. In this transgene, the differential DNA methylation at KvDMR1, proper imprinting of the downstream as well as upstream genes to KvDMR1 and even imprinting of *Cdkn1c* gene in placenta was observed (Cerrato, Sparago et al. 2005).*Cdkn1c* imprinting in the placenta was never observed before in the other transgene studies.

Chromatin studies at this domain showed that different histone modifications are present on different alleles at KvDMR1. The maternal allele shows presence of repressive histone modifications such as histone methylation at H3K9 and at H3K27, while the paternal allele possesses transcriptionally active chromatin with H3K4 histone methylation and histone acetylation of H3K9 and H3K14. However, in the KvDMR1 neighbouring region this differential histone modification was only observed in placental tissue, while embryo proper showed no difference between the alleles (Umlauf, Goto et al. 2004). Further to this, the repressive histone modification observed at maternal allele, was found to be independent of the DNA methylation at KvDMR1 (Lewis, Mitsuya et al. 2004). Both of the chromatin studies as well as the transgene studies showed that the KvDMR1 center though indispensable but is not sufficient to regulate the entire imprinting sub-domain.

KvDMR1 is a potential silencer, which is functional on the unmethylated paternal allele (Fitzpatrick, Soloway et al. 2002). This center also functions as an active promoter for the *Kcnq1ot1* long transcript (40-60kb long). Presence of unmethylated state at KvDMR1 and *Kcnq1ot1* transcripts are required for KvDMR1 center to be a functional silencer (Mancini-DiNardo, Steele et al. 2003; Mancini-DiNardo, Steele et al. 2006). There are discrepancies whether KvDMR1 also functions as an insulator and harbours potential CTCF binding (Chandrasekhar Kanduri and Victor Lobanenkov 2002; Kanduri, Fitzpatrick et al. 2002).

The *Kcnq1ot1* promoter (or KvDMR1) harbours conserved "CAAT" sites, to which *NY-F* transcriptional factor binds. These CAAT sites were shown to be essential to KvDMR1 for its promoter and silencer activity (Mancini-DiNardo, Steele et al. 2003; Du, Zhou et al. 2004; Pandey, Ceribelli et al. 2004).

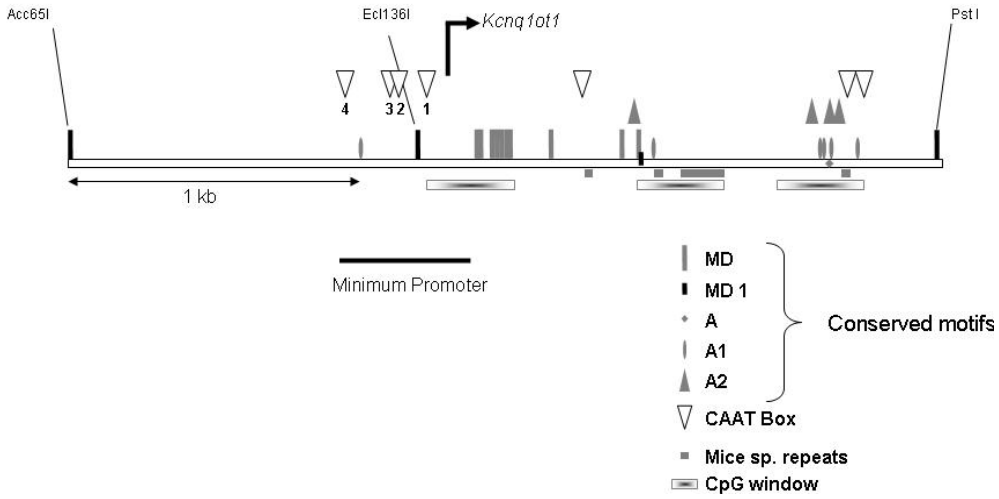


Figure 8: Overview of imprinting center 2 (KvDMR1) in mouse

Indicated is the minimum promoter mapped according to study performed by Marcini Dinardo et al, 2003 (nt 101453–102050; Gen. Bank accession number AP001295), while the conserved motifs and CAAT boxes are mapped according to the work by Paulsen et al 2005.

It was also observed that on pre termination of *Kcnq1ot1* (a long transcript app.40-60kb in length) transcript, the silencing activity at KvDMR1 is lost, even though KvDMR1 is unmethylated and is transcriptionally active (Thakur, Tiwari et al. 2004; Mancini-DiNardo, Steele et al. 2006)

Recently we have described highly conserved segmental regions in the KvDMR1 sub domain within different mammalian species. This conservation is specifically observed in intron 10 of *KCNQ1* gene, where KvDMR1 is also located. These segments show nucleotide identity of >70%, were >100bp long and their relative position to KvDMR1 was also conserved (Paulsen, Khare et al. 2005). It is assumptive that these elements in the introns of *KCNQ1* gene may function as *cis*-acting regulatory element either to the KvDMR1 or to the genes in the sub-domain. However at this moment their potential role in imprinting or on gene regulation is not yet clear.

I.5.2 The Imprinting center at Prader-Willi and Angelman syndrome region

I.5.2.1 Snrpn DMR

Another well studied imprinting domain is at PWS / AS imprinted region (on human 15q11-q13), and is regulated by *SNRPN*-DMR (PWS-SRO or IC). *SNRPN*-DMR is located at promoter to intron 1 of *SNRPN* gene in human and is at similar location in mouse. On this imprinting center paternal allele is unmethylated and is transcriptionally active for most of the imprinted genes such as *MKRN3, NDN* and *SNURF-SNRPN*. The maternal allele of this center is methylated and only *UBE3A* and *ATP10 C* genes are maternally expressed (in mouse *Atp10 c* gene is not imprinted) (Glenn, Porter et al. 1993).

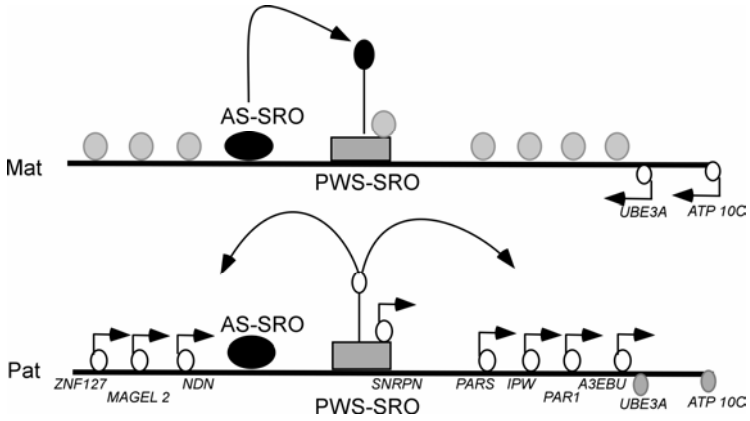


Figure 9: Regulation at PWS /AS imprinted domain

The SNRPN promoter on the paternal allele (Pat) remains unmethylated (open 'lollipop') and is transcriptionally active (horizontal arrow). Only UBE3A and ATP10C are maternally expressed in this imprinted region.

A second regulator in the region, the AR-SRO, is required to set the differential DNA methylation at the *SNRPN*-DMR (Shemer, Hershko et al. 2000). Interestingly, AS-SRO (35kb upstream of *SNRPN*-DMR) is methylated on both alleles but has an open chromatin (acetylated histones and access to DNase enzyme) only on maternal allele (Schumacher, Buiting et al. 1998; Buiting, Gross et al. 2003; Kantor, Kaufman et al. 2004). In contrast, at the *SNRPN*-DMR, the paternal allele is unmethylated and corresponds to open chromatin conformation, while maternal allele is methylated and

shows closed chromatin conformation (Schweizer, Zynger et al. 1999; Saitoh and Wada 2000).

I.6 Syndromes associated to imprinting centers

Defects in epigenetic modifications at imprinting centers may result in various syndromes. Two such syndromes mapped to above described imprinted regions are BWS and PWS /AS.

I.6.1 Beckwith Wiedemann syndrome (BWS)

Beckwith Wiedemann syndrome (Online Mendalian inheritance in Man (OMIM): 130650) was first observed by Beckwith (1963) and Wiedemann (1964). This syndrome is a growth disorder characterized by macrosomia (large body size), macroglossia, visceromegaly, embryonal tumors (e.g., Wilms tumour, hepatoblastoma, neuroblastoma, and rhabdomyosarcoma), omphalocele, neonatal hypoglycemia, and ear creases/pits. Additional diagnostic findings include polyhydramnios and premature enlarged placenta, and cardiomegaly.

BWS syndrome is sporadic in nature and the aetiology of this syndrome involves genetic and epigenetic factors. To date only one protein coding gene, *CDKN1C*, is known in which mutations alone can lead to the BWS phenotype (Hatada, Ohashi et al. 1996; Hatada, Nabetani et al. 1997; O'Keefe, Dao et al. 1997). *CDKN1C* is a cell cycle regulator and limits the cell proliferation. This gene is maternally expressed and is under the regulation of imprinting center 2 (KvDMR1) (Engemann, Strodicke et al. 2000; Horike, Mitsuya et al. 2000).

Misregulation of imprinting at centers *H19* DMR (upstream of *H19*) and KvDMR1 (located in intron 10 of KCNQ1 gene) also causes BWS syndrome. Disruption of DNA methylation mark on maternal allele at KvDMR1 center is observed as the major aetiological factor for BWS syndrome (>50% of sporadic cases). Presence of unmethylated DNA at KvDMR1 results in biallelic expression of *KCNQ1OT1* and silencing of the neighbouring maternally expressed imprinted genes, such as

CDKN1C (Lee, DeBaun et al. 1999; Sperandeo, Ungaro et al. 2000; Bliek, Maas et al. 2001; Weksberg, Nishikawa et al. 2001; DeBaun, Niemitz et al. 2002; Weksberg, Shuman et al. 2002; Murrell, Heeson et al. 2004; Martin, Grange et al. 2005). Deregulation of imprinting at *H19* DMR is observed in 10% of the sporadic cases of BWS patients. The DNA hypermethylation at *H19* DMR results in biallelic and over-expression of *IGF2* gene, a growth enhancing factor.

Genetic factors other than mutation in *CDKN1C*, involved are uniparental disomies (UPD) of chromosome 11p15 (10-20% of the sporadic cases) and micro-deletions. UPDs results from duplication of a chromosome or segmental duplication of a chromosome. The vast majority of BWS patients with UPD exhibits somatic mosaicism i.e. not all cells have the duplication in an individual and this is due to the post zygotic mutation (Reik, Brown et al. 1994; Itoh, Becroft et al. 2000). Micro-deletions have also been recently reported in BWS patients. Two familial case studies at *H19* DMR show that micro-deletion results in elimination of CTCF binding sites (Sparago A Nat genet 2004, Prawitt, Enklaar et al. 2005) . At KvDMR1, from the single case study on a patient harbouring a deletion of KvDMR1, the actual mechanism cannot be deduced. This deletion however disrupts and deletes the whole regulatory center KvDMR1 in BWS patient. The maternal transmission of the deletion at KvDMR1 causes BWS phenotype while paternal transmission has no effect. It should be noted that deletion is only within the *KCNQ1* gene and the *CDKN1C* gene is not affected (Niemitz, DeBaun et al. 2004).

BWS syndrome has also been shown in association to embryonic tumours. Among them Wilms tumour (kidney tumour) is quite often reported in BWS patients. Epigenetic lesions at *H19* locus and Wilm's tumour (WTs) was first observed by Moulton and Steenman (M J (Moulton, Crenshaw et al. 1994; Steenman, Rainier et al. 1994). They showed that DNA hypermethylation of *H19* promoter and inactivation of *H19* gene expression is often associated to Wilm's tumor in BWS patients.

I.6.2 **Prader-Willi (PWS) and Angelman (AS) syndromes**

Another well studied imprinting defect is Prader-Willi syndrome (PWS) and Angelman syndrome (AS) and is associated with neurogenetic diseases.

Angelman syndrome (AS; OMIM: 105830) results from lack of maternal contribution from chromosome 15q11-q13. Here the maternally expressed genes *UBE3A* and *ATP10C* are involved. *UBE3a* is indeed expressed in brain and is affected in AS while the involvement of *ATP10C* is not clear. This syndrome is characterized by mental retardation, abnormal behaviour, movement disorder and severe limitations in speech and language.

Prader-Willi syndrome (PWS: OMIM: 176270) is a clinically distinct disorder resulting from paternal deletion of the same 15q11-q13 region. This syndrome is characterized by diminished foetal activity, obesity, muscular hypotonia, mental retardation, hypogonadism, short stature, and small hands and feet.

Aetiologies of PWS and AS are, large chromosomal deletions (70%), uniparental disomy (29% and 1% respectively) and an imprinting defect at *Snrpn* DMR (PWS-SRO) (1%and 4% respectively). Maternal transmission of any of the above indicated defects leads to AS syndrome while paternal transmission causes PWS syndrome.

Recently concerns have been raised about the increasing incidences of imprinting syndrome in children conceived by assisted reproduction (ART). Several BWS patient cases were reported after the usage of ART, in particular after IVF (*In Vitro* Fertilization) treatment (DeBaun, Niemitz et al. 2003; Gicquel, Gaston et al. 2003; Maher, Afnan et al. 2003; Chang, Moley et al. 2005). Likewise 3 cases were also documented for Angelman syndrome after ICSI (Intra Cytoplasmic Sperm Injection) treatment (Cox, Burger et al. 2002; Orstavik, Eiklid et al. 2003).

I.7 DNA methylation in bovine and influence of IVF procedures

Similar to BWS overgrowth phenotype, in mice and in farm animals' (cattle and sheep) an overgrowth syndrome is often observed after the usage of ART procedures (Young, Fernandes et al. 2001; Dean, Bowden et al. 1998; Farin, Piedrahita et al. 2006). This overgrowth syndrome in farm animals is referred as **L**arge **O**ffspring **S**yndrome (LOS).

Since the current thesis work relates to the influence of ART on cattle (bovine) DMRs, hence here discussed in detail the various reproductive procedures used in cattle industry and the literature review on the LOS syndrome.

I.7.1 Cattle reproduction and assisted reproductive techniques in use

The progress of cattle genetics through assisted reproductive technology is impressive in the last two decades. Since 1970, artificial insemination (AI) and the associated techniques such as semen cryopreservation and ovulation synchronization dominated the cattle farm reproductive techniques. AI is defined as deposition of bull semen in the reproductive tract of the cow as means of impregnation. Here elite sires are selected on the basis of progeny testing, and then are extensively used to improve the traits like milk and meat production.

The female contribution to genetic progress was achieved with the advent of ET (embryo transfer) and the associated techniques such as non-surgical embryo collection, in vitro maturation, fertilization, and culturing of bovine oocytes. ET can be complimented with different strategies. Dams can be super-ovulated, artificially inseminated (AI) and later the resulted fertilized oocytes are collected by flushing. These fertilized oocytes are then cultured till blastocyst stage and are implanted in synchronized heifers. This technique is referred as multiple ovulation and embryo transfer (MOET). Oocytes can also be collected directly from mother's body by applying ultra-sonography, cultured for differentiation and then fertilized with spermatocytes (IVF), or in many cases a sperm is injected directly into an ovum, a process known as intra-cytoplasmic sperm injection (ICSI). If fertilization is

successful, similar to MOET, the fertilized ovum (or several fertilized ova) after undergoing several cell divisions, is either transferred to the surrogated mother's for normal development in the uterus, or frozen for later implantation. The purpose of commercial IVF is to obtain viable embryos from females that may not be able to produce descendants through conventional techniques.

Many factors influence the efficiency of the assisted reproductive techniques, and the main ones are the quality of donor sperm and oocyte and the technique used to culture the embryos from the zygote to the blastocyst stage.

I.7.2 Comparative placentation

One of the aspects for proper embryo development is placentation. Improper regulations of the imprinted domains are known to cause lesions in placental development. Placenta is a connective tissue which facilitates the nutrient exchange between mother and foetus during embryo development. Placenta can be classified according to the number of placental layers present for nutrient exchange or according to the site of placental attachment to the foetus.

Human and mouse possess a hemochorial placenta, where maternal blood is in direct contact with foetal chorion (see different layers of placenta Figure 10a). In dogs and cats, an endotheliochorial placenta (4 fetomaternal layers) is observed, while epitheliochorial placenta (6 fetomaternal layers) is present in pigs, cows, horses, and sheep.

Placenta can also be classified based on characteristics of the site of attachment between embryo and the endometrium of the uterus. These are diffuse, cotyledonary, zonary, and discoidal placentas (Figure 10b).

When the chorionic sac meets the uterine endometrium over its entire surface, it is known as diffuse placenta. This type of placentae is observed in horses, pigs, camels, lemurs, opossums, kangaroos, and whales. Chorionic villi can also be aggregated to form a broad band that circles about the center of the chorion such that the villi at the

ends regress, leaving only those in the center to be functional. This type of placentae is called as zonary placentae and is seen in carnivores.

In primates and mouse discoid placenta are seen where chorion remains smooth and the other part interacts with the endometrium to form the placenta. The maternal blood cells are in direct contact with the foetal chorion. In ungulates such as cows, deer, goat, and giraffe cotyledonary placentae is observed. Here the villi are clumped together into circular patches called cotyledons and meets with maternal caruncles to form the placentome.

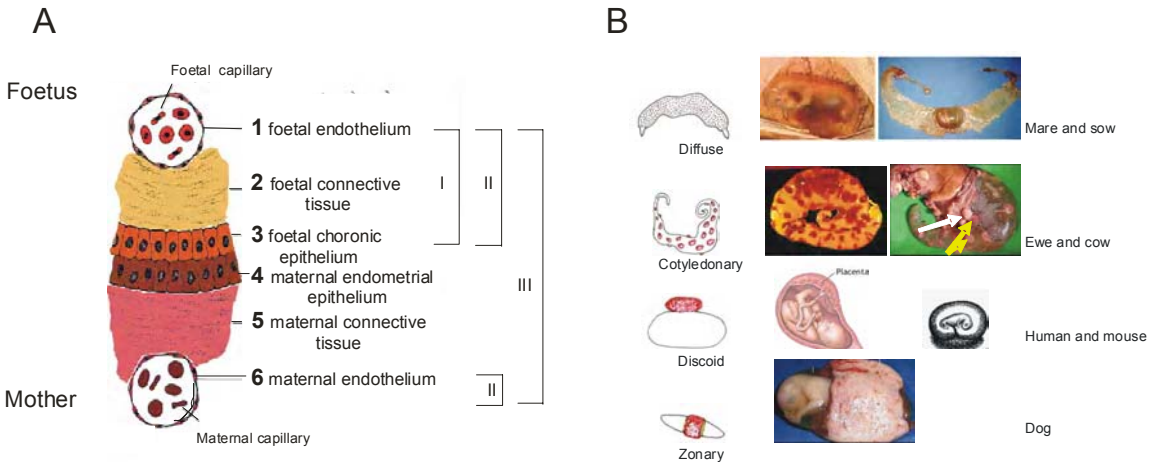


Figure 10: Comparative placentation

A) Classification according to the placental layers: I) Hemochorial; II) endotheliochorial and II) epitheliochorial

B) Classification of placenta according to the site of attachment. Arrows in the cow placentae represents cotyledon (yellow) and caruncles (white)

(Adopted and modified from "A companion to Developmental Biology"; seventh edition by Scott F.Gilbert: http://7e.devbio.com/index.php).

I.7.3 Placenta development in bovine

The development of the early embryos and placenta in bovine is markedly different in comparison to primates and rodents. In human, the blastocyst gets implanted to the mother's uterus at day 5-7 while in mouse implantation occurs at day 4 of gestation (Paria, Song et al. 2001; Vigano, Mangioni et al. 2003). In bovine as well as in sheep, the blastocyst undergoes an extended pre-attachment period. During this period there is extensive elongation of the trophoblast. Concomitantly with

trophoblast elongation, the inner cell mass also undergoes the first major differentiation process of gastrulation. Gastrulation gives rise to the different germ layers of the embryo (ectoderm, endoderm and mesoderm). In vivo, development of the zygote to the blastocyst stage in sheep and cattle takes about 6 days. Prior to the blastocyst stage there is no net growth of the embryo and during the pre attachment period, bovine embryo has access to nutrients within the oviduct and uterine fluid. The bovine placenta has many contact points in foetal membranes called placentomes. In bovine, placenta progressively attaches to the endometrium almost throughout the first trimester; this is in contrast to the more rapid and invasive attachment phase in human and rodents. The first sign of bovine implantation, takes place about two weeks later i.e. 21 dpi (days post insemination) (Guillomot 1995). By this time the bovine blastocyst has already initiated the gastrulation process and the trophoblast has elongated dramatically (Greenstein JS 1958; Maddox-Hyttel, Alexopoulos et al. 2003). The initial foetal placenta when comes in contact with maternal caruncles, it induces villous processes to undergo hypertrophy and hyperplasia and forms cotyledons which, by day 42 progresses to form larger and complex placentomes (The embryology of domestic animals; Noden DM 1990). Placentomes with extensive villous formation are the primary site of transport for easily diffusible molecules such as amino acids, glucose, oxygen and carbon dioxide, while macromolecules are transported in the inter placentomal areas adjacent to uterine glands. (Placentation in Marshel physiology of reproduction. 1994, and Kings GJ J exp zoology 1993)

I.7.4 IVF associated foetal and placental abnormalities

Though the feasible IVF technology has opened the possibilities to manipulate and cultivate the embryo, but on the other hand it has also been linked to many abnormalities in foetus. Reported abnormalities in foetuses or calves following transfer of in vitro cultivated embryos includes lower pregnancy rate, increased abortion, oversized calves, musculoskeletal deformities and abnormalities of placental development, which are often described as "**L**arge **O**ffspring **S**yndrome" (LOS). LOS has been described for bovine (Farin, Piedrahita et al. 2006), sheep (Sinclair, McEvoy et al. 1999) and mice (Eggan, Akutsu et al. 2001). These

abnormalities are more pronounced in cloned animals. Cloning procedures adopt to some extent the same protocols as *in-vitro* fertilization. Additional steps in cloning are enucleating the mature oocyte and thereafter transfer of the somatic nuclei in this enucleated oocyte. Later the cultivation and transfer to recipient heifers follows the same protocol as described for IVF derived foetuses.

Comparative studies on foetal and placental phenotype across the gestation interval and assisted techniques have revealed many interesting observations. Early as in 1995, Alexander BM on artificially inseminated and Hasler JM on in-*vitro* produced bovine calves performed systematic studies on early pregnancy losses (Alexander, Johnson et al. 1995; Hasler, Henderson et al. 1995). Alexander BM showed that embryonic losses from AI procedure in the first trimester of pregnancy precisely at 30 to 60 day of gestation are in comparable range to that of naturally mated pregnancy losses, i.e. 6.5% and 4.3% respectively. In contrast, Hasler JF showed that abortions in IVF pregnancies, within first 100 days of gestation may vary from 7% to 61% and these high incidences of abortion depend on the donor cells. The studied IVF calves were 20% heavier at birth and showed higher incidences of hydrallantois (water in allantoic membrane) and dystocia (difficulty in giving birth to the offspring).

Later other studies also showed that even usage of *in vitro* cultivation of fertilized oocytes may result in heavier calves, such as in MOET the calves were 8% heavier than AI calves at birth (van Wagtendonk-de Leeuw, Mullaart et al. 2000). Several groups have also reported placental defects in concepti from cultured bovine embryos derived from IVF (Behboodi, Anderson et al. 1995; Farin and Farin 1995; Crosier, Farin et al. 2000; Farin, Crosier et al. 2001; Bertolini and Anderson 2002; Bertolini, Mason et al. 2002; Bertolini, Moyer et al. 2004)) and nuclear transfer procedures (Hill, Burghardt et al. 2000; Hill, Edwards et al. 2001). These placental defects were reasoned for the major cause for early pregnancy failure and abnormal foetal growth in IVF and in cloned pregnancies. Often lower number of placentomes, thick and oversized placentomes and frequent incidences of hydrallantois and overweight placenta are observed in IVF and in cloned fetuses.

Quite interestingly, these IVF oversized calves at birth, eventually are no different than normal sized AI calves in subsequent development. Similarly NT embryo derived yearling cattle were not significantly heavier than control animals despite former being heavier at birth (Wilson, Williams et al. 1995). Added to the note that these IVF calves showed no difference in their productive or reproductive abilities when compared to normally conceived animals (van Wagtendonk-de Leeuw, Mullaart et al. 2000).

I.7.5 Imprinting defects in IVF derived foetuses

Altered expression of imprinted genes regulating foetal growth and development was initially proposed as one of the main cause for the occurrence of abnormal offspring syndrome. (Dean, Santos et al. 2001; Reik, Dean et al. 2001; Farin, Farin et al. 2004). It was proposed that inadequate *in vitro* culture conditions or improper genetic reprogramming during assisted reproductive procedures (ART) results in improper establishment of imprinting at the regulatory imprinting centers during the critical period of pre implantation development. Investigations on imprinted gene expression in bovine and ovine fetuses derived after ART usage supported this hypothesis. Bovine foetuses after IVF, showed elevated expression of *Igf2* in skeletal muscle and liver at day 70 of gestation. While in sheep the *Igf2*r imprinting center (located in intron 2 of *Igf2r*) was shown to have lower DNA methylation at the maternal allele and a lower expression of *Igf2*r in various embryonic tissues. (Blondin, Farin et al. 2000; Young, Fernandes et al. 2001)

Examination of gene expression patterns in embryos, foetuses, and placentas of cattle, sheep, and mice produced using in vitro culture procedures demonstrated altered expression of imprinted (Blondin, Farin et al. 2000; Doherty, Mann et al. 2000; Young, Fernandes et al. 2001; Bertolini, Beam et al. 2002; Wrenzycki, Herrmann et al. 2004), X-linked (Gutierrez-Adan, Oter et al. 2000; Niemann, Wrenzycki et al. 2002; Wrenzycki and Niemann 2003; Wrenzycki, Herrmann et al. 2005) and autosomal non-imprinted genes (Ravelich, Breier et al. 2004; Wrenzycki, Herrmann et al. 2004; Ravelich, Shelling et al. 2006). Changes in embryo-wide DNA methylation patterns and global DNA methylation levels were also reported in

association with adverse preimplantation development (Gabriela Gebrin Cezar and and Kenneth J. Eilertsen2 2003; Hiendleder, Mund et al. 2004).

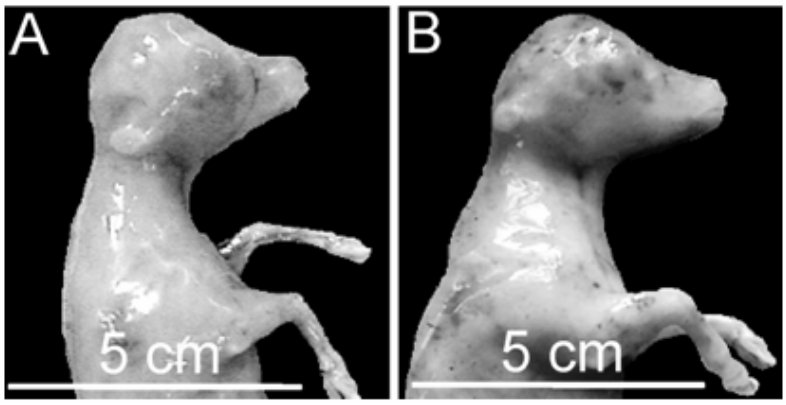


Figure 11: Bovine foetuses at Day 80 of gestation

Foetal weight comparison of day 80 A) AI derived foetus 82gm and B) SCNT cloned derived foetus 142 gm. The oversized IVF foetuses shows foetal weight of 100gm (not shown)

(reproduced from (Hiendleder, Mund et al. 2004)

Conclusions and aim of the thesis:

Regulation of imprinting clusters is a multi-factorial process. DNA methylation at the imprinting centers is the main regulatory modifications of the imprinting domain. It is clear that imprinting centers are not self sufficient in determining the regulation of the entire cluster. The regulation of genes controlled by imprinting center also requires additional *cis* acting elements such as enhancers, silencers, insulators and/or additional secondary DMRs. The mechanisms how these methylation marks are established and interpreted remain less clear.

Loss of imprinting at ICs (changes in DNA methylation) has been shown in association with multiple syndromes. Recent studies show a link between assisted techniques in reproduction and imprinting syndromes in human.

In my present thesis I am addressing two questions:

1) **Influence of ART procedures on imprinting centers.**

Here bovine is used as a model organism to study. It is so because in cattle farm industry, assisted reproduction is used routinely for producing calves. Secondly usage of ART in farm animal industry often results in large offspring syndrome (LOS). The LOS has the phenotypic similarities with human imprinting syndrome BWS. Moreover a systematic study can be performed on bovine owing to the easy accessibility to the sample material.

2) **Functional role of highly conserved short sequences flanking the KvDMR1 imprinting center**

The KvDMR1 imprinting center functions as an active promoter for a long transcript *Kcnq1ot1*. The long *Kcnq1ot1* transcript is essential for the regulatory functioning of KvDMR1 as a silencer. However it is not clear how the KvDMR1 promoter itself is regulated and what are the *cis* acting regulatory elements in this sub domain.

II Material and Methods

II.1 Materials

II.1.1 Reagents

Acrylamide-Bisacrylamide	Biorad GmbH, Munich, Germany
Agar	Life Technologies, Paisley, Scotland
Agarose	Biozym, Oldendorf Germany
APS	Fluka, Buchs, Switzerland
Acetic acid	Merck, Darmstadt, Germany
Bromphenol blue	Sigma, St. Louis, U.S.A
Coomassie-Blue R-250	Life Technologies, Paisley, Scotland
DTT	Sigma, St. Louis, U.S.A
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Sigma, St. Louis, U.S.A
Fetal bovine serum	Life Technologies, Paisley, Scotland
Formaldehyde	Carl Roth, Karlsruhe Germany
Formamide	Sigma, St. Louis, U.S.A
L-Glutamine	Life Technologies, Paisley, Scotland
Glycerin	Merck, Darmstadt, Germany
IPTG	Sigma, St. Louis, U.S.A
Isopropanol	Merck, Darmstadt, Germany
ß-Mercaptoethanol	Sigma, St. Louis, U.S.A
Methanol	Merck, Darmstadt, Germany
PBS	PAN Biotech, Aidenbach, Germany
PEG 4000	Fluka, Buchs, Switzerland
Penicillin/Streptomycin	Life Technologies, Paisley, Scotland
Sodium chloride	Merck, Darmstadt, Germany
SDS	Carl Roth, Karlsruhe Germany
TEMED	Invitrogen, Paisley Scotland
Tris-HCl	Carl Roth, Karlsruhe Germany
Xylencyanol	Sigma, St. Louis, U.S.A

II.1.2 Radioactive Substances

α 32P-dCTP	MP Bio Medicals Germany

II.1.3 Antibody

Hand1 antibody f(sc-22817)	Santa Cruz California USA

II.1.4 Enzymes

Restriction endo nucleases	New England Bio-labs, Frankfurt, Germany MBI Fermentas, Opelstrasse Germany Roche, Mannheim Germany
RNasein	Promega, Mannheim, Germany
DNase I	Boehringer, Mannheim Germany
Klenow-fragment	Boehringer, Mannheim Germany

RNase A	Boehringer, Mannheim Germany
Trypsin EDTA	PAN Biotech, Aidenbach, Germany
Shrimp alkaline phosphatase	USB, Cleveland USA

II.1.5 **Reagent Kits**

ABI Big Dye Terminator kit	Applied Bio systems, Foster City, CA, USA
Qia Quick Gel extraction kit	Qiagen, Hilden, Germany
Jetsorb	Genomed, Bad Oeynhausen Germany
Plasmid Isolation kit	Qiagen, Hilden, Germany
	Invitrogen, Paisley Scotland
TA cloning kit	Invitrogen, Paisley Scotland
	Promega, Mannheim, Germany
High Prime DNA labelling kit	Boehringer, Mannheim Germany
LipofectAMINE 2000	Invitrogen, Paisley Scotland
Dual Luciferase assay kit	Promega, Mannheim, Germany
ECL kit	Amersham, Biosciences NJ, USA

II.1.6 **Plasmids**

pGL3 basic	Promega, Mannheim, Germany
SV40 pGL3	Promega, Mannheim, Germany
CMV pRL	Promega, Mannheim, Germany
pCR2.1	Invitrogen, Paisley Scotland
pGEMT	Promega, Mannheim, Germany
pCDNA3 with Hand1	Gift from Prof Dr. Paul Riley
Bluescript SK+II	Stratagene, CA USA

II.1.7 **Standards, Markers and Ladders**

Protein marker	Biorad, Munich, Germany
DNA marker	
1-kb ladder	NEB, Frankfurt, Germany
100bp ladder	NEB, Frankfurt, Germany

II.1.8 **Miscellaneous**

Blotting paper	Schleicher and Schüll, Stuttgart, Germany
Blotting Membrane (nylon)	Roche, Basel, Switzerland
Dialysis filters, Type VS	
0,0025µm	Millipore, Schwalbach Germany
Pipette tips	STAR labs, Ahrensburg Germany
Plastic material (tubes etc)	Sarstedt, Numbrecht Germany
Quartz Cuvette	Sarstedt, Numbrecht Germany
Reaction tubes	Eppendorf,
X-ray film	Kodak (Biomax), New York USA
Ultra centrifuge tubes	Beckman, Krefeld Germany
Cell culture material	24 well plates: Becton Dickinson, NJ USA
	Pipettes and flasks: Sarstedt, Numbrecht Germany

II.1.9 **Instruments**

Centrifuges	Beckman, Krefeld Germany Sorvall (RC5B), Langenselbold Germany	
Gel documentation system	Biostep GmbH, Jahnsdorf Germany	
Spectrophotometer	Thermo Electron Corporation, Waltham, USA	
Luminometer	lumat Berthold, Bad Wildbad Germany	
CO_2 incubator	Sanyo, Bensenville USA	
Cell culture clean bench	Antair, Germany	
Microscope	TS 100, Nikon Ireland	
Balances	Sartoris, Bradford USA	
PCR machines	Eppendorf Master Gradient	Eppendorf
Electroblotter	Tank-Electro Blotter	PeqLab

II.1.10 **Biological material**

Bacteria

Escherichia coli

SURE 2	Stratagene, CA USA
TOP 10 F'	Stratagene, CA USA

Mammalian cell lines

HEK293T	Human kidney fibroblast
C2C12	Mouse foetal myoblast

II.1.11 **Buffer and solutions**

Buffers provided with the kits were used as per manufacture protocol. The other buffers used are listed below.

