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Transcriptional Silencing in the Imprinted *Igf2-H19* Loci: The Mystique of Epigenetics

BY

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ABSTRACT

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Genomic imprinting marks a subset of autosomal loci expressed in parent of origin-dependent monoallelic expression in a non-Mendelian fashion. To restore totipotency and to reset the imprint according to the sex of the individual, the mark must be erased during germline development. The imprinted *Igf2-H19* loci located distally on chromosome 7 in mouse and 11p15.5 in human, share common regulatory elements that regulate differential expression. Where the *H19* is silenced when paternally inherited, the *Igf2* is silenced when maternally inherited.

The differentially methylated 5'-flank of *H19* gene, termed imprinting control region (ICR), shown to display a unique chromatin organisation harbours hypersensitive sites in linker regions flanked by positioned nucleosomes on the maternal allele. This unique chromatin conformation functions as a methylation-sensitive and unidirectional chromatin insulator, which later was found to depend on the chromatin insulator protein CTCF.

The *H19* ICR exhibits default-silencing functions in promoter-proximal positions. The maximal distance between the *H19* ICR and the promoter of the reporter gene required for this effect was 1.2 ± 0.3kb which can be compared to the 1.9 kb distance between the endogenous *H19* ICR and *H19* promoter. Results suggest that the *H19* ICR adopts a chromatin conformation that must be separated by a minimal distance from pivotal *cis*-regulatory elements to avoid adverse effects on neighbouring promoters.

Poly(ADP-ribosy)lation represents a novel post-translational epigenetic mark that segregates with exclusively the maternal derived *H19* ICR and associated with factors that interact with the CTCF target sites. CTCF is itself poly(ADP-ribosy)lated and the poly(ADP-ribose) polymerase inhibitor 3-aminobenzamide relieves the insulator function of the *H19* ICR.

Designed zinc finger proteins were applied to examine if epigenetic marks provided an obstacle for targeted activation and silencing. The zinc finger protein ZFP809 with activator/repressor domain able to efficiently activate/silence the *IGF2* target. Murine hybrid cell lines of human chromosome 11, demonstrated that the ZFP809 overcame the epigenetic marks that repressed maternal *IGF2* and paternal *H19* allele, respectively. Results suggested that imprinted genes are not normally exposed to strong *cis*-regulatory elements and that the designed ZFPs can be exploited to develop a therapeutic method for rectifying epigenetic lesions.

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Paper IV:

Yann Jouvenot, Vasudeva Ginjala, Lei Zhang, Pei-Qi Liu, Mitsuo Oshimura, Andrew P. Feinberg, Alan P. Wolffe, Rolf Ohlsson & Philip D. Gregory. Targeted regulation of imprinted genes by synthetic zinc finger transcription factors**.** (*Gene Therapy,* accepted for publication)

CONTENTS

ABBREVIATIONS

AS	Angelman Syndrome
BWS	Beckwith-Weidemann Syndrome
CBP/p300	Creb Binding Protein/p300
ChIP	Chromatin Immuno-Precipitation/purification assay
CTCF	ccCTC binding Factor
DMD/DMR*	Differentially Methylated Domain/region
Dnmt	DnA methyltransferase
DOS	Dyad Octomer binding Sequence
HAT	histone acetyltransferase
HDAC	histone deacetylase
ICR*	Imprinting Control Region
IGF	insulin like growth factor
kb	kilobase
kd	Kilo Dalton
NHSSs	nuclease hypersensitive site
PWS	Prader-Willi Syndrome
PDGF	Platelet Derived Growth Factor
SNRPN	Small Nuclear Ribonucleoprotein Particle-associated peptide N
TSA	trichostatin A
UPD	Uni-Parental Disomy
WT	Wilm's Tumour
ZFP	Zinc Finger Protein

* Please note that ICR and DMD both refer to the same 2.2kb upstream region of H19 gene.

"It is as if the chromosomal proteins were invisible, revealing the underlying DNA without impediment to the transcriptional apparatus. Many have thought, some even stated that the master molecule has no clothes; that the structure of chromatin is irrelevant to transcriptional regulation. Emerging evidence, however, suggests the opposite".

Roger Kornberg (1991) *Cell*, 67(5):833-6

INTRODUCTION

The world of chromatin

Prior to the landmark discovery of structure of deoxyribonucleic acid (DNA, Watson and Crick 1953), it was proposed that a variation in gene expression was the basis of cell differentiation (Morgan 1934). Later on it was found the nucleic acid as the building blocks of a gene (Avery *et al*. 1944). The basic proteins associated with DNA were isolated and named as "histones" (Kossel 1928), which laid way to stimulating chromatin research. The histones are highly conserved throughout evolution having a very few basic forms (Fitzsimmons and Wolstenholme 1976). The earliest implication of histone modifications such as histone methylation and acetylation role in gene regulation (Allfrey *et al*. 1964) became a matter of concern for chromatin researchers.

Soon after the "bead on a string structure" of chromatin was proposed (Woodcock 1973; Olins and Olins 1974), a model of the nucleosome organization was elucidated as each 'bead' consists of DNA wrapped around an octamer amassed of two copies of histones H2A, H2B, H3 and H4 (Kornberg 1974; Kornberg and Thomas 1974; Bradbury 1976; Kornberg and Lorch 1999). In addition to core histones, linker histone (H1) was discovered (Newrock *et al*. 1978). Ten percent digestion of isolated nuclei by deoxyribonuclease I (DNase I) revealed that the accessibility to the nuclease depends on gene activity, suggesting an altered chromatin structure during transcription (Weintraub and Groudine, 1976). Moreover regulatory regions like promoters and enhancers were also shown to be hypersensitive to DNase I digestion (Wu *et al*. 1977a; Wu *et al*. 1977b). Several findings confirmed the necessity of RNA

polymerase for proper gene transcription (Parker and Roeder 1977; Jaehning and Roeder 1977). The first gene-specific factor necessary for the accurate transcription initiation isolated in 1980 (Engelke *et al*. 1980; Pelham and Brown 1980) paved a way for searching new factors. Subsequently DNA sequence and the histone octomer was found sufficient to generate a precisely phased nucleosome in which interactions of the two DNA strands with histones are not the same (Simpson and Stafford 1983). Subsequent reports showed that the gene-regulatory regions could be possible targets for ordered nucleosomal arrays (Almer and Horz 1986a; Almer *et al*. 1986b; Richard-Foy and Hager 1987) and histone specific modifications can lead to transcriptional activity. Chromatin environment hence plays a major role in gene regulation and maintenance of genome integrity (Megee *et al*. 1990; Durrin *et al*. 1991; Grunstein 1992; Megee *et al*. 1995; Vogelauer 2000).

The role of chromatin has emerged at the forefront of transcription research following the discovery of several key transcription factors (Arents and Moudrianakis 1993; Moudrianakis and Arents 1993; Clark *et al*. 1993; Kim 1993; Lai 1993; Ramakrishnan 1993; Hoffmann 1996; Xie 1996; Suto 2000; Fan 2002). The isolation of several variants of histone acetyltransferase (HAT), has infact revolutionised the chromatin research. Some of the HAT functioning transcription factor discoveries are; from Yeast Gcn5p (Brownell *et al*.1996), from mammalian cells PCAF (Yang et al. 1996), CBP/p300 (Ogryzko *et al*. 1996) and several other transcription factors shown to have HAT activity, such as TAFII250, a subunit of TFIID (Mizzen *et al*. 1996). The first histone deacetylase HD1p (HDAC1) was isolated from human cells by using trapoxin (TPX), a microbially derived cyclotetrapeptide that inhibits histone deacetylation *in vivo* (Taunton *et al*. 1996). The discovery of another potent histone deacetylase inhibitor called trichostatin A (TSA) as compared to sodium butyrate was an important advantage for chromatin researchers (Yoshida *et al*. 1990; Yoshida *et al*. 2001). In the past few years, the discovery and characterisation of the chromatin modifying machinery significantly advanced our understanding of the molecular activities that establish a transcriptionally competent substrate *in vivo*, and have underscored the importance of the part played by chromatin in the regulation of transcription.

"Epigenetic effects have often enough been viewed as verging on the mystical. It is a paradox of conventional genetics that two alleles can have the same genetic sequence but show different states of inheritance."

