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Spontaneous errors of imprinting in mouse embryos

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ABSTRACT

Genomic imprinting is a mechanism of fine regulation of gene expression. Imprinted genes are expressed from only one parental allele and many of them have critical roles in growth and development. Imprinting marks that distinguish the parental origin must be erased and re-established in germ cells according to the sex of the individual to ensure proper embryonic development. We investigated occurrence of imprinting errors in mouse embryos. Firstly, we tested the hypothesis that errors in resetting of imprints occur and lead to grandparental-origin effects on embryonic growth. Although we did not find statistically significant effects, we observed trends that should be confirmed by replication. Secondly, we examined expression of 5 genes located on the distal part of chromosome 12 in order to establish the incidence of spontaneous imprinting errors. We report a strain and parental-origin specific imprinting relaxation of *Dlk1* and *Dio3* genes.

RÉSUMÉ

L'expression des gènes soumis à l'empreinte génomique se limite à l'allèle hérité d'un parent en particulier. Les marques parentales doivent être effacées et réimplantées dans les gamètes pour refléter le genre de l'individu et permettre un développement normal des embryons. Nous avons étudié les erreurs spontanées de l'empreinte génomique dans les embryons de souris. Premièrement, nous avons testé l'hypothèse que les marques de l'empreinte génomique ne sont pas complètement effacée dans tous les individus et que l'origine grand-parentale des allèles influence la croissance embryonnaire. Malgré que nous n'ayons pas trouvé d'effet statistiquement significatif, nous avons observé des tendances qui pourraient être confirmées par réplication. Deuxièmement, nous avons examiné directement l'expression de cinq gènes de la portion distale du chromosome 12 pour déterminer l'incidence des erreurs spontanées de l'empreinte parentale. Nous rapportons un relâchement de l'empreinte sur les gènes *Dlk1* et *Dio3* dépendant de l'espèce et de l'origine parentale.

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Chemical compounds

BSA	Bovine serum albumine
dATP	dehydroxy adenosine triphosphate
dCTP	dehydroxy cytosine triphosphate
dNTP	dehydroxy nucleotide triphosphate
EDTA	Disodium ethylenediaminetetraacetate
IPTG	Isopropyl-[B]-D-thiogalactoside
KCl	Potassium chloride
$MgCl_2$	Magnesium dichloride
NaCl	Sodium chloride
PBS	Phosphate-buffered saline
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
X-Gal	5-bromo-4-chloro-3-indolyl-[b]-d-galactoside

Genes

Air	Antisense to insulin-like growth factor 2 receptor
ASCL2/Ascl2	Achaete-scute complex-like 2 (Drosophila)
Cdkn1c	Cyclin-dependent kinase inhibitor 1C (p57Kip2)
Dat	Dlk1 associated transcripts
Dio3	Deiodinase iodothyronine type III
Dlk1	Delta-like homologue
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT1o	DNA (cytosine-5-)-methyltransferase 1oocyte
DNMT3a	DNA (cytosine-5-)-methyltransferase 3a
DNMT3b	DNA (cytosine-5-)-methyltransferase 3b
DNMT3L	DNA (cytosine-5-)-methyltransferase 3L
GNAS/Gnas	Guanine nucleotide binding protein, alpha stimulating
Gnasxl	Guanine nucleotide binding protein, alpha stimulating, extra large
GRB10/Grb10	Growth factor receptor-bound protein 10
H19	H19, imprinted maternally expressed untranslated mRNA
HYMAI	Hydatidiform mole associated and imprinted
IGF2/Igf2	Insulin-like growth factor 2
IGF2R/Igf2r	Insulin-like growth factor 2 receptor
IMPT1	now called SLC22A1l
Ins2	Insulin 2
Jag2	Jagged 2
Kcnq1/Kvlqt1	Potassium voltage-gated channel, KQT-like subfamily, member 1
LIT1	now called KCNQ1OT1 (KCNQ1 overlapping transcript 1)
MAGEL2	MAGE-like 2
Mash2	Mammalian achaete scute homolog 2 (now called Ascl2)
Meg3/Gtl2	Maternally expressed gene 3/ Gene trap locus 2
MKRN3	Makorin, ring finger protein, 3
NDN/Ndn	Necdin homolog (mouse) / Necdin

Nesp	Neuroendocrine secretory protein
Nespas	Nesp antisense
Nnat	Neuronatin
Peg1/Mest	Paternally expressed gene 1
Peg3	Paternally expressed gene 3
Plg	Plasminogen
Slc22a1	Solute carrier family 22 (organic cation transporter) family member 1
Slc22a1l	Solute carrier family 22 (organic cation transporter) family member 1-like
Slc22a2	Solute carrier family 22 (organic cation transporter) family member 2
Slc22a3	Solute carrier family 22 (organic cation transporter) family member 3
snoRNAs	Small nucleolar RNAs
SNRPN	Small nuclear ribonucleoprotein N gene
SNURF	SNRPN upstream reading frame
Tnfaip2	Tumor necrosis factor, alpha-induced protein 2
Tssc3	Tumor suppressing subtransferable candidate 3
U2af1-rs1	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF), related sequence 1
U2AFBPL	U2(RNU2) small nuclear RNA auxillary factor binding protein-like
UBE3A/Ube3a	Ubiquitin protein ligase E3A
Vipr2	Vasoactive intestinal peptide receptor 2
Wars	Tryptophanyl-tRNA synthetase
WT1	Wilms tumor suppressor gene 1
WT1-as	Wilms tumor suppressor gene 1 antisense
Yy1	Yin Yang 1 transcription factor
ZAC/PLAGL1	Pleiomorphic adenoma gene-like 1
Zfy1	Zinc finger protein, Y-linked

Abbreviations

AS	Angelman syndrome
BWS	Beckwith-Wiedemann syndrome
CTCF	CCCTC-binding factor
d.p.c.	Days post coitum
DMR	Differentially methylated region
EGC	Embryonic germ cells
EST	Expressed sequence tag
IC	Imprinting center
LOI	Loss of imprinting
PCR	Polymerase chain reaction
PGC	Primordial germ cells
PWS	Prader-Willi syndrome
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
SRS	Silver-Russell syndrome
TNDM	Transient neonatal diabetes mellitus
TRD	Transmission ratio distortion
UPD	Uniparental disomy

CHAPTER 1

INTRODUCTION

1. Genomic imprinting

1.1 *What is imprinting?*

Genomic imprinting is defined as an epigenetic difference between alleles depending upon their parental origin. This difference leads to exclusive or preferential expression of one parental allele.

Parent-of-origin specific expression of imprinted genes requires that parental alleles are marked differently. The nature of imprints and the mechanisms involved have not yet been elucidated. Imprints have to be heritable through mitotic cell divisions and reversible in the germ cells. They must also be able to modify gene expression.

1.2 Characteristics of imprinted genes

One feature that characterizes imprinted genes is that they are assembled in clusters suggesting that imprinted genes within a cluster are co-regulated by regional control elements. Subsequently, such imprinting centers (ICs) have been identified for 3 imprinted gene clusters: *Igf2r* region (Wutz *et al.*, 1997), *H19/Igf2* region (Thorvaldsen *et al.*, 1998) and in the Prader-Willi/Angelman syndrome region of human chromosome 15q (Yang *et al.*, 1998) (Figure 1). Deletions or mutations of IC regions result in regional loss of imprinting. However, it remains unclear how parental origin is marked in ICs.

Differentially methylated regions (DMRs) were first found in the mouse endogenous *Igf2r* gene (Stoger *et al.*, 1993) and in the human PraderWilli/Angelman syndrome region (Dittrich *et al.*, 1992; Driscoll *et al.*, 1992). Imprinted regions are particularly rich in CpG islands (Engemann *et al.*, 2000) and DMRs were found in the majority of known imprinted genes (Bartolomei and Tilghman, 1997) (Table 1). CpG methylation regulates gene expression by silencing or activating different regulatory elements