Lysis buffer for genomic DNA isolation:

Solution A (10x):	EDTA	25 mM
	NaCl	75 mM
Solution B (10x):	EDTA	10 mM
	Tris-HCl (pH 8.0)	10 mM
	SDS	1%

NaI-Solution:	NaI	90 g
	Na2SO3	1 tea spoon
	Distilled water	100 ml
PBS-Buffer (1x): (without Mg2+, Ca2+)		pH 7. 4
	NaCl	137 mM
	KCl	2.7 mM
	Na2HPO4	10 mM
	KH2PO4	2 mM

PCR-Buffer (10x):

Tris-HCl (pH 8, 8)	75 mM
$(NH4)_2SO_4$	20 mM
$MgSO_4$	2 mM
Tween 20	0.1%

DNA loading dye (6x):

Bromophenolblue	0.25% (w/v)
Xylen-Cyanol	0.25% (w/v)
Saccharose	40%

TBE-Buffer (5x):

Tris-Base	54 g
Boric acid	27.5 g
EDTA	20 ml (0.5 M) pH 8,0
distilled water ad 1 l	

TE-Buffer (10x):

Tris-HCl (pH 8,0)	100 mM
EDTA (pH 8,0)	10 mM

20x SSC

NaCl	175.3 g
sodium citrate	88.2 g
distilled water	ad 1 litre (pH 7.0)

20% PEG 4000

PEG 4000	20 g
distilled water	100 ml

30% polyacyrlamide solution (37.5:1)

Acrylamide	150 g
Bis-acrylamide	4 g
distilled water	500 ml

Radio immuno precipitation Assay Buffer (*RIPA* lysis buffer)

Tris pH 7.5	20 mM
NaCl	150 mM
Nonidet P-40	1%
Sodium Deoxycholate	0.5%
EDTA	1 mM
SDS	0.1%

Protease inhibitors cocktail was mixed before performing protein extraction

Proteinase K (20mg/ml)

proteinase K	20 mg
distilled water	1 ml

Hybridization Solutions

Denaturation buffer:

NaCl	1.5 M
NaOH	0.5 M

Neutralization buffer

Tris –HCl (pH 7,4)	1 M
NaCl	1.5 M

0.5 M Sodium Phosphate Buffer (pH 7.2)

Solution A: Na_2HPO_4, $2H_2O$ (1M):	177.9 g
distilled H_2O:	1 litre.
Solution B: NaH_2PO_4, H_2O (1M):	137.99 g
distilled H_2O:	1 litre

Mix solution A and B for 1 litre of Sodium Phosphate Buffer (0.5 M pH 7.2) as follows:

Solution A:	342 ml
Solution B:	158 ml
distilled H_2O:	500 ml

Church Buffer:

Sodium Phosphate Buffer (0.5M pH 7.2)	0.25M
EDTA (0.5M)	1 mM
BSA	1%
SDS	7%

IPTG	Isopropyl-b,D-thiogalactoside (IPTG)	1.19 g
	distilled water	5 ml
	filter sterilize and Store at -20°C	

20% X gal	X gal	20 g
	Dimethyl Formamide	100 ml

For Bacterial cultures

Antibiotic	1000X Stock concentration
Ampicillin	100 mg/ml in ddH20
Chloramphenicol	34 mg/ml in 100% EtOH
Kanamycin	40 mg/ml in ddH20
Tetracycline	15 mg/ml in 70% EtOH

II.1.12 **Media**

Cell culture

DMEM	PAN Biotech, Aidenbach, Germany
RPMI	PAN Biotech, Aidenbach, Germany

Bacterial culture

LB media (pH 7, 0)

Bactro -trypton	20 g
Yeast extracts	5 g
NaCl	10 g
distilled water	1 litre and autoclave

For LB plates add 15 g bacto-agar in 1 l of LB media and autoclave

SOC-Medium: (pH 7, 0)

Bactro -trypton	20 g
Yeast extracts	5 g
NaCl	0.5 g
KCl	10 ml (250 mM)
distilled water ad	1 litre
Autoclave and then add	
Glucose	20 ml (1 M)
$MgCl_2$	5 ml (2 M)

II.1.13 **Primer Tables**

Primers	Sequence	Product Length	Tm	Acc. No.	Position
Bos IC1_143-For	5'-GTT CTG GGG GCT GCC ACA TCC A-3'	651bp	58°C	AC149767	143303-143325
Bos IC1-144-Rev	5'-ACG AGC GCC CTG TCT CCC ACA-3'				143933-143954
SN primer for IC1	5'-GGA GAG CTG TGA AAT CCT CCT-3'				
Bos-Lit1-379-For	5'-TCC ATC GCC ACA ACT TCT GTG G-3'	838bp	58°C	AC147396	37987-38008
Bos-Lit1-388-Rev	5'-CTA GCC GAG GTG AGC CCA GTG T-3'				38804-38825
Bos-Snrpn-For	5'-CGC GGG GAC CAC CAC AAC CA-3'	402bp	58°C	NW_935049	23748-23768
Bos-Snrpn-Rev	5'-TGC CAG CCG GAG ATG CGT GAC-3'				23346-23366
Bisulfite primers:				AC149767	143675-143688
Bos-BS-IC1 lower for	5'-GTA GTG TGG TTT GAT TTG GTA TT-3'	344bp	55°C		143941-143966
Bos-BS-IC1 lower rev	5'-AAC ATT AAA AAA TCC CTA TAC CTA A-3'				
IC2-BS- II.2-For	5'-GTT GGA TTT GTG TTT GGA GGT TAT-3'	511bp	50°C	AC147396	38127-38151
IC2-BS- II.2-Rev	5'-CTC AAC TCT TAA CTC AAA TTC CCA A-3'				38605-38630
Snrpn-BS-For	5'-ATA TTT TTA GTT TTG GAA GTT TAG GAT G-3'	465bp	55°C	NW_205360	23416-23391
Snrpn-BS-Rev	5'-CTC CAC TAA AAA ATA CCA CTC TAA ATT A-3				23040-23012
DMR0 IgF2-BS-For	5'-TGT TTT TGG AAG AAG ATA GAT TTG GT- 3'	386bp	50°C	AC149767.6	41444-41470
DMR0 IgF2-BS-Rev	5'-CCA ACT TAA TAA TAC TAA ACC TAC TA- 3'				41829-41803

Table 2: Primers Table for DNA methylation analysis of bovine DMRs

Primers used for polymorphism search and for bisulfite analysis for bovine putative imprinting centers. Here SN primer for *H19* DMR is the SNuPE primer used for analyzing the SNP in additional individuals.

RT-PCR primers:	Sequence	Product Length	Tm	Acc. No.	Position	Reference
ß actin						
Bos-ßactin-Exon4	5'-GAG AAG CTC TGC TAC GTC G-3'	255bp	58°C	BC102951	737-757	Lonergan, Gutierrez-Adan et al. 2000
Bos-ßactin-Exon5	5'-CCA GAC AGC ACC GTG TTG G-3'				981-999	
Gapdh						
Bos-Gapdh-for	5'-CCT TCA TTG ACC TTC ACT ACA TGG TCT A-3'	830bp	58°C	U85042	71–98	Ushizawa K et al 2005
Bos-Gapdh-rev	5'-GCT GTA GCC AAA TTC ATT GTC GTA CCA-3'				927-901	
18srRNA						
Bos-18srRNA-for	5'-AGA AAC GGC TAC CAC ATC CA-3'	169bp	62°C	AF176811		Goossens K et al 2005
Bos-18srRNA-rev	5'-CAC CAG ACT TGC CCT CCA-3					
Poly A						
Bos-PolyA-For	5'-GTT GCA GGG TAA CCG ATG AA-3'	361bp	55°C	X63436		Hall VJ et al 2005
Bos-PolyA-Rev	5'-TGT TGT GGG TAT GCT GGT GT-3'					
Igf2						
Bos-Igf2, 2-for	5'-CTG GCC CTG CTG GAG ACT TAC TG-3'	515bp	58°C	AC149767	57213-57236	
Bos-igf2,2-rev	5'-TCT GAT CCC CTC AGC CAG ATG G-3'				58010-58032	
Cdkn1c						
Bos-cdkn1c-for	5'-GCC GCT GCT GCC ACC ACT GC -3'	289bp	58°C	AC151858	148283 –148303	(BF039770 :474-494)
Bos-cdkn1c-rev	5'-GCT GCC GCG GGT TGC TGC TA-3'				147336- 147356	(BF039770:185-165)
Phlda2(Tssc3)						
Bos-Tssc3,2-for	5'-GCT GCG GAA GTC CTG CAT GG-3'	299bp	58°C	CB461779	70-92	
Bos-Tssc3-rev	5'-TGG AGA AGC GAA GTG ACA GCC TG-3'				349-369	
H19						
Bos av600-For	5'-AGC GGG ATC GGT GCC TCT GA-3'	128bp	58°C	AC149767	150448-150468	
Bos cb424-Rev	5'-GAT GGA CAC ACG TTC ATG CTT GGT G-3'				151651-151676	

Table 3: Primer Table for expression analysis in bovine BWS region

Primers		Acc. Number AJ271885	Base pairs	Tm
NICE 09_01 For	GCA GCC TGG GAA GAG ACC TGT GTT	185716 185739	463	58°
NICE 09_01 Rev	GGA AGG TTC CAG GCA GCA AGA GC	185253 185275		
polymorphic site	SD7(C) and C57/B6 (T)	185596		
NICE 10_04 For	CAG GCA CTG GAT GGC ACT TGC	163661 163681	493	58°
NICE 10_04 Rev	GCA GAG AAG TTG GGC GGG AAG	163168 163188		
NICE One For	TCG GCT GCT CGA GAA CAT GGC T	131413 131434	390	58°
NICE One Rev	CTG GAG CCA GCA GTG CTC TGC A	131023 131045		
NICE 10_13 For	GAG GCA CCG GGT GCT TCC GA	111976 111996	381	58°
NICE 10_13 Rev	GCC TGC TGC CTG TGG GTG GTC A	111595 111617		
polymorphic site	SD7(T) and C57/B6 (C)	111863		
NICE One Prom For	CCA GGA GAA GCC CAG CAA TCA TTT	131703 131726	703	58°
NICE One Prom Rev	CTC CAC CCA CAT CTT GCT CCT CAG	131023 131044		
polymorphic site	SD7(C) and C57/B6 (T)	131582		
Primers for bisulfite analysis				
NICE 10_13 BS For	TTT AAA GGG AAG AGG GTT TGG TT	111696 111718	359	55°
NICE10_13 BS Rev	ACT TCT CTA CCC ATA CCA AAA CCA A	112055 112079		
NICE One BS For	AGA TTT TAT GTT ATA ATG GGG TTA TAG	131138 131165	490	55°
NICE One BS Rev	CTA AAC CTA AAA ACC TTA AAT TCT AAC	131628 131654		
Primers for genotyping				
Mus-Tnni2 For	AGA AGC TAT TTG ACC TGA GGG GC	**NM_009405**		60°
Mus-Tnni2 Rev	GCC AAG TAC TCC CAG ACT GGA T			
Mus-Tnni2-SNuPE	TAA CTG CCC ACT TCC CCA GT (A/C)	nt 590		
Mus-Osbpl5 For	GCT CCC ACC CAC ACT TGT AGC C	**NM_024289**		60°
Mus-Osbpl5 Rev	GCT GCT CCT GGC AAC CCT CAC TC			
Mus-Osbpl5-Snupe (T/C)	GGG AGC ACT GGC CTC T (T/C)	nt 3141		
Mus-Osbpl5-Snupe (C/T)	CCT GGT AAT CTC AAG TG (C/T)	nt 3230		
Primers against pGL3 vector				
GL primer 2	CTT TAT GTT TTT GGC GTC TTC CA			55°
RV primer 3	CTA GCA AAA TAG GCT GTC CC			55°
RV primer 4	GAC GAT AGT CAT GCC CCG CG			55°

Table 4: Primers used for conserved NICE elements analysis

II.2 Methods

II.2.1 Sequence analysis and identification of putative imprinting centers in bovine and conserved elements at KvDMR1 imprinting sub domain

The human chromosome 11 genomic sequence (NT_009237.17: 1694216-755779) spanning from *MRPL23* till *CDKN1C* gene covering the entire human BWS region was used to screen the bovine sequences by BLAST (NCBI: http://www.ncbi.nlm.nih.gov/) or BLAT (Baylor college of medicine: http:// www.hgsc.bcm.tmc.edu) in the public server databases. Bovine BAC sequences AC150790, AC149767, AC169103.2 AC151859, and AC151858 covering the entire *Kcnq1* gene were obtained from NIH intramural sequencing center (http://www.nisc.nih.gov) (Paulsen, Khare et al. 2005). Similarly the entire human *Snrpn* locus (NT_026446.13:1503593-2099408) spanning app. 150 kb, 5'of the first exon to the last exon of the gene was used. Bovine sequences NW_205360 and AY743660 were obtained at the *Snrpn* gene locus. Sequence alignments were performed by pipmaker (http://pipmaker.bx.psu.edu/pipmaker/) software and CpG island searches were done by CpG Plot (http://www.ebi.ac.uk/emboss/cpgplot/) software. Annotated exons of the genes in the respective region and CpG island harbouring imprinting centers from human sequences were aligned against mouse sequences as well as bovine sequences by multipipmaker. CpG islands at the conserved location to imprinting centers in bovine sequence were selected as putative imprinting centers. Tandem repeat analysis on the selected CpG islands were performed by Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html) software.

Multiple sequence alignments at BWS region also revealed the presence of highly conserved segments of sequences within the introns of *Kcnq1* gene. These were also analyzed in multiple alignments for additional species such as galago (AC147392, AC148124, AC187417, AC149008, AC187174, AC148957, AC183696), bat (AC149442, AC146964, AC146963) dog (AC147594), armadillo (AC147402, AC147403, AC148922, AC151936, AC152478, AC184042, AC190096) and chicken (BX640540, BX640401, BX649221, BX649222, BX640404, AP003796, AP003795,

BX663531). These conserved segments were called NICE (Neighbourhood of Imprinting Center Element).

II.2.2 **Sample and sequence information**

Bovine sample material was obtained from Institute of Molecular Animal Breeding and Biotechnology, Gene Center of the Ludwig, Maximilian University Munich, Germany. Procedure for oocyte recovery, *in-vitro* maturation of oocytes and thereafter IVF, culturing and embryo transfer are described in Hiendleder et al 2006.

Heifers diagnosed pregnant were slaughtered in a local abattoir on day 80 after IVF or artificial insemination. Foetal parameters such as foetal weight and length, and foetal organ weights (heart, kidney, liver) were recorded. Liver samples were collected from the tip of the *Lobus hepatis sinister*, muscle samples were obtained from the *Musculus gluteus maximus* and brain samples were collected from the upper left hemisphere of the cerebrum. (Hiendleder, Wirtz et al. 2006)

Two sires, 22 dams and 45 foetuses, constituting different bovine families, were analyzed for DNA methylation studies at putative imprinting centers.

Mouse samples were collected from 9.5 dpc (days post coitus) in order to analyze the DNA methylation state of the conserved elements. Sample material was nomenclatured as "**p**lacental **e**mbryo" (PE), if extracted from placental part near to foetus, **p**lacenta **m**aternal (PM), when extracted from placental part near uterus and "**e**mbryo" (E) for embryo proper. Each sample was genotyped in order to check the parental contribution. Crosses between inbred strain C57/B6 and SD7 (strain containing a part of the distal chromosome 7 of *mus spretus* SEG (Hemberger, Redies et al. 1998)) were checked by genotyping for the *Tnni2* gene (performed by Joe Weber) while B6xSD7 embryos were checked by genotyping for the *Osbpl5* gene. SNuPE reaction protocol is same as performed for the polymorphism check at bovine *H19* DMR (discussed later). The *Tnni2* gene was analyzed for A/C polymorphism and *Osbpl5* for T/C and C/T polymorphisms. SNuPE primer and

polymorphism location is indicated in Table 4. Samples showing equal parental allele contribution were taken for DNA methylation analysis.

II.2.3 Polymorphism search by direct sequencing, restriction digestion and by SNuPE

PCRs were performed for polymorphism search in bovine foetuses with primers listed in Table 2. All PCRs were performed in presence of 1.5 mM $MgCl_2$, 1.3% DMSO and 1.3 M betaine as final concentration. Obtained PCR products were precipitated by PEG 4000 and were subjected for direct sequencing, restriction digestion and SNuPE analysis.

Direct sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit's from Applied Bio systems (Foster City, CA, USA). Sequencing reaction was performed according to manufacturer's protocol with the modification of 2 µl of terminator Dye in the sequencing reaction and with additives 1.3% DMSO and 1.3 M betaine as final concentration. Sequencing reactions were then purified by sodium acetate ethanol precipitation method (2 µl Sodium acetate 1.5 M and 250 mM EDTA to 50 µl 100% ethanol). Sequencing products were dissolved in 20 µl of loading dye (0.01 g crystal violet in 1ml deionised formamide). Before casting the sequencing gel, the sequencing plates were cleaned with 5 M NaOH, 2% alqinox detergent and distilled water. Thereafter gel solution was prepared by adding 250 µl of 10%APS, 35 µl of TEMED (N,N,N',N'-tetramethyl ethylene diamine) to the 5% polyacyrlamide solution, mixed, vacuumed and poured between the assembled plates. The comb was placed in reverse direction and fixers were tightened. The gel was left for 1 hour and 30 minutes to polymerize and was prerun for 30 minutes in TBE (10mM Tris HCl and 1mM EDTA) buffer. The samples were loaded on the gel after denaturation at 95°C for 5 minutes. The electrophoresis was performed in ABI Prism 377 DNA sequencer (version 3) at run voltage of 1.68 kV; current of 50 mA; electrophoresis power of 150 W; gel temperature of 51°C and laser power of 40mW and the running time was 7 hours for complete run. The analysis of the gel was done with ABI PRISM 100(version 3) with the instrument file dRhodamine and the sequences were analyzed for SNP by SeqAlign from Laser Gene software (DNASTAR, Inc, Madison, WI USA) and also manually.

The obtained SNP's (Table 5) were cross checked with parent allele information. Polymorphism at bovine KvDMR1 was localized at "AAT (T/C)"; therefore additional individuals for KvDMR1 polymorphism were checked by restriction digestion with *TasI* at 60°C for 3 hr. The digested PCR products were visualized on 2% agarose gel.

Polymorphism at *H19* DMR for additional individuals was checked by SNuPE reaction. The PEG 4000 precipitated PCR products were subjected to SNuPE primer (SN primer for *H19* DMR indicated in Table 2) with ddCTP and ddTTP as extension nucleotides, cycled for 2' 95°C followed by 50 cycles at 95° for 45", 55° for 1' and 60° for 2' in Eppendorf thermocycler (Eppendorff, Hamburg Germany). SNuPE reaction was carried out in 20µl of volume, with 3.6 µM SNuPE primer, 0.05 mM ddNTPs, 0.15 U Thermo Sequinase (Amersham) in reaction buffer supplied by the manufacturer. Extension products were later separated on dHPLC. (WAVE DNA Fragment Analysis System, Transgenomics)

Detected polymorphism and genotype for Semon (Se) or Simivit (Si) or Raser (Ra) sire						Position
IC1	G / A	Se (G / A)	Si (G / A)		AC149767	143854
	C / T	Se (C / T)	Si (C / T)			143755
Lit1 / IC2	G / A	Se (G / A)	Si (A / A)	Ra (G / A)	AC147396	38277
	A / -	Se (A / A)	Si (A / A)	Ra (?)		38470

Table 5: Location of detected polymorphism at *H19* DMR and KvDMR1 centers

Polymorphisms search in conserved elements was performed in mouse strains *C57BL/6, PWK, SD7* and *molossinus*. Primers and PCR conditions are listed in Table4. PCRs were performed on genomic DNA of the above stated strains, were PEG 4000 precipitated and later subjected for direct sequencing on automated sequencer from Beckman Coulter (Beckman Coulter GmbH, Krefeld, Germany) Polymorphism was observed between *C57BL/6* and *SD7* in two of the selected NICE elements (polymorphism site is indicated in Table 4) where CpG content was >10 CpG dinucleotides. The DNA methylation analysis was performed in at least 2 of the reciprocal crosses i.e. *SDxB6* and *B6xSD7* crosses.

II.2.4 Bisulfite modification, cloning, sequencing and analysis

II.2.4.1 Principle of bisulfite modification

The reaction between pyrimidines and sodium bisulphite was first described in early 1970s (Shapiro, Cohen et al. 1970; Shapiro and Weisgras 1970). In the first step of the bisulphite reaction, cytosines are sulphonated and deaminated and this reaction converts cytosine to uracil sulphonate. A subsequent desulphonation at a basic pH completes the conversion from uracil sulphonate to uracil (Figure 12).

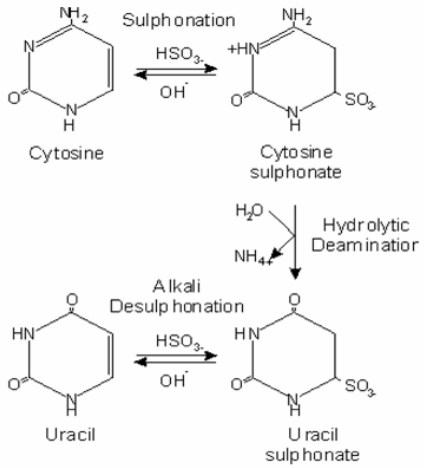


Figure 12: Chemical reaction for bisulfite conversion and modification

Methylation at cytosine residue protects from being converted to uracil on bisulfite treatment. The most critical step in bisulphite modification of the genomic DNA is denaturation. Sodium bisulphite can only react with cytosine which is not involved in base-pairing. It is so because only bases that can adopt a syn conformation can undergo substitutions at position 6 (Schweizer, Witkowski et al. 1971), and in double-stranded DNA, this is not possible because base-pairing locks the ring in an unreactive anti-conformation (Shapiro, Braverman et al. 1973). Once the cytosine residue has reacted, the base-pairing is no longer possible (Schweizer, Witkowski et al. 1971). Presence of high salt concentration in a standard reaction (3–5 M) favours the re-annealing of strands and this increases the risk of incomplete reaction (Rein, Zorbas et al. 1997). Various technical modifications to this procedure have been attempted to reduce strand annealing, such as digesting the DNA prior to modification and then performing the reaction in a thermocycler with repeated

heating steps to 95°C(Rein, Zorbas et al. 1997). This approach suffers from the problem that DNA is very prone to depurination and repeated heating to 95°C combined with a reaction at acidic pH 5 can severely degrade the DNA. A more successful approach was to embed the DNA in low melting point agarose blocks (Olek, Oswald et al. 1996). This approach not only restricts the re-annealing of DNA strands, but also eases the subsequent reaction steps and reduces DNA losses from downstream purification procedures.

II.2.4.2 **Protocol**

II.2.4.2.1 **Genomic DNA isolation**

Tissue samples were lyzed in 750µl lysis buffer (10 mM Tris HCl pH 8, 75 mM NaCl, 0.5M EDTA, 1% SDS) with 70 µl of proteinase K (20 mg/ml) and incubated at 50 °C overnight on a thermo shaker. Equal volumes of phenol (pH 8) were added to each sample and were placed on a rotator at room temperature for 15' for thorough mixing. Samples were then centrifuged at 14000 rpm for 5' and the supernatant was collected in a separate tube. Chloroform and isoamylalcohol (24:1 v/v) extraction was then performed twice on the sample before precipitating the DNA with isopropanol, and the pellets were washed twice with 70% ethanol. Air dried pellets were dissolved in an appropriate volume of 10 mM Tris (pH 8) and measured in spectrophotometer for the DNA concentration at 260 nm wavelength.
These DNA samples were later used for bisulfite treatment or for PCR amplification for the polymorphism search. Oocyte samples were treated in a different way (described below) for the bisulfite treatment.

II.2.4.2.2 **Bisulfite treatment on beads**

Genomic DNA (700 ng) was digested overnight with *HindIII* restriction endo nuclease (Roche) in 21 µl of reaction volume at 37°C. Restriction digested DNA was denatured in boiling water for 15 minutes and then immediately placed on ice for further treatment. Four µl of 2 M NaOH were added to each sample (final concentration 0.3M) and incubated at 50°C for 15 minutes. Fifty µl of 2% low melting agarose (SeaPlaque Agarose, FMC) were used per sample and of which 10 µl were taken to form the beads in ice cold heavy mineral oil (Heavy white mineral

oil, Sigma). One ml of freshly prepared 2.5 M bisulfite solution (pH 5) (1.9 g sodium bisulfite with 720 µl of 2M NaOH in 2.5ml distilled water was mixed with 55 mg of hydroquinone in 500 µl distilled water and was added to each sample tubes. Brief centrifugation was performed in order to reverse the phase, i.e. sodium bisulfite solution goes under the heavy mineral oil and the beads are then in bisulfite solution. Tubes were incubated initially on ice for 30 minutes and then at 50°C for 3.5hr. Beads were washed two times with TE (pH 8) for 15 minutes and then treated twice with 0.3M NaOH for 15 minutes. Later two washes with TE (pH 8) for 15 minutes were performed to remove the last traces of NaOH. All the solution from the tube was drained out and only the intact beads were stored at 4°C for further use in PCR amplification.

II.2.4.2.3 **Bisulfite treatment in liquid**

Genomic DNA (700 ng) was digested overnight with *HindIII* (Roche) in 21µl of reaction volume at 37°C. Thereafter the solution containing 187 µl of sodium bisulfite (1,9gm sodium bisulfite in 2,5ml distilled water and 750 µl of 2M NaOH) and 73 µl of scavenger solution (98.6 mg of 6 hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Aldrich Steinheim Germany) in 2,5ml of 1,4 dioxan (Mereck Damstadt Germany)) was added to each sample tube. This DNA-bisulfite solution was cycled in a PCR machine; 99°C for 15', 50°C for 1hr30', denatured at 99°C for 5' and again incubated at 50°C for 1hr and 30minutes. The desulphonation and purification steps were performed in Microcon 50 columns (Millipore corporation Bedford USA). In brief, samples were loaded on the column and centrifuged at maximum speed (14000rpm) for 15 minutes, and later were washed with 500 µl of TE (pH 8) and again centrifuged at maximum speed for 10 minutes. Samples in columns were then treated with 500 µl of 0.3M NaOH for 10 minutes and were centrifuged to remove the NaOH solution. One time wash with TE was done and then 50 µl of warm (50°C) TE was added, columns were turned upside down and transferred in the new tubes. Centrifugation at 3000 rpm for 20 minutes was performed in order to elute the sample DNA from the column.

II.2.4.2.4 **Bisulfite treatment on oocytes**

At least 50 oocytes were taken for a bisulfite reaction. These oocytes were mixed with 15 µl 2% LMP agarose and were heated in boiling water for 15 minutes. Before boiling, heavy mineral oil is overlaid on the oocyte-agarose solution. Samples were cooled down on ice in order to form one agarose bead where oocytes are already embedded. To this 750 µl of lysis buffer without SDS (10 mM Tris HCl pH 8, 75 mM NaCl, 0.5 M EDTA) and 70 µl of proteinase K (20 mg/ml) were added and incubated at 50°C for overnight at thermo shaker. Next day beads were washed with distilled water once and subjected to the protocol mentioned before for the bisulfite treatment on beads (section II.2.4.2.2).

II.2.5 **PCR amplification from bisulfite treated DNA**

Bisulfite modified genomic DNA beads were melted and 5 µl (or 3 µl from liquid bisulfite treatment) of it were added in 50 µl of PCR reaction mix for the amplification of the region of interest. PCR was performed with HotStarTaq DNA polymerase (QIAGEN GmbH, Hilden, Germany) at 95°C for 15' followed by 40 cycles of 95°C for 45", Tm for 45'' and 72°C for 1 minute. Last step of extension was performed at 72°C for 10 minutes.

The obtained PCR products were purified from gel by QIAEX II Gel Extraction (QIAGEN GmbH, Hilden, Germany) , cloned in pGEMT (Promega Madison, WI, USA) or pCR-TOPO2.1 (Invitrogen, Paisley Scotland) vector and were sequenced using standard methods either on Beckman Coulter (CEQ™ 8000 Genetic Analysis System, Fullerton, CA, USA) or at Berlin (Max Planck Institute for Molecular Genetics, ProScience Berlin). Sequence analysis of the bisulfite cloned sequences was performed with BiQ analyzer software. (Bock, Reither et al. 2005)

Bisulfite primers and PCR conditions for bovine putative imprinting centers are listed in Table 2 while for mouse conserved regions; they are listed in Table 4.