Benjamin Lewin (1998) The Mystique of Epigenetics, *Cell*, **93**, 301–303

Epigenetics and chromatin dynamics

Epigenetics is usually defined as "heritable changes in gene expression that occur without a change in DNA sequence" (Wolffe and Matzke 1999). Examples comprise changes in gene expression during development, differential segregation of gene activities (*i.e.* daughters of an individual cell have different patterns of gene expression), and mechanisms to allow the somatic inheritance of a specific set of active and quiescent genes (Holliday 1990). Epigenetic inheritance may also function to alter patterns of gene expression to safeguard the evolving biological system against changes in the environment. This adaptive epigenetic inheritance confronts with the "central dogma" that information is unidirectional from DNA to protein and the theory of Darwinian random mutation and selection is the sole mechanisms of evolution (Monk 1995).

Basically, gene expression can be regulated at multiple levels, like DNA/chromatin structural, transcriptional, and post-transcriptional modifications. Four genetic mechanisms: DNA point mutation, deletion, rearrangement, and amplification and two epigenetic mechanisms: DNA methylation and the preservation of DNA protein complexes (such as post-translational modifications of histones include acetylation and methylation and polycomb/trithorax group of proteins) appear to account for the majority of heritable alterations of gene expression (Gruenert and Cozens 1991). In this chapter, epigenetic mechanisms starting with chromatin modifications to DNA methylation and their role in gene regulation and disease will be discussed.

Chromatin modifications

The basic structural unit of chromatin is the nucleosome, which is formed by a histone octamer core around which 146 bp of DNA is wrapped. Further, the chromatin fiber is organized, either in euchromatin that contains actively transcribed genes, or in heterochromatin that contains inactive genes. It is usually stated that heterochromatin structure is more compact than euchromatin making the interaction of regulatory factors with their DNA targets difficult. However, a recent report has shown an interaction of general transcription factors with heterochromatin (Breiling *et al*. 2001). Elucidating the mechanisms by which DNA is packed into specialized chromatin domains is of profound importance for the understanding of gene regulation.

Transcriptional regulation plays an important role in development at different levels. The transcriptional initiation, DNA replication, repair and/or recombination necessitate DNA accessibility to factors involved in such processes. In addition, protein complexes, which are large in size as compared to a nucleosome, should be able to scan the DNA packed in chromatin. This requires sequential changes into chromatin structure through two major mechanisms a) the post-translational modification of histones; and b) the action of ATP-dependent chromatin remodelling complexes. The functional diversity of euchromatin and heterochromatin has been correlated with unique post-translational modifications of histone N termini, such as acetylation, methylation and phosphorylation; where acetylation and phosphorylation appear as transient marks, histone methylation may be a relatively stable epigenetic chromatin mark (Turner 2000; Jenuwein and Allis 2001).

Chromatin remodelling represents a change of nucleosome position and conformation, leading to chromatin assembly and disassembly. ATP-dependent chromatin remodelling complexes, especially the SWI/SNF and ISWI families, were initially found in yeast and *Drosophila* (Kingston and Narlikar 1999). The hSWI/SNF (human switch/sucrose non-fermenting gene) is an ATPase subunit of chromatin remodelling complex in human, which is a huge multi-molecular structure including either BRG1 or hBRM and tumor suppressor protein hSNF5/Ini-1, mainly activates gene transcription. The RSF heterodimer complex including hSNF2H is involved in

the initiation of transcription, while Mi2-NuRD complex has been reported to convert active to inactive chromatin due to both ATPase of Mi2 and HDAC activities. In addition, the HuCHRAC complex including hSNF2H and chromatin assembly factor hCAF1 is considered to associate with the replication and maintenance of heterochromatin (Poot *et al*. 2000). The chromatin assembly factor-1 (CAF1) has a function in the maintenance of chromatin coupled to DNA replication.

A post-translational modification performed by nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) is one of the well-known proteins with DNA-damage scanning activity, that signals DNA rupture and participates in base-excision repair (Herceg and Wang 2001). PARP-1 is a member of multifamily of poly(ADP-ribosyl)ating enzymes with distinct properties and subcellular localization (Chiarugi 2002; Smith 2001). Poly (ADP-ribosyl)ation has been proposed to function in genome repair by modifying architectural proteins proximal to DNA breaks, thus facilitating the opening of the condensed structure of chromatin required for the recruitment of the repairing complex revealed by *PARP-1* -/- mice (Shall and Murcia 2000; Liaudet *et al*. 2000). In addition to these functions PARP-1 can also induce cell death through NAD^+ depletion. The emerging concept of PARP-1 having a major role in the regulation of gene transcription and chromatin modifications is expanding. PARP-1 alters gene expression in both a positive and negative way, depending on the cell type, the gene and the transcription factor involved (Ziegler and Oei 2001). PARP-1 facilitates the recognition of transcription initiation DNA sequences (Slattery *et al*. 1983) and regulates the activity of transcription factors including AP-2, Oct-1, NF-κB, p53 and CTCF (Chiarugi 2002; Ginjala *et al*. 2002).

Histone acetylation

Histones can undergo post-translational modifications on their N-terminal tails. The emerging concept of `histone code' explains how combinations of N-terminal modifications on histones, including acetylation, methylation, phosphorylation, ubiquitination and poly(ADP-ribosy)lation have a control on gene regulation, DNA replication and chromatin-dependent processes (Strahl and Allis 2000). Histone acetylation takes place on N-terminal lysines and associated with transcriptional

activation in correlation with H3 phoshorylation and methylation (reviewed in Grunstein 1997; Kuo and Allis 1998; Struhl 1998). The H3 phosphorylation on Ser 10 has long been correlated with mitotic chromosome condensation (Hsu *et al*. 2000). Phosphorylation of histone H3 on serine 10 can also lead to transcriptional activation (Sassone-Corsi *et al*. 1999) and increases acetylation of the neighboring Lys 14 by GCN5 (Lo *et al*. 2001). Methylation of H3 on Lys 9, is catalyzed by SUV39H1 and this methylation interferes with phosphorylation of Ser 10 (Rea *et al*. 2000). It has also been reported that methylation of H3 on Lys 9 is linked to developmental changes in chicken β-globin locus (Litt *et al*. 2001). The most recent report suggests that histone acetylation acts protection against unmethylated state of DNA in chicken β-globin insulator locus (Mutskov *et al*. 2002), whereas DNA methylation often leads to histone deacetylation.

Histone hyperacetylation is usually correlated with transcriptional activation. However, recent reports point a role for histone acetyltransferase activity (HATs) in DNA replication and repair. Transcriptional co-activators such as CBP/p300 and PCAF are intrinsic HATs (Marmorstein and Roth 2001). Most of the HATs are part of large multiprotein complexes that are considered necessary to achieve correct histone acetylation within a nucleosome *in vitro*. Histone deacetylation is catalysed by histone deacetylases (HDAC) and is a rapid phenomenon. The human HDACs have been generally divided into three classes: HDAC1, HDAC2, HDAC3 and HDAC8 in class I, and HDAC4, HDAC5, HDAC6 and HDAC7 in class II. Based on the protein structure, classes I and II correspond to Rpd3 and Hda1 in yeast, respectively. Yeast Sir2, a NAD-dependent HDAC enzyme has been reported to be a new class of HDAC (Guarente, 2000). Two transcriptional co-repressor complexes, SIN3 and Mi2-NuRD (nucleosome-remodelling histone deacetylase), are known to interact with HDAC1/HDAC2 as component molecules (Knoepfler and Eisenman 1999; Ahringer 2000). The MBD3-MBD2 (methylated DNA-binding domain; MBD) interaction recruits the Mi2-NuRD complex to methylated DNA regions. Consequently, DNA methylation and histone deacetylation are co-operatively involved in transcriptional repression. Recent findings suggest that a novel complex called inhibitor of acetyltransferases (INHAT) complex, which binds to histones and masks them from

being acetyltransferase substrates, plays a regulatory role in chromatin modification and transcriptional regulation (Seo *et al*. 2001; Seo *et al*. 2002).

DNA methylation

One of the striking features of the vertebrate genomes is DNA methylation, *i.e.* 5-positioned carbon of cytosine in 5'-CpG-3' dinucleotide is frequently modified by a methyl group. The cytosine residue in complementary 3'-GpC-5' that makes the base pairs is symmetrically methylated, and these two methyl groups show a three-dimensional structure prominent in the major groove of the double-stranded DNA (Ohki *et al*. 2001). Around 60 to 90% of all CpG sequences in the vertebrate genome are methylated, while unmethylated CpG dinucleotides are mainly clustered in the CpG-rich sequence, termed CpG island, of the gene promoter region (Antequera and Bird 1993; Ng and Bird 1999). The fact that 5-methylcytosine, like cytosine, base pairs with guanine means that altered DNA methylation is not a mutation. In the following sections I will focus on DNA methylation as an epigenetic mechanism and its implications in gene regulation during mammalian development and disease.