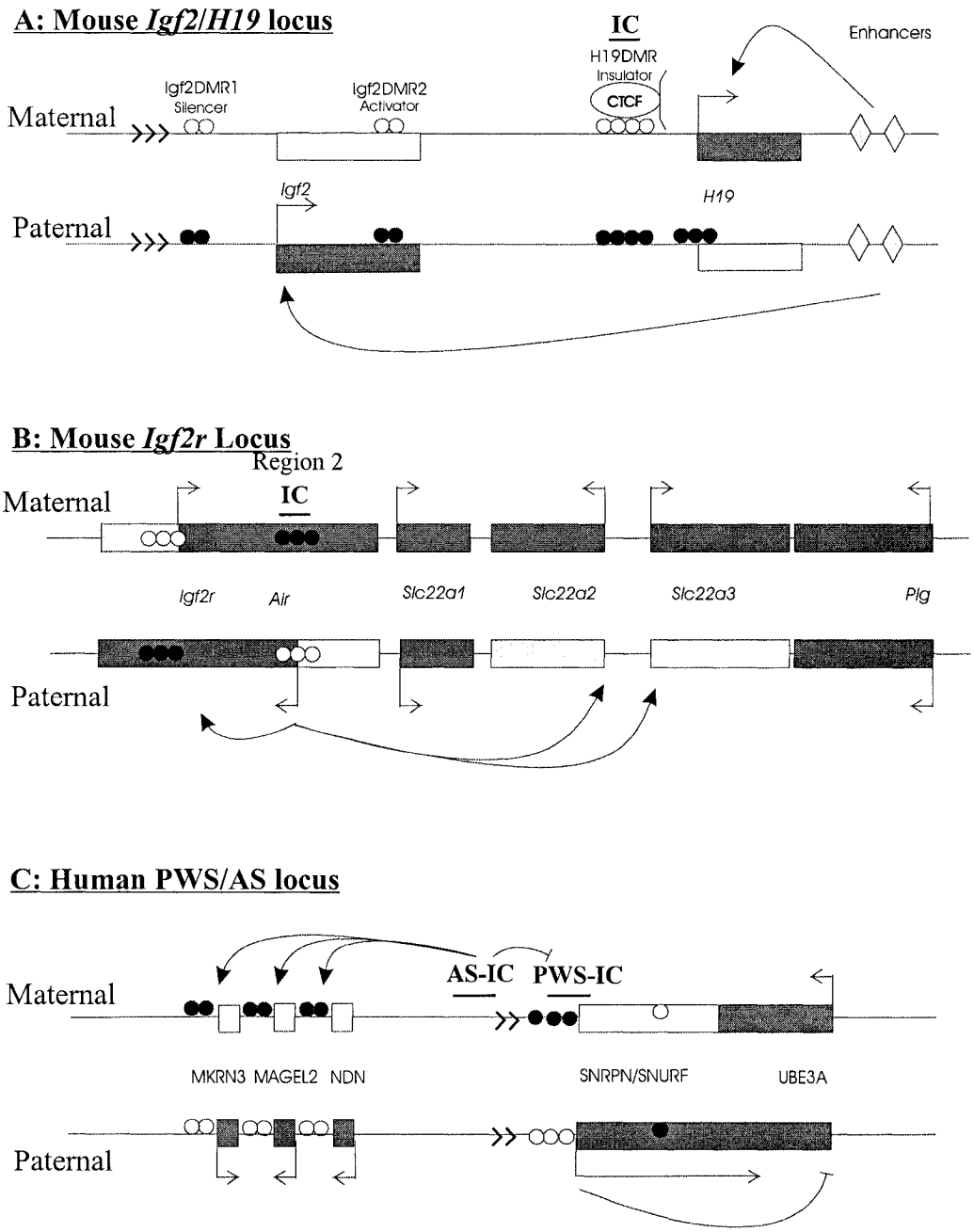

Figure 1: Schematic representation of 3 imprinted regions

Figure 1: Schematic representation of 3 well-studied imprinted regions. **A)** Mouse *Igf2/H19* region on the distal region of chromosome 7. On the maternal allele, CTCF protein binds to the unmethylated *H19* DMR and prevents downstream enhancers from interacting with the *Igf2* promoter (Bell and Felsenfeld, 2000; Hark *et al.*, 2000; Kanduri *et al.*, 2000; Srivastava *et al.*, 2000a; Szabo *et al.*, 2000). The unmethylated silencer in *Igf2* DMR1 silences *Igf2* gene (Eden *et al.*, 2001). On the paternal allele, *H19* promoter is methylated (Tremblay *et al.*, 1995) and silenced. *Igf2* expression is stimulated by the downstream enhancers (Leighton *et al.*, 1995) and by the methylated activator in *Igf2* DMR2 (Murrell *et al.*, 2001). **B)** Mouse Igf2r region on proximal chromosome 17. Region 2, located in *Igf2r* intron 2, is methylated on the maternal allele (Stoger *et al.*, 1993) and represses expression of the antisense transcript *Air* (Wutz *et al.*, 1997). Region 2 methylation and *Air* are essential for silencing *Igf2r*, *Slc22a2* and *Slc22a3* genes on the paternal allele (Sleutels *et al.*, 2002; Wutz *et al.*, 2001; Zwart *et al.*, 2001). **C)** Human PWS/AS region on chromosome 15q11-13. This region is controlled by 2 ICs: the AS-IC is deleted in a proportion of Angelman syndrome patients and the PWS-IC is deleted in a proportion of Prader-Willi syndrome patients. On the maternal allele, the AS-ICR directs methylation and silencing of upstream paternally expressed genes (Bielinska *et al.*, 2000; Moweryrushton *et al.*, 1996; Sutcliffe *et al.*, 1994). On the paternal allele, transcription of the *SNRPN/SNURF* unit is initiated in the PWS-IC (Runte *et al.*, 2001) and these antisense transcripts silence *UBE3A* expression (Rougeulle *et al.*, 1998).
Open circles: unmethylated CpGs. Black circles: methylated CpGs. Dark rectangles: expressed genes. Light rectangles: silenced genes. Arrows: gene expression. Curved arrows: action of regulatory regions. Oval: DNA-binding protein. Triangles: short tandem repeats.

Table 1 : Examples of imprinted mouse genes with associated DMRs

Chromosomal region	Gene	DMR location	Allele expressed	Allele methylated	References
12 distal	*Gtl2*	Promoter and exon 1	M	P	Takada *et al.*, 2002
17 proximal	*Igf2r*	Intron 2 (region 2)	M	M*	Stoger *et al.*, 1993
17 proximal	*Igf2r*	Promoter (region 1)	M	P	Stoger *et al.*, 1993
2 distal	*Gnasxl*	Promoter	P	M	Peters *et al.*, 1999
2 distal	*Nesp*	Promoter	M	P	Peters *et al.*, 1999
2 distal	*Gnas* locus	Upstream of *Gnasxl*	M	M*	Liu *et al.*, 2000
2 distal	*Nnat*	*Nnat* coding region	P	M	Kikyo *et al.*, 1997
6 proximal	*Peg1/ Mest*	Exon 1	P	M*	Lefebvre *et al.*, 1997
7 central	*Snrpn*	3' end (DMR2)	P	P	Shemer *et al.*, 1997
7 central	*Snrpn*	5' end (DMR1)	P	M*	Shemer *et al.*, 1997
7 distal	*H19*	Upstream region	M	P*	Bartolomei *et al.*, 1993 Tremblay *et al.*, 1995
7 distal	*Igf2*	Last exon (DMR2)	P	P	Feil *et al.*, 1994
7 distal	*Igf2*	Upstream region (DMR1)	P	P	Sasaki *et al.*, 1992 Feil *et al.*, 1994
7 distal	*Kcnq1* (*Kvlqt1*)	Intron between exons 10 and 11	M	M*	Engemann *et al.*, 2000
7 proximal	*Peg3*	Exon 1	P	M	Li *et al.*, 2000a

* Differential methylation inherited from the gametes.
M : Maternal P : Paternal

such as promoters, enhancers and silencers and by modulating the interaction of the region with DNA-binding proteins (Figure 1).

Imprinted genes are also characterized by the common presence of short tandem repeat sequences near or within CpG islands (Kende *et al.*, 1995; Reik and Dean, 2001) (Figure 1). Chaillet *et al.* (1995) demonstrated that short tandem repeats are necessary for parent-of-origin specific methylation of the transgene RSVIgmyc. Furthermore, short tandem repeats were found in proximity to the mouse imprinted *U2af1-rs1* gene (Shibata *et al.*, 1997) whereas the human homologous locus, *U2AFBPL*, which is not imprinted, does not contain such repeats (Pearsall *et al.*, 1996). Thus, tandem repeat sequences seem to have a role in imprinting.

Non-coding genes and anti-sense mRNA transcripts are also often found in imprinted regions. These transcripts affect imprinted gene expression in two ways: firstly, by competing for the same promoters and/or enhancers or secondly, by mediating regulation of transcription. For example, *Air* is a paternally expressed antisense mRNA and non-coding gene found to overlap with mouse *Igf2r* sequence (Wutz *et al.*, 1997). A targeted mutation resulting in a truncated *Air* transcript does not affect differential methylation of the region and imprinting of *Air* itself, but disrupts imprinting of 3 neighbouring maternally expressed genes: *Slc22a2, Slc22a3* and *Igf2r* (Sleutels *et al.*, 2002). This demonstrates that non-coding RNAs can play an active role in imprinting.

1.3 Epigenetic marks: DNA methylation and chromatin structure

Several lines of evidence suggest that DNA methylation is a good candidate for imprinting mark. Patterns of methylation of the cytosine residue of CpG dinucleotides are heritable, reversible and able to alter gene expression. The first series of experiments to demonstrate that DNA methylation plays a role in imprinting involved the generation of transgenic mice (Surani *et al.*, 1988). Several transgenes were methylated differently depending on which parent they were inherited from. Later, Li *et al.* showed that monoallelic expression of imprinted genes is disrupted in embryos homozygous for a loss-of-function mutation of *Dnmt1*, the enzyme responsible for methylation maintenance (Li *et al.*, 1993).