II.2.6 **COBRA and semi-quantification**

II.2.6.1 **Principle of COBRA**

Bisulfite modification of a genomic DNA converts cytosine residues to uracil and subsequent PCR amplification on the modified DNA, changes uracil to thymine in the amplicons. By this modification many of the restriction enzyme recognition sites are mutated. This mutation of cytosine to thymine residues most often disrupts the restriction sites; however in few instances it also creates a new recognition site. In contrast, if the methylated cytosines are present in restriction recognition sites, then there will be no conversion of cytosine to thymine, and eventually no disruption of the sites. When the obtained PCR products are subjected to the restriction digestion, it shows a distinct digestion pattern. This can be visualized on separating agarose or polyacyrlamide gels. Presence of digested or undigested amplicons represents the methylated or unmethylated templates in the unconverted genomic DNA (Figure 13)

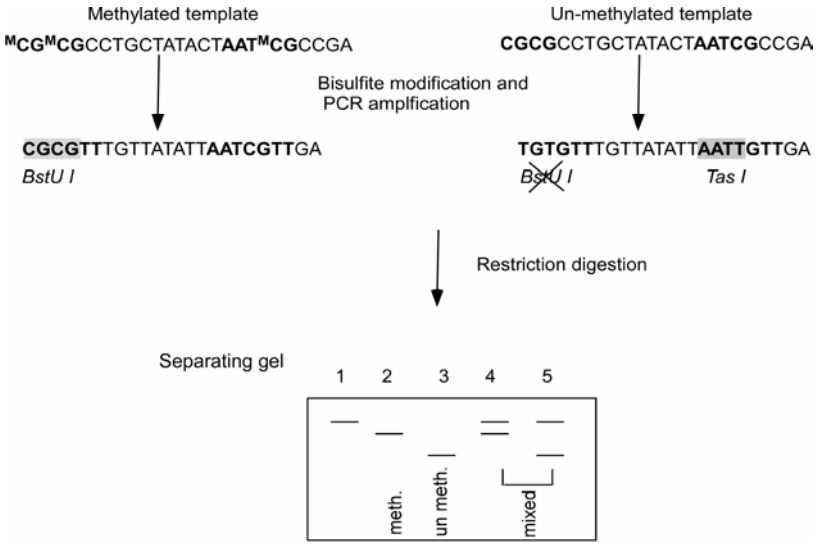


Figure 13: Principle of COBRA technique

Conversion of cytosine to thymine in unmethylated templates abolishes the *BstU I* site shown as cross. Lanes in the separating gel are undigested PCR product (1), digest with *BstU I* (2, 4) and digests with *Tas I* (3, 5). Each digestion pattern represents a different state of methylation at a locus in the genomic DNA e.g. lane 2 shows complete methylation state when digested with *BstU I* enzyme. Similarly lane 3 shows complete digestion with *Tas I* which is a new site created from bisulfite modification of unmethylated genomic DNA and then PCR amplification. While mixed means PCR amplicons (from bisulfite treated DNA) are generated from both types of templates methylated and unmethylated genomic DNA.

II.2.6.2 **Protocol**

PCR products from bisulfite treated DNA, were enriched by a second round of PCR for COBRA analysis. One µl of the first round PCR products were again amplified with same primers for another 20 cycles and were analyzed by different combination of enzymes for COBRA analysis (combinations and conditions are listed in Table 6). Restricted digest samples were run on 2.5% agarose gel in 1x TBE buffer (10 mM Tris HCl and 1 mM EDTA). Semi-quantification of digested bands was performed by calculating band intensities with the Total Lab software (Biostep GmbH, Jahnsdorf Germany). Band intensities of a single lane was taken in measurement but was also cross-checked with the intensity of undigested input. (Table 6)

Bovine putative imprinting centers					
	Enzyme	**Buffer**	**°C**	**Quantification formulae in a lane**	**Purpose**
IC1	BstU I	NEB buffer 2	65	(1-undigest)/ total band intensities	meth
IC2	Nru I	NEB buffer 2	37	(1-undigest)/ total band intensities	meth
	BstU I	NEB buffer 2	65	(1-undigest)/ total band intensities	meth
	EcoR I	NEB buffer 2	37	n.d	con.
Snrpn DMR	Tas I	MBI fermentas buffer B	65	(Band A (409bp) /total band intensities	unmeth
	Taq I	NEB buffer 3	65	(1-undigest)/ total band intensities	meth
DMR 0	Taq I	NEB buffer 3	65	(1-undigest)/ total band intensities	meth
	Tas I	MBI fermentas buffer B	65	n.d	con.
Conserved elements					
	Enzyme	**Buffer**	**°C**	**Quantification formulae in a lane**	**Purpose**
NICE One	Taq I	NEB buffer 3	65	(1-undigest)/ total band intensities	meth
KvDMR1	Taq I	NEB buffer 3	65	(1-undigest)/ total band intensities	meth

Table 6: Combination of restriction enzyme used in COBRA analysis

All COBRA digestions were performed for 3 hours at the indicated temperature in degree Celsius (°C). Semi quantification was performed according to the formulae stated in the Table. Conversion by bisulfite treatment disrupts the restriction sites, but is not affected if methylated (digesting amplicons from methylated templates). Likewise some additional sites are created (digesting amplicons from unmethylated templates). Conversion rate is checked either by creation of site or by disruption of a site indicated by "con".

II.2.7 **RT-PCR and hybridization**

Bovine tissue RNA was prepared by the conventional protocol of Chomczynski and Sacchi. (Chomczynski and Sacchi 1987) In brief, tissue material (100 mg) was homogenized on ice in 1 ml lysis buffer (4M guanidin-thiocyanat, 25 mM sodium citrate, 0.5% N-lauroylsarcosin, 0.1M beta mercaptoethanol dissolved in DEPC treated water). After complete homogenization, phenol chloroform extraction was performed in order to remove the proteins, lipids and genomic DNA. Equal volume of acidic phenol (pH 4) was added and centrifuged at 5000 rpm at 4°C; supernatant was collected and extracted twice with chloroform /isoamylalcohol (24:1). Extracted RNAs was precipitated by isopropanol / ethanol method and were dissolved in DEPC treated water. The obtained RNA was later treated with DNase enzyme in order to degrade any DNA present. One µl of DNase was added in 50 µl of RNA solution, incubated at 37°C for 30 minutes and then inactivated the DNAase enzyme by incubating at 95°C for 15 minutes. Thereafter RNA was once again extracted by the phenol-chloroform-isoamylalcohol procedure and this time RNA was precipitated by ethanol precipitation method. The obtained RNA pellet was dissolved in 25 µl of DEPC water and was quantified in a spectrophotometer at 260 nm of wavelength.

Reverse transcription was performed with M-MLV reverse transcriptase, RNase H Minus (Promega). Two µg of RNA were reverse transcribed in 1x buffer (provided by the manufacturer) supplemented with 6 µl dNTP (1,25mM of each dNTP), 0.5µl of RNAsin (40 units/µl), 0.2 µl random hexamers (500 µg/ml) and 1µl of reverse transcriptase (200 units/µl) (Promega). Reverse transcription was carried out at 37°C for 90 minutes and thereafter transcriptase was inactivated by heating to 90°C for 15 minutes.

Gene amplification was performed using gene specific primers listed in Table 4. All PCRs were performed for 95°C for 5' followed by 95° for 30 seconds, 58° for 30 seconds and 72° for 1 minute for 22 cycles (except for *PolyA polymerase* gene, the annealing temperature was 55°C). For *18srRNA* gene PCR amplification was performed for 20 cycles and 1/3rd volume of cDNA was taken for control genes *18srRNA*, *ß actin* and *Gapdh*. The *Igf2*, *Phlda2* and *Cdkn1c* genes were amplified in presence of 1.3 M Betaine and 1.3% DMSO as final concentration. Amplified

products were then run on 1.5% agarose gel, stained with ethidium bromide and photographed. Later the gels were subjected for denaturation for 15' (1.5M NaCl and 0.5M NaOH), neutralization for 15' (1M Tris pH 7.4 and 1.5 M NaCl) and the RNA was transferred (blotted) on a positively charged nylon membrane (Roche, Basel Switzerland).

Probes for different genes were obtained by amplifying from the genomic DNA. These probes were labelled with alpha ^{32}P dCTP (10 mCi/ml, MP bio-medicals Germany) using high prime premixed labelling kit from Roche (Roche Applied Science, Basel Switzerland) at 37°C for 30 minutes and later purified by Amersham G25 columns (Amersham Biosciences NJ, USA). Before hybridizing the membranes with probes for overnight at 60°C, membranes were prehybridized with Church buffer (sodium phosphate buffer 0.25M pH 7.2, EDTA 1 mM, BSA 1%, SDS 7%) at 65°C for 2 hr. Next day 3 washes with 2x SSC and 1%SDS and 3 washes with 0.2x SSC and 1% SDS were performed. Membranes were washed briefly with distilled water and wrapped in cellophane film before putting them for the exposure to Fujifilm super medical X ray films (Fujifilm UK). Exposures were performed for 1 hr, 4 hr at room temperature and overnight at -80°C. For *PolyA polymerase* and *Gapdh* the exposure time was also extended to 4 days at -80°C. Band intensity quantification was performed by Image Quant software using default parameters. (Amersham Biosciences NJ, USA)

II.2.8 **Plasmid Construct preparation for transient transfection assays**

Analysis of conserved elements for different regulatory functions such as promoter, enhancer and silencers were performed by dual luciferase assays.

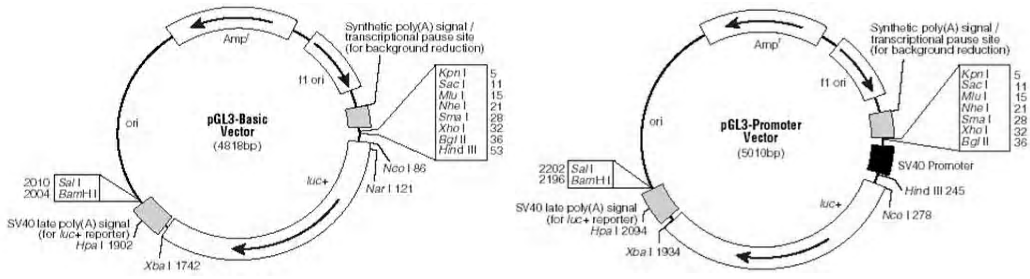
Plasmid constructs were made for promoter/ enhancer assay by utilizing pGL3 based vectors (Invitrogen, Paisley Scotland). NICE elements were initially cloned in pCR2.1 vector (Invitrogen, Paisley Scotland) and thereafter *HindIII* / *XhoI* as well as *Acc651 / XhoI* sites were used to insert the NICE elements in both orientations into pGL3 basic vector (Acc. No.U47295). Though these constructs contain parts of

vector pCR2.1, and these parts have some enhancing activity in HEK293T cells but they showed no promoter activity. In order to have endogenous condition, we cloned KvDMR1 promoter from mice into pGL3 basic vector. Initially KvDMR1 promoter was digested with *PstI* (AJ271885.2: 138671-142304) and a 3.6kb fragment was cloned in pCR2.1. Thereafter it was cleaved by *Acc65I* and *HindIII* (3.0 kb: AJ271885.2: 139280-142304) to clone in pGL3 basic vector in the known promoter orientation. KvDMR1 in pGL3 constructs were later digested with *SalI*, end filled and to it blunt end NICE elements were cloned. The end filling of the fragments were performed by Klenow fragment DNA polymerase (Boehringer, Mannheim Germany) according to the manufacturer's protocol. In brief, to the digested vector and insert DNA add Klenow buffer to 1x as final concentration, 40µM of each dNTP and 1 unit of Klenow fragment DNA polymerase. Incubate the reaction at 37°C for 10 minutes and then stop by heating the mixture for 10 minutes at 75°C. Later perform gel isolation of the linearized vector and insert for the appropriate ligation reactions.

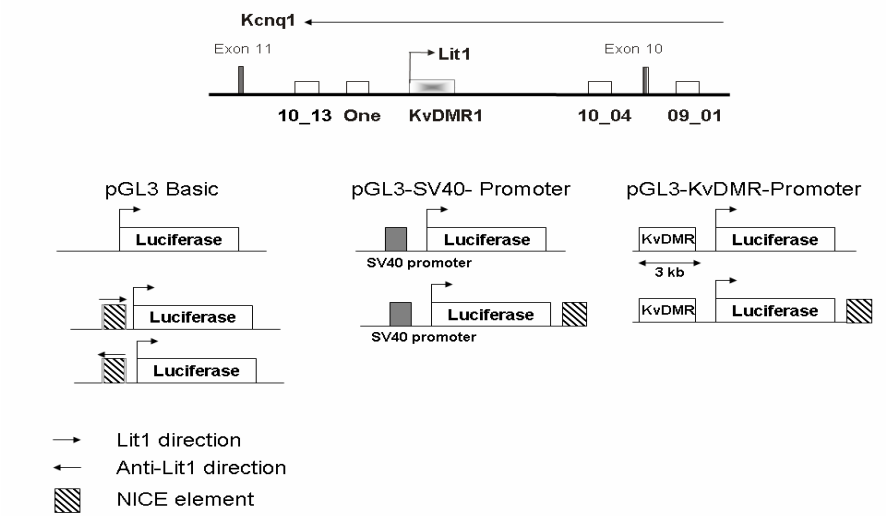
In order to check the promoter influence, KvDMR1 was replaced by SV40 promoter where SV40 was cleaved out from pGL3 SV40 promoter reporter construct (U47298).

To check the influence of tandem repeats present in KvDMR1, the repeats were cleaved from the KvDMR1 promoter at *Ecl136II* and *EcoRV* (AJ271885.2: 140488-142304) and religated. In this way approximately 2,0Kb from the promoter construct was removed. This deletion resulted in removal of almost all tandem repeats including conserved motifs. (Paulsen, Khare et al. 2005)

A)



B)



C)

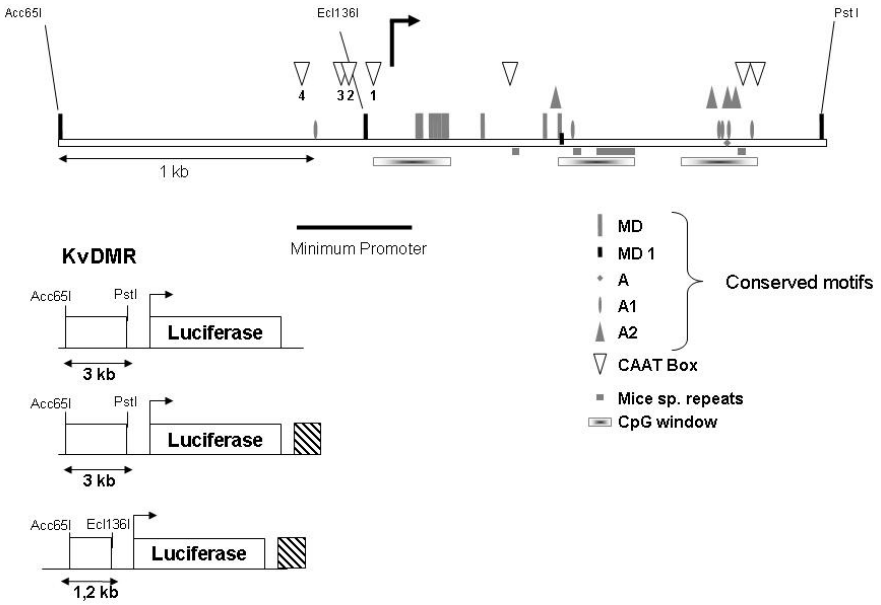


Figure 14: Plasmid constructs used in different transfection assays

A) Plasmid vector maps of pGL3 (Promega)

B) Constructs for promoter assay and for the influence of NICE elements on KvDMR1 or SV40 promoter activities.

C) Constructs with the KvDMR1 with deleted repeats as promoter.

Influence of Hand1 on NICE elements was performed by co-transfection assays, where Hand1 constructs were the kind gift from Paul Riley (Molecular Medicine Unit, Institute of Child Health; London. Described in (Scott, Anson-Cartwright et al. 2000)).

II.2.9 **Promoter/enhancer assays by Dual Luciferase assay**

Transient transfections were conducted with Lipofectamine 2000 (Invitrogen, Paisley Scotland). For single test plasmid transfection, 800 ng of construct DNA was transfected with 5ng of control renilla firefly luciferase (pRL having CMV promoter (Promega) for normalization) plasmid. Transfections were performed by standard protocol in 24 well plates with the modification of 1.5µl of Lipofectamine 2000 instead of 2 µl. In brief, $1x10^5$ cells were plated in 500 µl of growth medium without antibiotic one day prior to the transfection. At the time of transfection the cells were 70-90% confluent. The construct test DNA and control pRL plasmid DNA were diluted in 50 µl DMEM without serum or in OptiMEM (Solution A). In separate eppendorf tube 1.5 µl of Lipofectamine 2000 were added in 50 µl of media or OptiMEM and thereafter both the solutions were mixed after 5 minutes of incubation at room temperature. This solution mixture was then incubated at room temperature for 30 minutes so that complexes can be formed between plasmid DNA and the lipofectamine agent. Later, the mixture was added drop wise on to the cells and mixed gently by rocking the plate back and forth. Transfected cells were incubated at 37°C at 5% CO_2 for 30hr.

After desired incubation at 37°C, cells were washed twice with 500 µl of 1x PBS and were harvested by adding 200 µl of passive lysis buffer 1x (Promega) and incubated at room temperature for at least 30 minutes. Transfection analysis was carried out with 10µl of lysate in 50 µl of LARII and then 50µl of STOP and GLOW buffer (Promega). Luciferase readouts were carried on a Berthold lumat illuminometer (LB 9507; Bad Wildbad Germany) for 10 seconds. Transfections were carried out in triplicates and at least three independent transfections were made for each experiment.

In co-transfection assays, equal quantity of test constructs were transfected with control plasmid i.e. test plasmid I (400 ng) + test plasmid II (400 ng) and pRL control plasmid (5 ng). Harvesting of the cells was done after 72 hr of transfection, this time point was taken after optimization by a time assay (discussed in results).

Co-transfection was performed for each test construct i.e. NICE element, against Hand1 construct or with pcDNA3 vector in which Hand1 is cloned. Transfections in HEK293T cells were done according to standard protocol of transfections in 24 well plates.

Transfections in C2C12 cells were performed similarly with the modification that the media was replaced by OptiMEM before transfection. After transfection, cells were serum starved for 6hr at 37°C and 5% CO_2 and then only OptiMEM was replaced by complete media.

Cell Line	Transfection method	Co-Trans.	DNA construct (ng)	Renilla (control) (ng)	Serum starvation	Hrs of Incubation
HEK 293 T	Lipofectamine 2000	no	800	5	no	30 hrs
HEK 293 T	Lipofectamine 2000	with ***Hand 1***	400+ 400	5	no	72 hrs
C2C12	Lipofectamine 2000	no	800	10	yes	30 hrs

Table 7: Transient transfection methods used in different cell lines

II.2.10 **Western blot**

The trypsinized cells were spin down after 1 time wash with PBS. Collected cells were lyzed in 20µl of RIPA lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium Deoxycholate, 1 mM EDTA, 0.1% SDS) with proteinase inhibitor cocktail (SIGMA) and incubated on ice for 30 minutes. During incubation, samples were tapped at every 5 minutes. Lyzed cells were incubated for 10 minutes in boiling water and were centrifuged at 14000 rpm for 10 minutes. The supernatant was collected and now the samples were ready for loading on SDS PAGE.

SDS PAGE (polyacyrlamide gel electrophoresis) gel is comprised of running gel and stacking gel. Stacking gel concentrates all the protein before they can be resolved on running gel. After assembling the glass plates, 10% resolving gel (10% acrylamide mix, 0.4 M tris (pH 8.8), 0.1% SDS and for polymerization 100 µl of 10% APS & 4 µl TEMED for 10ml resolving solution) was poured to about 1cm below the wells of the comb (app. 5 ml). The gel was sealed with water saturated 1-butanol and left for at least 1hr for gel polymerization. Later water saturated 1-butanol was poured off and 5% stacking gel solution (5% acrylamide, 0.125M Tris (pH 6.8), 0.1% SDS and

for polymerization 50 µl of 10% APS & 2 µl TEMED for 5 ml stack solution was added and was increased according to the volume) was added and the comb was inserted. When the stacking gel was polymerized, the plates with the casted gel were placed in the gel rig and were immersed in running buffer (0.025 M Tris pH 8.8, 0.192 M glycine, and 0.1% SDS). Wells were thoroughly washed before loading the samples with 2x Laemmli loading buffer (130 mM Tris HCl pH 6.8, 10% Glycerol, 4% SDS, 0.02 % bromophenolblue and 4% ß mercaptoethnaol). Twenty µg of protein extract was loaded and the gels were run at constant current of 35-37mA. Before transferring proteins from SDS PAGE to PVDF (polyvinylidenedifluoride) membrane, the membrane was wetted with 100% methanol for 30 minutes on rocker. After pre-wetting in 1x transfer buffer (0.025 M tris pH 8.3, 0.192 M glycine) membrane and the gel were assembled as a "sand-witch" for Tank-Electro Blotter (PeqLab, Erlangen Germany) in the following manner "sponge-filter paper-gel-membrane-filter paper-sponge". Transfer of proteins from gel to membrane was performed in was performed at 2 mA/cm^2 for overnight at 4°C on stirrer.

The pre-stained protein marker lane was cut off from the membrane after the transfer of the proteins to the membrane and the membrane was incubated in blocking buffer (10% BSA in 0.02% Tween 20 PBS) at room temperature (22°C) for 4 hours. Thereafter the first antibody (1:1000; Hand1 polyclonal antibody raised in rabbit) in 2.5% BSA was added and incubated overnight at 4°C with constant agitation. Next day the membrane was washed 3x with 0.05% Tween 20 in PBS before adding the second antibody (1:10000; horse radish peroxidase (HRP) conjugated anti rabbit antibody) and incubated for 4 hours at room temperature. Membrane was later washed 3x with 0.05% Tween 20 in PBS and was processed using the ECL detection kit (according to the manufacturer protocol).

II.2.11 **Statistical analysis**

Chi square statistical analysis was performed to analyze DNA methylation data obtained by cloning and sequencing. Only informative individuals were taken in account for the statistical analysis. At least 15 clones per sample with an allele clone ratio of 0.4-0.6 (allele clones/ total number of clones) was considered for analysis.

Pair wise chi square test was executed to compare individual tissues, with the degree of freedom as 1.

Student t test (paired comparison) was used to analyze the significance of the constructs readouts in different transfection assays. Comparison was performed against the reference promoter such as, KvDMR1 promoter, KvDMR1-R promoter or SV40 promoter in the respective transfection assays. In co-transfection assay the comparison was against the co-transfection with control vector. Triplicates of a transfection assay was averaged and considered as one read out of an experiment and at least three transfections were analyzed for the statistical analysis

III Results

III.1 DNA methylation at bovine imprinting centers

III.1.1 Identification of imprinting centers and informative individuals in bovine

Human assisted reproduction (ART) has been shown to affect epigenetic modifications of imprinting centers of the BWS and PWS/AS regions. Localization of the relevant genomic elements in bovine using comparative genome analysis was initially performed in order to identify the homologous putative DMRs in bovine.

The entire genomic region homologous to the human Beckwith Wiedemann syndrome (BWS) region is located on bovine chromosome 29 while the location of the PWS / AS gene cluster is still not clear. On comparative sequence analysis of the bovine BWS region to human and mouse, we found similar arrangement and order of the genes. This also holds true for the location of evolutionary conserved elements and known regulatory elements such as the endodermal enhancer elements downstream of *H19* (Figure 15, Appendix Table 1 & 2) (Ishihara, Hatano et al. 2000; Paulsen, Khare et al. 2005).

The chromosomal region of the PWS/AS region is currently neither present in a contiguous annotated sequence nor completely covered by overlapping BAC clones. Therefore it was not possible to verify the entire structure of the PWS/AS gene cluster in bovine. However we identified BAC sequences containing the entire *Snrpn* gene. Performing comparative sequence analysis at PWS / AS region among bovine, mouse and human sequences, only *Snrpn* exons shows sequence conservation (refer appendix Table 2 & 4 for the location of the DMR and exons of the *Snrpn* gene).

In order to identify the putative DMRs, we analyzed various characteristics of known imprinting centers in human and mouse.

The *H19* DMR is a CpG island which is located about 3 kb upstream of the putative *H19* transcriptional start. In human, *H19* DMR consists of long tandem repeats; i.e. 400bp A and B repeats while in mouse, different repeats are present: a G rich repeat

and so called K-repeats. (Jinno, Sengoku et al. 1996; Stadnick, Pieracci et al. 1999; Thorvaldsen, Mann et al. 2002; Lewis, Mitsuya et al. 2004). This center also harbours CTCF protein consensus binding sites 7 in human and 4 in mouse (Bell and Felsenfeld 2000). The putative bovine *H19* DMR region has similar relative location to the *H19* gene i.e. 3 kb upstream of the 1st exon of the gene and also possesses long tandem repeats of 1,6kb length (Appendix Table 3). The 1,6kb long tandem repeat is repeated 3 times and the third replicate is truncated. The bovine putative *H19* DMR harbours 7 CTCF consensus binding sites, similar to human *H19* DMR, and consensus binding sites numbered 2 to 6 are present on the 1,6kb long tandem repeats. (Figure 16) This center in bovine also contains G rich repeats between CTCF binding site and *H19* transcriptional start site, similar to the mouse *H19* DMR. Overall the bovine *H19* DMR exhibits considerable structural similarities to both, the corresponding *H19* DMR in human (large repeated substructures and number of CTCF sites) and mouse (G-rich repeats).

We also located the second BWS imprinting center (KvDMR1) at a conserved position in intron 10 of the bovine *Kcnq1* gene (Figure 15). For human and mouse it has been shown that this imprinting center is a promoter of the *Kcnq1* antisense transcript *Kcnq1*ot1 (Lit1). Also in bovine the KvDMR1 comprises a CpG island like promoter structure (including CAAT boxes) linked to an extensive cluster of short tandem repeats. We have shown that the CpG island itself and the repeats exhibit only marginal sequence conservation among all mammals. However in different mammals the KvDMR1 imprinting center is flanked by a number of highly conserved elements. These characteristics i.e. presence of short tandem repeats and flanking conserved elements in bovine sequences demarcates the CpG island at similar location as the putative KvDMR1 (Figure 16 & Figure24) (Paulsen et. al 2005)

Similarly in the PWS / AS imprinting region, the DMR is a CpG island and located at the 1st exon of the *Snrpn* gene in human and in mouse. In bovine sequences, the corresponding DMR (which is a CpG island) within exon1 region of *Snrpn* is well conserved on the sequence level (Figure 16).

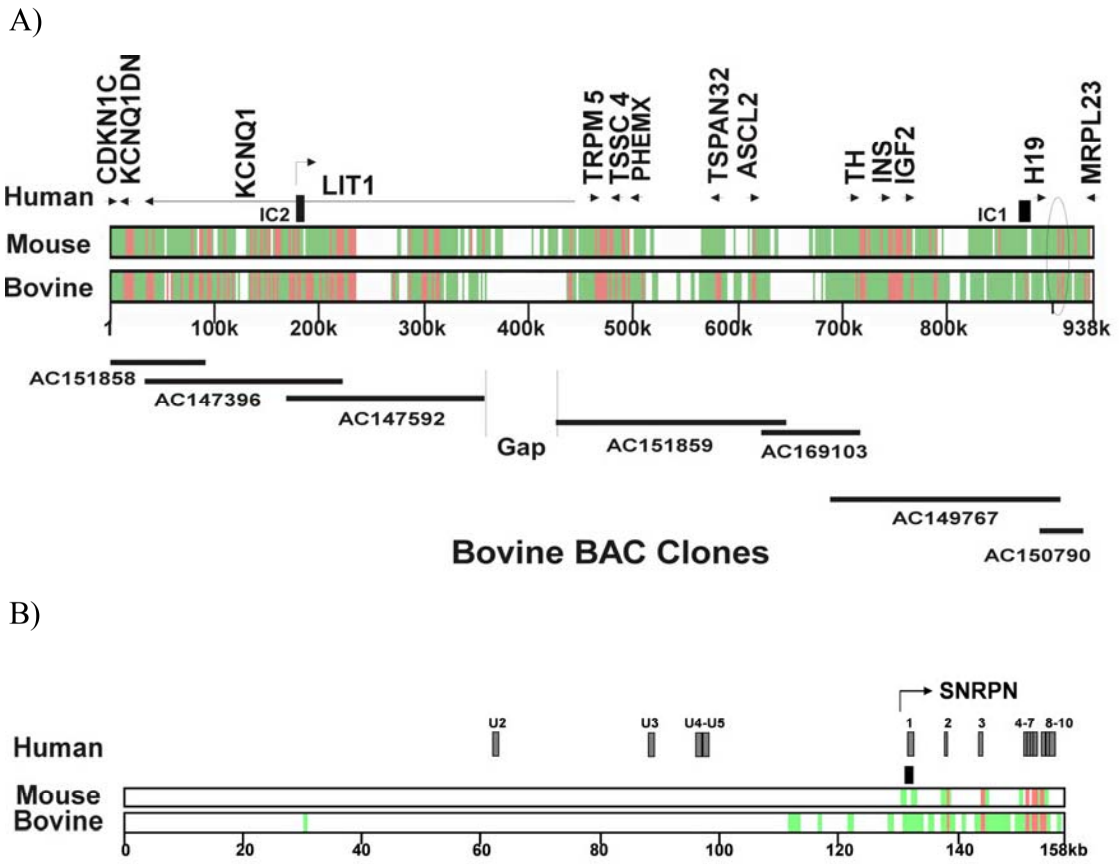


Figure 15: Putative DMRs in bovine compared to human and mouse imprinting domains

A) BWS region and B) SNRPN gene aligned against mouse and bovine sequences. Overlapping BAC clones are shown as horizontal line for bovine BWS region and the indicated gap is of approximately 50kb. Multipip plot analysis was performed with human sequence as reference against the mouse and bovine sequences. Alignments of >70% sequence identity with >100bp sequence length are represented as red bars, while lower than the indicated threshold are shown as green bar. The oval at BWS plot represents the location of endodermal enhancers.

Having located all relevant imprinting control elements within the BWS and PWS/AS regions in bovine, I then screened our collection of foetuses, corresponding dams (if known) and sires for informative polymorphisms. In total 45 foetuses and corresponding sires and dams (22 in number) were analyzed in this study. For *H19* DMR and KvDMR1, only one completely informative polymorphisms (i.e. with an unambiguous assignment of foetal alleles to their sire and dam origin) and a few foetuses in which alleles can be distinguished but were not assigned to the parent allele information, were identified. Unfortunately no informative polymorphism within the S*nrpn* DMR or in the imprinted genes at BWS region of all samples analysed was found.

III.1.2 **Identification of allele specific methylation at the imprinting centers**

The selected imprinting centers are primary DMRs (Differentially Methylated Regions) i.e. they acquires different DNA methylation marks in the germ line and maintain the difference through out the development. Hence initial DNA methylation analysis was performed on germ cells i.e. spermatocytes and oocytes. It has been shown that the DNA methylation imprints at the imprinting centers are established at different stages of oocyte development. Moreover this time dependent imprint establishment at an imprinting center was shown to vary between human and mouse (Lucifero, Mertineit et al. 2002). Therefore initially the GV stage oocytes were analyzed for methylation imprints and later metaphase II oocytes DNA methylation imprints were also examined (next section).

The DNA methylation analysis was performed by cloning and sequencing of PCR products derived from bisulphite treated genomic DNA. In addition, the DNA methylation imprints at *H19* DMR and KvDMR1 for the allele specific DNA methylation in completely informative individuals in several foetal and placental tissues were also examined. For the foetal tissues DNA methylation analysis another technique, COBRA (combined bisulfite and restriction analysis, refer methods section II.2.6.1), was also used

At *H19* DMR, the DNA methylation study included two CTCF sites located within the repeated 1,6kb segments of this DMR. Hence amplification resulted in two distinct size products (I) harbouring the 3rd CTCF binding including an allele specific polymorphism and (II) the 5th CTCF binding site with no informative polymorphism (Figure 16). These PCR products can be distinguished by a length polymorphism, where product II has an insert "TCTTGAGCTGACCCTGCCTGC". As with human and mouse *H19* DMR DNA methylation, the bovine *H19* DMR was completely methylated in sperm DNA and complete absence of methylation was observed in oocytes (GV stages) DNA. This differential DNA methylation was also observed in various tissues of the foetus and (partially) in placental tissue (Figure 17 & Figure 20).

Methylation analysis at KvDMR1 in bovine revealed that sperm DNA was unmethylated and the DNA methylation imprints were already present at GV stage oocyte DNA i.e. they were completely methylated. The differential DNA methylation was also observed at foetal stages in both the embryo and placenta (Figure 18 & Figure 20). The third imprinting center the *Snrpn* DMR also showed complete DNA methylation in GV stage oocytes and complete unmethylated state in sperm DNA. Here too different foetal tissues and placental tissue were analyzed by COBRA (combined bisulfite and restriction analysis) and by cloning and sequencing of bisulfite treated template PCR products. (Figure 19 & Figure 20) Both independent assays confirmed a DNA methylation level of about 50%. Moreover, individually sequenced clones revealed an almost one to one distribution of unmethylated and methylated sequences suggesting an allele specific imprint. Due to the absence of polymorphisms I was unable to assign the parental allele identity.