DNA methylation is an enzymatic modification performed by DNA methyltransferases. Two different types of DNA methyltransferases have been characterized in eukaryotes, *de novo* methyltransferases such as Dnmt3a and Dnmt3b (Okano *et al*. 1998) that use non-methylated DNA as a substrate; and maintenance methyltransferases, such as Dnmt1, that methylate hemimethylated DNA generated by replication of methylated sites (Bestor *et al*. 1988; Bestor 2000). The *de novo* methylation during embryonic stem (ES) cell differentiation *in vitro* involves the *Dnmt3a* and *Dnmt3b* genes and appears to be important for the establishment of methylation marks of imprinted genes (Okano *et al*. 1999). The DNA hypomethylation can lead to chromosomal instability in *Dnmt1*-/- embryonic stem cells and in cells treated with the demethylating agent 5-azadeoxycytidine (Chen *et al*. 1998). The recent investigations of mutants in *Arabidopsis*, mutations of chromatin remodelling factor termed *DDM1* were found to cause hypomethylation of the genome (Mittelsten and Paszkowski 2000). It is thus suggested that *DDM1*-mediated chromatin formation may be a requisite for the maintenance of genome-wide methylation in plants

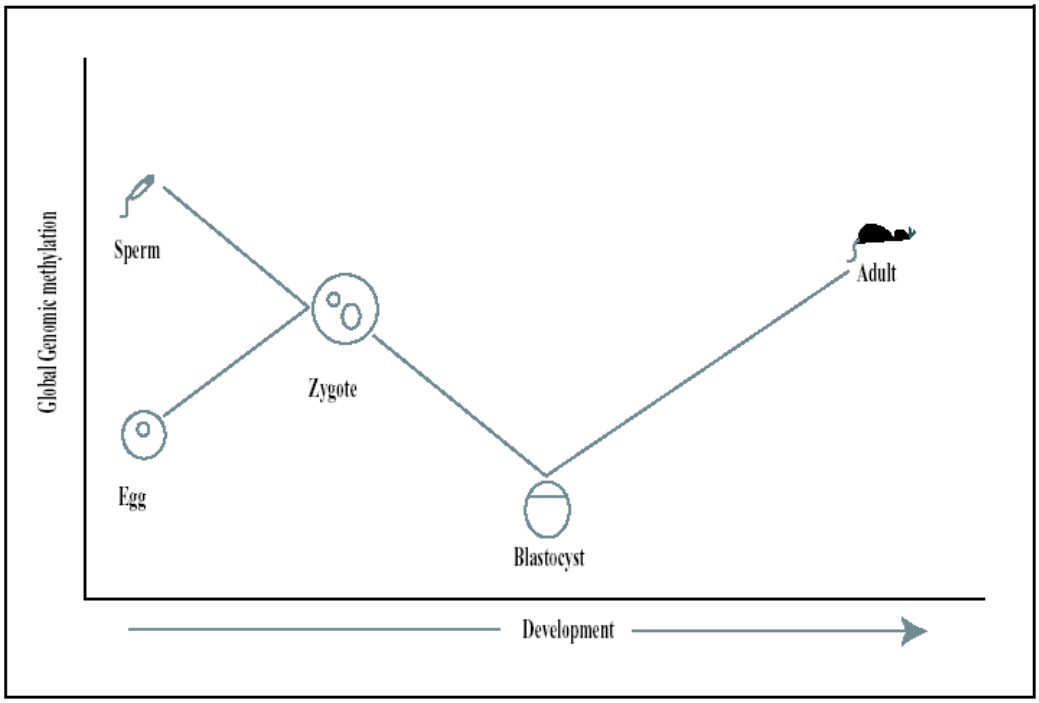
(Bourc'his and Bestor 2002). The human homologue for *DDM1* is *Lsh* (Lymphoid specific helicase), *Lsh* -/- mice shown disruption of genome-wide methylation including the imprinted region of the *H19* gene (Dennis *et al*. 2001). *Lsh* is a member of SNF2 family of chromatin remodelling factors. A comparable factor to *Lsh* called *Dnmt3L* has homology with *Dnmt3a* and *Dnmt3b*, but lacking enzymatic activity (Aapola *et al.* 2001). Interestingly, *Lsh* is essential for the establishing maternal methylation of imprinted genes (Hata *et al*. 2002).

DNA methylation during mammalian development

Methylation of cytosine (5'CpG) is heritable and can be correlated with the potential for a gene to be expressed, with chromatin structure, replication timing, X-chromosome inactivation and imprinting (Bird 1986; for reviews, see Bird 1987; 1995; 1997). The oocyte and sperm genomes are undermethylated compared to somatic tissue, but sperm DNA is more methylated than that of the oocyte (Tada *et al*. 1997; Reik *et al*. 2001). This differential methylation of the sperm and ovum DNA makes it a candidate gametic imprint. Methylation changes have been associated with the appearance of different embryonic and extra-embryonic lineages early in development (Monk *et al*. 1987). The mature male and female gametes acquire differential methylation patterns: These methylation differences are maintained in pre-implantation and raised abruptly during post-implantation (Figure 1) (Chaillet *et al*. 1991). Significant loss of methylation occurs between the 8-cell embryo and the blastocyst stage (Kafri *et al*. 1992). This process may provide a means of easing the gametic epigenetic programme with the exception of individual genetic loci that are subject of imprinting, before the formation of somatic lineages and re-establishment of the appropriate gametic imprint in reproductive tissues. It has been shown that mammalian primordial germ cells, embryonic stem cells, and the blastocyst seem to develop normally without noticeable DNA methylation. However, as soon as stem cells initiate the differentiation process, DNA methylation becomes essential for proper development (Jaenisch 1997). During development, genome-wide methylation of somatic (except extra-embryonic) tissues occurs during implantation, first in the inner cell mass of blastocyst stage and later in the gastrula.

The existence of CpG islands is the most salient feature of vertebrate DNA methylation patterns, that is, unmethylated GC-rich regions that have high relative densities of CpG and are located at the 5' ends of many human genes. The computational analysis of the human genome sequence calculates 29,000 CpG islands (Lander *et al*. 2001; Venter *et al*. 2001). A small proportion of all CpG islands become methylated during development, and when this happens the associated promoter is stably silenced. Developmentally programmed CpG-island methylation of this kind is involved in genomic imprinting and X-chromosome inactivation. The *de novo* methylation events take place in germ cells or the early embryo (Jaenisch *et al*. 1982), suggesting that *de novo* methylation is predominantly active at these stages. There is evidence, however, that *de novo* methylation can also occur in adult somatic cells. A significant fraction of all human CpG islands are prone to progressive methylation in certain tissue types during aging and in cancer cells (Baylin and Herman 2000; Issa 2000) and permanent cell lines (Harris 1982; Antequera *et al*. 1990; Jones *et al*. 1990). However, the underlying mechanisms and consequences for maintaining DNA methylation status, gain and loss are yet to be fully understood.

Figure 1. Relative global methylation changes in early, late embryogenesis and development.



The DNA methylation alone hinders DNA-binding activities of several methylation-sensitive transcriptional factors comprising AP2, CREB, cMyc/Myn, cMyb, CTCF, E2F, NF-κB and ETS. In addition to methylated DNA, methyl-CpG binding proteins are hypothetically required to inhibit transcription by methylation-insensitive transcriptional factors such as Sp1 and YY1. Methyl-CpG binding proteins decipher methylation patterns to mediate interactions between DNA methylation, histone deacetylation, and chromatin components. At present, five families of methyl-CpG binding proteins with a conserved methylated DNA-binding domain (MBD) have been identified. MeCP2, MBD1, MBD2 and MBD3 can be involved in methylation-mediated transcriptional repression, and MBD4 has a DNA glycosylase activity for removing a thymine from T-G mismatch sites (Bird and Wolffe 1999; Ballestar and Wolf 2001). The MeCP1 complex includes MBD2 and other proteins, and some components may be different in cell types.