Although it has been established that DNA methylation is required for maintaining imprinting in somatic cells, it remains unclear if methylation provides the initial imprint. The role of other factors remain to be established. It has been shown that parental *H19* DMR alleles acquire methylation at different moments in male gametogenesis although both alleles are devoid of methylation marks in 13.5 d.p.c. embryos (Davis *et al.*, 2000). This suggests that parental identity of imprinted alleles is marked by other epigenetic mechanisms in addition to methylation.

Chromatin structure and histone acetylation are also candidates for imprinting marks. There is evidence for chromatin structure and histone acetylation differences between parental alleles on imprinted genes (Bartolomei *et al.*, 1993; Ferguson-Smith *et al.*, 1993; Moore *et al.*, 1997; Pedone *et al.*, 1999). Furthermore, in the presence of trichostatin A, an inhibitor of histone deacetylation, normally silenced alleles of imprinted genes are expressed (Hu *et al.*, 2000; Hu *et al.*, 1998b; Svensson *et al.*, 1998).

1.4 DNA-methyltransferases

Trans-acting factors essential for establishment and maintenance of imprints were extensively investigated. Three DNA methyltransferases were isolated: *Dnmt1*, *Dnmt3a* and *Dnmt3b* (Bestor *et al.*, 1988; Okano *et al.*, 1998). *Dnmt1* is the predominant form in mammals and is generally responsible for maintenance of DNA methylation. Targeted mutations of this gene are lethal and result in loss of imprinting (LOI) (Li *et al.*, 1993). An oocyte specific isoform of *Dnmt1* was isolated, *Dnmt1o* (Mertineit *et al.*, 1998). This form is present during oocyte maturation and early embryonic development (Mertineit *et al.*, 1998). Mice homozygous for a null mutation of *Dnmt1o* appears to be normal, but embryos from homozygous mothers do not develop to term (Howell *et al.*, 2001). Although methylation imprints are established normally in *Dnmt1o*$^{-/-}$ females, their offspring display loss of parental-origin specific methylation and loss of imprinting (Howell *et al.*, 2001). Dnmt1o protein is localized in the nucleus only at the eight-cell stage suggesting that it is responsible for methylation maintenance of imprinted loci only for one cell division (Howell *et al.*, 2001).

Dnmt3a and *Dnmt3b* are essential for *de novo* DNA methylation in germ cells and early postimplantation embryos (Okano *et al.*, 1999). Thus, *Dnmt3a* and *Dnmt3b* are likely

candidates to establish imprints in germ cells. However, it remains unclear how these enzymes recognize target sequences for imprinting and how different methylation patterns are established in male and female germlines. Investigation of proteins binding to these enzymes led to the identification of *Dnmt3L* (Aapola *et al.*, 2000). *Dnmt3L* has sequence similarity with other *Dnmt3* enzymes but lacks DNA-methyltransferase activity (Klimasauskas *et al.*, 1994). It binds to and colocalizes with *Dnmt3a* and *Dnmt3b*. Targeted mutation of *Dnmt3L* prevents establishment of maternal imprints in oocytes and causes defects in spermatogenesis (Bourc'his *et al.*, 2001; Hata *et al.*, 2002).

2. Imprint resetting

Imprinting marks are established in gametogenesis and/or early embryogenesis. In the developing germ cells, imprints have to be reset to reflect the sex of the individual. This process consists of two steps: imprints are first erased from both alleles and then re-established before being transmitted to the next generation (Figure 2).

2.1 Erasure of imprints

Mouse primordial germ cells (PGCs) are specified around 6.5 d.p.c. and migrate into the genital ridge between 8 and 11 d.p.c. (Anderson *et al.*, 2000). At this stage, PGCs undergo a series of epigenetic modifications including X chromosome reactivation in females and a general decrease in global DNA methylation (Monk *et al.*, 1987). Promoters of several house-keeping genes become demethylated in the germ cells of 12.5 d.p.c. and 13.5 d.p.c embryos (Kafri *et al.*, 1992). These changes are thought to be necessary for the totipotency of germ cells.

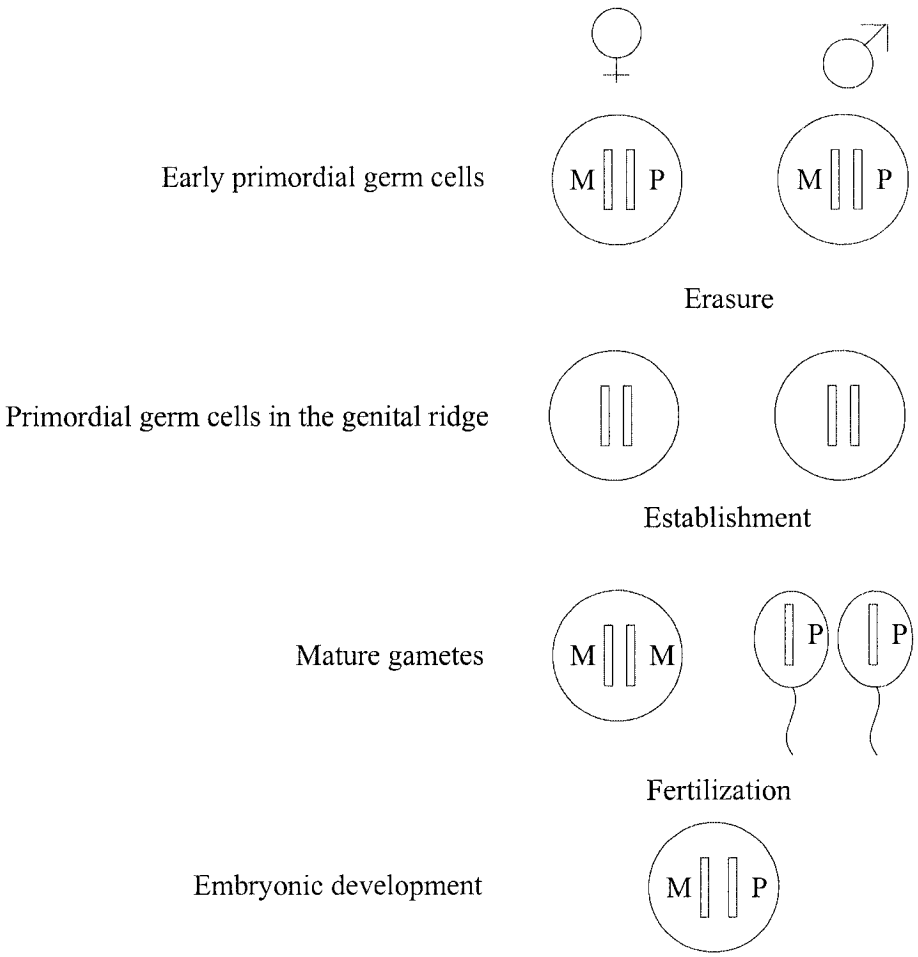

Figure 2: Primordial germ cells (PGCs) carry somatic imprints that distinguish the paternal and maternal chromosomes. As PGCs enter the genital ridge, imprints are erased. Subsequently, imprints are re-established as paternal during late gametogenesis in male germ cells and as maternal in oocytes during follicular growth.

Several groups used different strategies to investigate how imprints are modified during mouse gametogenesis. Experiments using purified PGCs revealed that imprinted genes are biallelically expressed at 11.5 d.p.c. (Szabo and Mann, 1995). Methylation status of various DMRs was also examined in PGCs from 10.5 to 13.5 d.p.c. embryos. Methylation was absent from DMRs in 12.5 and 13.5 d.p.c. PGCs (Brandeis *et al.*, 1993; Lee *et al.*, 2002; Reik *et al.*, 2000). Two studies demonstrated that in male and female PGCs, demethylation of imprinted regions occurs between 11.5 and 12.5 d.p.c. and is not simultaneous for all DMRs (Hajkova *et al.*, 2002; Lee *et al.*, 2002).

Other groups studied imprinting in embryonic germ cells (EGCs) derived from PGCs at different embryonic stages. In agreement with observations made in PGCs, EGCs derived from 11.5 and 12.5 d.p.c. PGCs have hypomethylated DMRs (Labosky *et al.*, 1994; Tada *et al.*, 1997; Tada *et al.*, 1998). Finally, expression of several imprinted genes was analyzed in embryos derived by nuclear transfer of PGC nuclei to enucleated oocytes. Although it is unclear how these manipulations affect imprints, these results also suggested that erasure does not proceed simultaneously for all imprinted genes (Lee *et al.*, 2002).