In conclusion, all three DMRs (*H19* DMR, KvDMR1 and the *Snrpn* DMR) showed a parent of origin specific differential DNA methylation imprint that is established in germ cells and maintained after fertilization. However in placenta both *H19* DMR and KvDMR1 imprints are apparently not fully maintained (Figure 20). This partial absence of DNA methylation imprints in placenta contrasts the situation in mouse where I (data not shown) and others observed a complete maintenance of differential DNA methylation at all three imprinting centers.

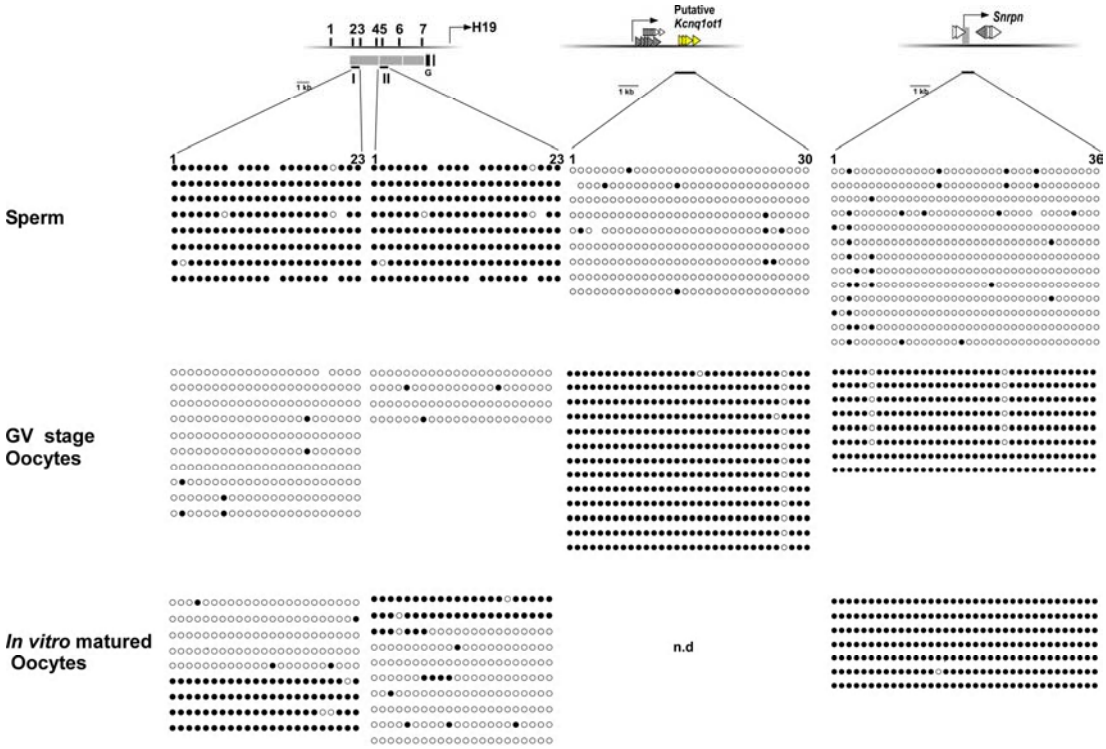


Figure 16: Bovine putative imprinting centers show differential DNA methylation in germ cells

Imprinting centers in bovine were identified by comparison with the characteristics described in human and in mouse for the respective homologous center. Upper most panel shows the imprinting centers in bovine, indicated with the location of nearest known transcript, presence of CTCF binding sites (consensus binding site: CCGCNNGGNGNC) and the presence of tandem repeats. Refer to appendix Table 2 for the genomic location of the DMRs and for tandem repeats.

Methylated CpGs are indicated by filled circles (●), unmethylated by open (○) and ambiguous CpGs by the absence of circles ( ) and each horizontal lane represents the analysis of a single clone. *H19* DMR bisulfite treatment and amplification has resulted in two products shown as product I and II. Here G at *H19* DMR is the location of G rich repeat. Numbers above the circles indicate the number of CpG analyzed and at KvDMR1 center analysis of 30 CpGs from 47 is shown here. Here n.d. refers to not determined.

III.1.3 **Disruption of imprinting mark at *H19* DMR in *in-vitro* matured oocyte**

In bovine, IVF procedures also involve the *in-vitro* maturation of oocytes before subjecting them to fertilization with spermatozoa. Several studies indicate the influence of culturing (during maturation of oocytes or post-fertilization culturing of embryos) on DNA methylation imprints and hence I next examine the stability of DNA methylation imprints in *in-vitro* maturated oocytes.

In *in-vitro* matured oocytes, the DNA methylation mark at *Snrpn* DMR was unaffected and no significant difference in DNA methylation level was observed when compared to GV stage oocytes (p=0.29). Both stages were showing complete DNA methylation at the *Snrpn* DMR. In contrast *H19* DMR showed absence of DNA methylation in GV stage oocytes, which significantly appears in *in-vitro* matured oocytes (p<0.001). Bisulfite PCR products obtained from matured oocyte DNA displayed mostly complete methylated or unmethylated clones and only few mosaic clones. (Figure 16)

Similar observations of incorrect imprints at *H19* DMR have been reported for *in vitro* maturation of human oocytes. (Borghol, Lornage et al. 2005) Due to the lack of PCR amplicons, KvDMR1 DNA methylation was not analyzed for the *in vitro* matured oocyte.

III.1.4 **Aberrant DNA methylation in placenta of IVF foetuses**

After knowing that the DNA methylation imprints are well conserved in bovine at the selected imprinting centers, I next analyzed the stability of DNA methylation imprints in IVF derived foetuses.

For an initial screen of different individuals and tissue samples, I used the COBRA (combined bisulfite and restriction analysis) technique. In short, bisulfite PCR products were subjected to the restriction enzymes digestion and later analyzed by agarose gel electrophoresis. After bisulfite treatment and PCR amplification, most of the restriction sites are “mutated” due to bisulfite induced base changes, and also a few new sites are created. Presence of methylation on cytosine however prevents the disruption of some restriction sites. The digestion pattern of PCR amplicons, obtained from methylated and unmethylated template, can be easily distinguished.

COBRA analysis revealed that the methylated patterns at the imprinting center at PWS / AS region, the *Snrpn* DMR in AI and in IVF families were no different. Both groups showed identical patterns indicative for the presence of both methylated and unmethylated sequences (Figure 19). Moreover these patterns do not change in

different tissues. The selected PCR products were also cloned and sequenced, and the results obtained were consistent to the COBRA results i.e. *Snrpn* DMR was showing equal representation of methylated and unmethylated clones in different embryonic and extra-embryonic tissues. Hence the studied IVF foetuses showed no influence of the IVF treatment on DNA methylation at *Snrpn* DMR (Figure 20)

The COBRA analysis at *H19* DMR also revealed similar differential DNA methylation patterns in different embryonic tissues of AI and in IVF animals. However placental tissues were showing variation with mostly reduced DNA methylation in IVF animals as compared to the AI animals. Three IVF foetuses showed reduced DNA methylation in placenta while one IVF foetus was normal for DNA methylation (Figure 17). Cloning and sequencing of bisulfite products revealed a substantial loss of paternal allele methylation in one informative IVF foetuses as compared to AI placenta ($p< 0.001$) (Figure 20). One of the control foetuses (AI-F37) also revealed reduced DNA methylation in placental tissue ($p< 0.001$). This foetus was obtained after super ovulating dam and performing AI and later transferring embryos to recipient heifers, termed as **M**ultiple **O**vulation and **E**mbryo **T**ransfer (MOET).

Similar to *H19* DMR, KvDMR1 also showed DNA methylation differences in placenta. AI foetuses showed mosaic DNA methylation on maternal allele. Maternal DNA methylation within placenta of AI and MOET control foetuses varies from 36 to 68%. Interestingly, the AI foetus showing lower DNA methylation by cloning and sequencing revealed proper differential methylation in COBRA analysis. It should be noted that the later technique does not take in account of the DNA methylation at respective alleles, but gives an overall DNA methylation at a locus. However, in placenta of IVF foetuses, a complete absence of DNA methylation was observed in two of four samples on the maternal allele, IVF _F58 and IVF-F59 ($p< 0.05$) (Figure20 and Figure 18). One of the IVF placental samples (IVF F12) showed a gradual loss of DNA methylation on the supposedly maternal allele (due to the presence of higher DNA methylation marks i.e.21%). This tissue sample was showing most of the clones with partial DNA methylation, having a gradient from right to left (Figure22). Unfortunately due to the lack of sufficient amount of PCR

products, I could not cross check the IVF-F12 sample by COBRA analysis. Placenta tissue from IVF-F14 showed presence of proper differential DNA methylation between two alleles (79%). This foetus has also shown a normal DNA methylation imprint at *H19* DMR in the placental tissue sample. As for *H19* DMR imprinting center, here too foetal tissues revealed no significant DNA methylation difference between control AI and IVF animals (Figure 20).

***H19* DMR**

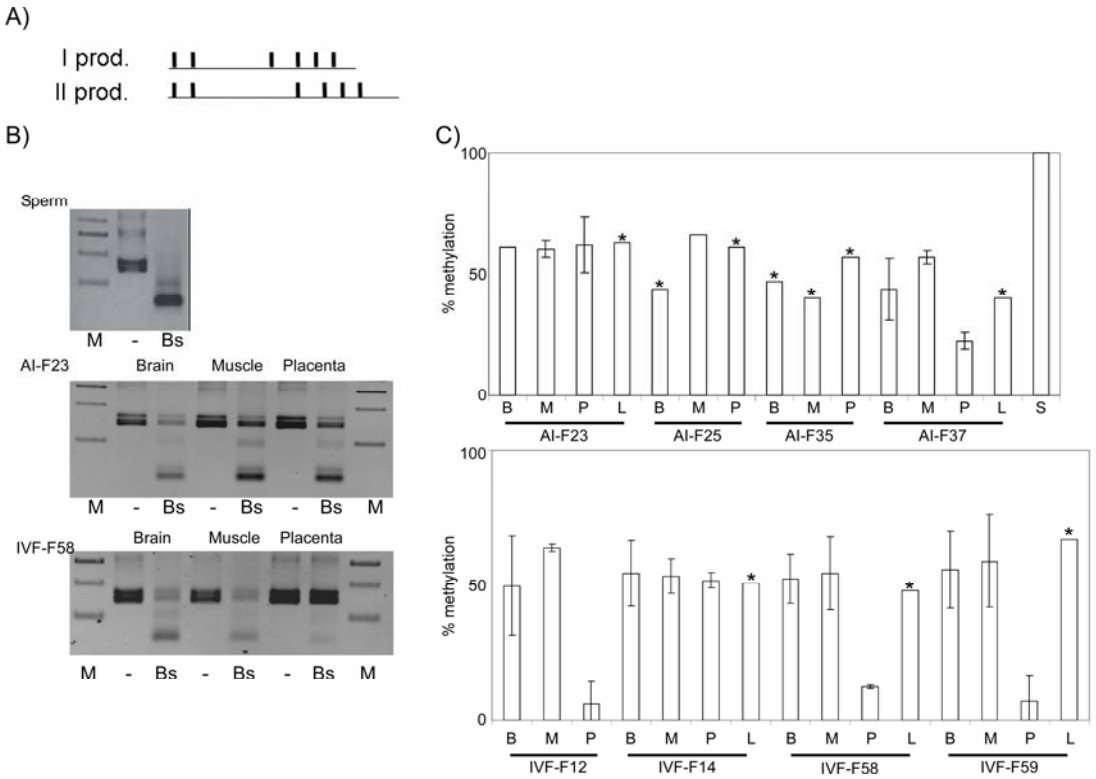


Figure 17: COBRA on PCR product at *H19* DMR

Bisulfite converted DNA (PCR products) was digested with *BstUI*. Digestion with *BstUI* indicates the presence of methylated cytosines within the restriction sites.

A) The number of restriction *BstUI* sites are indicated on a bisulfite treated and amplified PCR product at *H19* DMR. The PCR amplification has resulted in two amplicons: product I and II.

B) Agarose gels showing the restriction digestion pattern of the PCR product (from bisulfite treated DNA). Where undigested PCR product is indicated by "**-**"sign.

C) The band intensities from the agarose gels were quantified by TOTAL Lab software (Bio Step) and were used to roughly estimate the DNA methylation level of individual tissues. This estimation is presented as bar graph of two independent COBRA experiments. Refer to Table 6 for formulae used for band intensity estimation analysis.

Asterisk(s) indicates that the sample was analyzed by COBRA only once, while B, M, L, P means brain, muscle, liver and placenta (foetal part of placenta i.e. cotyledon).

KvDMR1

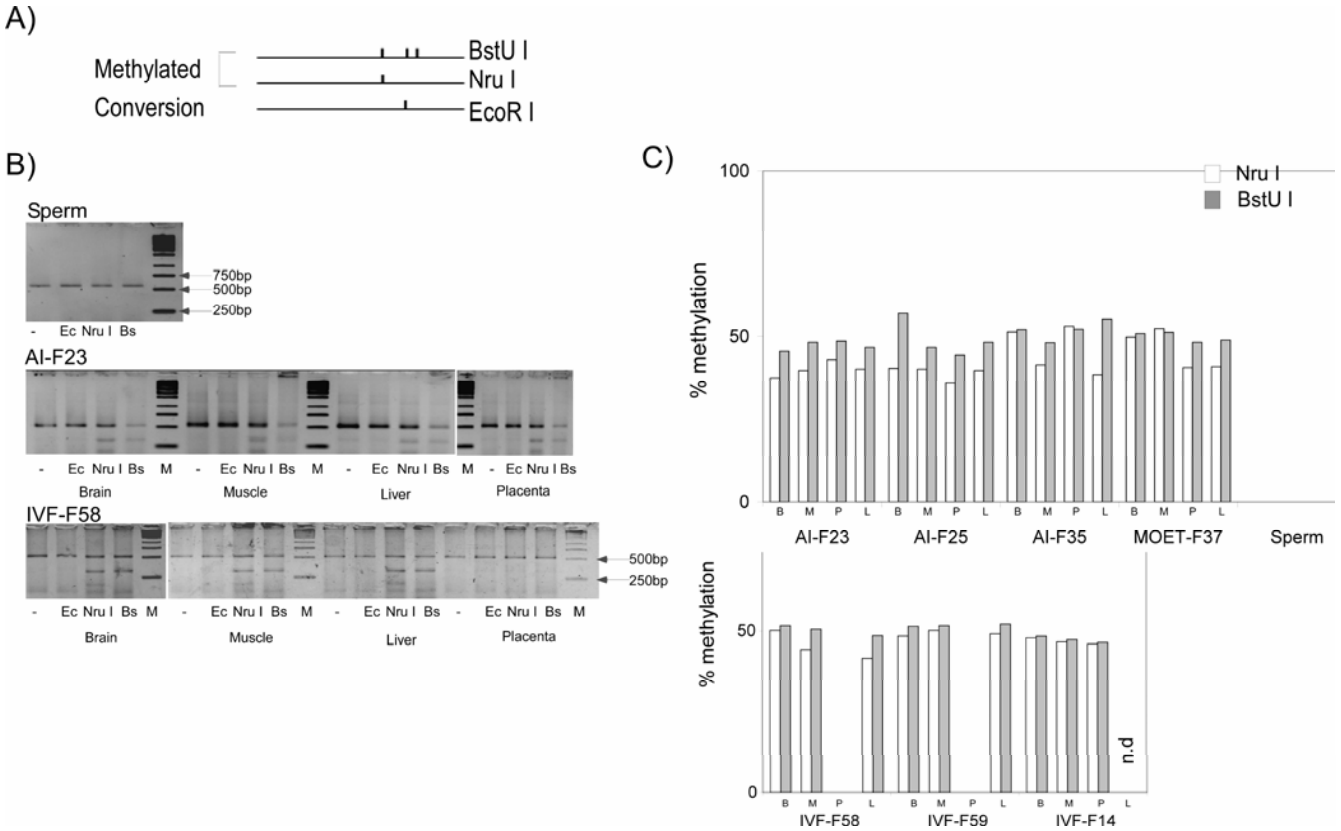


Figure 18: COBRA on PCR product at KvDMR1 center

A) Schematic drawing of bisulfite converted and PCR amplified products at KvDMR1. Indicated are the number of *BstUI (Bs)*, NruI (Nru) and *EcoRI(Ec)* restriction sites. *BstUI* and *NruI* digested PCR products represents methylation in these restriction sites. Improper bisulfite converted and PCR amplified products will be digested by *EcoRI* restriction enzyme. Hence non digested PCR products in EcoRI digest, will represents the proper bisulfite conversion of genomic DNA.B) Agarose gels showing the restriction digestion pattern of the PCR product (from bisulfite treated DNA). C) The band intensities from the agarose gels were quantified by TOTAL Lab software (Bio Step) are presented in bar graph as described in Figure 17. Here B, M, L, P means brain, muscle, liver and placenta (foetal part of placenta i.e. cotyledon)

Snrpn DMR

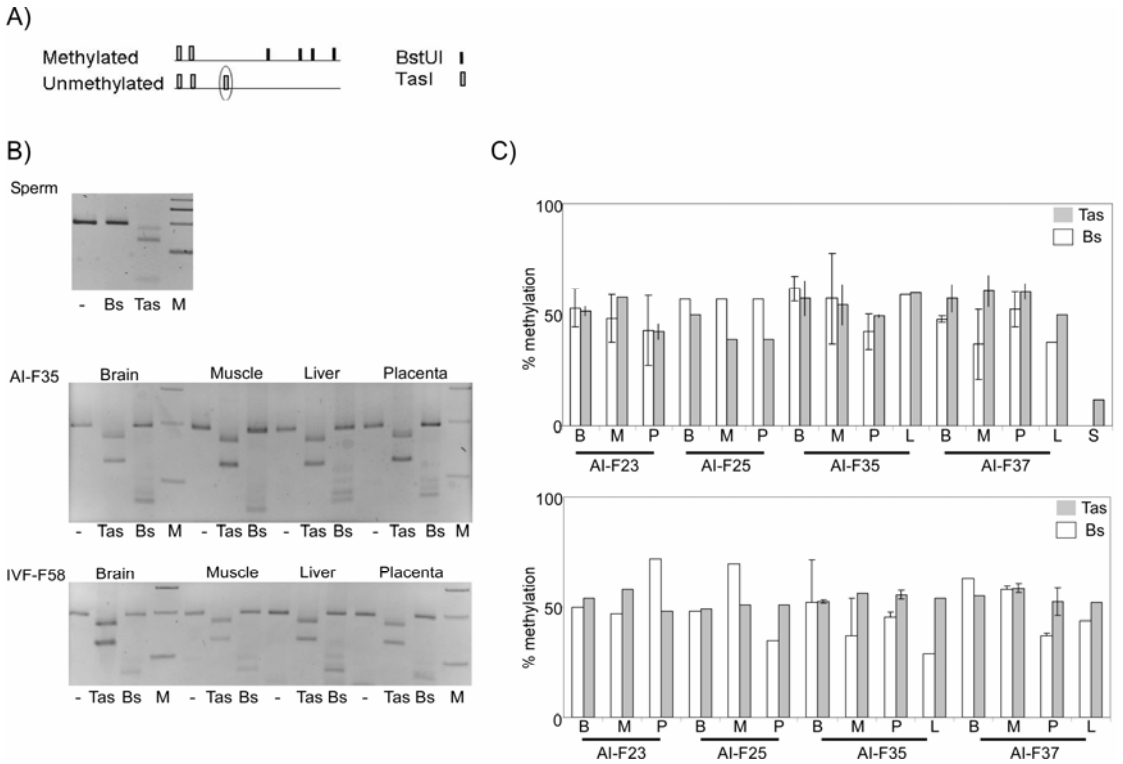
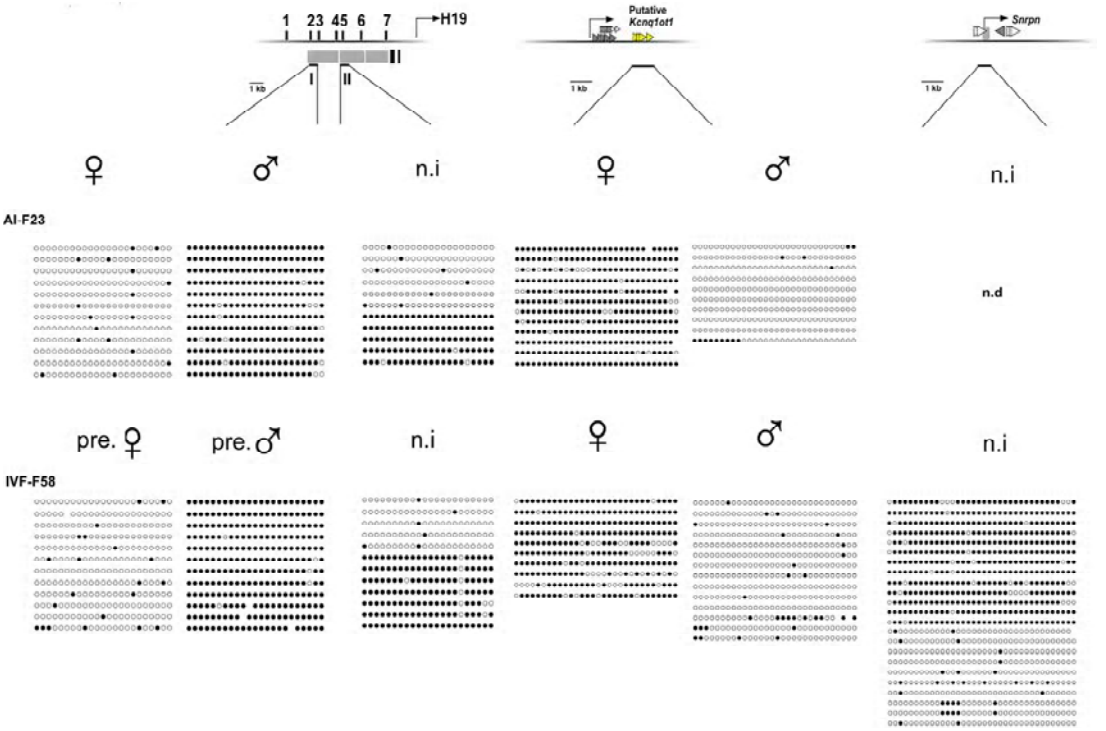


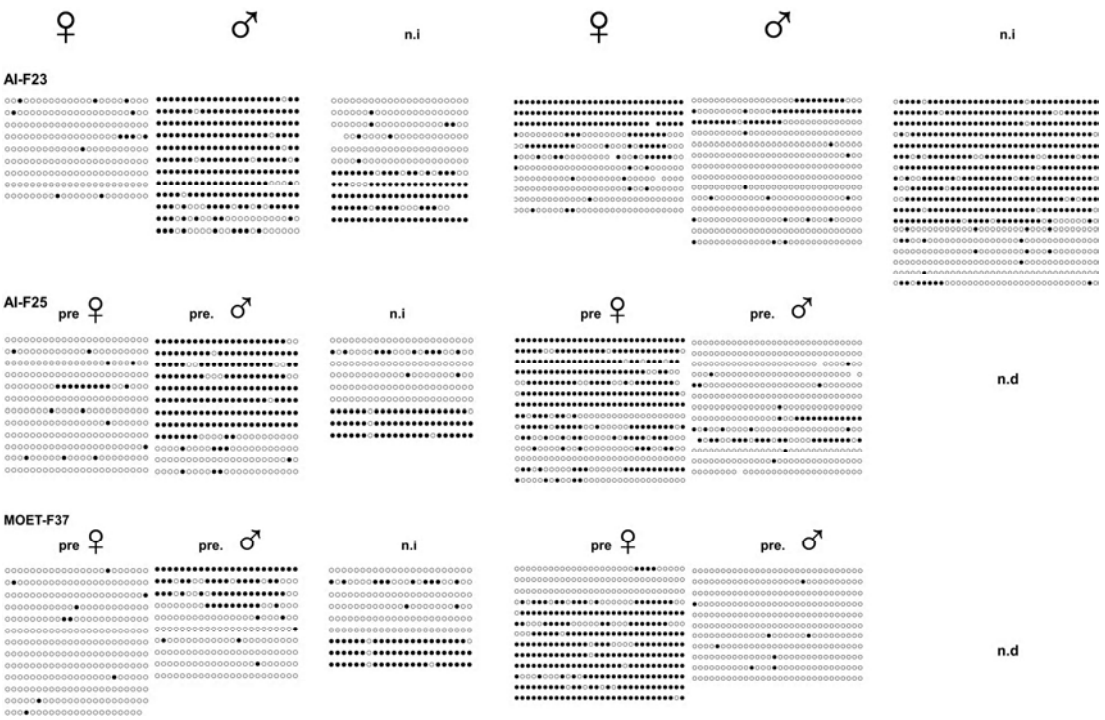
Figure 19: COBRA on PCR product at *Snrpn* DMR center

A) The number of restriction *BstUI* and *TasI* sites are indicated on a bisulfite treated and amplified PCR products at *Snrpn* DMR. PCR amplification (after bisulfite conversion) from unmethylated DMR will have an additional TasI site indicated by circle. B) Agarose gels showing the restriction digestion pattern of the PCR product (from bisulfite treated DNA). Here the "-" represents undigested PCR product; "Bs"*BstUI* digested (represents methylated DNA in the genome) and "Tas" as *TasI* digested PCR products (represents unmethylated DNA in the genome). C) The band intensities from the agarose gels were quantified by TOTAL Lab software (Bio Step) are presented in bar graph as described in Figure 17. Asterisk(s) indicates that the sample was analyzed by COBRA only once, while B, M, L, P means brain, muscle, liver and placenta (foetal part of placenta i.e. cotyledon)

A) Foetal tissue brain



B) Placental tissues of control foetuses



C) Placenta tissues of IVF derived foetuses

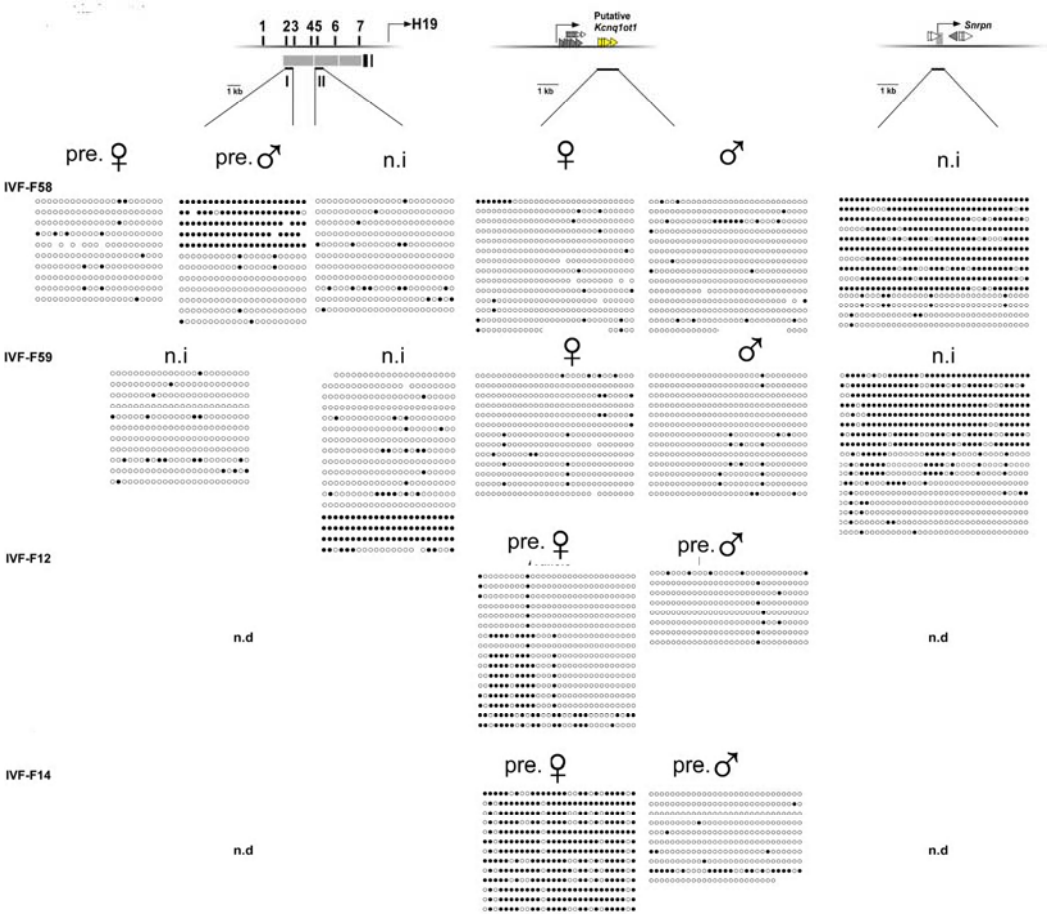


Figure 20: DNA methylation analysis on three imprinting centers *H19* DMR, KvDMR1 and *Snrpn* DMR

Foetal brain and placental tissues analyzed from day 80 foetuses derived by different fertilization procedures i.e. AI, MOET and IVF. Parental specific alleles are indicated with respective symbols (♂♀) while if the parental of origin information is ambiguous then alleles were assigned as presumed maternal (pre♀) or presumed paternal (pre ♂) alleles depending on the methylation levels on the alleles. The sample tissues lacking polymorphism are indicated by "n.i" (non informative) and all the clones are grouped together. Here filled circle represents methylated CpG, open circle for unmethylated CpG and ambiguous CpG with absence of circle. Foetal tissues showed no difference in DNA methylation between AI control animals and IVF derived foetuses (A). Placental tissues also showed presence of differential methylation in control foetuses (B), while aberrant DNA methylation was observed in IVF derived placental tissues at H19 DMR and KvDMR1 (C). n.d is not determined

III.1.5 DNA methylation at CpG islands in the *Igf2* gene

So far the DNA methylation analysis showed aberrant methylation on primary DMRs only in placental tissues of IVF derived foetuses. Here too only imprinting centers in the BWS region showed incorrect imprinting, while the imprinting center *Snrpn* DMR was indifferent as compared to AI control foetuses. Next I asked the stability

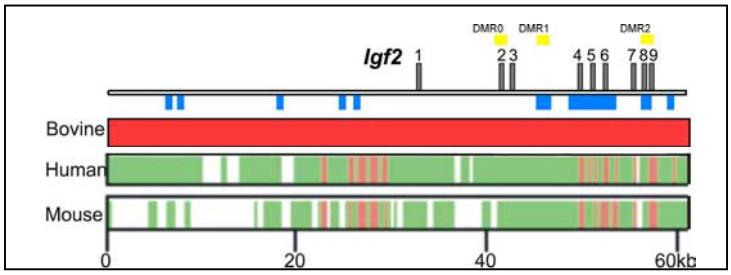
of methylation imprints in other DMRs in BWS region. Other than *H19* DMR and KvDMR1, the known DMRs in BWS region are located in different introns of *Igf2* gene and in the introns of *Cdkn1c* gene. The three well characterized DMRs at *Igf2* gene locus are DMR0 (in intron1), DMR1 (in intron 3) and DMR2 (spans a region from exon 8 till exon 9 in mouse). The DMR1 and DMR2 specify a CpG island definition (DNA of length of minimum 200bp, contains >50% C+G and the calculated Observed /Expected (observed CpG / [number of C's * number of G's) / window length]) ratio over 0.6(Gardiner-Garden and Frommer 1987), while DMR0 is a CpG rich region (Monk, Sanches et al. 2006). In human and mouse DMR0 is maternally methylated, while DMR1 and DMR2 are paternally methylated. The DMR0 is differentially methylated in placenta tissue in mouse only; however in human it shows differential DNA methylation in all foetal tissues as well as in placental tissue (Moore, Constancia et al. 1997; Lopes, Lewis et al. 2003; Monk, Sanches et al. 2006).

In bovine, the putative DMRs within in *Igf2* gene were observed at the similar location i.e. DMR0 in intron 1; DMR1 in intron 3 and DMR2 in the last exons of the *Igf2* gene. As in human and mouse, the bovine DMR1 and DMR2 also constitute a CpG islands, while DMR0 was enriched in CpG dinucleotides but showed no clear CpG island by definition (Gardiner-Garden and Frommer 1987). The DMR0 shows high sequence conservation with the human and mouse sequences (Figure 21, location indicated in appendix Table 2).