Epigenetics and disease

Several human diseases have been described that involve epigenetic defects, as listed in Table 1. The DNA methylation defects, for example, includes the ICF (immunodeficiency, centromeric instability and facial anomalies), Rett's and fragile X syndromes (Robertson and Wolffe 2000). It has been observed that the mutations in *Dnmt3b* gene lead to methylation defect in ICF syndrome (Hansen *et al*. 1999; Xu *et al.* 1999). The *MeCP2* gene locates on the X-chromosome mutated in Rett's syndrome patients (Amir *et al*. 1999) and the targeted deletion of *Mecp2* generates Rett's syndrome symptoms (Guy *et al*. 2001). This syndrome is the most frequent of the female neurodevelopmental disorders, with loss of speech, autism, ataxia, erratic hand movements and mental retardation, being recognized from 6 to 18 months after birth. The MBD4 gene is altered in tumors with microsatellite instabilities (Riccio *et al.* 1999). A recent gene knockout strategy in mice found that *MBD3* is required for embryonic development, whereas *MBD2*-deficient mice are viable (Hendrich *et al*. 2001). *MBD2*-deficient cells lack MeCP1 complex and cannot efficiently repress exogenous methylated promoter. However, it is necessary to further analyze the

inactivation of the endogenous methylated gene by MBD-containing proteins, and their functional interrelationship and redundancy *in vivo*.

In Coffin–Lowry syndrome (CLS) individuals there were deletions, nonsense and missense mutations in the gene encoding *RSK2*, part of a family of growth factor-regulated serine/threonine kinases acting distally in the ras-dependent MAPK signalling cascade (Trivier *et al.* 1996). The *RSK2* is required for phosphorylation of H3 during the nucleosomal response stimulated by epidermal growth factor (EGF) and shown that purified RSK2 can phosphorylate H3 on Ser10 *in vitro* (Sassone-Corsi *et al.* 1999), CLS individuals are deficient in this histone modification. The X-linked α-thalassemia/mental retardation syndrome (ATR-X syndrome) has been shown to result from mutations in the *ATRX* gene located at Xq13 (Gibbons *et al*. 1995).

Mutations and/or uniparental disomy (UPD) in imprinted genes are not inherited in a regular Mendelian fashion. Three congenital human disorders are known to be origin by errors in the expression pattern of imprinted genes: Prader-Willi syndrome (PWS), Angelman syndrome (AS) and Beckwith-Wiedemann syndrome (BWS) (Cassidy 1997; Clayton-Smith and Pembrey 1992; Li *et al*. 1997). In addition to these, a number of cancers are also caused by errors in imprinted genes (Ohlsson 1999).

Table 1. Epigenetics and human diseases

Features	Disease
Imprinting	
IGF2, IGF2R	Wilm's tumor (*WT1*), Beckwith-Wiedemann syndrome, Hepatoblastoma
SNRPN, UBE3A	Prader-willi and Angelman syndrome
X-linked	
FMR-1	Fragile X mental retardation
X-inactivation centre	Functional disomy of X-linked genes
DNA methylation	
DNMT3b	ICF syndrome
MBD2	Colon cancer
MBD4	Tumors with microsatelite instability
MeCP2	Rett's syndrome
Chromatin modifications	
a) Histone acetylation	
CBP	Rubinstein-Taybi syndrome
MLL-CBP	Leukemia
MOZ-CBP	Acute myelocytic leukemia
p300	Colon cancer, Gastric cancer and tumors
b) Chromatin remodelling	
ATRX	α-Thalassemia
BRG1	Tumors
HSNF5/Ini-1	Rhabdoid tumor
Mi2	Auto-antibody in dermatomyosis
MTA1	Metastatic potential of cancer
c) Phosphorylation of H3	Coffin-Lowry syndrome
Transcriptional control	
PML-RARα	Acute promyelocytic leukemia

Transcription therapy

Gene transcription process requires a transcription factor that recognizes and binds to a specific DNA sequence associated with a particular gene and by causing the activation or repression of that gene. Transcription factors generally consist of two components: DNA binding domain that recognizes a specific DNA sequence and thereby directs the transcription factor to the proper chromosomal location; and a functional domain that determines whether the gene is activated or repressed.

Human genome projects have revealed a large number of novel genes whose functions have yet to be determined (Lander *et al*. 2001; Venter *et al*. 2001). Assigning biological functions to these newly identified genes and genes involved in specific diseases are currently one of the most important research objectives. This process is often divided conceptually into two steps: target discovery and target validation. The engineering of a particular class of transcription factors known as zinc finger DNA-binding proteins (ZFPs) can regulate candidate disease genes will help to facilitate target validation.

Table 2. Outline of designed ZFPs diverse applications

Technology	Application	Advantage
Diagnostics	DNA Diagnostics Polymorphism Detection	-Fast, Inexpensive Sensitive, Reliable
Genomics	Functional Genomics - Gene Discovery - Target Validation	-Allows both up and down regulation of target genes
Therapeutics	Molecular Medicine - Transgene Therapy	- Highly efficient gene regulation
Transgenic	Agricultural & Industrial Biotechnology in genetically modified organisms - Metabolic engineering in microbes, plants and animals	- Conditional and reversible regulation - Targeting endogenous genes

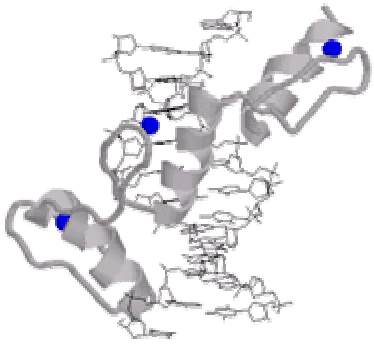
The designed ZFP technology can also be used to generate genetically modified organisms in which the expression of endogenous genes is regulated by zinc-finger transcription factors. The summary of designed ZFPs applications and

advantages are highlighted in table 2. The ZFPs act as highly efficient and specific genetic switches in cells (Kim *et al*. 1997a; Kim and Pabo 1997b; Kim and Pabo 1998) and are capable to both up- and down-regulate target genes (Beerli 2000). Zinc finger proteins can be targeted to endogenous genes (Choo *et al*. 1994; Zhang *et al*. 2000) to achieve both conditional and reversible gene transcription (Kang and Kim 2000).

ZFP interaction with DNA motif

Zinc finger transcriptional factors have been identified in a wide variety of organisms, including microbes, fungi, plants, animals, and humans. Unlike other DNA binding domains, zinc fingers are highly adaptable with respect to DNA recognition; naturally occurring zinc finger proteins demonstrate varied DNA-binding specificities, recognizing many different DNA sequence elements. Using powerful design and selection methods, its possible to construct novel zinc finger proteins that bind to nearly any predetermined DNA sequence of 10 to 20 nucleotides in length. When these zinc finger proteins are fused to a transcriptional activator or repressor domain, sequence-specific transcriptional regulatory proteins are produced having ability to recognize a single target gene within the entire genome.

Figure 2. Zinc finger protein bound to DNA



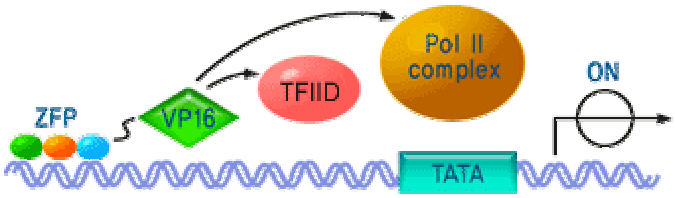
The first structural framework for understanding how zinc fingers recognize DNA motif by Pavletich and Pabo provided a useful basis for the designing of novel DNA-binding proteins (Pavletich and Pabo 1991; Figure 2, with kind permission of the publisher). Study shows that the three zinc fingers bind similarly in the major groove of B-DNA and their amino-terminal portion of an alpha helix makes primary contacts in a three-base pair target wrapping around the double helix.

Mechanisms of ZFP gene switches

The designer transcription factors would then be expressed *in vivo* and can function specifically to switch target genes on or off (see Figure 3, reprinted with kind permission from Kangsik Yun). The ZFPs can be fused to a transcriptional activation or repression domain to create designer transcription factors. These designer transcription factors can be used in a variety of systems to turn genes of interest on or off. Efforts to design proteins that regulate specifically the expression of disease-associated genes represent a logical therapeutic strategy. The envisions that designer genes encoding ZFPs that target disease-associated genes can be delivered to patients via appropriate gene therapy protocols might however be compromised by epigenetic barriers (Yann Jouvenot *et al*. 2002).