Overall, these data were obtained by using different experimental approaches and therefore one should be careful in comparing them. Cell purification and culturing methods can influence developmental speed and DNA methylation levels. Nevertheless, they demonstrate that imprint erasure starts as mouse germ cells reach the genital ridge at 11.5 d.p.c. and is completed by 13.5 d.p.c. Little is known about the mechanisms that erase imprints. Imprints are not erased simultaneously in different imprinted genes or clusters or even in different DMRs within the same cluster. This suggests that imprints are erased in a stepwise manner by a specific mechanism rather than as a consequence of the general lack of maintenance methylation in dividing germ cells which would cause simultaneous progessive demethylation of all sites.

2.2 Establishment of imprints

Once somatic imprints are erased in the germ cells, new parental marks have to be established. Several groups examined how imprints are established on the maternally expressed *H19* gene in male mouse germ cells. *H19* DMR, located upstream of *H19* promoter is critical for proper monoallelic expression of *H19* and *Igf2* genes (Figure 1) (Thorvaldsen *et al.*, 1998) and methylation of the paternal allele is inherited from sperm and maintained through gametogenesis (Tremblay *et al.*, 1997). Thus, this region is likely to harbour the primary imprinting mark.

Methylation of *H19* DMR is detected in mitotically arrested male germ cells at 15.5 d.p.c. and postnatally in the spermatogonial stem cells (Davis *et al.*, 2000; Ueda *et al.*, 2000). However, methylation of both alleles is only completed in pachytene spermatocytes (Lucifero *et al.*, 2002). Although both alleles are completely unmethylated in germ cells of 13.5 d.p.c. males, the paternal allele acquires methylation more rapidly than the maternal allele (Davis *et al.*, 1999; Davis *et al.*, 2000). In comparison, methylation of a secondary DMR in the promoter region of *H19* is acquired after implantation (Srivastava *et al.*, 2000b).

In contrast, the *Snrpn* gene is paternally expressed; its DMR1 is methylated on the maternal allele, and is postulated to represent the primary imprint (Shemer *et al.*, 1997). Methylation of this region occurs during oocyte follicular growth and is completed by metaphase II (Lucifero *et al.*, 2002).

2.3 Maintenance of imprints in early embryogenesis

In mice, genome-wide DNA methylation level decreases after fertilization until blastocyst stage (Monk *et al.*, 1987). This demethylation occurs in two phases. First, the paternal genome is actively demethylated prior to DNA replication (Mayer *et al.*, 2000; Oswald *et al.*, 2000). Then, the global methylation level decreases with each cell cycle because of the absence of maintenance methyltransferase in the nucleus (Carlson *et al.*, 1992; Howlett and Reik, 1991; Monk *et al.*, 1991; Rougier *et al.*, 1998). Primary DMRs must be preserved from this process as it was demonstrated for the *H19* DMR (Olek and Walter, 1997; Tremblay *et al.*, 1995).

DNA methylation increases in the embryo after implantation (Monk *et al.*, 1987). Unmethylated alleles from DMRs need to be protected from *de novo* methylation. This can be achieved by specialized chromatin structures or DNA-binding proteins. For example, the binding of CTCF protein appears to be necessary to maintain the maternal *H19* DMR allele unmethylated (Schoenherr *et al.*, 2003).

3. Imprinting variation

3.1 Tissue-specific and stage-specific imprinting

The imprinting of several human and mouse genes varies with cell type and developmental stage (Table 2). For example, the mouse *Igf2* gene is not imprinted and expressed biallelically in 8-cell stage embryos (Latham *et al.*, 1994), but is expressed monoallelically from the paternal allele after implantation. However, in the choroid plexus and leptomeninges it is expressed biallelically (DeChiara *et al.*, 1991). In adult rats *Igf2* is expressed only in the choroid plexus and leptomeninges (Stylianopoulou *et al.*, 1988). Moreover, *Igf2* imprinting is regulated by tissue-specific elements. For example, imprinting of a placenta-specific *Igf2* transcript depends upon regulatory elements not required for imprinting of fetal *Igf2* transcripts (Moore *et al.*, 1997).

3.2 Polymorphic imprinting

Polymorphic imprinting refers to the variability among individuals with regard to the imprinting status of particular genes. This phenomenon was first observed for the human *IGF2R* gene, which was shown to be imprinted in 3 out of 14 fetuses (21%) and expressed biallelically in the others (Xu *et al.*, 1993). Genes that show polymorphic imprinting include: *WT1* (Jinno *et al.*, 1994), *IGF2* in blood cells (Giannoukakis *et al.*, 1996) and *IMPT1* (Dao *et al.*, 1998).

Table 2: Examples of mouse and human genes with tissue and stage-specific imprinting regulation

Gene	Tissue & Time specificity	Reference
Mouse genes		
Nespas	Biallelic expression in adrenal and testis tissues; paternal expression in other tissues.	Li *et al.*, 2000b
Grb10	Maternal expression in embryos, adult liver, kidney and muscle; biallelic expression in fetal brain; paternal expression bias in adult brain.	Miyoshi *et al.*, 1998 Hitchins *et al.*, 2002 Hikichi *et al.*, 2003
Igf2	Biallelic expression in adult and fetal choroid plexus and leptomeninges; biallelic expression before implantation; paternal expression in other tissues.	DeChiara *et al.*, 1991 Latham *et al.*, 1994
Igf2r	Maternal expression in peripheral tissue; biallelic expression in central nervous system.	Hu *et al.*, 1998a
Slc22a1l (*Impt1*)	Maternal expression in fetal tissues; biallelic expression in adult tissues.	Dao *et al.*, 1998
Ins2	Maternal expression in yolk sac at 14.5 d.p.c.; biallelic expression in embryos and in yolk sac at 12.5 d.p.c.	Deltour *et al.*, 1995
Kcnq1	Maternal expression in all fetal tissues; biallelic expression in adult brains (also strain-specific effects)	Jiang *et al.*, 1998b
Ube3a	Maternal expression in hippocampus and cerebellum; biallelic expression in other regions of the brain.	Albrecht *et al.*, 1997
Human genes		
GNAS	Maternal expression in pituitary; biallelic expression in other tissues.	Hayward *et al.*, 2001
GRB10	Paternal expression of most isoforms in fetal brain; maternal expression of one isoform in fetal muscles; biallelic expression of other isoforms.	Blagitko *et al.*, 2000 Hikichi *et al.*, 2003
SLC22A1 (*IMPT1*)	Maternal expression in placenta, chorioamnion, liver and adrenal gland; biallelic expression in fetal and adult kidney. Strong inter-individual variations.	Dao *et al.*, 1998
KCNQ1	Tissue-type specific imprinting: biallelic expression in heart and maternal expression in other tissues.	Lee *et al.*, 1997
UBE3A	Maternal expression in the brain; biallelic expression in other tissues.	Rougeulle *et al.*, 1997 Vu and Hoffman, 1997
WT1	Maternal expression in fetal brain and in 5/9 preterm placenta; biallelic expression in kidney.	Jinno *et al.*, 1994

Giannoukakis *et al.* (1996) examined family clustering and inheritance of *IGF2* imprinting in lymphocytes. Two individuals with *IGF2* monoallelic expression in blood cells were found in the same family. These individuals inherited different alleles of *IGF2* suggesting that *IGF2* imprinting depends upon *trans*-acting factors (Giannoukakis *et al.*, 1996). Another group investigated imprinting of 3 genes, *IGF2*, *SNRPN* and *SLC2A1L* (*IMPT1*), in blood leukocytes from 262 Japanese individuals. They found four unrelated individuals with biallelic expression of *IGF2* out of 38 informative cases (10.5%) (Sakatani *et al.*, 2001).

4. Abnormal expression of imprinted genes

4.1 Uniparental disomy and chromosomal aberrations

Androgenotes (two paternal genomes) and gynogenotes (two maternal genomes) are uniparental conceptuses created by the transfer of two pronuclei from the same sex into an enucleated oocyte. Although they have a normal diploid number of chromosomes, they have abnormal expression dosage of imprinted genes and most of these conceptuses have major anomalies and die during early post-implantation development (McGrath and Solter, 1984; Surani *et al.*, 1984).

Uniparental disomy (UPD) defines the inheritance of both homologous chromosomes from one parent. UPD can arise spontaneously in humans by different mechanisms such as trisomic and nullisomic rescues (Spence *et al.*, 1988). The phenotypic consequences of UPD may arise because of abnormal dosage of imprinted gene products or expression of recessive traits if the two homologous chromosomes are identical (isoUPD). Mosaicism for aneuploid cells is common and also affects the phenotype.

Deletion or duplication of the active allele of imprinted genes results in silencing or double dose of expressed product.

4.2 Loss of imprinting

Loss of imprinting (LOI) is defined as biallelic expression or silencing of both parental alleles of imprinted genes despite biparental inheritance and the presence of two alleles. LOI may arise from errors in erasure, establishment, maintenance or interpretation of imprints and results in abnormal gene product dosage.

Mice lacking *Dnmt1* enzyme fail to maintain parent-of-origin specific methylation and imprinting (Li *et al.*, 1993). This is an example of a global imprinting defect caused by a mutation in a gene essential for imprinting. In humans, familial complete hydatidiform moles are characterized by recurrent loss of conceptuses of biparental origin lacking maternal imprints (Judson *et al.*, 2002). This suggests a maternal failure to establish germline imprints leading to a global imprinting defect in humans.