The COBRA analysis at the bovine DMR0 showed presence of high DNA methylation in sperm DNA. This was also confirmed by direct sequencing the PCR product amplified from bisulfite modified sperm DNA (Figure 22c). Unfortunately oocyte samples were not available at this time and hence were not analyzed for the DNA methylation. COBRA analysis on different foetal tissues as well as on placental tissues in various individuals revealed similar digestion patterns. This indicates the presence of methylated and unmethylated template in the genomic DNA at DMR0. COBRA analysis showed presence of almost 1:1 ratio of methylated and unmethylated templates, which might be due to the allele specific methylation. When IVF animals were compared with AI control animals, only 1 of 4 IVF derived

foetuses showed aberrant digestion pattern in COBRA analysis. The IVF-F58 foetus showed almost complete absence of methylation in placenta while in liver it was completely methylate (Figure 22 a, b). On cloning and sequencing of bisulfite PCR products, DMR0 showed mosaic pattern of DNA methylation in different foetal tissues and placenta. Due to the lack of polymorphism, alleles cannot be assigned to the methylation state (data not shown). Nevertheless individual clones can be classified in 2 classes based on the methylation level, which indicates presence of differential methylation at this DMR.. Presence of mosaic DNA methylation pattern at bovine DMR0 is in consistence with the mouse and human DMR0 DNA methylation (Moore, Constancia et al. 1997; Monk, Sanches et al. 2006).

A)



B)

```
Bovine          ------------------------------------TGTCTCTGGAAGA-AGACAGACCT 23
Human           GACCAGTGAAGTGAAAAACCCCCCAGCTGAGTACCTTGCTCATGGAAGATGGAAGGACAT 75
Mouse           CAGATGACTGATGACAGAGAAGAATTCTAAATTGAGTGTCCATGAAACATGAGGCGACAA 240
                                                 **    **  **  *      ***
Bovine          GGCCGCACTGTGCTG-------GTGCTGCACTGGGAGCGCCCGAGGGAA-GATTTTTCTG 75
Human           AACTGCACAGTACAT-------A-ACTGTACCGGGAGCACCTAAAATCC-AATTTTTCTG 126
Mouse           GATGGCGCTGTGCCACAAGGATACACCACACCCGGAAGACCTTAGAAAGTAATTTTAATA 300
                    **  *  **  *           *   **  ***   **  *       *****  *
Bovine          ACTGTCCTCGAACCCGGATTT-TTCCCAAGATGCCCCGCCCCC----ATTTTACCCGTGC 130
Human           CTGATTCTTGAACCCTGCTTTGTCCCCCTTATTCCCCCCCCCCCGCCATTTTACCAGTGC 186
Mouse           TTTATTTTAAATCCGGACCCTAATTTGCATAAGCCACGCCCCT-----TTTTACCTGTGC 355
                    *   *   * **      *          *  ** * ****      ******* ****
Bovine          CACACGCACTGGCATGCCCGGATGTGTCCAGTGATTGCCAAGTGTCAATC-AATTGAGGC 189
Human           CACGTCCACCAACATTCCAGG--GTGTCAAGTAACTGCCAAGTGTCACTCTAAGTAAAGC 244
Mouse           CACGCCCACAGACATTCCAGG--GTGTCAGGTGACTGCCAGGTGTCAATC-CAGTGAAGC 412
                ***   ***   *** ** **  *****  ** * ***** ****** **  * * * **
Bovine          CCCGCCCACCCTCAACCCCCCTCCGCAGAGCCCC-ACCTCCCAACTGGCAGCCAGTACTT 248
Human           TACACCCACTCCCCACCACC-TCCACATAGCCCCCACCTCCTAGCTGGCAG--GGAGCTT 301
Mouse           CCCACCCACTCTCCACCCC--TGCACATAGTCCCTACCCCCTAGCTAACAGGAAGTGCTT 470
                  * ***** * * *** *  * * ** ** *** *** ** * **  ***   *  ***
Bovine          CTGGCT-AATGCCCATGCCCACTAACGCCTTTCTGCCAGGCTAAGGGTGGGCCTAGTCTC 307
Human           CTGGCT-TATGCCCACGCCCACAGGCGCCTTTCTGCCAGGTCAGGGGTGGGCCAAACCTC 360
Mouse           CTAGCTTAATTCAAACCTGCATAGACGCCTTCCTGTCTG-TCAGGCAGGGGCCAAAGCCC 529
                ** ***  ** *  *    **    ****** *** * *   * *   ***** *  * *
Bovine          CACCCTCTGATGCGCC-ATACCCTCACGTGGGAAGGTGCCAGAGCCAGCTGTTCCAGCAG 366
Human           CACCCGCTAATGTACC-ATGCCCTGGTGCTGGAAAGTGCCTGAGCCAGCTGCCCCAGCGG 419
Mouse           CACCCTCTAATGCCCCCATACCCTAGTGTAGGAAAGCGCCATAGCCAGCTGCCCCATAAG 589
                ***** ** ***  ** ** ****   *  **** * ***  *********  ***   *
```

Figure 21: Identification of putative DMRs in *Igf2* gene

A) Multiple sequence alignment of bovine *Igf2* as reference sequence with human and mouse. Refer to Figure 15 for the colour code for the alignment. The *Igf2* exons are in grey bars, CpG islands in blue bars, putative DMRs highlighted in yellow. Co-ordinates for the presented DMRs in *Igf2* are indicated in appendix Table 2. B) Sequences of the DMR0 region exhibits high similarity between human, mouse and bovine, where * means identical nucleotide. The analyzed CpG are highlighted with green colour and the *TaqI* sites with the open boxes. The first site "CCGA" will be converted in *TaqI* site after bisulfite treatment.

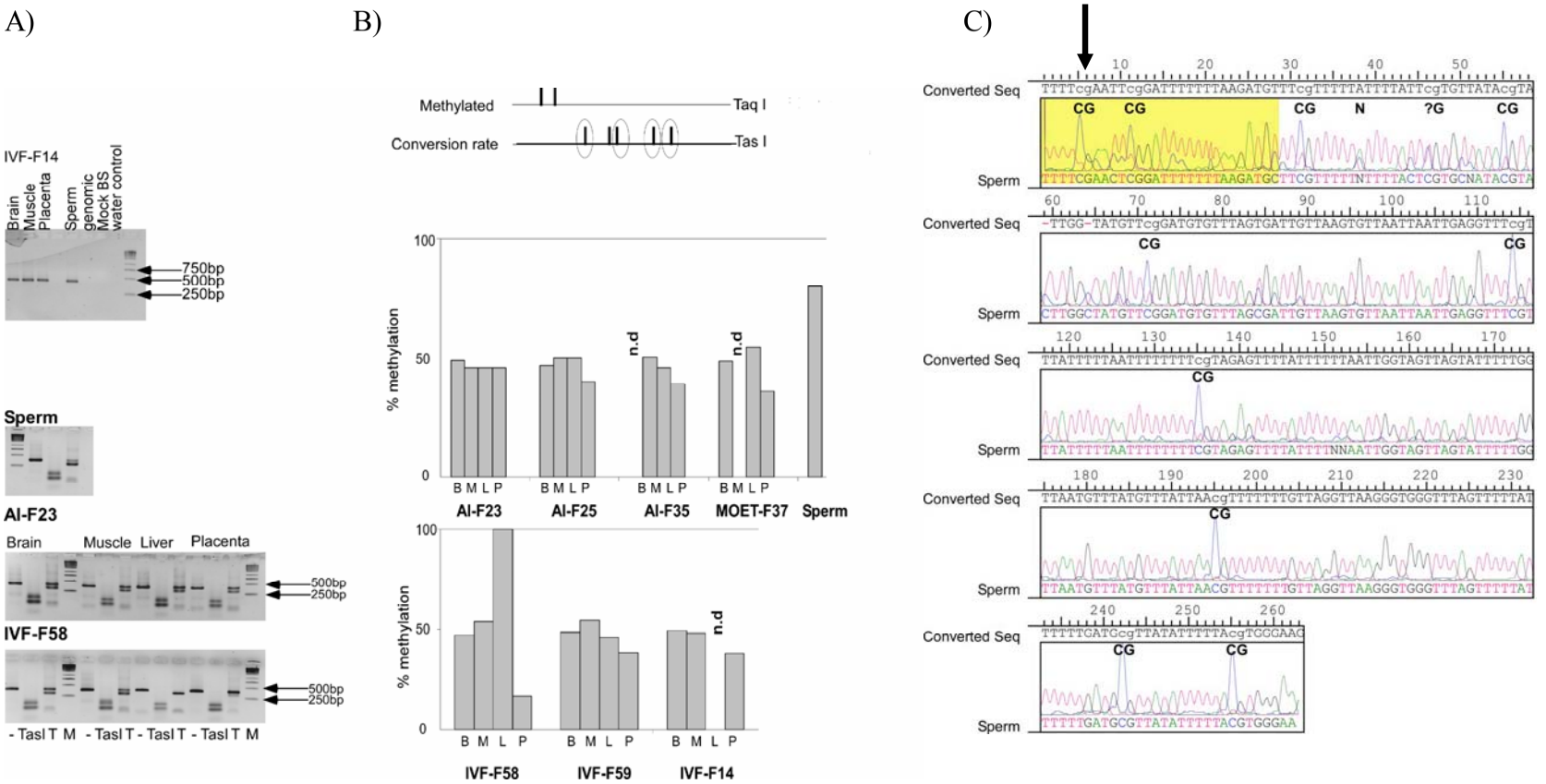


Figure 22: DNA methylation analysis at putative DMR0 in bovine

A) The top panel shows bisulfite PCR products of DMR0 region (386bp) and the lower panel shows digestion pattern on agarose gel. Indicated T is *Taq I* (TCGA) enzyme and M is a size marker. B) Indicated are the restriction sites (TaqI and TasI) on PCR product (from bisulfite treated DNA). Circled *TasI* sites represents additional *TasI* sites (AATT) created on proper bisulfite conversion from "C" to "T". The bar graph represents the COBRA analysis with *Taq I* enzyme at putative DMR0. The indicated B, M, L and P are Brain, Muscle, Liver and placenta respectively. C) The theoretical converted sequence is aligned to the direct sequenced sperm DNA PCR product and the arrow indicates the *TaqI* site. The PCR product consist 14 CpG dinucleotides and of which the DNA methylation state of 11 CpG dinucleotides is displayed here.

III.1.6 Expression analysis of genes regulated by imprinting center *H19* DMR and KvDMR1

Having observed the defects in DNA methylation at the studied imprinted centers, the next aim was to check the expression of the imprinted genes regulated by the affected imprinting centers. Since *Snrpn* DMR has not shown any significant DNA methylation differences, hence expression of genes associated with this center was not analyzed. Imprinted genes such as *Igf2* and *H19* which are regulated by *H19* DMR and *Phlda2* (*TSSC3*, *Ipl*) and *Cdkn1c* regulated by KvDMR1 were analyzed by semi-quantitative RT PCR. For normalization I used four genes namely *PolyA polymerase, Gapdh, 18sRNA* and *ß actin* genes. RT-PCR was conducted for 22cycles and the PCR products were blotted and hybridized with radio labelled probe for semi-quantification.

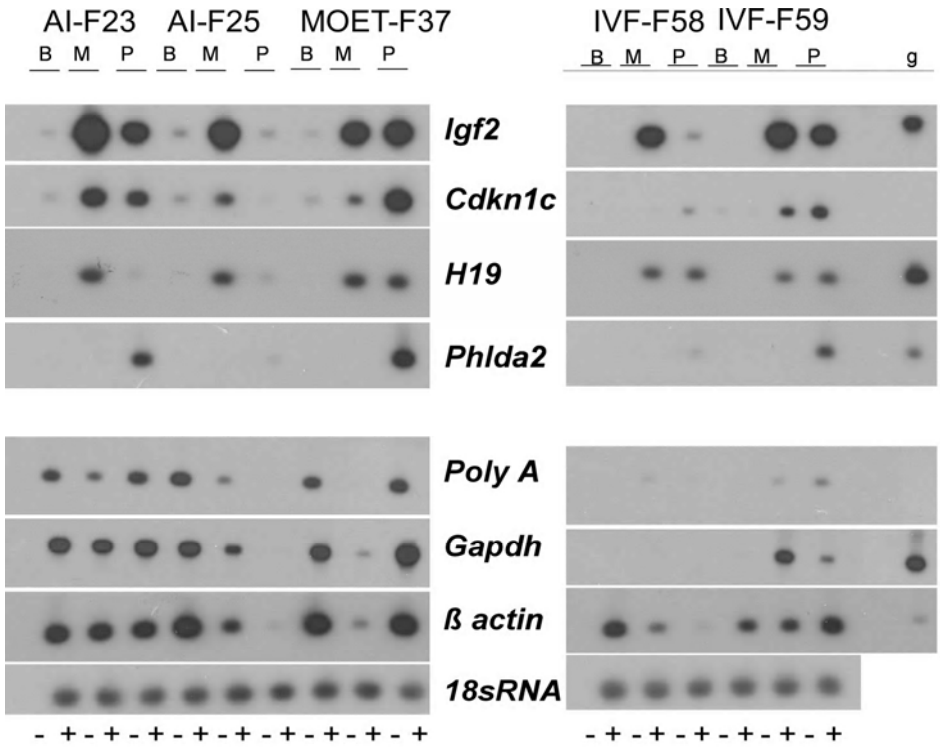
Expression analysis on the control gene *18sRNA* showed even quantities of RNA in all analyzed tissues (AI and IVF). Within AI tissue samples all other control genes showed presence of even amounts of RNA except for 2 tissue samples (AI F25 placenta and MOET F37 muscle). Both samples showed weak amounts of RNA of *ß actin, PolyA polymerase* and *Gapdh*. When control genes were compared within IVF samples, high variation in the amounts of RNA was observed. The control gene *PolyA polymerase* showed low level to the complete absence of amounts of RNA in IVF derived foetal brain and muscle tissues respectively. Similarly *Gapdh* gene expression was also low in several IVF derived foetal and placental tissues. Therefore, if taken in account the expression of all control genes, it will be difficult to normalize the gene expression in IVF tissue samples.

Here I observed that none of the control gene used were reliable for normalizing the expression of gene of interest. All the control genes showed high variability among different tissue sample derived from control foetuses. This might be due to either high variation in the gene expression within different control foetuses or due to the presence of poor quality of RNA in few samples. Only *18s RNA* control gene was well amplified from all tissue samples derived from IVF foetuses. The *Gapdh* and *PolyA polymerase* control genes showed aberrant expression after IVF treatment and hence were unsuiTable for the normalization. Only *ß actin* control gene was well

amplified from different bovine tissue samples (AI) and showed variations depending on the presumed RNA quality in few samples (AI-F25 and IVF-F58 placental tissues, Figure 23a). Moreover it seems that in this study the *ß actin* gene expression was not affected by the IVF treatment and hence was used for further analysis.

When different genes from BWS region were compared after normalizing against *ß actin* gene or by visual evaluation, I did not observe any gross difference between AI control samples and IVF tissue samples. However, in this analysis I did confirm that *Phlda2* is expressed in placenta in bovine, as known from human and mouse expression data. And that the *H19* gene shows over-expression in the placental tissues, where DNA methylation defects at *H19* DMR was also observed.

A)



B)

	ß actin	Gapdh	18sRNA	Poly A		Igf2	H19	Phlda2	Cdkn1c
AI-F23 Brain	++	++	+	++		-	-	-	-
AI-F23 Muscle	++	++	+	++		++++	+++	-	++
AI-F23 Placenta	++	++	+	++		++	-	+++	++
AI-F25 Brain	+++	++	+	++		-	-	-	+/-
AI-F25 Muscle	++	+	+	+		+++	++	-	+
AI-F25 Placenta	-	-	+	-		-	-	+/-	-
MOET-F37 Brain	+++	++	+	++		-	-	-	+/-
MOET-F37 Muscle	+/-	+/-	+	+/-		++	++	-	+
MOET-F37 Placenta	+++	++	+	++		++	++	++++	+++
IVF-F58 Brain	+++	-	+	-		-	-	-	-
IVF-F58 Muscle		-	+	+/-		+	+	-	+/-
IVF-F58 Placenta	-	-	+	-		-	+	+/-	-
IVF-F59 Brain	+	-	+	-		-	-	-	-
IVF-F59 Muscle	+	++	+	+/-		+++	+	-	+
IVF-F59 Placenta	++	+	+	+		++	+	++	++

++++	Very high expression
+++	High expression
++	Moderate expression
+	detecable
+/-	weak expression
-	not detected

Figure 23: Semi-quantitative RT-PCR of the imprinted genes

A) Southern blot images of RT-PCR products hybridized to the gene specific probes. The underneath plus and minus sign indicates RT performed in presence and absence of transcriptase respectively. RT-PCR was performed for 22 cycles (except *18sRNA* 20cycles) before analyzing by southern hybridization. Hybridized membranes blots were incubated for 4hr at room temperature before developing except for *Phlda2, Poly A* and *Gapdh* where exposure was extended to over night at -80°C. Here different foetal tissues and placental tissues are represented as B (brain); M (muscle); P (placenta) and g (for genomic DNA amplification.

B) Summarizes the visual quantification of results in A.

III.2 Characterization of conserved elements at imprinting center 2

Review of literature

The imprinted domain at the telomeric end of chromosome 7 in mice is regulated by the imprinting center 2 (KvDMR1), which is located in the intron 10 of *Kcnq1* gene. On the paternal allele KvDMR1 is unmethylated, functions as an active silencer and harbours a promoter for *Kcnq1ot1* transcript (Mancini-DiNardo, Steele et al. 2003; Thakur, Tiwari et al. 2004). As discussed earlier in the introduction, various studies on chromosomal breakpoints in human BWS patients, mouse transgene and chromatin studies at this domain indicate the involvement of cis and/or trans regulatory elements (Reid, Davies et al. 1997; John, Ainscough et al. 2001; (Cerrato, Sparago et al. 2005; Umlauf, Goto et al. 2004; Lewis, Mitsuya et al. 2004). We have previously reported the presence of high sequence conservation in intron 10 of *Kcnq1* gene (Paulsen, Khare et al. 2005). It is assumptive that this high conservation has regulatory function. In order to understand the functional role of highly conserved DNA elements at this imprinting sub-domain, transient transfection assays as well as DNA methylation studies were performed.

III.2.1 **Identification and selection of NICE elements**

As shown before that the bovine BWS region has similar arrangement and order of the genes when compared to human and mouse sequences (Figure 15. This comparative sequence analysis also revealed high conservation within *Kcnq1* gene in all three species human, mouse and bovine when compared to entire BWS region. When this comparative analysis was expanded in different species such as galago, armadillo, bat, dog and chicken where later two species are not shown here in the alignment (Figure 24), it supported our previous finding. In all species except of chicken, high sequence conservation was observed in intron 9, 10 and 14. In most of the species the sequence information for the entire BWS region was not available; hence the search for conserved elements in the entire BWS region was performed only for human, mouse and bovine sequences. However where ever the sequence information for above stated species was available, it was cross checked.

The sequence comparison at *KCNQ1* gene revealed 32 conserved elements. When given the criteria of >90% if < 300bp or >80% if >300bp within human, mouse and bovine sequence comparison, highly conserved elements could be identified. We coined the term NICE (**N**eighbouring the **I**mprinting center **C**onserved **E**lements) for these conserved elements. These highly conserved elements were localized mostly in intron 10 of *KCNQ1* gene (12 in number), while 1 was in intron 9 and 5 were in intron 14 (Figure 24, Table 9). These conserved elements were named as numbers if they were highly conserved among different mammalian species and are located in intron 10 (eg. NICE One and were also described in Paulsen et al, 2005), while were named as exon number_conserved element number (e.g. NICE 09_01) if they were conserved among most of them i.e. if one mammalian species is not showing high conservation. Four elements were found highly conserved (numbered One, Two, Three, and Four) and two of them (One and Four) were also found to be conserved in chicken *KCNQ1* (Table 9)(Paulsen, Khare et al. 2005).

In order to analyze these conserved elements by *in vitro* and *ex vivo* experiments (transient transfection assays), 4 of these highly conserved elements were selected

after performing various *in silico* analysis (stated below). These were NICE 09_01, NICE 10_04, NICE 10_08 and NICE 10_13 and the plasmid constructs were made after amplifying the conserved sequences from the mouse genomic DNA.

These selected 4 conserved elements were equally distributed on either side of the KvDMR1 imprinting center. The conserved element NICE 09_01 and NICE 10_04 are located down stream of *KCNQ1OT1* transcript, while NICE One and NICE 10_13 are upstream of the transcript. The conserved elements NICE One and NICE 10_13 contains >10CpG dinucleotides and hence were also ideal for DNA methylation analysis in different embryonic tissues. Conserved elements NICE 09_01 and NICE 10_04 are the longest elements showing very high conservation among different mammalian species. The conserved element NICE 10_04 also lies within the *Kcnq1ot1* transcript (Umlauf, Goto et al. 2004). Other NICE elements do have interesting properties but we decided to start our analysis with the above stated NICE elements.

On *in silico* analysis on these conserved elements, following were the interesting facts: 1) NICE One is a potential promoter having a score of 0.9 from promoter prediction searches (Promoter prediction 2.0 http://www.cbs.dtu.dk/services/Promoter/). One of the bovine EST AV592964, also map to this element and shows splice variant with exon 11, 12 and 13 of *Kcnq1* gene. Many promoter specific elements such as CAAT box, GC box and TATA boxes were also mapped and found to be conserved between different species, here demonstrated for human, mouse and bovine (Figure 25).

2) None other than E box motifs were found to be enriched in these conserved NICE elements. Basic helix-loop-helix proteins bind to E boxes and are known to play important role during development. We also looked for the expression pattern known from literature on the selected candidate factors binding to E boxes. Among them, the protein Hand1 was often seen as potential target for non-canonical E boxes at NICE elements (appendix Figure 1). Hence this protein was taken under consideration for further analysis. Hand1 is expressed during preimplantation stages

in placenta and is also involved in patterning expression in cardiac ventricles.(Scott, Anson-Cartwright et al. 2000; Togi, Kawamoto et al. 2004)

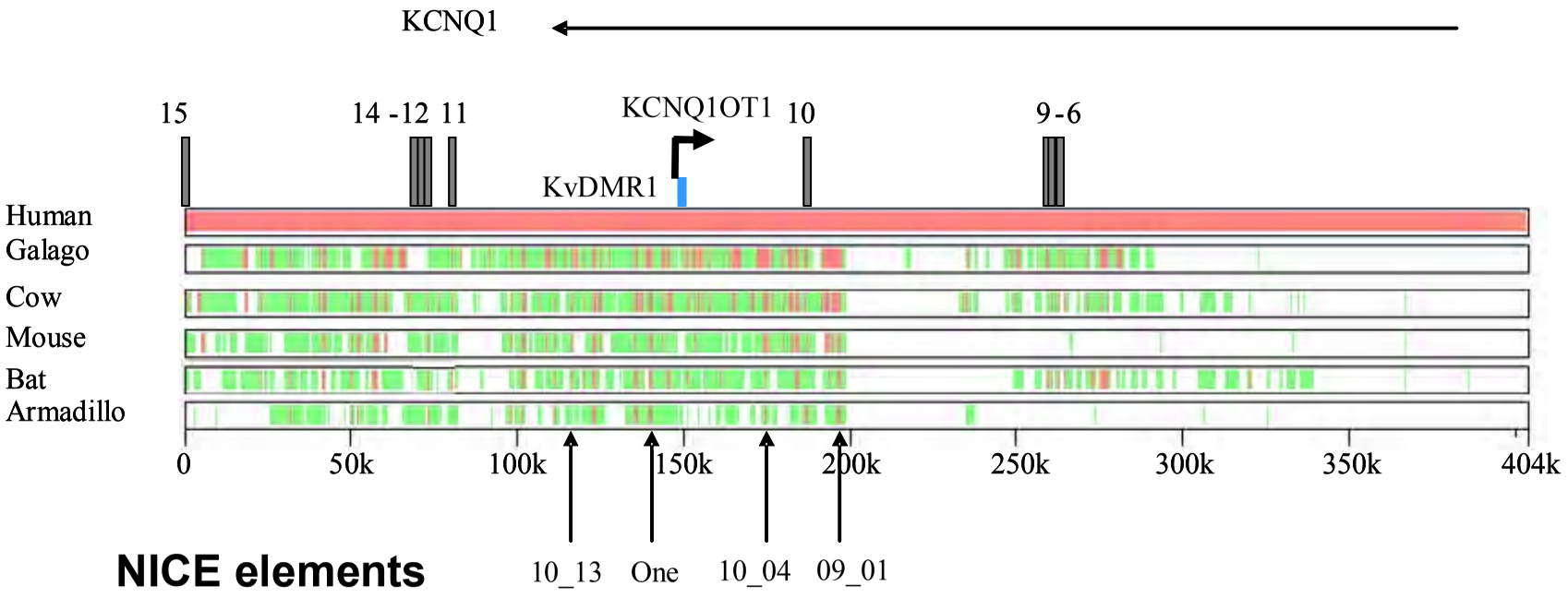


Figure 24: Conserved NICE elements at the KvDMR1 domain

Multiple sequence alignment of the *KCNQ1* gene, where human sequence was taken as reference sequence and compared to different mammalian species, such as galago, cow, mouse, bat and armadillo. Nucleotide identity >70% and >100 bp are marked by red colour in the plot while green colour shows the alignment only. Conserved elements are observed in intron 10 of KCNQ1 gene, while few of them are also present in intron 9 and 14.

Human KCNQ1 gene				Human AJ006345				Comparision against Human Mouse AJ251835					Comparision against Human Bovine AC147592				
Exon 1a	1001	1393															
Exon 1	83848	83938				bp				bp	%				bp	%	
				95668	95891	223	NICE 01_01		38060	37831	229	81%		4400	4599	199	70%
Exon 2a	119671	119933															
Exon 2	126525	126651															
Exon 3	127222	127300															
Exon 4	127910	128006															
Exon 5	128743	128883															
				131062	131270	208	NICE 05_01		10837	10625	212	81%		38208	38274	66	98%
Exon 6	139334	139444															
Exon 7	141113	141208															
Exon 8	143472	143594															
Exon 9	144615	144756						AJ271885									
			++	206978	207707	729	**NICE 09_01**		185929	185183	746	91%		117459	118152	693	91%
				209081	209357	276	NICE 09_02		183921	183637	284	87%		119407	119672	265	86%
				210227	210452	225	NICE 09_03		182789	182564	225	83%		120754	120979	225	89%
				210877	211058	181	NICE 09_04		182134	181953	181	84%		121473	121603	130	82%
Exon 10	217008	217128															
				216975	217135	160	NICE 10_01		175902	175721	181	88%		128472	128594	122	89%
			++	219673	220062	389	NICE 10_02		173319	172929	390	86%		132169	132562	393	85%
				221909	222116	207	NICE 10_03		171248	171042	206	87%		134321	134523	202	85%
			++	229113	230152	1039	**NICE 10_04**		164143	163052	1091	84%	AC147396	8637	9560	923	80%
			++	252410	253160	750	NICE 10_05		143662	142920	742	82%		36942	37662	720	83%
			++	258377	258950	573	NICE 10_06		137187	136626	561	81%		43075	43518	443	80%
				260293	260597	304	NICE 10_07		134929	134631	298	79%		44566	44869	303	83%
			++	263810	264378	568	**NICE 10_08**		131534	130885	649	86%		48055	48573	518	86%
				266772	266965	193	NICE 10_09		128251	128053	198	78%		51164	51306	142	87%
			++	268102	268464	362	NICE 10_10		126834	126461	373	80%		52350	52648	298	87%
			++	268757	268920	163	NICE 10_11		126129	125965	164	89%		52963	53123	160	90%
				279964	280414	450	NICE 10_12		116774	116326	448	83%		63243	63527	284	86%
			++	287611	287894	283	**NICE 10_13**		112008	111723	285	96%		69322	69595	273	92%
			++	292467	292789	322	NICE 10_14		108737	108401	336	87%		72326	72557	231	90%
				297569	297746	177	NICE 10_15		104069	103845	224	80%		77847	78000	153	84%
			++	301866	302447	581	NICE 10_16		96623	96019	604	90%		85090	85492	402	91%
				305675	305955	280	NICE 10_17		93312	93031	281	87%		88367	88628	261	85%
Exon 11	323883	323958															
				330943	331065	122	NICE 11_01		74582	74447	135	86%		115585	115686	101	94%
Exon 12	330963	331057															
Exon 13	331989	332035															
Exon 14	332980	333041															
				333687	333799	112	NICE 14_01		72631	72517	114	80%		118155	118254	99	78%
				336701	336823	122	NICE 14_02		70631	70512	119	78%		120998	121115	117	94%
			++	343584	343911	327	NICE 14_03		64284	63899	385	86%		130290	130610	320	88%
				346324	346543	219	NICE 14_04		61798	61597	201	85%		134040	134213	173	88%
				346919	347107	188	NICE 14_05		61121	60903	218	82%		134637	134785	148	87%
			++	353503	353822	319	NICE 14_06		55079	54753	326	91%		140465	140722	257	94%
			++	361968	362320	352	NICE 14_07		48201	47845	356	86%		147529	147842	313	90%
			++	372172	372297	125	NICE 14_08		37208	37057	151	82%		158305	158414	109	94%
			++	380876	381386	510	NICE 14_09		28606	28153	453	82%		168993	169447	454	90%
				398593	398862	269	NICE 14_10		14742	14472	270	82%		183307	183538	231	86%
Exon 15	402674	402910															

Table 8: List of conserved NICE elements

Co-ordinates for exons of the human KCNQ1 gene (AJ006345) as well as the highly conserved elements (NICE elements) are indicated in three species human, mouse (AJ251835, AJ271885) and bovine (AC147592, AC147396). Percentage indicates the sequence identity of mouse or bovine against human sequence respectively. Open boxed and bold NICE elements are here under analysis (namely NICE 09_01, NICE 10_04, NICE 10_08 and NICE 10_13). The conserved elements NICE 10_08 is also referred as NICE One in the publication from Paulsen et al (2005). Here ++ indicates the criteria NICE >90% if < 300bp or >80% if NICE >300bp

Hand1 protein is known to possess anti *Ascl2* properties, where later protein is important for placentation (as Hand1 is) and is mapped to KvDMR1 sub-domain. The *Ascl2* gene is also imprinted in mice in placenta (Alders, Hodges et al. 1997; Scott, Anson-Cartwright et al. 2000).

III.2.2 Transient transfection assays to characterize the murine conserved elements

III.2.2.1 Promoter assays

Earlier, the *in silico* analysis has already revealed that NICE One may be a potential promoter. At this conserved element the bovine EST AV592964 is spliced to exon 11, 12 and 13 of the *Kcnq1* gene. Analyzing the conserved element NICE One and neighbouring region in human, mouse and bovine, I could also detect potential regulatory sites such as TATA box, GC Box and CAAT boxes (Figure 25). None other conserved elements showed any significant score for promoter prediction.

Hence next I addressed the same question in transient transfection assays. Transfections were performed in two different cell lines HEK293T cells and C2C12 cells. In this analysis I could not detect any promoter activity in any of these cell lines, for any of the conserved elements.