Figure 3. Use of designer transcription factor as "gene switches"

a) Activating transcriptional initiation by designer transcriptional activator protein (ZFP-VP16)



b) A designer transcriptional repressor protein (ZFP-KRAB) interferes with transcriptional activator system

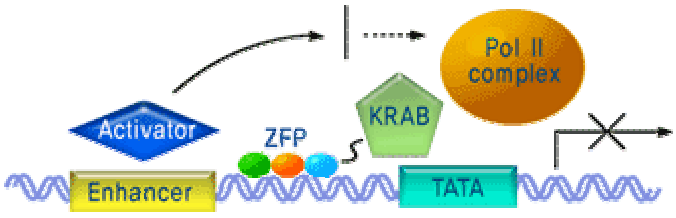


Figure 3 a and b: VP16: transcriptional activation domain; **TFIID**: transcription factor required for transcription initiation; **Activator**: transcriptional activator protein; **KRAB**: transcriptional repressor domain; **Pol II complex**: RNA polymerase II

Genomic Imprinting

One of the most intriguing discoveries in mammalian genetics during the last decades is genomic imprinting. Imprinting is the epigenetic process that differentially modifies the parental alleles at certain genetic loci and is generally considered to occur in gametogenesis. Such epigenetic modifications of DNA and chromatin are somatically heritable and generate the uneven expression of the parental alleles during development. Thus, imprinted genes are expressed monoallelically with one parental allele being persistently silenced. About fifteen years ago, the first evidence of imprinting was documented (Barton *et al*. 1984; McGrath and Solter 1984; Surani *et al*. 1984), although the formal demonstration of imprinted genes was not achieved until 1991 when the selective maternal expression of *Igf2r*, the paternal expression of *Igf2* and the maternal expression of *H19* in mice were reported by different research groups (Barlow *et al*. 1991; Bartolomei *et al*. 1991; DeChiara *et al*. 1991). Nevertheless, to my surprise the parent-of-origin effects were evidently familiar by the mule breeders in Asia Minor >3000 years ago (Savory 1970).

So far, over 50 imprinted genes have been identified in the human and mouse genomes (http://www.mgu.har.mrc.ac.uk/imprinting/imprin-viewdatagenes.html), with estimates that these genes account for 0.1% of genes in the mammalian genome (~50-100). Imprinting has been shown to exhibit roles in development, sex determination, mammalian evolution, cancer and the inheritance of certain genetic diseases. A number of imprinted genes are associated to genetic disorders in human (Morison and Reeve 1998). However, genomic imprinting is not the only principle that generates monoallelic expression. Both the autosomal and the sex-linked genes that include immunoglobuiln, Olfactory receptor, T cell receptor and X-inactivated genes may undergo monoallelic expression with random parental allele expression; where as imprinted genes are atypical, autosomal genes expressed predominantly non-random manner *i.e* either the paternal or maternal inherited in somatic cells, reviewed in Table 3 (Chess 1998; Ohlsson *et al*. 1998; Ohlsson *et al*. 2001a).

Imprinted genes put forth imperative effects principally on foetal development and their deregulation is implicated in a variety of pathologies comprising sporadic, inherited and induced growth disorders. Imprinted genes are distinctly modified during gametogenesis and each parentally derived allele functions differently in the early-fertilized zygote. The sperm cell compared to the ovum undergo differential packaging also plays an important role. The chromatin structure and epigenetic mechanisms superimpose on the sequence of a gene to determine the timing, location and level of expression. These modifications, rather than genetic divergence of the gametes, are likely to be the signal that marks the sperm and egg alleles as different.

In general the imprinted loci illustrate a number of atypical structural and functional features that may be associated with the mechanistic facets of mono-allelic expression or to modes of their evolution. Distinctively, imprinted genes are clustered in certain genomic regions and associated regulatory regions normally have a mixture of features comprising tandem repeats concurrently with differentially methylated CpG islands and overlapping transcription of coding or non-coding RNAs.

Table 3. An overview of monoallelically expressed genes

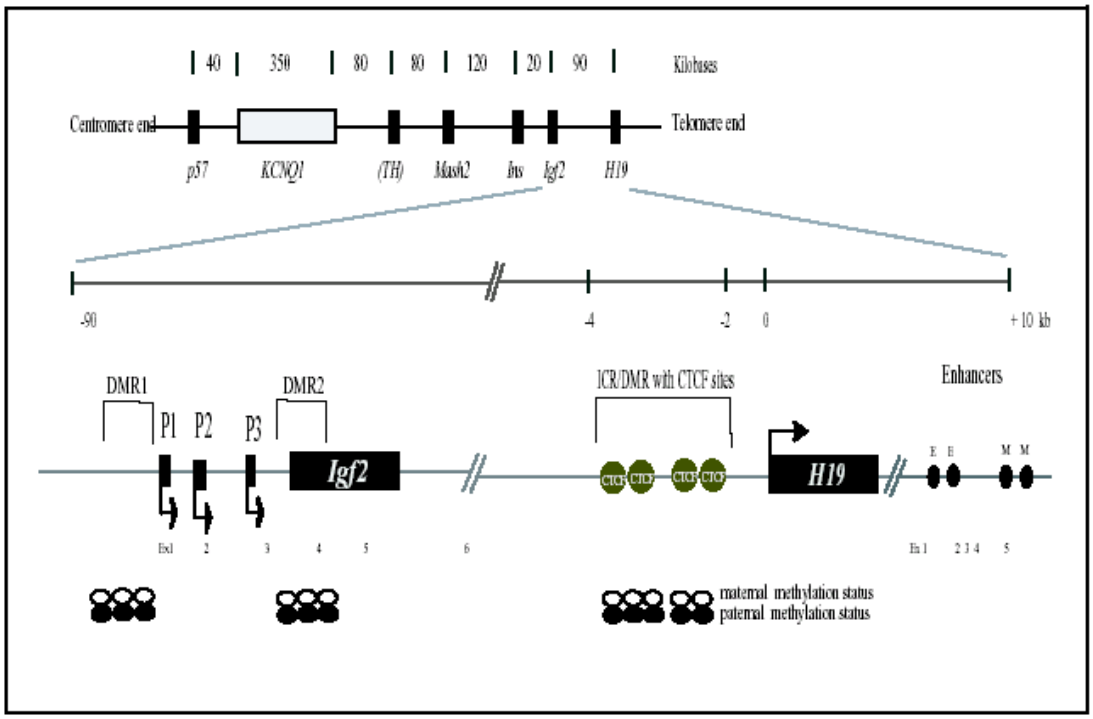
Gene	**Chromosome**	**Parental allele**
Imprinted genes	Autosomal	Nonrandom
X-inactivated gene	X-linked	Random (embryo), non-random (placenta)
T cell receptor genes	Autosomal	Random
Olfactory receptor genes	Autosomal	Random
NK cell receptor genes	Autosomal	Random
Cytokine genes	Autosomal	Random
Immunoglobulin genes	Autosomal	Random

In the following sub-sections I will try to expound different aspects of genomic imprinting including mechanisms, disease and its evolutionary significance in general and with a special focus on the well-studied *Igf2-H19* imprinted loci.

Regulation of Igf2 and H19 imprinted region

Well-studied insulin-like growth factor 2 (*Igf2*) and *H19* genes in mouse and human are reciprocally imprinted. The *H19* gene in maternal chromosome is preferentially transcribed while *Igf2* is inactive; whereas the *H19* in paternal chromosome is silent and *Igf2* is active. The maternally derived *H19* transcript does not encode any protein. The two genes are separated by ~90 kb on human chromosome 11p15.5 and mouse distal chromosome 7, and show remarkable co-regulation (Reik and Walter 1987). During development, both genes share regulatory elements that are located upstream and downstream of *H19.* Two sets of endoderm-specific enhancers and mesoderm-specific enhancers are located ~10 kb downstream of *H19* and these have been shown to regulate the expression of *H19* as well as *Igf2* (Leighton *et al*. 1995; Kaffer *et al*. 2000). The imprinting regulatory domain could be narrowed down to ~4 to 2 kb upstream of *H19,* and consists of the *H19* promoter, which is separated from a differentially methylated "imprinting control region" (ICR) further upstream by a G-rich repeat. The ICR located 2kb upstream of the mouse/human *H19* gene has been a major spotlight of research, due to its vital role in *Igf2-H19* imprinting (see Figure 4) (Tremblay *et al*. 1995; Jaenisch 1997; Lyko *et al*. 1997; Tremblay *et al*. 1997; Thorvaldsen *et al*. 1998; Brenton *et al*. 1999; Frevel *et al*.1999; Drewell *et al*. 2000; Kanduri *et al*. 2000a). The *H19* ICR is hypomethylated on the maternal allele, whereas the paternal allele acquires a methylation imprint during spermatogenesis and maintained in later development (Tremblay *et al*. 1995). The deletion of entire ICR results in loss of imprinting (Elson *et al*. 1997). However, the conditional deletion of the paternal ICR in differentiated skeletal and cardiac muscle cells did not result in activation of the paternal *H19* allele or changes in the methylation of the *H19* promoter region (Srivastava *et al*. 2000). The maternal ICR harbours several hypersensitive sites and functions as a unidirectional insulator, which is regulated by CpG methylation (Kanduri *et al*. 2000a; Holmgren *et al.* 2001). Therefore, the ICR appears to be crucial for the establishment of the methylation imprint and the silencing of the paternal *H19*, and maternal *Igf2*. While the ICR is necessary for the continuous silencing of the maternal *Igf2* allele, it is not vital for the maintenance of paternal *H19* silencing.