Environmental conditions are also believed to alter imprinting in preimplantation embryos. Prolonged culture of embryonic stem cells in fetal calf serum complemented medium leads to methylation changes in DMRs and aberrant imprinted gene expression (Dean *et al.*, 1998). Components of the culture medium are particularly important: *H19* methylation increases in preimplantation embryos cultured in M16 medium with serum (Koshla *et al.*, 2001) and decreases in Whitten's medium (Doherty *et al.*, 2000). The influence of environmental factors on imprinting maintenance is particularly important with the wide use of assisted reproduction technologies (ART). Recent data suggest that children born after ART are more likely to have imprinting disorders such as Angelman syndrome (Cox *et al.*, 2002) and Beckwith-Wiedemann syndrome (DeBaun *et al.*, 2003) due to incorrect establishment of methylation imprints.

Locus-specific LOI is also observed. 2-4% of Prader-Willi syndrome (PWS) and Angelman syndrome (AS) patients have biparental inheritance of chromosome 15q and normal karyotype. However, their paternal chromosome carries a maternal methylation pattern (PWS) or the maternal chromosome carries a paternal methylation pattern (AS). In some of these patients, a genetic mutation in the imprinting center (IC) prevented correct establishment of imprints (Buiting *et al.*, 1995; Reis *et al.*, 1994; Saitoh *et al.*, 1996; Sutcliffe *et al.*, 1994). In a proportion of PWS and AS patients, no genetic mutations were found (Buiting *et al.*, 1998; Burger *et al.*, 1997). This suggests that

epigenetic mutations occur stochastically to cause spontaneous imprinting errors in mice and in humans.

5. Consequences of incorrect dosage of imprinted genes

5.1 Consequences of incorrect dosage of imprinted genes in mice

In 1990, Cattanach and Beechey used mice with characterized Robertsonian translocations to generate mice with uniparental disomy for specific chromosomal regions (Cattanach and Beechey, 1990). By analysing phenotypic consequences of UPD, they constructed a mouse imprinting map. Phenotypic consequences of UPD range from early embryonic lethality to abnormal postnatal growth rate and behavioural anomalies (Beechey et al. 2001) (Table 3).

The targeted disruption of individual imprinted genes has also revealed that imprinted genes affect placental development, growth regulation and embryonic survival (Table 4).

5.2 Consequences of incorrect dosage of imprinted genes in humans

UPD of chromosomes with imprinted genes are associated with developmental, endocrine and growth abnormalities in humans (Table 5). UPD that have not been identified may have no phenotypic consequences. However, they may also not have been identified because of embryonic lethality.

Beckwith-Wiedemann syndrome (BWS) has been mapped to chromosome 11p15 (Koufos *et al.*, 1989). This condition is characterized by somatic overgrowth before and after birth, birth defects and a predisposition to embryonic tumors such as Wilms tumors. The etiology of the disease is complex and more than one gene appears to be involved. Some patients have incomplete BWS phenotypes. For example, LOI of *IGF2* seems to increase the risk of developing cancer whereas LOI of *KCNQ1OT1* (*LIT1*) causes birth defects (DeBaun *et al.*, 2002).

Table 3: Phenotypes associated with UPD in mice

Chromosomal region	Parental origin	Phenotype
Proximal 2	Maternal	Variable fetal viability with growth reduction
Proximal 2	Paternal	Placental overgrowth
Distal 2	Maternal	Neonatal behaviour and lethality, hypokinetic
Distal 2	Paternal	Neonatal behaviour and lethality, hyperkinetic
Proximal 6	Maternal	Early embryonic lethality
Proximal 6	Maternal	Prenatal growth retardation
Proximal 7	Maternal	Neonatal lethality
Central 7	Maternal	Postnatal lethality
Proximal 7	Paternal	Postnatal growth/viability/behaviour
Distal 7	Maternal	Late fetal lethality
Distal 7	Paternal	Early embryonic lethality
Distal 9	Maternal	Reduced postnatal growth
Proximal 11	Maternal	Reduced postnatal growth
Distal 12	Maternal	Late embryonic/neonatal lethality & reduced growth
Distal 12	Paternal	Late embryonic lethality & growth enhancement
Proximal 17	Paternal	Neonatal lethality (Tme)
18	Maternal	Fetal growth retardation (unclear)
18	Paternal	Fetal growth retardation (unclear)

Adapted from Beechey et al. 2001: http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html

Table 4: Targeted disruption of mouse imprinted genes

Gene	Allele expressed	Phenotype	Reference
Igf2	Paternal	Growth retardation.	DeChiara et al., 1990
Dlk1	Paternal	Growth retardation and obesity.	Moon et al., 2002
Peg1	Paternal	Reduce pre and post-natal growth, reduced survival and altered maternal behaviour.	Lefebvre et al., 1998
Peg 3	Paternal	Reduce placental as well as fetal growth and altered maternal behaviour.	Li et al., 1999
Ndn	Paternal	Reduced postnatal survival and behaviour abnormalities.	Gerard et al., 1999 Tsai et al., 1999 Muscatelli et al., 2000
Igf2r	Maternal	Fetal overgrowth and late embryonic lethality.	Lau et al., 1994
Ube3a	Maternal	Neurological deficits.	Jiang et al., 1998c
Ascl2 (Mash2)	Maternal	Placental abnormalities and mid-gestation lethality.	Guillemot et al., 1995
Kcnq1 (K_VLQT1)	Maternal	Deafness and loss of balance.	Lee et al., 2000a Casimiro et al., 2001
Cdkn1c ($p57^{kip2}$)	Maternal	Developmental abnormalities, late embryonic or neonatal lethality.	Yan et al., 1997 Zhang et al., 1997
Tssc3	Maternal	Placental overgrowth.	Frank et al., 2002

Table 5: Phenotypes associated with UPD in humans

Chromosome	UPD	Phenotype	Reference
6	Maternal	Intrauterine growth restriction (2 cases).	van den Berg-Loonen et al., 1996 Spiro et al., 1999
6	Paternal	Transient neonatal diabetes mellitus and growth retardation.	Whiteford et al., 1997
7	Maternal	SRS, pre and post natal growth retardation.	Mergenthaler et al., 2000 (Eggermann *et al.*, 2001b)
11p	Paternal	BWS and growth enhancement when partial or mosaic, possibly lethal when complete.	Li et al., 1998 Dutly et al., 1998 Slatter et al., 1994
14q	Maternal	Low birth weight, short stature, obesity, mental and motor delay.	Reviewed by Healey et al., 1994
14q	Paternal	Developmental delay, polyhydramnios, facial and skeletal abnormalities.	Reviewed by Cotter et al., 1997
15q	Maternal	PWS (low birth weight, hyponia, obesity and short stature and behavioral and endocrinal abnormalities).	Reviewed by Nicholls, 1993
15q	Paternal	AS (mental retardation and ataxia).	Reviewed by Nicholls, 1993
20q	Maternal	Pre and post-natal growth retardation.	Chudoba et al., 1999 Eggermann et al., 2001

Chromosomal abnormalities or imprinting defects in human chromosomal region 15q11-q13 are associated with two human syndromes: Prader-Willi (PWS) and Angelman (AS). PWS condition is caused by the loss of paternal products of 15q11-q13 while AS is associated with loss of the maternally expressed *UBE3A* gene (Jiang *et al.*, 1999; Nicholls *et al.*, 1998; Rougeulle *et al.*, 1998). PWS is manifested as hypotonia, short stature, obesity and behavioural abnormalities including learning disabilities (Prader *et al.*, 1956). AS is characterised by developmental delay, mental retardation, sleep disorders, hyperactivity, ataxia and seizures (Angelman, 1965).

Silver-Russell Syndrome (SRS) is caracterized by low birth weight and post-natal growth retardation, triangular facies, asymmetry and fifth finger clinodactyly (Russell, 1954; Silver *et al.*, 1953) although symptoms vary among patients (Price *et al.*, 1999; Wollmann *et al.*, 1995). Genetic causes for this disease are heterogeneous and different patterns of transmission have been observed in families with more than one affected individuals (Price *et al.*, 1999). Association with an imprinted region was proposed to

explain the predominant maternal transmission of the disease (Preece *et al.*, 1999). Approximately 10% of SRS patients have maternal UPD of chromosome 7 (Bernard *et al.*, 1999; Kotzot *et al.*, 2000). Two candidate regions were identified: 7p11.2–p14 (Monk *et al.*, 2002) and 7q31-qter (Hannula *et al.*, 2001).

The imprinted genes on chromosomal region 20q13.3 are involved in endocrine functions. This region contains the complex *GNAS* locus which contains different genes transcribed from alternative promoters but sharing exons 2 to 13. LOI and genetic mutations in the *GNAS* locus create different hormone resistance diseases depending upon the transmitting parent (Davies and Hughes, 1993).