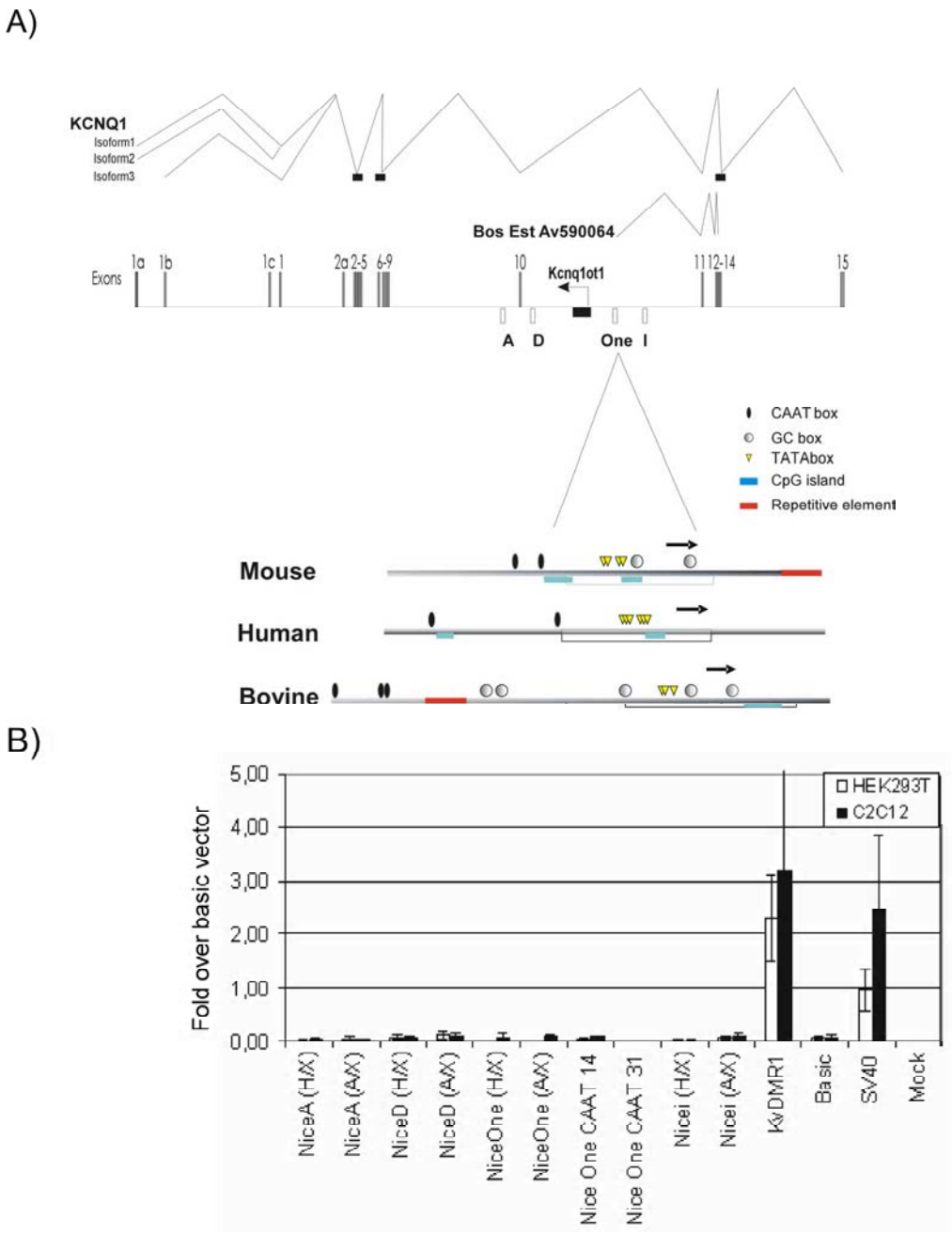


Figure 25: Promoter analysis of the NICE elements

A) Various human KCNQ1 transcripts are presented on the top. The known bovine transcript AV592964 is also mapped to it. Horizontal bars below represent a 1.5kb DNA sequence analysis of NICE One element in human, mouse and in bovine. Indicated on these bars are various in silico predicted regulatory elements such as TATA box, CAAT box and GC box. Arrows indicate the start of the bovine transcript AV592964.

B) The bar diagram represents the fold increase over the basic luciferase vector. Different restriction sites used for cloning the NICE elements from pCR2.1 to pGL3 vector are indicated as HindIII (H); ACC65I (A) and XhoI (X). Number 14 and 31 represents both orientations of the 0,8kb mouse DNA segment extending from CAAT boxes to NICE One element. Each transfection was performed in triplicates and the graph is a representation of at least three independent experiments.

III.2.2.2 Co-Influence of NICE elements on different promoters

The next question was to check whether any of these conserved elements have any enhancer or silencer activity. Hence the selected conserved elements were placed downstream of the *Luciferase* gene in pGL3 vectors where different promoters such as KvDMR1 or SV40, were already present at promoter site. Here I asked for the influence of these conserved elements on different promoters and in different cell line i.e. HEK293T (human embryonic kidney fibroblast.) and C2C12 (mouse foetal myoblast.) cells.

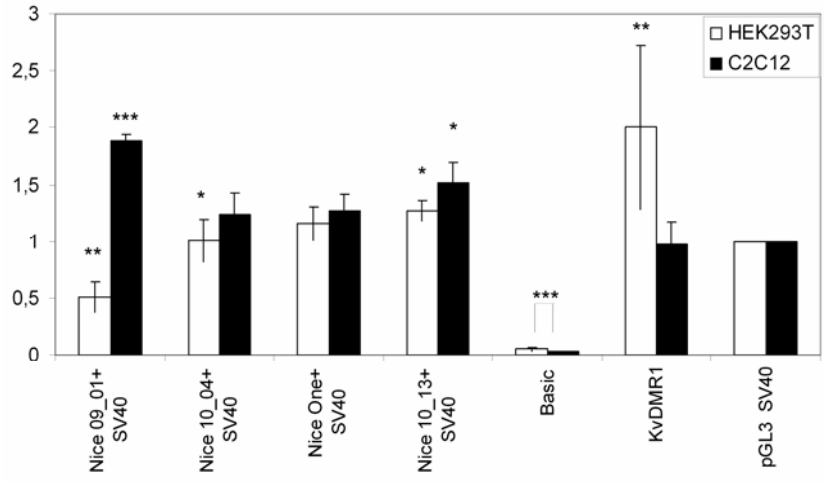
In transient transfection assays, I observed a 2 fold increase in the promoter activity of KvDMR1 when compared to SV40 promoter in HEK 293T cells. However when the same promoters were analyzed in C2C12 cells, both KvDMR1 and SV40 showed equal strength of promoter activities. High variation within the promoter activity for the KvDMR1 was observed in different transfection assays in both HEK293T and in C2C12 cell lines.

When these promoters were subjected against different conserved elements in transient transfection assays, only NICE 09_01 showed significant influence on SV40 or on KvDMR1 promoters in both cell line HEK293T and C2C12 cells. The conserved element NICE 09_01 showed the highest suppression on KvDMR1 promoter ranging from 50 to 60 % in C2C12 and HEK 293T cells respectively ($p<0.01$). NICE 09_01 also showed suppression to SV40 promoter in HEK 293T cells ($p<0.01$). Interestingly in contrast to its suppression effect in HEK293T cells, NICE09_01 showed almost 2 fold enhancement of SV40 promoter activity in C2C12 cells ($p<0.001$) (Figure 26 a, b).

Likewise the conserved element NICE 10_04 also showed suppression effect in HEK293T, for either SV40 or KvDMR1 promoter activities ($p<0.05$). However this conserved element had no significant effect on any of the promoter activities in C2C12 cells (Figure 26 a, b).

Some interesting facts were also observed for the conserved elements NICE One and NICE 10_13. The conserved element NICE One showed no significant influence on SV40 promoter in any of the cell lines studied. However slight but not significant suppression in HEK293T cells and almost 1.5 fold enhancement in C2C12 cells ($p<0.001$) was observed against KvDMR1 promoter activity. In contrast NICE 10_13 showed enhancement ($p<0.05$) for SV40 promoter activity in both cell lines, while it only showed suppression for KvDMR1 promoter activity in C2C12 cells ($p<0.05$). NICE 10_13 had no influence on KvDMR1 promoter in HEK 293T cells (Figure 26 a, b).

A)



B)

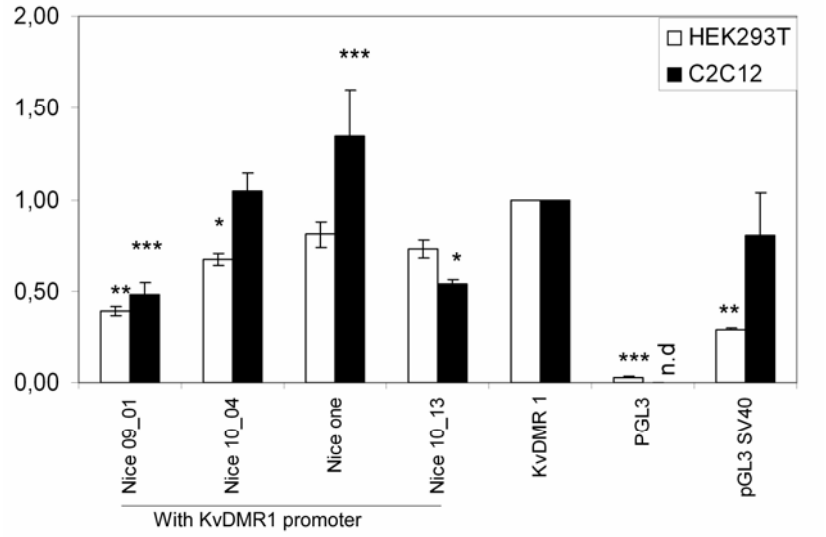


Figure 26: Influence of NICE elements on different promoters in HEK293T and C2C12 cells

Graphs were plotted by giving the reference value as 1 to the pGL3 SV40 promoter (A) and the KvDMR1 promoter (B). All the other luciferase /renilla readouts were compared to the reference promoter activity. Statistical analysis was done by "student t test" where each sample was compared to the reference promoter. Here * means $p<0.05$; ** $p<0.01$ and *** is $p<0.001$. Vector pGL3 means basic construct without having any promoter element and NICE 09_01+SV40 means constructs with SV40 as promoter. Each experiment was conducted three times and triplicate transfection for each sample was carried out in each experiment.

III.2.2.3 **Influence of tandem repeats in KvDMR1 promoter and conserved elements**

The imprinting center KvDMR1 also harbours different tandem repeats and conserved motifs downstream of the transcriptional start site. (Figure 21c) Presence of tandem repeats and its functional significance at imprinting centers is controversial. (Pearsall, Plass et al. 1999; Lewis, Mitsuya et al. 2004; Reinhart, Paoloni-Giacobino et al. 2006) It has been shown that KvDMR1 promoter activity is not affected from the absence of these tandem repeats or the conserved motifs (Mancini-DiNardo, Steele et al. 2003).

Hence I was interested to address the influence of conserved elements on KvDMR1 without tandem repeats and to compare with full length KvDMR1 promoter.

On removal of tandem repeats and conserved motifs from KvDMR1 promoter, I did not observe any reduction or enhancement of promoter activities in any of the studied cell lines i.e. HEK293T or in C2C12 cells in comparison to full length KvDMR1 (Figure27). It should be noted that the KvDMR1 without repeats construct, is lacking one of the CAAT boxes (numbered 4 in Figure 27) and is 26 bp away from the transcriptional start site, as earlier stated by Marcini DiNardo. (Mancini-DiNardo, Steele et al. 2003)

Combining the KvDMR1 promoter lacking tandem repeats, to different conserved element constructs, I observed significant suppression of the promoter activity. Three of four analyzed conserved element showed suppression on the promoter activity in HEK293T cells and these were NICE 09_01, NICE 10_04 and NICE One (p values of <0.01, <0.01 and <0.05 respectively). One conserved element, NICE10_13 showed no influence to KvDMR1 promoter either in presence or in absence of repeats. When the same constructs were analyzed in C2C12 cells, a significant removal of suppression earlier seen against full length KvDMR1 was observed for elements NICE 09_01 and NICE 10_13. The conserved element NICE 09_01 still shows suppression of 30% on promoter activity, which with full length KvDMR1 was 50%. In addition NICE 10_13 showed no suppression on the KvDMR1 promoter having no repeats. Interestingly

conserved elements NICE One and NICE 10_04 showed significant suppression on the promoter activity of KvDMR1 lacking tandem repeats ($p<0.001$ and $p<0.01$ respectively). In earlier study on full length KvDMR1 promoter, the NICE 10_04 conserved element showed no influence, while NICE One had 1,5 fold enhancement of KvDMR1 promoter activity in C2C12 cells.

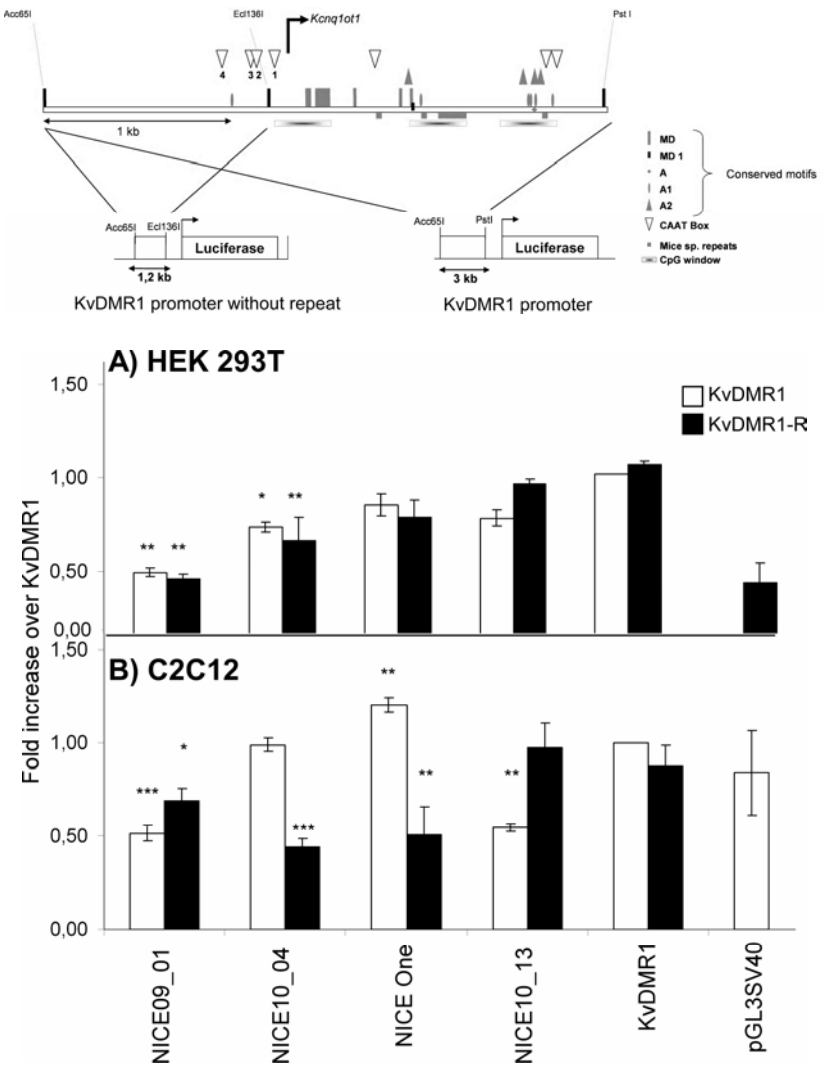


Figure 27: Influence of NICE elements on KvDMR1 promoter without repeats

Upper panel shows the schematic drawing of the promoters used here. For comparison, the influence of conserved elements on KvDMR1 was plotted again (refer Figure 26). The bar graphs are plotted for the respective cell lines i.e. HEK293T (A) and C2C12 cells (B). The KvDMR1 promoter activity was used as reference for both types of promoter, KvDMR1 with repeats and without repeats and was normalized to the value 1. Statistical analysis was done by "student t test" where each sample was compared to the reference promoter. Note that presence or absence of repeats (KvDMR1 or KvDMR-R) has no influence on the KvDMR promoter activity in HEK293T and C2C12 cell lines. Indicated asterisk(s) represent p value $p<0.05$ (*); $p<0.01$(**) and $p<0.001$(***). Each experiment was conducted three times and triplicate transfection for each sample was carried out in each experiment.

III.2.2.4 **Analysis of transcriptional factor Hand1 on NICE elements in HEK293T cells**

Multiple sequence alignments among different mammalian species showed presence Hand 1 factor binding sites ("NRTCTG") in highly conserved NICE elements. The 4 consensus binding sites of Hand1 protein on NICE 09_01, two each on NICE 10_04 and NICE One and only one site in NICE 10_13 was observed (appendix Figure 1). Other NICE elements (not analyzed in this study) also showed the presence of Hand1 binding sites (already discussed), hence the next question was to analyze the candidate Hand1 transcriptional factor and its influence on the activity of conserved NICE elements.

Transient co-transfections were performed in order to analyze the influence of Hand1 protein on conserved elements. The expression construct of Hand1 was a kind gift from Prof. Paul Riley (Hill and Riley 2004) and for control co-transfection, vector backbone where Hand1 cDNA was cloned, in this case pCDNA3 was used.

Initially the influence of Hand1 protein on SV40 and KvDMR1 promoter was analyzed. In silico analysis showed presence of Hand1 consensus binding at both promoters. Only the SV40 promoter, in transient transfections in HEK 93T cells showed influence of Hand1 protein on its promoter activity. Although KvDMR1 full length promoter also contains number of non conical E boxes, but on transient transfection it showed no significant influence of co transfection with Hand1 protein expressing construct when compared to the control vector co transfection read outs. Though promoter activity of KvDMR1 itself varies from transfection to transfection, but this has never shown consistent significant influence in the presence of Hand1 protein. Hence KvDMR1 promoter was used in presence or absence of conserved elements for further analysis.

The next task was to check the influence of Hand1 protein at different time points and at different concentrations. In order to do so pCDNA3-Hand1 constructs were titrated against NICE 09_01 KvDMR1 full length construct as well as these transfections were conducted for different incubation time. Optimization was performed with NICE 09_01 with KvDMR1 as promoter with increasing amount of Hand1 (1:1, 1:2 and 1:3)

construct and for different time scale (24hr, 48hr and 72 hr). In both types of optimization, I could not detect any significant influence of KvDMR1 promoter itself when compared to the pcDNA3 control vector. These experiments showed that the Hand1 protein at 1:1 ratio (i.e. 1 time Hand1 expressing construct and 1 time conserved element with KvDMR1 promoter) was sufficient and no improvement was observed with 1:2 or 1:3 co-transfection ratio (data not shown). However the influence of Hand1 protein on KvDMR1 promoter in presence of conserved NICE 09_01was significant only after 72 hr of post transfection (Figure 28).

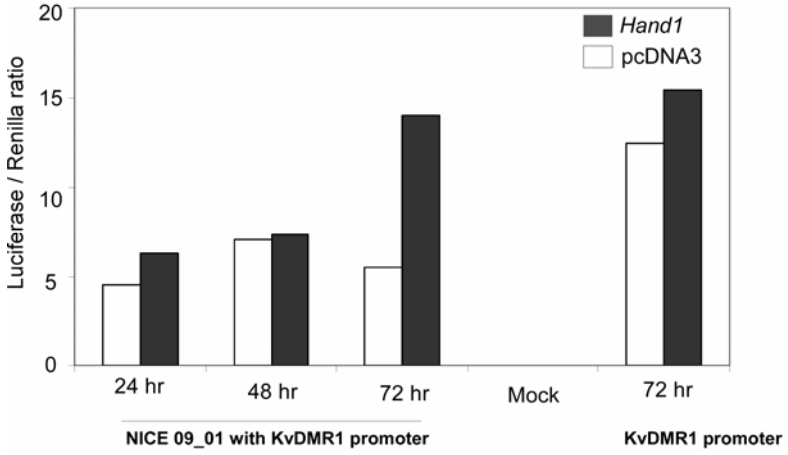
The Hand1 protein expression was also confirmed by western blot analysis in these co-transfection assays. Transfected cells were divided at the time of harvesting and one half was used for luciferase activity and another half was utilized to perform western blot with Hand1 antibody (sc-22817, Santa Cruz California USA.). The result of western blot showed cross reactivity of Hand1 antibody with some unknown proteins (visible as high molecular weight bands in the blot), but at the expected size of Hand1 protein (27+5 kilo dalton (kd)), a single band was observed. A second band was also observed at 27kd and this represents the endogenous translational start sites in the Hand1 expressing construct. In a control co-transfection with pcDNA3 construct, no Hand1 protein was detected. Interestingly maximum protein expression of Hand1 was seen at 24hr which reduces with elongated post transfection time. At 72 hr post transfection Hand1 band was still visible but was very weak in comparison to 24 hr band intensity. However the influence of Hand1 is only visible after 72 hr in dual luciferase assay, and at this time point the Hand1 protein expression is weaker when compared to 24 and 48 hr incubation. It should be taken in account that control loading of the proteins is not performed and all the loading is compared with non specific reactivity of the antibody (higher molecular weight bands on western blot Figure 28b).

The titration and time dependent experiments revealed that 1:1 ratio of plasmid constructs and 72hr post transfection incubations are the optimum conditions. Hence further analysis on conserved elements was performed with these two criteria.

As earlier shown that conserved elements NICE 09_01 and NICE 10_04 had suppression effect on the KvDMR1 promoter activity, a similar observation was seen when these conserved element constructs were co-transfected with control pcDNA3

vector. However when NICE 09_01 was co-transfected with Hand1 expressing construct, there was a significant removal of suppression (<25%) ($p<0.01$) Similar results were obtained with NICE 10_04 co-transfected with Hand1 expressing construct, which showed a complete removal of suppression ($p<0.05$).

A)



B)

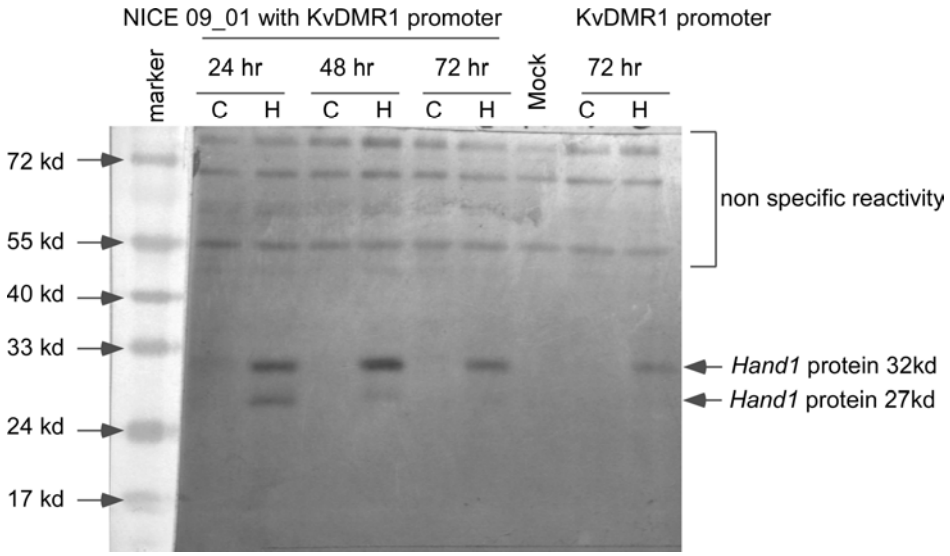


Figure 28: Optimization for co-transfection and expression of Hand1 protein

a) Dual luciferase transfection assay read outs are presented in the bar graph. The ratios of reporter luciferase gene with renilla luciferase gene activity were plotted in the graph. Renilla luciferase is an internal control to normalize the transfection efficiency. Co-transfections were performed with Hand1 expressing construct and the readouts were compared to the co-transfection with pcDNA3 vector.

b) Western blot of the same transfection assay, where C means co-transfections with pcDNA3 and H means co-transfections with Hand1 expressing construct

Influence of Hand1 showed no statistical significance on NICE one, even though 1.5 fold enhancement was observed for KvDMR1 promoter activity ($p<0.1$). This might also reflect the high variability within the KvDMR1 promoter activity. In contrast NICE 10_13 did not showed any influence in presence of Hand1 protein.

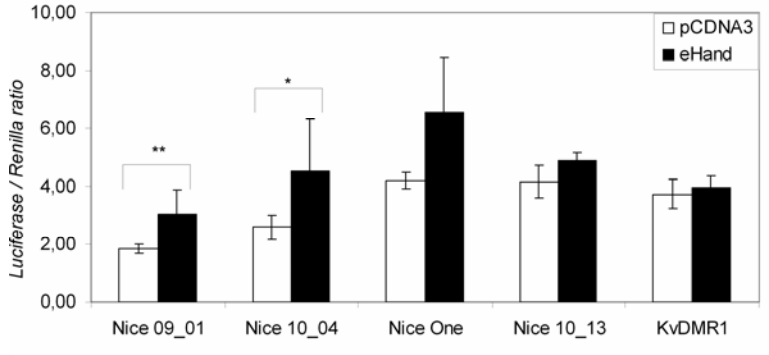


Figure 29: Co-transfection with Hand1 expressing construct

The constructs containing NICE elements with KvDMR1 as promoter were analyzed by co transfecting with Hand1 expressing construct. The analysis shows removal of suppression from NICE 09_01 and NICE 10_04 while NICE one shows enhancement of 1.5 fold. KvDMR1 alone or with NICE 10_13 shows no influence in presence of Hand1 protein. Here student t test was applied to compare constructs with conserved NICE elements co-transfected with Hand1 against co-transfection with control pcDNA3. Single asterisk(s) * is for p value < 0.05 while double asterisk(s) ** means $p<0.01$. Each experiment was conducted at least three times and duplicate transfection for each sample was carried out in each experiment.

III.2.2.5 DNA methylation analysis of NICE elements

The next question addressed in this study was the DNA methylation imprints on these NICE elements in mouse. The KvDMR1 imprinting domain is placental specific and consists of many genes which are exclusively expressed in placenta such as *Phlda2* and *Ascl2*. Hence DNA methylation analysis was performed in embryo proper as well as in placental tissues.

Among the selected NICE elements, only NICE One and NICE 10_13 have more than 10CpG dinucleotides, therefore these were selected for DNA methylation analysis.

The samples of tissue material were collected at 9,5dpc, and three different tissue samples were collected. These were embryo proper (E), placental tissue near the uterus

(PM) and placental material near to foetus (PE). In order to check the parental contribution, first generation (F1)crosses between inbred strain *C57/B6* and *SD7* were checked by genotyping for *Tnni2* gene (performed by Joe Weber) while *B6xSD7* embryos were checked by genotyping for *Osbpl5* gene. Samples showing equal contribution were taken for the DNA methylation analysis. Placental tissues towards uterus (PM) samples were not analyzed for DNA methylation by cloning and sequencing since they showed higher maternal contribution in genotyping this is due to higher contribution of maternal tissue in these samples. Therefore PM sample tissues were only analyzed by COBRA analysis.

DNA methylation analysis in germ cells (sperm and matured oocytes (metaphase II stage)) revealed complete DNA methylation at both conserved elements NICE One and NICE 10_13. Analyzing foetal and placental tissue at NICE 10_13 revealed partial DNA methylation, where both the parental alleles at NICE10_13 were indifferent i.e. most of the clones were methylated and only few clones were not methylated. When NICE One was analyzed for the embryo, it showed complete DNA methylation on both alleles and this was also confirmed in reciprocal crosses. However NICE One analysis for placental tissue, showed biased DNA methylation towards the maternal allele. The maternal allele showed 70% DNA methylation while paternal allele was only 30% methylation ($p<0.05$) (Figure 30b). Presence of complete methylation in embryo and having unmethylated and methylated state in placenta for NICE One was also confirmed by COBRA analysis, where only placental samples were clearly showing the presence of methylated and unmethylated templates (Figure 30 a). Even the placental tissue PM (which contains some maternal tissue) also showed in COBRA analysis the presence of both type of templates methylated and unmethylated (data not shown). All DNA methylation analysis on foetal and placental tissue was also performed on reciprocal crosses, which exhibited the similar tendency for DNA methylation. As a control, DNA methylation study at the KvDMR1 imprinting center was also performed. COBRA results on KvDMR1 in mice showed presence of PCR amplicons representing unmethylated and methylated templates in embryo proper as well as in placental tissue. The bisulfite PCR product of one placental sample was cloned and sequenced and it revealed complete DNA methylation on the maternal allele while complete absence of

methylation on paternal allele. Here observed DNA methylation state on KvDMR1 is consistent with the previous observations (Lewis, Mitsuya et al. 2004).

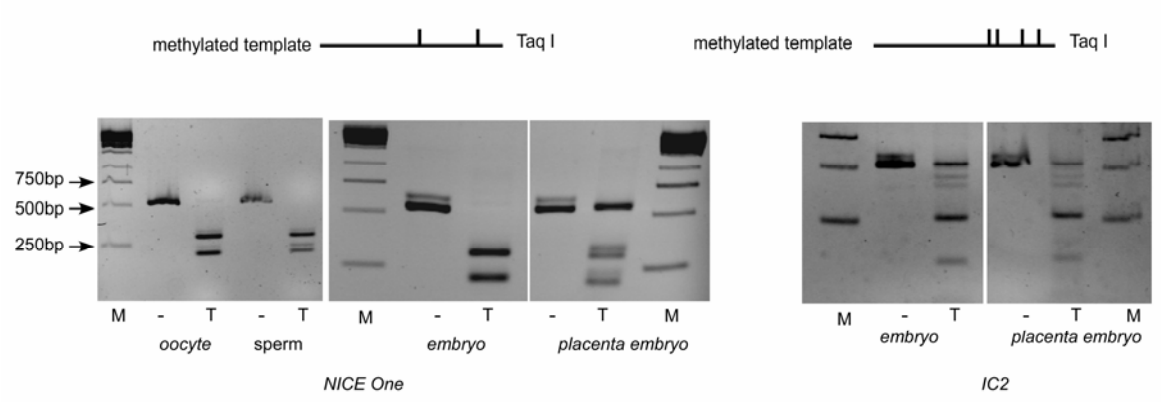


Figure 30 a: COBRA analysis at NICE One and control KvDMR1 center in mouse

COBRA analysis was performed with *Taq I* endonuclease restriction enzyme. This enzyme digests only the PCR amplicons representing the methylated state of template in the genome. Here T is the digest with *Taq I* while M represents the DNA size marker.

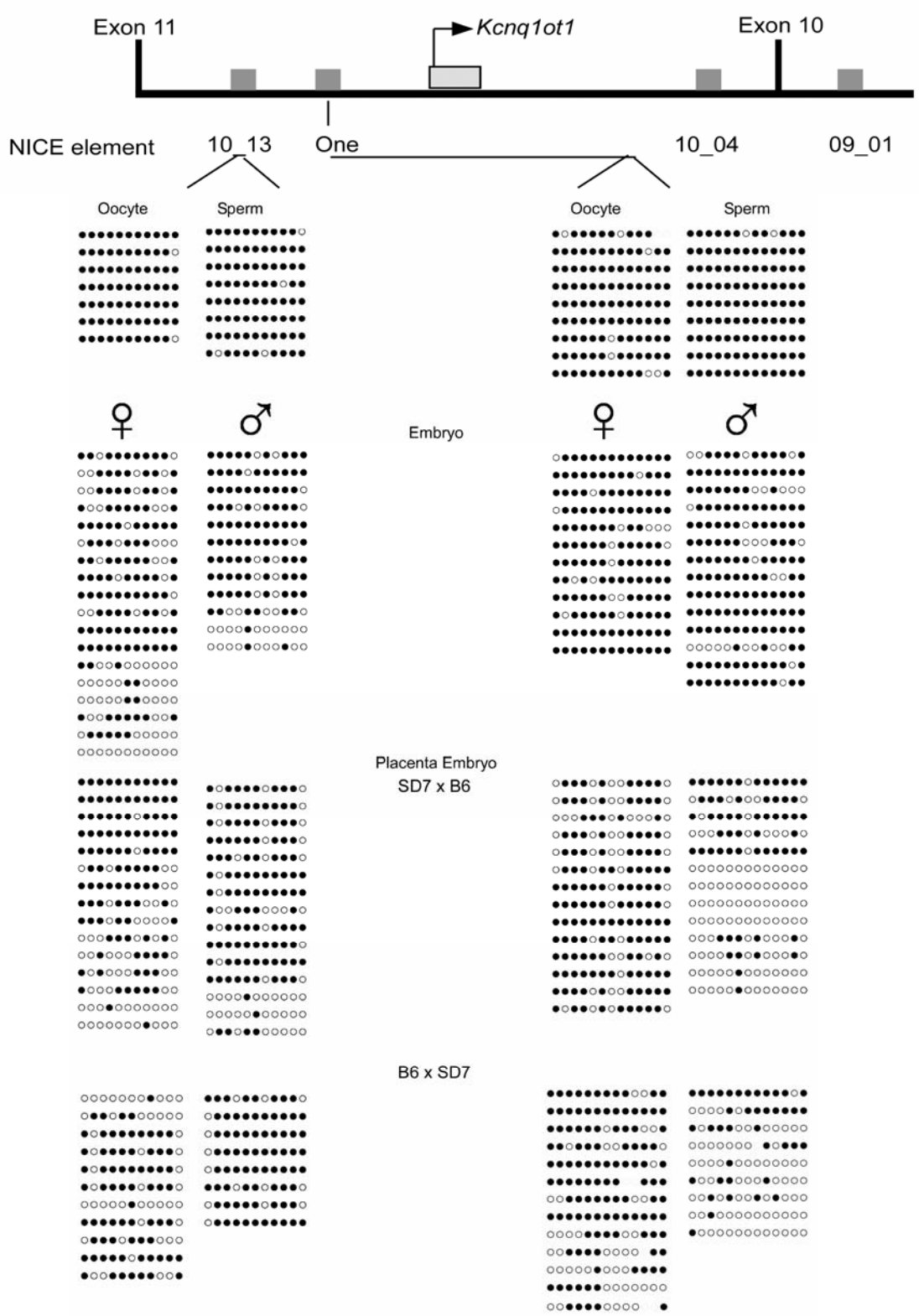


Figure 30 b: DNA methylation analysis of NICE One and NICE 10_13

Schematic map illustrates the positions of NICE elements with respect to the KvDMR1 on the top. Below, the filled black circles indicate the methylated CpGs while white circles represent the unmethylated CpGs. Absence of circle represents non-informative CpGs. Each horizontal row of circles represents a single clone.