Figure 4. Physical structure of the *Igf2-H19* region in the mouse. The ecto-(E), meso-(M) dermal enhancers and the four CTCF binding sites of the imprinting control region (ICR) are indicated in ovals. Maternal and paternal differentially methylated status is shown in open (un-methylated) or closed (methylated) circles.



The 1.2 kb long element in ICR that exhibits silencing effects in *Drosophila* (Lyko *et al*. 1997) is necessary for the imprinting of *H19* reporter transgenes in mice (Brenton *et al*. 1999). Paternally inherited deletion of this element resulted in partial loss of silencing (Drewell *et al*. 2000), whereas the maternal *Igf2* was not reactivated by maternal inheritance of the deletion, which suggested the presence of a boundary element in the ICR. Recently, binding sites for a methylation-sensitive protein CTCF having a 11-zinc finger DNA binding domain (Ohlsson *et al*. 2001b) have been identified within the boundary element (Bell *et al.* 2000, Hark *et al.* 2000, Kanduri *et al.* 2000b and Szabo *et al*. 2000). This boundary element is insulating the interacting *Igf2* from the enhancers downstream of *H19*. Methylation-sensitive binding of CTCF prevents binding to methylated paternal allele (Holmgren *et al*. 2001), thereby allowing induction of *Igf2* expression by interaction with the *H19* downstream enhancers. The deletion of *Igf2* DMR1 results in loss of imprinting and biallelic

expression of *Igf2* in most mesodermal tissues but did not affect *H19* expression (Constancia *et al*. 2000). Similarly, the deletion of 40 kb skeletal muscle-specific repressor downstream of *Igf2* resulted in biallelic expression of *Igf2* in skeletal muscles but did not effect *H19* expression (Ainscough *et al*. 2000). This specific activation of the maternal *Igf2* allele could reveal the location of the boundary that separates this muscle-specific repressor from the *H19* promoter, and hence causes tissue-specific differences in *Igf2* expression, but leaves *H19* unaffected.

Evolutionary significance of imprinting

The evolution of imprinting can be understood as the stable outcome of sexual selection acting differently on the parental alleles of genes that influence parental investment in offspring. Consistent with this explanation, imprinted genes are expressed predominantly during embryonic and postnatal development in mammals and in the developing endosperm of plants. Maternal or paternal expression at imprinted loci is associated with reduced or increased parental investment, respectively. Such selective forces have implications for understanding mechanistic aspects of genome reprogramming in the early mammalian embryo. Hypothetically, genomic imprinting has evolved because an embryo's parental genome is selected to receive more resources from maternal tissue (through placenta) than is the embryo's maternal genome (Haig and Trivers 1995). Paternal imprinted genes will therefore be designed to take out maximum resources from the mother while maternally imprinted genes will limit such transfer of resources (Moore and Haig 1991).

AIMS OF THE PRESENT INVESTIGATION

The aim of this thesis focused on the chromatin structure and gene expression at imprinted *Igf2-H19* loci, characterise the *cis* and *trans* acting factors and their modifications role in gene regulation. Specifically the study was concentrated on answering the following questions.

- Does maternal and paternal imprinting control region of *H19* has differential chromatin structure?

- What are the mechanistic features of the *H19* ICR and its role in differential expression of the *Igf2-H19*?

- Is there any cis regulatory element in ICR or the spacer between ICR and *H19* promoter and is the conserved spacer distance required for proper *Igf2-H19* expression?

- Does the chromatin insulator CTCF or other factors at *H19* ICR undergo post-translational modifications and if so what are the implications?

- Is it possible to override the silenced imprinted gene regulation and correct epigenetic lesions?

RESULTS

Paper I

Maternal-specific nuclease hypersensitive sites (NHSSs) found at H19 ICR.

Here we used intraspecific mouse crosses *i.e*. *Mus musculus musculus* (Mus or M) and *Mus musculus domesticus* (Dom or D) to elucidate the chromatin conformation status at *H19* differentially methylated imprinting control region (ICR). A polymorphic restriction endonuclease *Bbs*I site in ICR specifically present in *M.m. musculus* allele was used in this study to distinguish maternal or paternal specific ICR. The micrococcal nuclease (MNase) treated DNA was digested with restriction enzymes *Afl*III, *Bbs*I and *Bcl*I. The indirect end-labelling method identified specific hypersensitive sites present only on maternal ICR. These maternal-specific nuclease hypersensitive sites (NHSSs) were further fine mapped, revealing that maternal ICR harbours two regions of strong nuclease hypersensitive sites called NHSS I and NHSS II and a third weaker one NHSS III. It was observed that MNase has no target preferences *in vitro* to methylation status of the ICR, and no difference was found between *in vitro* methylated ICR fragment and a purified control when subjected to MNase digestion.

The NHSSs in *H19* ICR mapped to the linker regions between the positioned nucleosomes. In order to study whether or not the nucleosome positioning and hypersensitive sites are organized by default, we developed an episomal assay with 6.5 kb *H19* minigene. The episomal vector (pREPH19), having 3.5 kb of the 5' flanking region and 0.8 kb of the 3' region covering entire ICR with *H19* promoter was transfected into human hepatoblastoma cell line (Hep3B). We conducted indirect end-labelling experiment in stably transfected cells propagated for three months, the ICR of the episomal vector exhibited the hypersensitive sites and nucleosome positioning as well. Similar results were obtained with human chorio carcinoma cell line (JEG-3) in cells propagated just a week. There was no difference in the NHSSs positions, generated by nuclease treatment between *in vivo* maintained and transfected cells of *H19* 5' flank. Methylation analysis by bisulphite sequencing and Southern analysis

showed that pREPH19 remained largely unmethylated during the propagation of the transfections.

The maternally inherited NHSSs in H19 DMD function as an insulator element.

The *H19* minigene was used as reporter gene in the pREPH19 plasmid construct. Several other plasmids were generated with or without SV40 enhancer and ICR to 3' of the *H19* gene (Figure 5a, Paper I). The transient transfections of several episomal vectors carrying the *H19* minigene were examined to see if ICR harbours any intrinsic silencing activity and to find out if the ICR could act an insulator. To analyse the putative silencing function, the constructs pREPH19 and pREPH19H (ICR deleted) were transfected and selected for stable episomal uptake into Hep3-B cell line. Ribonuclease protection assays showed no difference in the levels of reporter gene expression between these vectors. Challenging the promoter with SV40 enhancer (pREPH19G and pREPH19A) positioned downstream of the *H19* minigene resulted in more than 200 fold up-regulation of *H19* expression.

Next, we tried to answer the second question, if maternal *H19* ICR functions as an insulator or boundary element by blocking promoter-enhancer interaction. A new episomal construct (pREPH19B) was made from pREPH19A by adding an additional ICR placed between the *H19* minigene and SV40 enhancer. If the maternal ICR functions as an insulator, it would inhibit the communication between the SV40 enhancer and the *H19* promoter. The total RNA collected from transfected JEG-3 cells propagated for 7 days was subjected to RNase protection assay. The results showed a significant down regulation of *H19* expression in pREPH19B where the ICR was positioned in between the *H19* promoter and SV40 enhancer. The control having a neutral fragment in place of ICR in between the promoter and the enhancer (pREPH19D) showed a normal high level of *H19* expression. This result indicated that the maternal unmethylated ICR allele was responsible for the down-regulation of *H19* expression in pREPH19B construct. Finally the ICR directionality was examined by inverting the orientation of the 3' ICR (pREPH19C) in between the *H19* promoter and SV40 enhancer. Surprisingly, this construct did not inhibit the SV40 enhancer from

activating the *H19* promoter, conveying that ICR functions as a position and orientation-dependent insulator.

Paper II

A repressive property of the H19 ICR is position-dependent.

In this article, we focused on the ~2 kb region that separates the *H19* ICR from the neighbouring *H19* promoter. Strikingly, the ICR possesses a silencer function in promoter-proximal, but not in distal positions. Transient transfections of different CAT reporter vectors carrying *H19* ICR and *PDGF-B* promoter, revealed that the basal activity of the *PDGF-B* promoter was reduced 5-fold when ICR was inserted in either orientation in a promoter-proximal position. The silencing effect was relieved when the natural spacer was inserted between the *H19* ICR and *PDGF-B* promoter. Similar results were obtained when the spacer-*H19* ICR fragment was reversed. Hence, we conclude that the effect of spacer on the ICR silencing property is position-dependent, but not orientation-dependent.