Transient neonatal diabetes mellitus (TNDM) is associated with an imprinted gene cluster located on chromosome 6 (Temple *et al.*, 1996). Finding of several patients with paternal UPD or paternal duplication of the region 6q24 suggests that TNDM results from functional excess of paternal genes (Temple *et al.*, 1996). Two paternally expressed imprinted genes in this region, *PLAGL1* (*ZAC*) and *HYMAI*, are likely candidates for this disease because they were found to be biallelically expressed in fibroblasts from a TNDM patient (Mackay *et al.*, 2002).

Loss of imprinting is one of the most common features found in tumors (Feinberg, 2000). Relaxation of *IGF2* imprinting is detected in several tumor types, e.g. Wilms tumors (Ogawa *et al.*, 1993; Rainier *et al.*, 1993), rhabdomyosarcoma (Pedone *et al.*, 1994; Zhan *et al.*, 1994), lung cancer (Suzuki *et al.*, 1994), hepatoblastoma (Rainier *et al.*, 1995) and leiomyosarcoma (Vu *et al.*, 1995). Biallelic expression of *IGF2* is also found in normal tissues from which tumors originated, suggesting that it may increase cancer susceptibility (Cui *et al.*, 1998). LOI of *H19, WT1-as and PEG1* is also reported in tumors (Chen *et al.*, 2000; Malik *et al.*, 2000; Ogawa *et al.*, 1993; Rainier *et al.*, 1993).

In summary, consequences associated with aberrant expression of imprinted genes in humans are important. They include growth, developmental, endocrine and behavioural abnormalities. It is also plausible to suggest that aberrant expression of imprinted genes essential for embryonic or placental development, such as *CDKN1C* and *ASCL2*, may be incompatible with the development of an embryo.

6. Objectives and hypothesis

The goal of this study was to examine spontaneous imprinting errors in mouse embryos. We suggested that imprinting errors in embryos may cause miscarriage, infertility and developmental defects in humans and embryonic death and developmental anomalies in mice.

As described earlier, imprinting marks are erased and re-established during gametogenesis. We proposed that imprint erasure stochastically fails in a proportion of gametes in which grandparental imprints remain (Figure 3). For example, a proportion of the grandpaternal alleles transmitted by a female would retain the paternal imprinting pattern. Offspring that inherited such an incorrectly imprinted allele from their mother and a normally imprinted allele from their father would therefore have two alleles with paternal imprints. Affected imprinted genes would therefore be biallelically expressed or silenced.

Phenotypic consequences of failure to reset imprints depend upon the function of the affected genes. If errors of imprint resetting affect a growth factor that is normally expressed from the paternal allele such as *Igf2*, a proportion of embryos that inherited the grandpaternal allele from their mother would produce twice the normal dosage of Igf2 and display enhanced growth rate. Hence, we tested the hypothesis that embryonic growth would be influenced by the grandparental origin of the inherited allele in chromosomal regions harbouring imprinted genes, which regulate embryonic growth.

If an imprinted gene essential for embryonic survival is affected, such stochastic errors in imprint resetting would lead to embryonic lethality. Hence, a proportion of offspring that inherited a grandpaternal allele from their mother may die because of imprinting errors. We expected this loss to cause transmission of grandparental alleles to deviate from Mendelian ratio of 1:1. We previously examined transmission ratios of microsatellite marker alleles in three imprinted regions harbouring genes essential for

embryonic survival in three-week old mice (Croteau *et al.*, 2002). We found a significant distortion from expected Mendelian ratios for the distal region of chromosome 12. This suggested that a proportion of embryos that inherited the grandpaternal distal chromosome 12 allele from their mother were lost during gestation. To demonstrate that these embryos are lost because of errors in imprint resetting in the maternal germ line, we investigated expression of individual genes in this region (Figure 4). The objective was to identify one or more candidate genes for the embryonic lethality associated with imprint resetting failure.

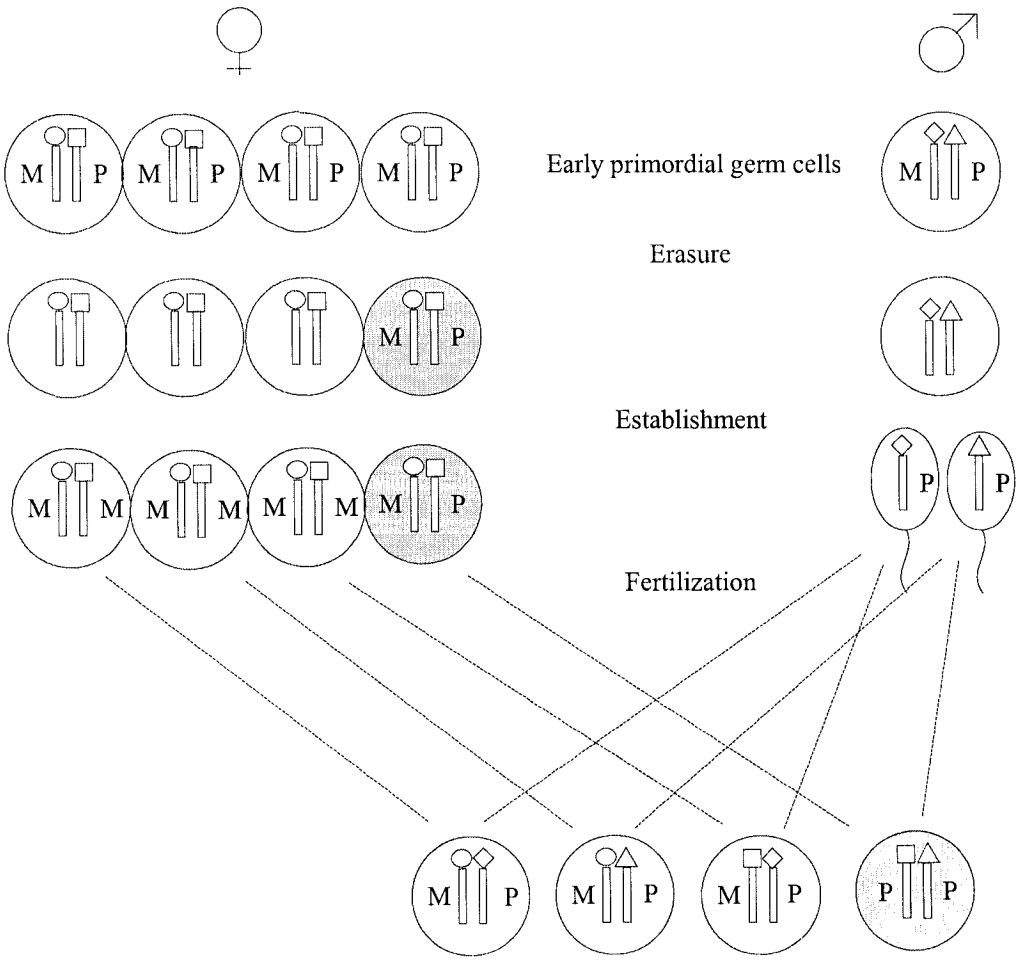

Figure 3: Schematic representation of failure in resetting of imprints. Imprints are not erased properly in a proportion of gametes in which grandparental imprints remain (grey oocyte). Upon fertilization, a proportion of offspring would inherit 2 paternally marked alleles: one improperly reprogrammed allele from their mother and a normally imprinted allele from their father. Shapes above the chromosomes indicate their identity.

7. Experimental approach and expectations

7.1 Grandparental-origin effect on embryonic growth

First, we examined grandparental-origin effect on embryonic growth. Three chromosomal regions were chosen: distal chromosome 2, distal chromosome 7 and distal chromosome 12. Embryos with paternal UPD2 are significantly heavier than their littermates from 14.5 d.p.c. to 16.5 d.p.c. (24% heavier at 16.5 d.p.c.). This difference is due to swelling and it decreases after the embryonic kidney starts to function at 16.5 d.p.c. (Williamson *et al.*, 1998).

Despite the fact that embryos with paternal UPD7 die early in development (around 10.5 d.p.c.), we chose to include distal chromosome 7 because of the presence of *Igf2* gene that promotes embryonic growth (DeChiara *et al.*, 1990) and because 15.5 d.p.c. embryos chimeric for normal cells and distal chromosome 7 paternal disomic cells display growth enhancement (Ferguson-Smith *et al.*, 1991).

Finally, we included distal chromosome 12. UPD for distal chromosome 12 results in late fetal lethality and abnormal growth phenotypes (Georgiades *et al.*, 2000). Embryos with paternal UPD12 have heavier placentas than their normal littermates at 18.5 d.p.c. Embryos with maternal UPD12 have fetal and placental growth retardation from 15.5 d.p.c. (Georgiades *et al.*, 2000). Furthermore, humans with maternal UPD for the homologous region, 14q32, also show intrauterine growth retardation (Healey *et al.*, 1994).