IV Discussion

The first objective of the experimental work of my thesis was to identify the imprinting regions in bovine and to examine the imprinting status. Imprinted regions in bovine were identified by sequence comparison of the homologous regions with human and mouse. The identified imprinted centers were then analyzed for the DNA methylation status. With a few exceptions, bovine ICs and DMRs showed the presence of methylation imprints, a typical feature for mammals. Exceptions were found in embryo proper and in placental tissues in few DMRs for the DNA methylation. Another focus of this study was to compare the DNA methylation on the selected DMRs in foetuses derived by different fertilization procedures. Here the ART (IVF) derived foetuses showed extensive hypomethylation in DMRs located in the BWS region in placental tissue only. The aberrant methylation pattern was restricted to the BWS region and was not observed in PWS / AS region.

The second objective of my thesis was to characterize conserved elements flanking one of the imprinting centers, the KvDMR1. The presence of short highly conserved sequence elements in the introns of *Kcnq1* suggests that they might act as additional regulators within KvDMR1 imprinting sub-domain. Indeed 4 of these conserved elements tested in transient transfection assays showed effect on promoter activity. Furthermore one of these conserved elements shows a placental specific methylation pattern, making it a primary candidate for a placental specific regulatory function.

IV.1 DNA methylation at imprinting centers in bovine and influence of IVF procedures

IV.1.1 Identification of imprinted centers in bovine

In this study I analyzed the imprinted regions in the bovine, human and mouse. With the main focus on a detailed DNA methylation analysis in bovine, the three species (bovine, human and mouse) were compared for the imprinted region at BWS and PWS /AS regions. On comparative study, it was observed that the gene order and the location of the imprinting centers (for bovine these were putative centers) were conserved in all three species in the respective imprinting region. A detailed

comparison of the putative center in bovine to its homologous human and mouse imprinting center revealed many structural similarities in all three species. All the DMRs shared few common structural similarities, such as presence of CpG islands associated to tandem repeats within the imprinting center and that the centers were at a similar distance from the nearest gene. Some specific features of mouse and human imprinting centers were also observed at the bovine *H19* DMR and KvDMR1. The bovine *H19* DMR showed presence of conserved CTCF consensus binding sites similar to human and mouse *H19* DMR architecture (Bell and Felsenfeld 2000). A comparison of sequences flanking KvDMR1 revealed the presence of several highly conserved elements (NICE, discussed below) at similar distances to the KvDMR1 imprinting center in different mammalian species (Paulsen, Khare et al. 2005). In contrast to the conservation of NICE elements, the CpG islands or the associated tandem repeats in the imprinting centers show only marginal sequence homology, despite having the same epigenetic mark (imprints).

IV.1.2 **DNA methylation imprints are conserved in bovine, mouse and in human**

The prerequisite step in determining the imprinting status in bovine (as differentially methylated), was the search for informative polymorphic sites in the bovine putative DMRs. This was required in order to distinguish the parental alleles in the DNA methylation analysis. However only a limited number of individuals showed presence of polymorphisms at the *H19* DMR and KvDMR1 centers, and complete absence of polymorphisms for the *Snrpn* DMR or in the exons of the bovine putative imprinted genes was observed. This low degree of polymorphism might be due to the extensive use of artificial insemination (AI) in bovine farm industry. With the use of AI, only few elite sires (father) were used during the last decades to improve the economically important traits. This eventually has resulted in high homogeneity in the genomes of specific breeds. Hence the alternative approach is to use hybrid lines to study the imprinting in bovine e.g. progeny from *Bos taurus* and *Bos indicus*, which currently many labs are performing (Dindot, Farin et al. 2004).

In this study the DNA methylation analyses were performed by two independent methods i.e. COBRA and by cloning and sequencing of PCR products obtained from bisulfite treated genomic DNA. COBRA technique is a quantitative method for the

assessment of DNA methylation. It only analyses methylation of cytosines within the restriction sites and cannot distinguish between the methylation of alleles. On the other hand cloning and sequencing is quantitative as well as qualitative method for analyzing the DNA methylation. The later technique not only distinguishes the respective parental alleles but also determines the methylation levels at each CpG dinucleotide. However due to the involvement of many technical steps, cloning and sequencing sometimes show bias towards the methylated or unmethylated templates. Hence both techniques COBRA and cloning and sequencing, are treated as a compliment to each other in the present DNA methylation analysis.

In the present investigation, I demonstrate that DNA methylation imprints are present at bovine *H19* DMR, KvDMR1 and *Snrpn* DMRs in germ cells. The differential methylation at different DMRs is well maintained in foetal tissues such as brain, liver and muscle. Extra-embryonic tissue i.e. cotyledon (placenta), also show presence of differential DNA methylation at the *H19* DMR and *Snrpn* DMR in control AI animals. However at the KvDMR1 imprinting center, placental tissue showed mosaic but biased DNA methylation at maternal allele. This mosaic and hence incomplete DNA methylation in bovine placental tissue at the KvDMR1 is different from mice and human placental tissues, where a firm methylation is observed (Table 9) (Lewis, Mitsuya et al. 2004, own observation; Monk, Arnaud et al. 2006). It is not clear whether these DNA methylation differences arise from the different placental structures or to the different physiology (refer introduction I.7.2).

It is known that DMR0 located in *Igf2* gene, shows placental specific differential methylation in mouse. In contrast human DMR0 shows differential methylation in all foetal and placental tissues (Moore, Constancia et al. 1997; Monk, Sanches et al. 2006). The obtained bovine DNA methylation results show consistency with the published data on human tissues i.e. presence of methylated and unmethylated templates in different foetal tissues and in placenta tissues at DMR0. The Table 9, below summarizes the DNA methylation in bovine, human and mouse for the analyzed DMRs.

	Bovine		Human		Mouse	
	Foetus	**Placenta**	**Foetus**	**Placenta**	**Foetus**	**Placenta**
H19 DMR	Diff. Meth	Diff. Meth	Diff. Meth	Diff. Meth	Diff. Meth	Diff. Meth
KvDMR1	Diff. Meth	Diff. Meth (mosaic)	Diff. Meth	Diff. Meth	Diff. Meth	Diff. Meth
Snrpn DMR	Diff. Meth*	Diff. Meth*	Diff. Meth	Diff. Meth	Diff. Meth	Diff. Meth
DMR0 (*Igf2* gene)	Diff. Meth*	Diff. Meth*	Diff. Meth	Diff. Meth	Meth	Diff. Meth

Table 9: Conserved differential methylation at homologous imprinted centers

Analyzed bovine DNA methylation results were compared with the published human and mouse DNA methylation at different DMRs. The DNA differential methylation was conserved in all three species in foetus (for bovine brain, muscle, liver tissues are treated together as foetus) and in extraembryonic placental tissues at the analyzed DMRs. Few deviations (highlighted with boxes) were also observed in this comparative study. These were at mouse DMR0 which is documented as completely methylated in foetal tissues and the present study where bovine placental tissues show mosaic DNA methylation at KvDMR1. The asterisk means absence of polymorphism in the study, and complete methylation is indicated by "Meth" while, differential methylation by "Diff.Meth".

Interestingly DMR0 showed complete DNA methylation in bovine sperm DNA. Presence of methylation in sperm DNA is indicative that paternal allele might be methylated in foetal tissues. This seems to be in contrast to the DNA methylation state in mouse where paternal allele is unmethylated and serves as functional promoter for the placental specific *Igf2* gene. It can be speculated that this DMR is a secondary DMR and the oocytes DNA also has high methylation state. In later stages of embryo development one allele looses the DNA methylation marks, while the other allele maintains it. This will eventually results in a differentially methylated state at DMR0 in foetal and extraembryonic tissues. To validate the present assumption on DMR0, the methylation studies on the oocytes as well as on the early embryo developmental stages will be the next steps. Methylation analysis on early embryonic stages will give more insight as how the indifferent methylation marks on parental alleles acquires the differential state. It is known that both parental genomes undergoes demethylation after fertilization (Oswald, Engemann et al. 2000; Reik, Dean et al. 2001). Therefore it will be also interesting to know the dynamics of methylation on this DMR, whether both the alleles shows demethylation and only one allele gets methylated in later stages of development or only one allele is demethylated after fertilization.

IV.1.3 DNA methylation at imprinting centers in IVF derived foetuses is aberrant

Our study was also focused on determining the DNA methylation differences between foetuses produced by different fertilization procedures at the selected imprinting centers. The subsequent methylation analysis was hence performed on a pre-selected overgrown IVF derived foetuses and the control foetuses derived by AI or MOET fertilization procedures. The analyzed IVF derived bovine foetuses showed reduced DNA methylation at imprinting centers of BWS region. The paternally imprinted *H19* DMR and at maternally imprinted KvDMR1 imprinting centers showed reduced DNA methylation only in placental tissues. However, the embryo proper of IVF derived foetuses showed normal differential DNA methylation, and this was also observed for the control foetuses. In contrast imprinting center in PWS /AS region, *Snrpn* DMR, showed no DNA methylation lesions.

Interestingly, one of the control animals (MOET-F37) derived after multiple ovulation and embryo transfer (MOET) had proper differential DNA methylation at all analyzed imprinting centers except at *H19* DMR. Here the placental tissue showed a reduced overall DNA methylation. Unfortunately only one MOET individual was analyzed in this study and hence no general conclusions can be drawn on the observed effect.

Beside the MOET placenta, I also observe changes in DNA methylation in *in-vitro* matured oocytes. Again the hormonal stimulation is involved in the maturation of oocytes. However, here the changes at *H19* DMR were opposite to expected i.e. high DNA methylation in oocytes. On the other hand MOET fertilization procedure also involves steps of *in vitro* culturing and embryo transfer. These additional steps may play a role in causing lesions on DNA methylation at *H19* DMR. In conclusion, *H19* DMR was found susceptible to IVF procedures. The factors involved or the consequences of this aberration (DNA methylation) at *H19* DMR are not yet clear. These observations are in line of the earlier findings of aberrant DNA methylation at *H19* DMR on hormonal treatment or during oocyte maturation in human (Thomassin, Flavin et al. 2001; Sato, Otsu et al. 2006; Borghol, Lornage et al. 2005).

In summary all the above findings indicate that the influence of IVF technology 1) varies on different imprinting centers, here *H19* DMR and KvDMR1 are affected while *Snrpn* DMR and DMR0 were not, 2) differs between embryo proper and extra-embryonic tissues, and 3) in-vitro maturation of oocytes and hormonal treatment may play a role in epigenetic misregulation at certain imprinting loci. During my investigation on DNA methylation in bovine, some intriguing questions also came into light. Is there any link between the appearance of DNA methylation during oocyte maturation at the *H19* DMR and the reduced DNA methylation in extra-embryonic tissue in IVF foetuses at paternal allele? Or is it the culture condition, which leads to the reduced DNA methylation?

Earlier study demonstrated that IVF foetuses produced by similar procedures show elevated DNA methylation, especially in liver tissues. In this publication no obvious DNA methylation difference for placenta was observed between IVF foetuses from control AI animals. (Hiendleder, Mund et al. 2004) This difference to my findings may be explained by the fact that the earlier study was performed by quantifying the total ^{m}C (methylated cytosine) in the genome (including repetitive elements) while in present study the focus was on DNA methylation of a single copy imprinted DMRs. However at DMR0, in 1 of the IVF derived foetuses I do observe high methylation in liver tissues.

IV.1.4 Correlation of phenotype or the expression data to the observed aberrant DNA methylation

Analyzed IVF foetuses displaying reduced to absence of DNA methylation at H19 DMR and KvDMR1, also show elevated growth characteristics such as birth weight, different organ weight, and crown rump length. However not all IVF conceived foetuses showed defects at *H19* DMR and KvDMR1 imprinting centers (IVF-F14). This individual was also normal for growth parameters when compared to control AI animals (refer to the Table 10).

Foetal overgrowth phenotypes have been documented for mice and sheep in IVF or SCNT derived foetuses. In cloned sheep foetal overgrowth was attributed to the loss of DNA methylation at the imprinting center located in intron 2 of *Igf2r*. As a

consequence *Igf2r* gene expression was dramatically reduced, while there was no deviation in *Igf2* expression when compared to control animals (Young, Fernandes et al. 2001). In cloned mice the overgrowth phenotype was attributed to DNA hypermethylation at *H19* DMR imprinting center which eventually resulted in over-expression of *Igf2* (Dean, Bowden et al. 1998). However, several other studies observed a reduced growth in IVF derived mice litter (Van der Auwera and D'Hooghe 2001). In the present study the bovine *H19* DMR and KvDMR1 are affected (unmethylated) in placental tissues of IVF derived foetuses.

Treatment	Imprinting defect in DMRs at BWS region				Phenotype						
	Brain	Placenta	Muscle	Liver	Fetus wt. (gm)	Liver wt (gm)	Lung wt. (gm)	Heart wt. (mg)	Kidney wt. (mg)	Crown-Rump (cm)	Thoraxcircumf. (cm)
AI-F23	no	no	no	no	84,2	3,33	3,60	654	460	12,9	9,4
AI-F25	no	no	no	no	77,1	2,93	3,01	514	472	12,6	9,6
AI-F35	no	no	no	no	61,5	2,53	1,83	416	518	11,3	9
MOET-F37	no	H19 DMR	no	no	77,3	3,41	2,46	672	536	12,65	9,3
IVF-F58	no	H19 DMR / KvDMR1/ DMR0	no	DMR0	100,9	4,69	3,83	975	769	13,2	10,3
IVF-F59	no	H19 DMR / KvDMR1	no	no	103,3	5,02	4,18	821	665	13,1	10,1
IVF-F12	no	H19 DMR / KvDMR1	no	no	98,3	3,58	3,24	656	568	12,9	9,8
IVF-F14	no	no	no	no	72,3	2,91	2,50	480	410	12,8	8,9

Table 10: Summary of methylation defects in ART derived bovine foetuses and associated phenotype

The grey shaded area indicates the higher growth measurements in IVF derived foetuses as compared to the AI control animals. These results are the summary of COBRA and clone and sequencing analyses, where both techniques showed consistent results. One exception was AI-F23 which showed normal differential methylation in COBRA analysis but reduced maternal methylation at KvDMR1 region. Refer to the discussion section IV.1.1 for the explanation of these differences between COBRA and clone and sequencing of bisulfite PCR products.

It seems that IVF protocols occasionally results in abnormal foetal growth in different species and that the associated mechanisms also vary at the molecular level. Above, two studies were performed on cloned sheep and mice, which quite often demonstrate pronounced phenotypes. On the other hand IVF derived bovine foetuses show significant but not pronounced abnormal growth when compared to the control AI animals. Our study indicates the possibility of reduced *Igf2* (a growth enhancing gene) expression due to the loss of DNA methylation at paternal *H19* DMR allele. Reduced *Igf2* expression would cause a reduced growth. In contrast here the IVF derived foetuses showing reduced DNA methylation at *H19* DMR have also the overgrowth phenotype. Also in the other imprinting center, KvDMR1, the reduced maternal methylation should lead to reduced expression of *Cdkn1c* (growth

suppressing gene), *Ascl2* and *Phlda2* (involved in placental development) genes. This is expected due to increased silencer activity around the KvDMR1 (Du, Beatty et al. 2003; Mancini-DiNardo, Steele et al. 2003). Thus aberrant decreased expression of the genes regulated by KvDMR1 like *Cdkn1c*, *Ascl2* and *Phlda2* would co-relate with the observed growth defects and placental abnormalities in IVF derived foetuses. However my preliminary expression analyses do not show any reduction in the expression of *Cdkn1c* or *Phlda2* genes in the foetuses showing incorrect DNA methylation imprint. In summary the current knowledge on the changes of methylation and expression do not give a conclusive picture.

The present analysis on *Igf2* gene expression in IVF derived bovine foetuses does not support the previously shown elevated *Igf2* expression at day 70 of gestation of bovine IVF derived foetuses (Blondin, Farin et al. 2000). In the publication the authors normalized the *Igf2* gene expression against *18sRNA* expression. In reference to the publication, I also analyzed 1*8sRNA* along with 3 other control genes for normalizing the expression results. The *18sRNA* is ubiquitously expressed and was also found to be well expressed in all the analyzed tissues in the present analysis. However control genes such as *ß actin, Gapdh* and *PolyA polymerase* showed variable gene expressions in the control animal tissues. The placenta of AI-F25 and muscle of MOET-F37 (refer result section Figure 23) showed low expression of all the control genes except of *18sRNA*. This indicates either high variability of control genes expression within tissues and in individuals or the presence of poor quality RNA. Even a higher variation in the expression of control genes was observed in IVF derived foetal tissues. Here again the IVF derived foetal tissues showed even expression of *18sRNA* in all analyzed tissues.

Therefore the future expression analysis requires a control gene or a combination of genes which have low variability within individuals, after IVF procedures and also shows expression dependency on the RNA quality. These criteria's might also include a tissue specific control gene, e.g. a placental specific control gene. This will be done by checking the additional control genes, which are routinely in use for human and mouse gene expression. These are ubiquitin C, TATAA-box binding protein, cyclophilin A, and ß glucuronidase (Vandesompele, De Preter et al. 2002; Goossens, Van Poucke et al. 2005). In addition few other human genes are included

since they show low variability among different human tissue samples. These are *PolR2A* (polymerase RNA II (DNA directed) polypeptide A), *Rplp0* (ribosomal protein, large, P0) and *Fn1* (fibronectin1) (personnel communication with Gregor Reither, Medical Faculty of Saarland University, Homburg/Saar, Germany). All the selected control genes will be tested on different bovine tissues samples and the genes which meet the criteria stated above will be selected for future expression studies.

IV.1.5 **Possible mechanisms of overgrowth phenotype in ART derived foetuses**

In summary so far there is no conclusive model including all three effects i.e. changes in DNA methylation of imprinting centers, expression of imprinted genes and the observed phenotype. It has been shown that most of the pregnancy losses in IVF conceived foetuses occur in the first trimester of pregnancy i.e. from day 30 to day 90 and this is mostly attributed to abnormal foetal membrane (placenta) development (Hasler, Henderson et al. 1995). It has also been reported that IVF foetuses show reduced foetal growth in the initial phase of gestation i.e. day 37 to day 58, and this difference from control animals disappears by day 72 (Bertolini, Mason et al. 2002). However, these reports have to be taken with ultimate caution since these observations were performed by ultra-sonography which is a relative measurement. The tendency of initial reduced growth of the foetus which eventually results in foetal overgrowth at the time of birth is also documented for cloned calves (Hashizume, Ishiwata et al. 2002). Other investigators reported that the foetal overgrowth in LOS calves shows significant increment only in the last (third) trimester of pregnancy (Bertolini, Mason et al. 2002; Hashizume, Ishiwata et al. 2002).

My study shows that the *H19* DMR has a reduced DNA methylation in placenta of IVF foetuses, and this will result in reduced expression of *Igf2* gene. The *Igf2* gene is involved in foetal growth enhancement. Any reduced expression of *Igf2* gene in placental tissue will eventually result in lower number of placentomes or abnormal development of placentomes. This effect is quite often seen in IVF and cloned animals. It is also known that a compensatory mechanism develops in foetuses with abnormal development of placental tissue, in order to balance the undernourished

foetus (Bertolini, Mason et al. 2002). Hence, the late first trimester of gestation in bovine seems to be critical for such compensatory mechanisms. At the molecular level it could be co-related as a compensatory mechanism generated either by maternal or by foetal tissues, to overcome the deficient growth promoting genes (such as *Igf2*) for the proper placentome development. This may be reflected at day 72 of gestation when IVF and control animals have similar foetal growth measurements (Bertolini, Mason et al. 2002). In the present study the time point for bovine sample collection was performed at day 80 of gestation, which may coincide with the speculated compensatory phase. This might explain why there were no gross differences at the expression level of the *Igf2* or *Cdkn1c* genes (growth related genes) in the analyzed samples.

It is well known that *Cdkn1c* and *Igf2* genes are the key players in BWS related overgrowth phenotype. The *Igf2* gene is a growth enhancer, while *Cdkn1c* is a checkpoint control gene indirectly regulating the cell proliferation (and also the growth). Hence it is expected that any disturbances in the balance between these two genes might lead to an abnormal growth phenotype. Secondly it is also possible that other imprinted genes are involved in the fine tuning of the growth parameters. Recent study on interactions of Zac1 protein (also imprinted gene) indicates the existence of imprinted genes network regulating the embryonic growth (Varrault, Gueydan et al. 2006).

Hence a further investigation on networking of not only imprinted genes but also the interactions between the DMRs is required. It is clear that *H19* DMR interacts with secondary DMRs such as DMR1 and DMR2, in regulating *Igf2* imprinted gene in embryo. However *Igf2* gene shows a placental specific splice variant in mouse. In mouse DMR0 is known to regulate *Igf2* gene expression in parent of origin specific manner in placenta. Therefore it will be interesting to investigate whether *H19*DMR also interact with DMR0 in mouse placenta for the *Igf2* gene regulation. In contrast to mouse, human (Monk, Sanches et al. 2006) and our study on bovine species show presence of differential methylation at DMR0 in embryo and in placental tissues. Both the studies suggest that there might be a different regulatory mechanism of *Igf2* gene where not only *H19*DMR, DMR1 and DMR2 but also DMR0 is involved.

Nevertheless, to gain more knowledge on the influence of assisted reproductive technology (e.g. IVF) on imprinted regions, a systematic analysis is required. This will consider all the required parameters such as, foetal and placental measurements, plasma protein assessment, normalized gene expression, global DNA methylation level and DNA methylation at other imprinted centers at different days of gestation.

Aforementioned facts are that the large offspring syndrome 1) is an abnormal foetal growth observed only during prenatal development 2) after parturition the defect is rectified 3) the placental abnormalities are often associated with LOS foetuses and finally 4) results from present investigation show epigenetic lesions (DNA methylation) in IVF derived foetuses at imprinting centers, only in placental tissues while embryo proper is normal. This study will be continued on a large population of bovine in order to confirm the DNA methylation defects observed on indicated imprinting centers as molecular markers for pre-diagnosis of LOS in cattle industry. In addition primary imprinting centers such as imprinting center at the *Igf2r* gene locus and the secondary DMRs at *Igf2* gene, DMR1 and DMR2 will also be analyzed.

The LOS animals share similarities with BWS human patients' i.e. phenotypic features and the post parturition development. Hence, it will be interesting to analyze human placental tissues for DNA methylation at imprinted centers in BWS patients. If affected, they can serve as early markers for pre-diagnosis of BWS foetuses in human.

IV.2 Functional role of conserved elements at KvDMR1 imprinting sub-domain

In parallel to the DNA methylation study on bovine DMRs, I also studied the functional significance of the *cis* acting conserved elements in the KvDMR1 sub-domain of the BWS region.

The main observations in this study were:

1) On sequence alignments of the BWS region in different mammalian species, I show that there is pronounced sequence conservation in the introns 9, 10 and 14 of *KCNQ1* gene. Since these conserved elements are flanking the imprinting center KvDMR1 in BWS region, they were named as NICE elements.

2) Using transfection assays, I demonstrate that selected conserved NICE elements do not have any promoter activity in human embryonic fibroblast HEK293T or in mouse embryonic myoblast C2C12 cells.

3) Few of the analyzed conserved elements show a significant influence on heterologous SV40 or homologous KvDMR1 promoters in HEK293T or in C2C12 cell lines

4) The KvDMR1 promoter contains tandem repeats. Earlier investigations as well as this study shows that these tandem repeats are not required for the KvDMR1 promoter activity (Mancini-DiNardo, Steele et al. 2003). However in mouse myoblast C2C12 cells, the NICE element influences the KvDMR1 promoter in a tandem repeat dependent manner.

5) Co-transfection of Hand1 expressing constructs showed influence of the regulatory activities of some NICE elements on KvDMR1 promoter.

6) The DNA methylation of one of the analyzed conserved elements varies significantly between embryo and placenta. In placenta it exhibits a biased DNA methylation of the maternal allele (imprinted).

It was clear from the investigation that none of the analyzed conserved elements has any promoter activity. Hence their influence as being the putative alternative promoters e.g. as start points of antisense transcripts can be ruled out. However the possibility that a strong enhancer regulating any of these conserved elements for being a promoter cannot be challenged.

In this investigation I show that the promoter activity of KvDMR1 is specifically modulated by the presence of different conserved elements located in different introns of the *Kcnq1* gene. The observed influence seems to be regulated by the tandem repeat array present in the promoter region. This is the first investigation supporting the active regulatory role of tandem repeats at KvDMR1 locus. Other investigators as well as my study show that the promoter activity of KvDMR1 is not affected by the repeats alone (Mancini-DiNardo, Steele et al. 2003). KvDMR1 itself shows no sequence conservation among different species except the presence of CAAT boxes and the conserved motifs, which vary in number in different species (Du, Zhou et al. 2004; Paulsen, Khare et al. 2005). In addition, I also demonstrate that one of the CAAT boxes in KvDMR1 promoter region is not essentially required for its promoter activity in HEK293T and C2C12 cell lines. This conclusion is based on the fact that I did not observe any difference between promoter activities of KvDMR1-R (without repeats), which also lacks CAAT box numbered 1 (result section Figure 27a), when compared to full length KvDMR1 promoter.

The possible functional role of tandem repeats at imprinted centers is elusive. Mixed opinion exists in terms of tandem repeats as being requisite for imprinted centers (Pearsall, Plass et al. 1999; Lewis, Mitsuya et al. 2004; Walter, Hutter et al. 2006). However our results indicate that the promoter activity of KvDMR1 can be altered in presence of conserved elements. When tandem repeats are deleted from the KvDMR1, the influence of conserved elements on KvDMR1 promoter was also removed (refer to results section).These results suggest the possibility of direct or indirect interactions of KvDMR1 with the conserved NICE elements. The ongoing investigation will provide us with more evidences in support to the possible regulatory role of tandem repeats at KvDMR1 imprinting center. In the present study the conserved NICE elements on mouse KvDMR1 promoter showed influence in a repeat dependent manner in mouse myoblast cells. Our results on the KvDMR1 center or the associated tandem repeats showed no sequence conservation among different mammalian species (including human and mouse) (Paulsen, Khare et al. 2005). Therefore influence of NICE elements on human KvDMR1 promoter in HEK293T, NIH3T3 and C2C12 will be tested. This later experiment will show whether NICE elements influences on specific type of tandem repeats or it only requires a promoter possessing the tandem repeats.

The above investigation was conducted in cell lines originating from different species (human and mouse) and different tissues (fibroblast and myoblast). Therefore a third cell line e.g. a mouse fibroblast cell line (NIH 3T3), will be used to confirm the consistency in the results obtained from human embryonic kidney fibroblast (HEK293T). Since the present investigation (also discussed later) gave clues that NICE elements might be involved in placental specific gene regulation. Therefore another cell line originating from mouse placental tissue (a trophoblast cell line) will also be used for further analysis.

When the *in silico* predicted candidate transcriptional factor Hand1 was tested in co-transfection experiments, assays demonstrated significant removal of suppression from NICE 09_01 or NICE 10_04 promoter activity in HEK293T cells after 72 hr post transfection. Note that Hand1 co-transfection experiments did not show any significant influence of Hand1 protein on KvDMR1 promoter activity. Interestingly the number of conserved Hand1 consensus sites present in these NICE elements also corresponds to the effect seen in transient transfection assays i.e. maximum influence is from NICE 09_01 having 4 sites and no influence from NICE 10_13 which harbours only one site (appendix Figure 1). When Hand1 protein expression was checked in above co-transfections experiments by Western blot, it showed presence of Hand1 protein at all time points i.e. 24hr, 48 hr and 72 hr post transfection. However, the significant influence can be observed after 72 hr post transfection in luciferase read outs. It should be noted that Hand1 protein also undergoes post translational modifications such as phosphorylation, which influences its biological activity (Firulli, Howard et al. 2003). Another possibility for the influence of Hand1 protein at 72hr might be the presence of two Hand1 peptides (32 and 27kd band) expressed from the constructs. These two proteins might counter-acts and hence the effect is not visible at 24 and 48 hrs, or that the interacting partner is missing at these early time points. Hence to rule out all these possibilities we wish to continue the same experiments in a mouse trophoblast cell line where Hand1 protein expression is induced to higher levels upon differentiation (Scott, Anson-Cartwright et al. 2000). Here, if required then the sTable cell lines will be established in order to understand the regulatory mechanisms involving the conserved elements in trophoblast cell line. Study on sTable lines will limit the technical steps and also the associated experimental errors.

The Hand1 transcriptional factor is expressed exclusively in placenta and in the heart ventricles. It is known to interact with other factors to form hetero dimers for its functional activity (Scott, Anson-Cartwright et al. 2000; Firulli, Howard et al. 2003; Hill and Riley 2004). It is known that defects in *Kcnq1*, harbouring KvDMR1 center, leads to cardio myopathy such as Long QT syndrome1, Romano-Ward syndrome or Jervell and Lange-Nielsen syndrome. Therefore it's highly likely that the influence of Hand1 protein in cardiomyocytes or in placental tissue will reveal pronounced functional significance of the conserved elements on KvDMR1 promoter activity. Interestingly the KvDMR1 imprinting domain exhibits different imprinting mark in embryo proper and in placenta. In placenta this domain shows faithful differential imprint marks on histone modification in the entire domain whereas in embryo proper these marks are restricted to the KvDMR1 center only. It should be also noted that *KCNQ1,* which harbours the KvDMR1 imprinting center, is not imprinted in human foetal heart and in adult tissues (Lee, Hu et al. 1997).Therefore it will be interesting to know the functional role of Hand1 in regulating the KvDMR1 imprinting domain in different tissues. These findings will also highlight the antagonistic behaviour of Hand1 and *Ascl2* (in placental tissue), where later is regulated by the KvDMR1 imprinting domain.

When these NICE elements were analyzed for the epigenetic modifications (DNA methylation), I observed substantial differences between embryo proper and placenta in one of the conserved elements. NICE One showed the existence of partially differential DNA methylation (maternal allele being more methylated) in placenta. The embryo proper however showed complete DNA methylation at both alleles. If the unmethylated or methylated state on the conserved element is recruiting the transcriptional factors for its regulatory functioning, then the different functional state on the alleles can only be predicted for placenta tissue. This is in concurrence with the differential histone modification observed in placenta and not in embryo proper, in the KvDMR1 imprinting dom ain. It also indicates that the importance of DNA methylation in placenta might have been underestimated.

In addition we are planning to check these conserved elements for the presence of insulator or silencer activity. This will be performed by colony forming assays (Bell

and Felsenfeld 2000) and here all the plasmid constructs for the selected NICE elements are ready and experimental conditions are also optimized.