We also examined the performance of ICR silencing in these constructs following treatment with trichostatin A (TSA), a histone deacetylation inhibitor. The TSA treatment enhanced basal activity of *PDGF-B* promoter 10-fold, but little effect was noticed when *PDGF-B* promoter was placed immediately following ICR. We further examined the histone acetylation status at *PDGF-B* promoter region with chromatin immuno-purification (ChIP) assays in co-transfected JEG-3 cells (+/- TSA) of ICR with and without spacer followed by *PDGF-B* promoter. The immuno-purification was performed using the antibodies against the acetylated forms of histones H3 and H4 pulled down sequences specific regions of natural spacer. The results show that the proximity of ICR directly controls acetylation status at the promoter of the reporter gene.

The Insulator domain of Igf2/H19 organizes a distance-dependent silencer.

To examine the minimal distance effect, we generated a series of deletion mutants of the natural spacer region that successively positioned the ICR closer to the *PDGF-B* promoter. The *H19* ICR repression effect was abolished when positioned more distally

from the promoter, the critical distance mapped between 856 and 1456 bp. The supporting positive control for this experiment was made from 1.94 kb anonymous spacer inserted in place of natural spacer, the results showed that the repressor function was abrogated in this instance. Further, we examined a section of the ICR fragment that harbours two different CTCF target sites, which independently and together acted as silencers in promoter-proximal position with or without TSA. Interestingly the mutated CTCF construct (pSISS1S2) and 5' region of CTCF target sites (pSISNH) revealed that the silencer function does not require the CTCF target sites.

Paper III

Maternal-specific Poly(ADP-ribosy)lation at H19 imprinting control region.

We addressed here how poly (ADP-ribosy)lation, a post translation modification mark, is associated with the *H19* ICR alleles. The *H19* ICR's distance-dependent silencing (Ginjala *et al*. 2002) might be the cause of poly (ADP-ribosy)lation, creating bulky chromatin complex. To this end, we performed chromatin immunopurification (ChIP) assays of allele specific distribution of poly(ADP-ribosy) lated protein/s, using a highly specific antibody against polymers containing 10 or more ADP-ribose units. Mouse foetal liver cells of intra-specific hybrid crosses of *M.m.domesticus* and *M.m. musculus* was used for ChIP assay, the PCR analysis showed that ADP-ribosylated proteins interact specifically with the maternal *H19* ICR allele.

Poly (ADP-ribosy)lation of CTCF

Since the chromatin insulator protein CTCF interacts only with maternal *H19* ICR allele similar to that of maternal specific poly(ADP-ribosy)lation mark, we next examined the possibility that CTCF was poly(ADP-ribosy)lated. This was explored by immunoprecipitation analysis from human cancer cell line T47D with Western blot where CTCF and PAR antibodies recognised a common band of ~140 kd. We conclude that CTCF is PARlated *in vivo*. It was also found that N-terminal portion of the CTCF is preferentially poly(ADP-ribosy)lated, leaving the DNA-binding zinc finger domain unmodified. Interestingly the immunoaffinity-purified poly(ADP-ribosy)lated CTCF efficiently interacted with one of the CTCF target sites within the *H19* ICR, showing that PARlation did not compromise its DNA-binding properties.

Poly(ADP-ribosy)lation is required for H19 insulator function.

Based on the above results we next asked if poly(ADP-ribosy)lation is essential for the *Igf2/H19* insulator domain function. To resolve this question efficiently, we developed a new assay based on a green fluorescent protein (GFP) reporter gene in control of human *IGF2* promoter 3 and equipped with a destabilization signal. Flow cytometry analysis of JEG-3 cells transfected with various vectors (Figure 3c, Paper III) with an internal control of red fluorescent protein (RFP) reporter for plasmid transfection efficiency has revealed the GFP signal was extinguished when *H19* ICR (pB-GFP) was between the promoter and SV40 enhancer. This effect was dramatically relieved by poly(ADP-ribose) polymerase inhibitor 3-aminobenzamide (3-ABA) treatment for 14 hours of the 48 hour transfected cells. The normally low number of GFP expressed cells, when transfected with pB-GFP, was augmented to more than 50 fold after 3-ABA treatment to reach 68% of the GFP positive cells transfected with *H19* ICR-less plasmid (pA-GFP). The 3-ABA treatment did not enhance GFP expression in control constructs with or without SV40 enhancer (pA-GFP and pO-GFP). These results imply that the *H19* ICR insulator role depends on poly(ADP-ribosy)lation. Directness of this effect was assessed by ChIP assays followed by real time PCR analysis; two independent experiments showed that 3-ABA treatment robustly reduced the poly (ADP-ribose) content of the *H19* ICR, while CTCF association remained mostly unaltered.

Paper IV

The imprinted gene regulation through engineered zinc-finger transcription factors.

In this publication, we have engineered synthetic zinc-finger proteins (ZFPs) that specifically interact with the sequences within the imprinted *IGF2/H19* domain and selectively regulate these genes. Here we show that a designed ZFP can both positively and negatively regulate the expression of the *IGF2/H19*. The specificity of ZFPs activation or repression depends upon the functional domain used and the direct interaction at specific sites proximal to the relevant promoter/s. A synthetic transcription factor named ZFP809 was particularly potent in reactivating the transcriptionally silent and naturally imprinted alleles *IGF2/H19*. Our results suggest

that the designed ZFPs can be exploited to develop a rapid and robust therapeutic method for controlling epigenetic lesions.

The transcription factor "ZFP809" can activate and repress the IGF2 and H19.

The target promoters were scanned for 9-bp recognition sequences by a previously reported library of validated 3-finger ZFP transcription factors (Liu *et al.* 2002). Five different synthetic ZFPs were chosen based on a binding site within ± 500bp of one or more of the four *IGF2* promoters having the dissociation constant of <100 pM. To promote gene activation, the VP16 activation domain was fused to these DNA-binding moieties. The selected ZFPs, encoding plasmids transfected in to human embryonic kidney cells (HEK 293), increased the level of *IGF2* mRNA between 1.8 to 50 fold as compared to the control plasmid with no zinc finger domain. The ZFP809 has achieved highest level of activation having high affinity to the target site *in vitro*. The chimera of p65 activation domain with ZFP809 without functional domain failed to produce *IGF2* mRNA to the levels of VP16, suggests the importance of functional domain.

A binding site for ZFP809 was also found downstream of the *H19* transcription start site. Real time RT-PCR analysis with target primers and probes for this region of the *H19* gene reveals that both the VP16 and p65 of ZFP809 are able to increase *H19* expression 40-120 fold respectively, and the presence of functional domain is essential for this up-regulation. For the ZFP-driven repression studies, we have used a carcinoma-derived cell line U2OS, which expresses a significantly higher levels of *IGF2* and *H19* as compared to low basal level expression in HEK293 cells. The ZFP DNA recognition domain was linked to the v-ErbA transcriptional repressor protein. A stable U2OS cell line was generated to express the ZFP transcription factor under the control of a tetracycline repressor (TetR), which negatively regulated the CMV promoter. Doxycycline (DOX) treatment of these cells released the CMV promoter from TetR repression and allowed activation of the ZFP transcription factor. Over a 48 hr of DOX treatment concentrations from 0 to 2ng/ml to the cells induced the ZFP809-vErbA expression. The *IGF2* and *H19* steady-state levels are strongly repressed under these conditions: The *H19* and *IGF2* RNA levels were repressed to 11% and 20% of

its starting level in non-induced cells respectively. The repression was observed for both genes correlated well with the increasing levels of DOX treatment.

The designed transcription factor "ZFP809" interacts with IGF2/H19 promoters.

Chromatin immuno-precipitation (ChIP) assay was performed exploiting the flag epitope carried by the ZFP transcription factor to demonstrate the binding of ZFP809 to the consensus sites in the *IGF2* and *H19* promoter regions. DOX concentration increased selective enrichment of ZFP809 in the *IGF2* and downstream *H19* promoter regions. Concomitant with enrichment of factor, the robust repression of *IGF2* and *H19* was observed at the highest DOX concentration used (2ng/ml). The two independent control (*GAPDH* and *VEGF-A*) fragments that do not contain consensus-binding sites for ZFP809, showed no significant enrichment upon ZFP induction. Hence, these results confirm the *in vivo* binding of ZFP809 to the proposed target binding sites.

The reactivation of imprinted IGF2 and H19 alleles endogenously, by ZFP809.