Thus, 15.5 d.p.c. and 18.5 d.p.c. embryos were generated. Two reciprocal backcrosses were designed to detect grandparental-origin effects. Offspring from both backcrosses inherited the same genetic material from their father and mother. However, identical alleles inherited from the mother were from different grandparental origin. Thus, effects of parental origin were distinguishable from allelic effects. These embryos were weighed, sexed and genotyped upon collection.

7.2 Distal chromosome 12 genes

We previously found a grandparental-origin dependent transmission ratio distortion (TRD) of maternal alleles in distal chromosome 12 (Croteau *et al.*, 2002). These data suggest that establishment and/or maintenance of imprints in this region are not equally accurate in all embryos. In this study, we examined expression of genes located in this region (Figure 4A). We investigated known imprinted genes and genes whose function is related to embryonic development. We determined if both or only one allele of these genes was expressed in individual embryos using transcribed polymorphism to determine allelic origin.

We analyzed the expression of the paternally expressed Delta-like 1 homologue (*Dlk1*) gene (Schmidt *et al.*, 2000). *Dlk1* is located 82 kb upstream of the Maternally expressed gene 3/ Gene-trap locus 2 *(Meg3/Gtl2)* (Figure 4B). Imprinting of *Meg3/Gtl2* gene was investigated in embryos from reciprocal crosses between C57BL/6 and MOLF/Ei mice (Croteau *et al.*, 2003). *Meg3/Glt2* was found to be maternally expressed in embryos from one cross and biallelically expressed in about 30% of the embryos of the reciprocal cross. Here, we tested *Dlk1* expression in the same embryos using 2 single nucleotide polymorphisms (SNPs) between C57BL/6 and MOLF/Ei strains that we found by sequencing.

We also analyzed expression of Deiodinase, iodothyronine type III (*Dio3*) gene which is involved in thyroid hormone metabolism and located 704 kb distal to *Meg3/Gtl2* on chromosome 12 (Figure 4B). It was recently shown to be imprinted as expression from the paternal allele represent 80% of total expression in embryos (Tsai *et al.*, 2002). In this assay, parental origin of *Dio3* transcript was identified by using a SNP between a *Mus musculus molossinus* strain (JF1) and C57BL/6. We tested this polymorphism on MOLF/Ei and found that it is the same as in JF1.

More genes located on distal chromosome 12 were chosen for their function in embryonic development. We investigated Tumor necrosis factor, alpha-induced protein 2 (*Tnfaip2*), Vasoactive intestinal peptide receptor 2 *(Vipr2),* Jagged2 (*Jag2*), Yin Yang 1 transcription factor 1 (*Yy1*) and Tryptophanyl-tRNA synthetase *(Wars)* genes (Figure 4).

Tnfaip2 is involved in angiogenesis and possibly spermatogenesis (Wolf *et al.*, 1994). *Vipr2* is a neuropeptide possibly implicated in apoptosis and cell proliferation (Pankhaniya *et al.*, 1998). *Jag2* is a member of the Notch signalling pathway and is involved in limb, craniofacial and thymic development in mice (Jiang *et al.*, 1998a). *Yy1* is a transcription factor present during embryonic development. Lack of *Yy1* expression leads to peri-implantation lethality (Donohoe *et al.*, 1999). *Wars* is an ubiquitous enzyme involved possibly in translation regulation (Clemens, 1990) and in splicing (Kittle *et al.*, 1991). An ES-cell specific splice form of *Wars* coding for a protein with a COOH terminus extension was found (Pajot *et al.*, 1994).

Sequencing of *Jag2* and *Tnfaip2* did not detect polymorphisms between strains MOLF/Ei, C57BL/6 and BALB/c. However, we found one SNP in *Vipr2* between BALB/c and C57BL/6. We also found SNPs between MOLF/Ei and C57BL/6 strains in *Yy1* and *Wars* mRNA. Therefore, we could further study the allelic expression of *Dlk1*, *Dio3, Vipr2*, *Yy1* and *Wars*.

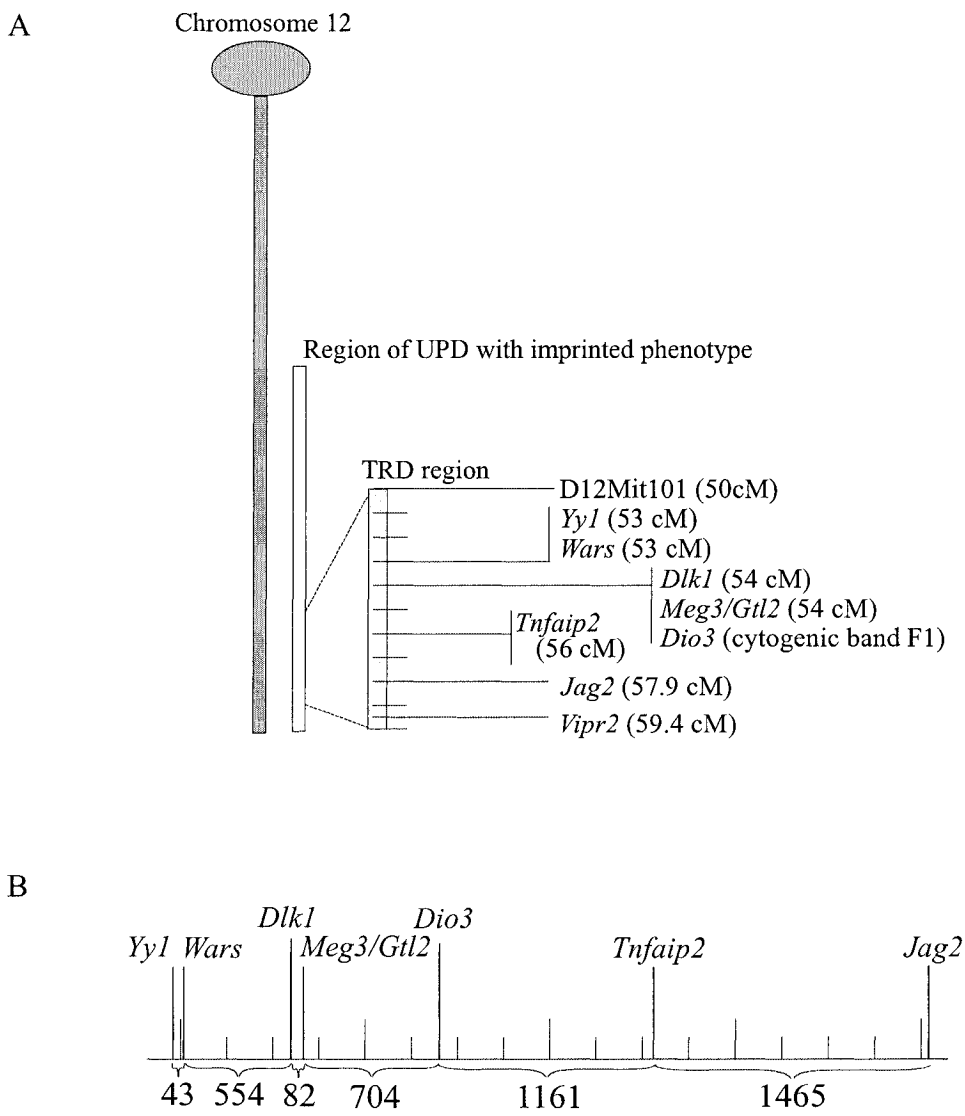

Figure 4: A) Schematic representation of the mouse chromosome 12. The region with imprinted phenotype (Georgiades *et al.* 2000) is depicted as an open rectangle. The TRD region (Croteau *et al.* 2002) is depicted as a grey rectangle. Genes investigated in this study are indicated. **B)** Physical map of the region from 53 to 58 cM from mouse contig GI:28522618. The distance between the studied genes is indicated below the scale in kb.

CHAPTER 2

MATERIALS AND METHODS

1. Mouse strains and crosses

C57BL/6NCrlBR (C57BL/6) and BALB/cAnNCrlBR (BALB/c) mice were obtained at Charles River Canada, Saint-Constant, QC, Canada. Two reciprocal backcrosses were generated to examine grandparental origin of the alleles inherited from the mother (figure 5):

A: (C57BL/6 x BALB/c) F1 females mated to C57BL/6 males

B: (BALB/c x C57BL/6) F1 females mated to C57BL/6 males

For the A cross, 33 F1 females were generated and 24 F1 females for the B cross.

For expression studies, crosses between C57BL/6 and MOLF/Ei mice were used. (C57BL/6 x MOLF/Ei) F1 mice were kindly provided by Dr. Danielle Malo. Crosses between MOLF/Ei and C57BL/6 mice and collection of embryos and tissues were conducted by Dr Sylvie Croteau. Reciprocal crosses (Figure 6) were used to obtain embryos that inherited a C57BL/6 allele from their mother and a MOLF/Ei allele from their father (BM) and embryos that inherited a MOLF/Ei allele from their mother and a C57BL/6 allele from their father (MB). These embryos were collected at 11.5 d.p.c.