Additional experiments will also be performed in order to get complete understanding of the regulation at KvDMR1 imprinting domain. These will include identification of DNase hypersensitivity regions by real time PCR at KvDMR1 imprinting sub domain. This experiment will help in selecting tissue specific candidate NICE elements, which have a regulatory role at this sub-domain. The experiments such as Chromosome Conformation Capture (3C) will highlight the interaction of conserved elements with the KvDMR1. Investigation will also be performed on additional conserved elements located in *KCNQ1* introns as well as in combination with other promoters from KvDMR1 imprinting sub-domain. The transfection assays will be performed in HEK293T cells, C2C12 cells, NIH3T3 cells and in a trophoblast cell line. Other experiments such as electro mobility shift assay (EMSA) to show interaction of Hand1 with conserved element will supplement the findings. Identifying the DNase hypersensitive region by real time PCR will be performed at KvDMR1 imprinting sub-domain. If NICE elements are enriched in this assay then it will also confirms the regulatory potential of NICE elements at KvDMR1imprinting sub-domain

Summary

The epigenetic mark such as DNA methylation, at imprinted centers is often disrupted in imprinting syndromes in human. Recently these epigenetic alterations have also been implicated in assisted reproduction technology (ART) derived syndromes. The DMRs (sometimes also called imprinting centers) are indispensable for gene regulation in the imprinting domains. However the details of tissue and allele specific gene regulation in imprinted regions are poorly understood. In this study I investigated the Beckwith Wiedemann syndrome Region (BWS) in order to gain more knowledge on the influence of ART on maintance of imprinting in cattle and on the regulatory role of conserved elements in the vicinity of KvDMR1, one of the imprinting centers in the BWS region.

For investigation of epigenetic aberrations caused after the usage of ART, cattle was used as a model organism because of the easy accessibility of sample material and also frequent occurrence of overgrowth phenotypes similar to BWS syndrome. In cattle, the putative imprinting center regulating BWS and PWS /AS imprinting clusters are well conserved in genome in location when compared to human and mouse sequences. These centers also faithfully maintain differential methylation imprints in the germ lines as well as in embryo proper and in placental tissues. On comparative methylation analysis between foetuses derived from different fertilization procedures, i.e. AI derived (control animals) and IVF derived foetuses (day 80 of gestation), a substantial loss of methylation in placental tissues was detected at imprinting centers in BWS region i.e. *H19* DMR and KvDMR1. In contrast the embryo proper showed no methylation defect in IVF derived foetuses. It was also observed that not all imprinting centers were affected by the usage of ART. Imprints at the *Snrpn* DMR, in the PWS / AS region, were well maintained in IVF derived and control AI derived foetuses.

The functional regulation of the *cis* acting conserved elements was analyzed at KvDMR1 imprinting sub-domain. The KvDMR1 sub-domain is characterized by the presence of high sequence conservation in intron 10 of the *Kcnq1* gene (Paulsen, Khare et al. 2005). When 4 of highly conserved elements were linked to a reporter

gene and tested in transient transfection assays, they showed varying influence on the KvDMR1 promoter. When tandem repeats were deleted from the KvDMR1 promoter, release of the influence of conserved elements on promoter activity was observed. This is the first report showing that tandem repeats in KvDMR1 promoter modulate its promoter activity by interacting with conserved elements in the BWS domain. Interestingly, co-expression of the placental and heart specific transcriptional factor Hand1 has resulted in enhanced KvDMR1 promoter activity only in presence of conserved elements. In particular potential functioning of one conserved element in placenta is also indicated by an unusual allele specific DNA methylation pattern.

In conclusion, the presented data on methylation in bovine embryo and placenta and to some extent the functional analysis on cis acting conserved elements support the hypothesis that the KvDMR1 sub-domain behaves differently in the embryo proper and in placental tissues.

Zussamenfassung

Im Menschen sind bei Imprinting-assoziierten Syndromen epigenetische Markierungen an Imprinting-Zentren wie z. B. die DNA-Methylierung oft verändert. Kürzlich wurde entdeckt, dass diese Veränderungen auch bei durch ART (assisted reproduction technology, künstliche Befruchtung) erworbenen Syndromen auftreten. DMRs (manchmal auch Imprinting-Zentren genannt) sind für die Regulation von Genen in Imprintingdomänen unverzichtbar, die Details der allel- und gewebsspezifischen Genregulation jedoch kaum verstanden. In dieser Studie habe ich die Beckwith-Wiedemann-Syndrom (BWS) Region analysiert, um den Einfluß von ART auf Imprinting in Rindern, sowie die regulatorische Rolle konservierter Elemente in der Nachbarschaft der KvDMR1, eines der Imprinting-Zentren der BWS-Region zu untersuchen.

Um die epigenetischen Veränderungen nach Einsatz von ART zu untersuchen, wurde das Rind als Modell-Organismus gewählt, da hier leicht zugängliches Probenmaterial zur Verfügung steht und sich nach ART dem BWS-Syndrom ähnliche Phänotypen (Überwuchs) zeigen können. Im Rind sind die putativen Imprinting-Zentren der BWS bzw. PWS/AS Imprinting-cluster hinsichtlich ihrer Lage im Genom beim Vergleich zu Maus bzw. Mensch gut konserviert. Diese Zentren behalten ihre differentiellen Methylierungen in der Keimbahn, sowie im Embryo und plazentalen Geweben. Vergleichende Methylierungsanalysen an Föten, die durch unterschiedliche ART-Techniken (d.h. durch AI bzw. IVF empfangene Föten) entstanden, zeigten im plazentalen Gewebe einen substanziellen Methylierungsverlust an den Imprinting-Zentren der BWS-Region, d.h. die *H19*-DMR und die KvDMR1. Im Embryo fand sich dagegen keine veränderte Methylierung in mitlles IVF generierten Föten. Auch zeigte sich, dass nicht alle untersuchten Imprinting-Zentren durch ART betroffen sind. So wurden die Imprints der *Snrpn* DMR in der PWS/AS Region sowohl in Kontrolltieren (AI) als auch in IVF-Föten aufrecht erhalten.

Die regulatorische Funktion konservierter, in *cis* wirkender Elemente wurde an der KvDMR1 Imprinting-Subdomäne untersucht. Die KvDMR1 Subdomäne zeichnet

sich durch eine hochkonservierte Sequenz im Intron 10 des *Kcnq1* Gens aus (Paulsen, Khare et al. 2005). Vier der hochkonservierten Elemente wurden mit einem Reportergen gekoppelt und in transienten Transfektionen getestet. Sie zeigten unterschiedlichen Einfluß auf den KvDMR1 Promotor. Wurden die Tandem Repeats vom KvDMR1 Promotor entfernt, verringerte sich der Einfluß der konservierten Elemente auf die Promotoraktivität. Dies ist der erste Hinweis dafür, dass die Aktivität des KvDMR1 Promotors durch Interaktion seiner Tandem Repeats mit konservierten Elementen der BWS-Region moduliert wird. Interessanterweise zeigte sich bei Ko-expression des plazenta- und herzspezifischen Transkriptionsfaktors Hand1 eine verstärkte KvDMR1-Promotoraktivität nur bei Anwesenheit der konservierten Elemente. Eine mögliche Funktion dieser konservierten Elemente in der Plazenta wird ferner durch ein ungewöhnliches DNA Methylierungsmuster eines der Elemente in eben diesem Gewebe unterstrichen.

Zusammenfassend lässt sich sagen, dass die vorgelegten Daten zur DNA Methylierung in Embryo und Plazenta des Rindes und zum Teil die Funktionsanalysen der in *cis* wirkenden, konservierten Elemente die Hypothese unterstützen, dass die KvDMR1 Subdomäne sich im Embryo und in der Plazenta unterschiedlich verhält.

Abstrakt

Der Mechanismus des Imprinting ist eine in Säugetieren bekannte Erscheinung. Genomisches Imprinting beinhaltet multiple Faktoren. Es ist bekannt, dass fehlreguliertes Imprinting im Menschen verschiedene Syndrome verursacht. Gegenwärtige Untersuchungen zeigen einen Zusammenhang zwischen künstlicher Befruchtung und Imprinting-Syndromen im Menschen. In der vorliegenden Arbeit wurden Föten von Rindern untersucht, um den Einfluß künstlicher Befruchtungstechniken (ART) auf Imprinting-Regionen zu überprüfen. Es wurden verschiedene Imprinting-Regionen gewählt und deren Imprinting-Zentren durch Sequenzabgleich mit Mensch und Maus identifiziert. An allen untersuchten Imprinting-Zentren waren die Imprints (DNA-Methylierung) vorhanden. Beim Vergleich der Imprints von Föten einer Kontrollgruppe (AI) mit denen von IVF-generierten Föten, zeigte sich ein substanzieller Verlust paternaler Methylierung in der *H19*DMR, wohingegen sich maternale Methylierung der KvDMR1 nur in plazentalen Geweben IVF-generierter Föten (ART) fand.

Der zweite Fokus der vorliegenden Arbeit lag darin, das regulatorische Potenzial hoch-konservierter, kurzer Sequenzen (NICE), die das KvDMR1-Zentrum flankieren, zu untersuchen. Vier der hochkonservierten Elemente wurden getestet und zeigten einen Einfluß auf die Promotor-Aktivität. Dabei zeigte sich, dass dieser Effekt durch die Anwesenheit von Tandem Repeats in der KvDMR1 moduliert wurde. Dies ist die erste Untersuchung, die eine mögliche Rolle der konservierten NICE-Elemente und der Tandem Repeats auf die Promotor-Aktivität des KvDMR1-Imprinting-Zentrums aufzeigt.

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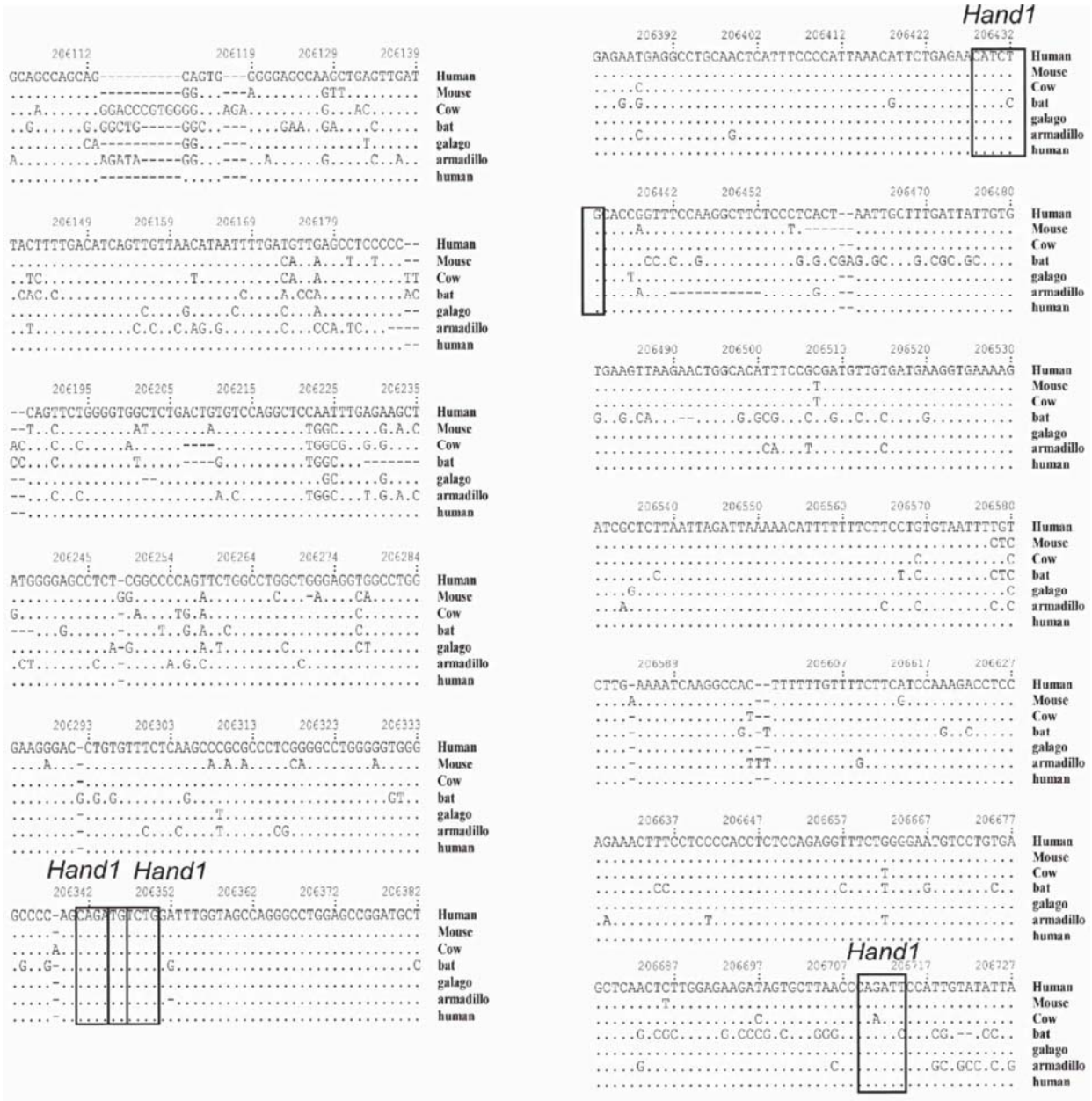
VII **Appendix**

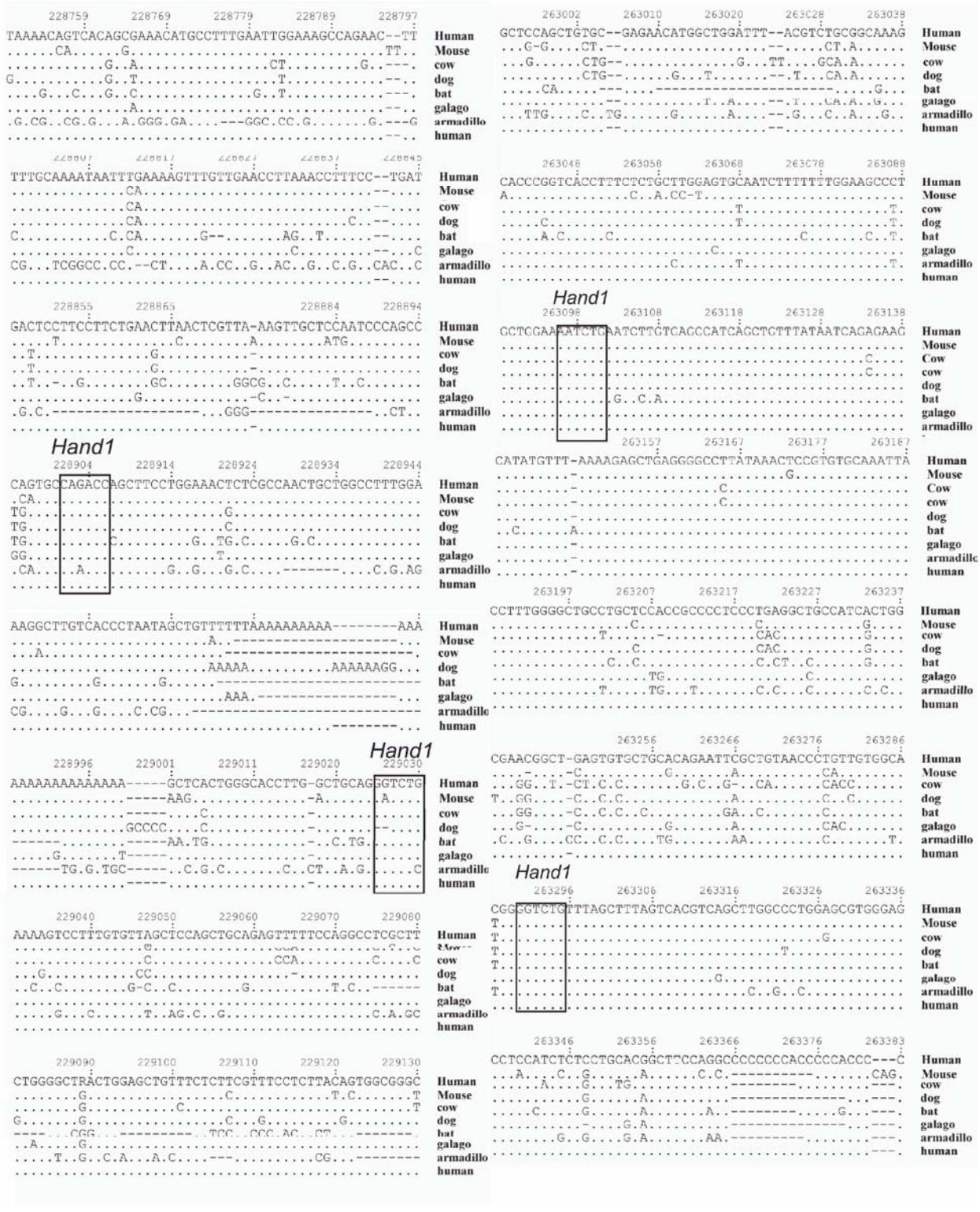
Appendix Figure 1:

Multiple sequences alignment between different mammalian species at the NICE elements

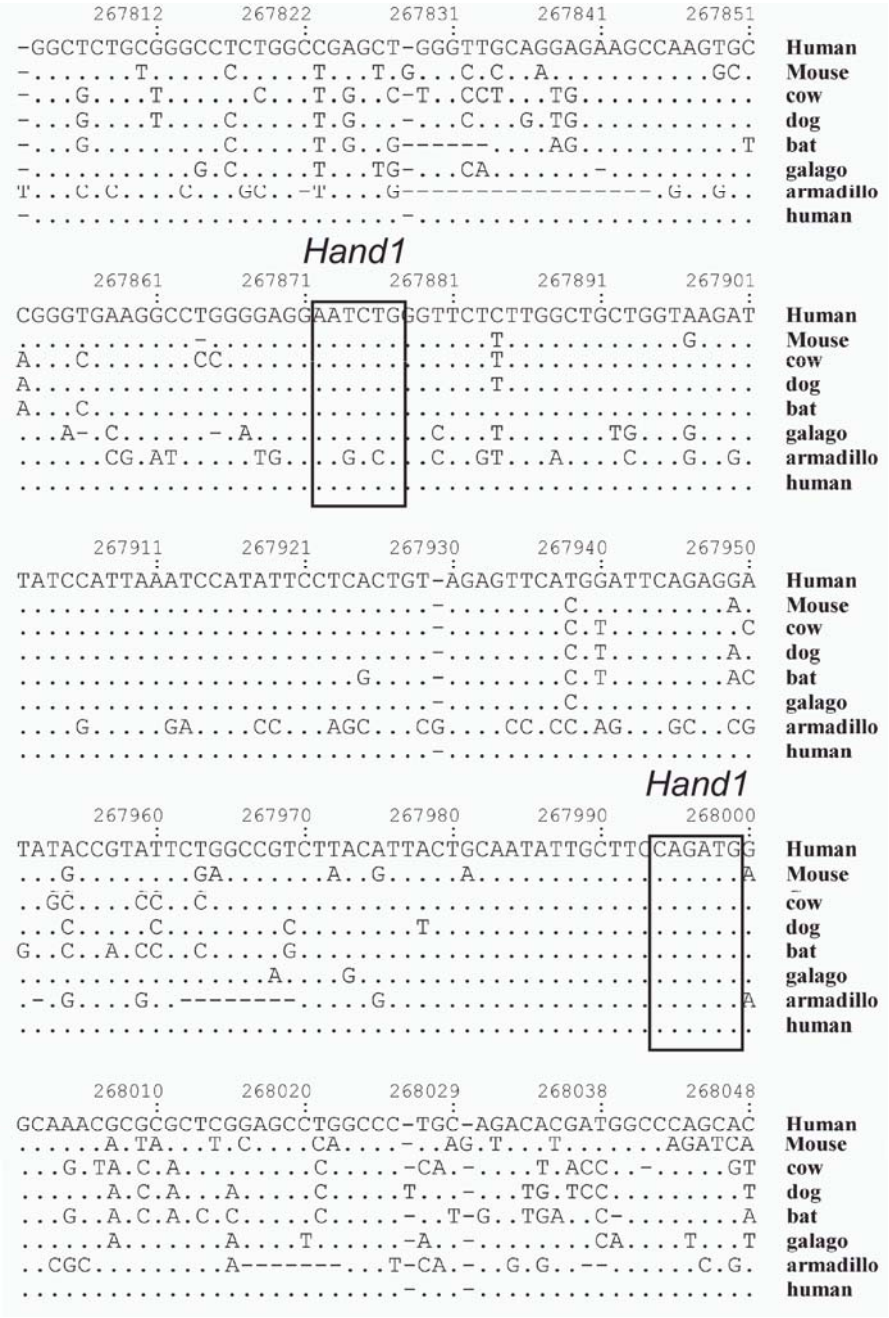
The identical nucleotides in the alignment is represented by a dot (.), gap by a dash (-) and mismatches by the respective nucleotides (A, C, G, T). Mapped Hand1 sites (highlighted with open boxes) are according to the consensus binding sites "NRTCTG" or "CACGYN", where R represents G or A; N represents any nucleotide and Y is for C or T.



Multiple sequence alignment at NICE 09_01



Multiple sequence alignments at NICE 10_04 and NICE One



Multiple sequence alignment at NICE 10_13

Appendix Tables

Human NT_009237.17		1694216 755779		Bovine			
	Exon	Exon length		BAC clone		Exon	Exon length
H19					**H19**		
890061 889950	5	111		AC149767.6	151170 151267	5	97
889868 889746	4	122			150963 151090	4	127
889665 889553	3	112			150778 150884	3	106
889457 889323	2	134			150575 150698	2	123
884526 884274	1	252			144507 144758	1	251
IGF2					**IgF2**		
753018 752521	9	497			57593 58093	9	500
752227 752079	8	148			57140 57288	8	148
750377 750215	7	162			55527 55689	7	162
747515 746351	6	1164			51620 52740	6	1120
745609 745445	5	164			50729 50893	5	164
744878 744514	4	364			49807 50162	4	355
738159 738077	3	82			43476 43561	3	85
736618 736426	2	192			42024 42211	2	187
727480 727370	1	110			33436 33547	1	111
KCNQ1					**Kcnq1**		
440645 440260	1a	385		AC151859.1	72073 72443	1a	370
424037 424030	1b	7			61077 61084	1b	7
364265 364174	1c	91					
357816 357726	1	90			GAP		
321970 321708	2a	262					
315116 314990	2	126		AC147592.2	172109 172235	2	126
314419 314341	3	78			171601 171679	3	78
313731 313635	4	96			170977 171073	4	96
312898 312758	5	140			170245 170385	5	140
302309 302199	6	110			162152 162262	6	110
300532 300437	7	95			161789 161884	7	95
298174 298052	8	122			159792 159914	8	122
297017 296890	9	127			158741 158890	9	149
223783 223663	10	120			78097 78217	10	120
116900 116825	11	75		AC147396.2	110407 110482	11	75
109784 109690	12	94			115591 115685	12	94
108758 108712	13	46			116575 116621	13	46
107768 107707	14	61			117446 117501	14	61
37977 36634	15	1343			187000 188212	15	1212
CDKN1C				AC151858.1	**Cdkn1c**		
2109 1829	3	280			35001 35277	3	276
1745 1610	2	135			34709 34806	2	97
1074 13	1	1061			33314 34225	1	911

Appendix Table 1: Human-bovine BWS region

Human NT_009237.17		1694216 755779		Bovine BAC clone							
IC1		882714 885776		AC149767.6	143383 151298						
IC2		185152 186760		AC147396.2	38700 38000						
Endodermal enhancers		892949 893117		AC150790.4	176185 176301	Ref:	Ishihara K Genome Research 2000.				
		894421 894665			174753 174840						
		902423 902492			167445 167687						
		905802 906029			166094 166212						
		908385 908524			164054 164292						
		910208 910478			161241 161280						
		917809 918062			157472 157601						
IGF2	DMR2	752519 861849		AC149767.6	57162 90313		AY849923				
	DMR1	740600 740829			45922 46184		AY849918				
	DMR0	735954 736626			42036 42215						
	P4	748477 748498			53679 53711		Amarger V Mammalian Genome 2002				
	P3	747278 747400			52505 52625						
	P2	744552 744676			49856 49903						
	P1	727372 727482			33523 33553						

Appendix Table 2: Regulatory elements at Human-bovine BWS region

IC1		AC149767.6									
1,6 kb re	1st	143406 144924									
	2nd	144753 146307									
	3rd	146120 147630									
G like repeat		GTTCACCCATGGTCAAAGCTGGGATTTTTCCAATGAAATCTGGGG									
		GGGGAAACT									
CTCF consensus		CCGCNNGGNGNC							Bell, 2000		
IC2		AC147396.2									
▶		GGGCTNCTYNRTGAGGAGA									
▷		GAGCGCAGCCGTGAG									
▶		conserved motifs							Paulsen, Khare et al. 2005		
Snrpn DMR		NW_205360		23047 23877							
▶		GGCGGCGGTGGGCATT			Repeat present in Human SNRPN DMR						
▷		CCACCACGG									

Appendix Table 3: Tandem repeats at the analyzed imprinted centers

	Human-Bovine SNRPN Gene					
	Human	ref: mRNA NM_0033097.3			Bovine	
	NW_925783.1 1452000-1610000				NW_205360	
		Exon	Exon length			Exon length
	620708 62781	U2	73			
	88540 88707	U3	167			
	96189 96302	U4	113			
	96651 96725	U5	74			
DMR	131099 131173	1	74		23554 23628	74
	138216 138311	2	95		17673 17765	92
	144035 144185	3	150		14296 14413	117
	150415 150560	4	145		6409 6554	145
	151462 151613	5	151		5037 5188	151
	152409 152520	6	111		2294 2405	111
	152981 153133	7	152		1683 1835	152
	153882 154020	8	138		1116 1254	138
	154297 154422	9	125		623 746	123
	154511 154686	10	175		347 516	169

Appendix Table 4: Human-bovine Snrpn gene

Abbreviations

µl	: microlitre
AI	: Artificial Insemination
APS	: Ammonium persulfate
Ascl2	: Achaete-scute complex homolog-like 2
AS-SRO	: AS- Shortest Region of Overlap
bp	: basepair
BWS	: Beckwith Wiedemann Syndrome
C2C12	: Mouse myoblast cell line from C3H strain
CBF	: CCAAT box-Binding Factor
Cdkn1c	: cyclin dependent kinase inhibitor 1c
CMV	: CytoMegalo Virus
CO_2	: Carbon Dioxide
CTCF	: CCCTC-binding factor
CTF	: CCAAT-binding Transcription Factor
ddNTP	: Didioxy Nucleotide TriPhosphate
DMEM	: Dulbecco's Modified Eagle Medium
DMR	: Differentially Methylated region
DMSO	: Dimethyl Sulfoxide
Dnmt	: DNA methyl transferase
dpi	: Days post insemination
ECL	: Electrochemiluminescent
EDTA	: Ethylenediaminetetraacetic acid tetrasodium salt dihydrate
Hand 1	: Heart and Neural Crest Derivatives expressed 1
ESTs	: Expressed Sequence Tags
ET	: Embryo transfer
FCS	: Foetal Calf Serum
Gapdh	: Glyseraldehyde-3-phosphate dehydrogenase
H (no) K (no)	: Histone (no.) amino acid lysine (number)
HEK293T	: Human embryonic kidney fibroblast cell line
hr	: Hour
HS	: Hyper Sensitive
ICF	: Immunodeficiency, Chromosomal instabilities, and Facial abnormalities syndrome
ICR	: Imprinting control region
ICSI	: Intra cytoplasmic sperm injection
Igf2	: insulin like growth factor 2
Igf2r	: insulin like growth factor 2 receptor
IPTG	: Isopropyl-ß-D-thiogalactopyranosid
IVF	: In vitro fertilization
Kcnq1	: potassium voltage-gated channel, KQT-like subfamily, member 1
Kcnq1ot1	: KCNQ1 overlapping transcript 1
KvDMR1	: KCNQ1 Differentially Methylated Region 1
LCRs	: Long range control regions
LH	: luteinizing hormone
Lit1	: long QT intronic transcript 1
LOS	: Large offspring syndrome

mM	: milli Molar
MOET	: Multiple Ovulated Embryo Transfer
mRNA	: Messenger ribonucleic acid
NaCl	: Sodium Chloride
NaOH	: Sodium hydroxide
NF-Y	: nuclear transcription factor Y
NICE	: Neighbouring the Imprinting center Conserved Elements
O_2	: Oxygen
OMIM	: Online Mendelian Inheritance in Man
PCR	: Polymerase chain reactions
PGC	: Primodial Germ Cells
Phlda2	: pleckstrin homology-like domain, family A, member 2
pRL	: plasmid renilla
Pu	: purine
PWS / AS	: Prader Willi syndrome / Angelman syndrome
PWS-SRO	: PWS-Shortest Region of Overlap
Py	: Pyrimidine
SCNT	: Somatic Cell Nuclear Transfer
SNuPE	: Single Nucleotide Primer Extension
Spm	: suppressor mutator
SV40	: Simian virus 40
TEMED	: N,N,N',N'-Tetramethylethylenediamine
TESS	: Transcriptional element search software
tRNA	: transfer ribonucleic acid
UPD	: Uniparental disomy
WT	: Wilm's tumour

Curriculum vitae of Tarang Khare

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Date of birth:	18 May 1974
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Academic background

Schooling

1979-1991	Campus school, Pantnagar India

Research and training

1991-1996	Bachelor of veterinary medicine and animal husbandry G. B. Pant University of Agriculture and Technology
1998-2000	Masters in Molecular Biology Interuniversity program of Molecular Biology (IPMB), conducted by VUB Brussels, KUL Leuven and University of Antwerp, Belgium. Master thesis was performed in the lab of Prof. Michael Georges, department of animal genetics, University of Liege, Belgium. (Master thesis: RH mapping and detection of SNPs in the GH gene: a positional QTL candidate)
2001-2006	PhD student under the supervision of Prof. Jörn Walter, department of genetics/epigenetic, University of Saarland, Germany. (phD thesis: Influence of assisted reproductive technologies on imprinting centers and functional characterization of conserved elements in Beckwith Wiedemann Syndrome (BWS) region

Employment history

1996-1997	Field veterinarian in Haldwani, India
1997-1998	Research assistant in the joint project under the supervision of Dr. B.D.Lakhchura (G.B.P.U.A&T, Pantanagar India) and Dr.D.C.Kaushal (CDRI, Lucknow India). (Theme: development of monoclonal antibody against progesterone hormone in bovine)

2000-2001 Research assistant in the lab of Dr.Gerhard Behre; KKG Leukaemia, Munich, Germany.
(Theme: identification of the target and interacting proteins in AML-ETO (leukaemia) diseased patients by utilizing 2D-Gel and Mass spectrophotometer techniques)

Oral presentations

1) "Analysis of Imprinted domains in Bovine"
Workshop of the DFG priority program 1129 2004
4th Nov-6th November, 2004 Überherrn, Germany

2) "DNA methylation analysis at imprinting centers and IVF derived foetuses in bovine"
"Downunder Embryo Symposium" A Symposium on the Cell Biology of the Early Embryo 23rd- 25th August, 2006 Brisbane, Australia

Poster presentations

1) "Down under Embryo Symposium":

A Symposium on the Cell Biology of the Early Embryo
Brisbane, Australia 23rd- 25th August, 2006

2) "International Genomic Imprinting Workshop 2006"

Tokyo, Japan 30thNov – 1st Dec. 2006

Publications

1) **Khare T**, Hiendleder S ,Paulsen M and Walter J
DNA methylation analysis at imprinting centers and IVF derived foetuses in bovine. (Manuscript in preparation)

2) Walter, J., B. Hutter**, Khare, T**.,Paulsen, M.et al. (2006)
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Cytogenetic Genome Research 113(1-4): 109-15.

3) Paulsen, M. **Khare, T.** Burgard, C. Tierling, S. Walter, J. (2005)
Evolution of the Beckwith-Wiedemann syndrome region in vertebrates
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