With the help of restriction site polymorphism at exon V of the *H19* gene in cultured HEK293 cells, we asked the question if it was possible for ZFP809 to overcome the imprinted state of *IGF2* and *H19*. Normally HEK293 cells maintain mono-allelic expression of *H19* and *IGF2*. The results from transfection of ZFP809 linked to VP16 in HEK293 cells demonstrate a strong increase of both the parental *H19* alleles. As a control, non-transfected HEK293 cells showed mono-allelic expression of *H19* and *IGF2*.

In addition to the above experiment, we examined the reactivation of *IGF2* and *H19* in murine hybrid cell line containing a single human chromosome 11 of known parental origin, having mono-allelic expression status of both *IGF2* and *H19*. The transfection of ZFP809 linked to the VP19 into maternal/paternal murine hybrid cell line confirmed the previous analysis of *H19* expression. We could observe the reactivation of the silent alleles of *H19/IGF2* in the cell line having only the paternal/maternal copy of the chromosome 11 respectively. These results evidenced that the engineered transcription factor ZFP809 is capable of reactivating the epigenetically silenced states of two imprinted genes.

DISCUSSION AND CONCLUSIONS

In **Paper I**, we addressed diverse characteristics of the imprinting control region or differentially methylated domain of mouse *H19* maternal allele. The studies conveyed maternal specific hypersensitive sites, nucleosome positioning and chromatin insulator as major features engaged in a key task of organizing the imprinted state of the *H19* and *Igf2* genes. The foremost significance in the study is that the 5' flank of *H19* ICR classified the differential chromatin conformation between paternal and maternal ICR alleles. The multiple hypersensitive sites were found exclusively in the un-methylated maternal ICR, which has unidirectional enhancer-blocking activity. Although the hypersensitive sites we have found in these studies are also supported by others (Hark *et al*. 1998, Khosla *et al*. 1999), the chromatin conformation characteristics recently reported (Hark *et al*. 2000; Khosla *et al*. 1999) that the 5' flank H19 is nucleosome free is now ruled-out (Kanduri *et al.* 2002). This can be explained with experimental disparity, as our indirect end-labelling approach with short probe size corresponding to one nucleosome linker unit, offered a higher resolution to argue against earlier interpretations (Khosla *et al*. 1999) based on the use of long probes.

The unidirectional insulator feature of the *H19* ICR in this study contradicts with previous findings (Lyko *et al.* 1997) where the mouse ICR functions as a bi-directional silencer in transgenic *Drosophila* embryos; this could be due to presence/absence of the decisive factors. The feasible role of the insulator could be to obstruct the communication between the *H19* enhancers and the upstream *Igf*2 gene on the maternal allele in a methylation-sensitive manner, thus silencing the maternal *Igf2* gene. A note worth to mention is the existence of a 21-base repeat sequence in hypersensitive linker regions conserved between the 5' flank of mouse and human *H19* ICRs (Stadnick MP *et al*. 1999). The recent studies show that the target methylation-sensitive binding of transcription factor CTCF is to the conserved 21-base region of *H19* DMD (Bell *et al.* 2000, Hark *et al.* 2000, Kanduri *et al.* 2000b and Szabo *et al*. 2000). CTCF is also reported to play a role in chicken β-globin (Bell *et al*. 1999) and *apoB* (Antes *et al*. 2001**)** gene regulation.

In **Paper II**, we documented that the *H19* ICR possesses a silencer function in proximal to it's promoter, but not in distal position or *H19* ICR represses *cis*-regulatory elements in a distance-dependent mode but autonomous of the intervening DNA sequence. The results strongly suggest that the conserved distance or space between the ICR and the *H19* promoter in mouse and man suggests a pivotal function in transcriptional activity. The mouse and human *H19* ICRs are separated by ~2kb requisite "spacer" in spite of no similarity in the sequence it self to circumvent adverse consequence on the *H19* expression. Our finding illustrates that the minimum distance for defusing the silencer function was 1.34 kb between the *H19* ICR and the promoter of the reporter gene and the silencing motion was TSA-insensitive. This could be explained in a way that the *H19* ICR-specific recruitment of a TSA-insensitive histone deacetylase that locally alters the histone acetylation status to organize a repressive chromatin conformation.

The ICR-spacer deletions suggest the minimal distance required for strongest silencing, these findings raise the question whether there is another unknown group of histone deacetylases still to be defined, those that are responsible for the positive effect on silencing and whose activity inhibitors like TSA can't overcome. The deletion and mutation analyses reveal that the silencing feature may not entirely depend on CTCF target sites; nevertheless this situation may be less probable. The multiple *cis* elements including the CTCF target sites within the *H19* ICR together organize a chromatin conformation that sterically prevents accessibility of regulatory factors to juxtaposed promoter and enhancer elements, might both arrange a protecting guard around the maternal *H19* allele against *de novo* methylation.

Paper III focussed on a form of post-translation modification of transcription factor CTCF structure, involving the transfer to protein multiple copies of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) and how these modifications affect the *Igf2*/*H19* insulator complex. Our chromatin immuno-purification assays of mouse foetal liver cells from intra-specific hybrid crosses of *M.m. domesticus* and *M.m. musculus* were immuno-purified using a highly specific antibody against polymers containing 10 or more poly ADP-ribose units. The PCR analysis of immuno-

purified DNA suggests that poly(ADP-ribosy)lated protein/s interact with exclusively maternal *H19* ICR allele. Since the chromatin insulator protein CTCF also interacts with only unmethylated maternal *H19* ICR allele, we asked if CTCF is PAR modified; *in vitro* modification assays followed by Western blot analysis revealed a 140 kd band, which was recognized by both antibodies against poly(ADP-ribose) and CTCF. Following DNA binding assay showed PAR modified CTCF capably interacted with one of the CTCF target sites within maternal *H19* ICR allele. Further flow cytometry analysis of JEG-3 transfected with GFP reporter vectors explains poly (ADP-ribosy)lation is indispensable for insulator function.

The recent reports (Hori *et al*. 2002, Tada *et al*. 2001) have shown a dyad Oct-binding sequence (DOS) maintains the maternal specific unmethylated state of at least one CTCF-binding site within the *H19* ICR allele at post-implantation stages. It has been also shown that Oct-1 requires the PARP-1 (poly ADP-ribosyl polymerase) to stabilizes the binding of Oct-1 to the octamer motif (Nie *et al.* 1998), in addition to this PARP-2 gene transcription controlled by Oct-1(Amé *et al*. 2001). In view of these findings, there is a possibility that the insulator protein CTCF which is poly(ADP-ribosy)lated and DOS both are required to maintain insulator complex and unmethylated state of maternal *H19* ICR allele. In conclusion, the putative involvement of the poly(ADP-ribosyl)ated CTCF binding at its target sites ICR seems to be essential for the maintenance of the insulator function. Observations in episomal plasmid transfection experiments reveal that the presence of poly(ADP-ribose) polymerase (PARP) inhibitor 3-aminobenzamide (3-ABA) is capable to relieve insulator effect of ICR.

In **Paper IV**, we have elucidated a novel method to activate or repress the imprinted *IGF2-H19* genes through an engineered synthetic zinc finger transcription protein/factor (ZFP) competent to interact with target DNA sequences in the promoters. Activation domains VP16 or p65 and repressor domain v-ErbA are joined with ZFP809 to up-regulate or down-regulate both *IGF2* and *H19* genes. The reactivation/repression of silent alleles of both *IGF2/H19* by ZFP809 is due to direct interaction with target DNA binding and domain-dependent.

Imprinted genes are mono-allelically expressed and mechanistic determinants are moderately understood. Imprinting control region located in the 5'-flank of the *H19*, which averts the enhancers located 3'-flank to the *H19* gene from interacting with the *IGF2* promoters 90 kb away on the maternal chromosome, while silencing the *H19* gene on the paternal chromosome. The repression of the maternally derived *IGF2* may engage the chromatin insulator protein CTCF to interact with the *H19* ICR in a methylation-sensitive and allele-specific manner.

Our ChIP data reveals that the proximal promoter of the *H19* is *laissez-faire* to the binding of ZFP809 resulting in up-regulation of the paternally silent allele. Nevertheless, the ZFP809 transcription factor target-binding site does not include a CpG dinucleotide; hence it is implausible to be directly affected by the methylation status of the region, imperative to bring up that a generally repressive chromatin structure averts expression of the *H19* is revealed erroneous here. The results suggested possible therapeutic application of engineered zinc-finger protein (ZFP) transcription factors to rectify the epigenetic lesions.

"Knowing trees, I understand the meaning of patience. Knowing grass, I can appreciate persistence"

Hal Borland (1900-1978)

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