2. Embryo collection

Males were caged with one or two females for one night. The next morning, successful mating was assessed by vaginal plug and this day was considered as 0.5 d.p.c. At day 15.5 or 18.5 d.p.c., pregnant females were sacrificed and embryos dissected out of the uterus and rinsed in PBS. For each embryo, uterine tissue was carefully removed, placenta was isolated with extraembryonic membranes and excess fluid was removed with absorbent paper. Embryos and placentas were kept on ice and weighed individually. The posterior legs and tail were used for DNA extraction while the rest of the embryo and the placenta were transferred in individual tubes and frozen at –80 °C.

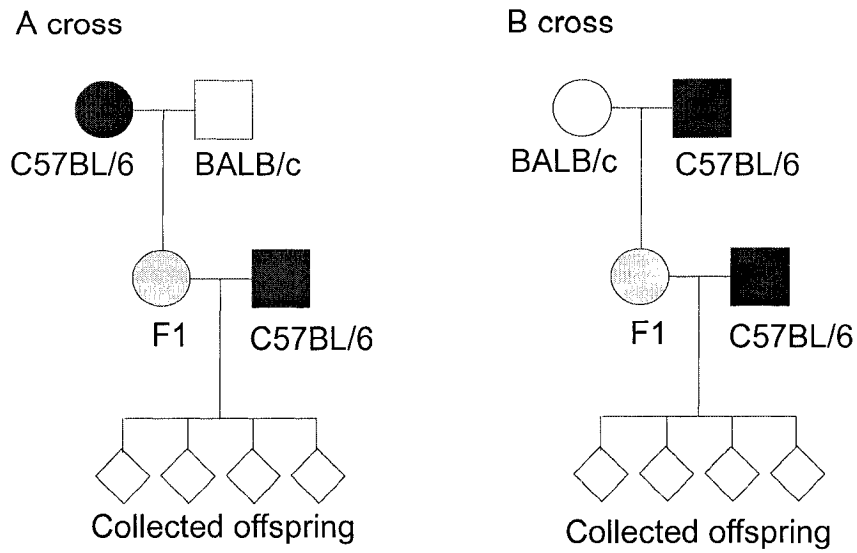

Figure 5: Reciprocal mouse crosses used to study grandparental origin effects. **A.** C57BL/6 females are mated to BALB/c males. F1 females are backcrossed to C57BL/6 males and generated offspring are collected and studied. **B.** For the reciprocal cross, BALB/c females are mated to C57BL/6 males and F1 females are backcrossed to C57BL/6 males. Generated offspring are genetically identical to those collected in the reciprocal cross except the grandparental origin of alleles inherited from their mother.

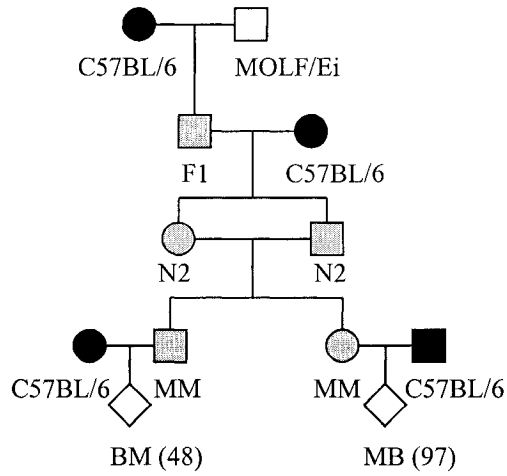

Figure 6: Mouse cross used to generate embryos heterozygote for C57BL/6 and MOLF/Ei in the distal region of chromosome 12. 48 embryos inheriting a C57BL/6 allele from their mother and a MOLF/Ei allele from their father (BM) and 97 embryos inheriting a MOLF/Ei allele from their mother and a C57BL/6 allele from their father (MB) were collected.

3. DNA extraction

Legs and tails were incubated overnight at 55°C in 700 µl of lysis buffer with Proteinase K (50 mM Tris-HCl, pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS and 0.5 mg/ml Proteinase K).

DNA was purified by performing two consecutive extractions with one volume of phenol (pH 8) and one purification with one volume of chloroform/isoamyl alcohol (24:1). The samples were then precipitated in three volumes of ethanol. DNA was recovered with a pipet tip and resuspended in 300 µl of water. DNA was left for a minimum of 3 hours to dissolve under gentle agitation. Samples were reprecipitated in 200 mM sodium acetate and 3 volumes of ethanol. Spooled DNA samples were then dried on filter paper and dissolved in 1 ml of water.

4. Genotyping

Genotypes were determined by PCR amplification of alleles at microsatellite loci. Microsatellite marker primer pairs were chosen for their location in regions of interest and their polymorphisms between C57BL/6 and BALB/c (Table 6). Primers were chosen using the Mouse Genome Informatics web site (http://www.informatics.jax.org), and ordered at Research Genetics (Huntsville, Ala., USA) or Sigma-Aldrich (Oakville, Ont., Canada). Each PCR reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 µM dNTP, 1.5 mM $MgCl_2$, 0.3-0.5 µM of primers and 0.1 unit of Taq polymerase (MBI Fermentas, Vilnius, Lithuania). PCR reactions were carried out in a Biometra T3 thermocycler (Montreal Biotech Inc, Montreal, Qc, Canada), starting with 3 min at 95°C, then doing 35 cycles of the following program: 30 sec at 95°C, 30 sec at 55 °C and 1 min at 72 °C; and the reaction ended by 10 min at 72 °C. PCR products were separated by electrophoresis in 2.5% agarose gels and visualized by ethidium bromide staining. For markers resulting in bands too close to be distinguished in agarose, radioactive PCR reactions were done with 0.1 µC α [^{32}P] dCTP (Perkin Elmer, Boston, MA, USA) and bands were separated in 5% denaturing polyacrylamide gels.

Table 6: Microsatellite PCR markers used for genotyping

Marker	Position (cM)	Size (bp)	
		C57BL/6	BALB/c
D2Mit 230	107	139	131
D12Nds2	59	195	165
D7Mit174	69	109	123

5. Sex determination

Sex of individual embryos was determined by PCR amplification of the Y-chromosome specific gene *Zfy1*, using the following primers:

F: 5' - AAGATAAGCTTACATAATCACATGGA - 3'

R: 5' - CCTATGAAATCCTTTGCTGCACATGT - 3'

PCR was done in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 μM dNTPs, 2.5 mM MgCl2, 0.5 μM of primers, 0.1 unit of Taq polymerase from MBI Fermentas (Vilnius, Lithuania) and going through 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C and ending with 10 min at 72°C. After electrophoresis in 2 % agarose gels and ethidium bromide staining, 600 base pair bands indicated male samples.

6. Statistical analysis

Statiscal analysis were performed using XLStat-Pro 6 program (Addinsoft, Paris, France). Two tests were performed: 1) Fetal weight mean of fetuses inheriting alleles of each grandparental origin were compared by performing the Kruskal-Wallis test. This approach allowed us to compare means from samples with different variances; 2) Deviation of weight distribution of each group from normal distribution was tested using the Kolmogorov-Smironov goodness-of-fit test. The size of the embryos for these particular crosses and the magnitude of the effect possibly caused by imprinting errors were unknown, hence we could not perform power calculations for the tests nor the sample size needed to obtain significant results.

7. RNA extraction and RT-PCR

Embryos and placentas were crushed manually and other tissues were homogenized using a PowerGene 125 homogenizer (Fisher Scientific, Nepean, ON, Canada). RNA

was extracted using trizol (Life Technologies, Burlington, ON, Canada) and chloroform and precipitated in isopropanol.

cDNA was synthesized using M-MLV reverse transcriptase (Life Technologies, Burlingtion, Ont., Canada). Oligo dT ($pd(T)_{12-18}$) were used as primers. Reactions were done as recommended for the reverse transcriptase: 10 min at 72°C in the absence of reverse transcriptase and 60 min at 37°C with the enzyme in the following conditions: 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM Dithiothreitol (DTT) and 0.5mM dNTPs.

8. Allelic expression assays

Following reverse transcription, cDNA were amplified by PCR. The primers for PCR amplification (Table 7) were designed according to published sequences (*Dlk1* GI:13365690, *Wars* GI:13097425, *Vipr2* GI:6678574 and *Yy1* GI:6678620) and were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Standard PCR conditions were used (see the genotyping section).

PCR fragments were labelled with trace amount of α [^{32}P] dCTP (Perkin Elmer, Boston, MA, USA). PCR products were separated by electrophoresis in a denaturing 5% polyacrylamide gel. Quantification of PCR products was done using a Storm 860 phosphoimager (Amersham Canada Ltd, Oakville, ON, Canada).

Amplification of genomic *Dlk1*, *Vipr2*, *Wars* and *Yy1* genes resulted in fragments larger than those amplified from mRNA due to the presence of introns. *Dio3* is an intronless gene (Tsai *et al.*, 2002), hence control amplifications without reverse transcriptase were performed to confirm the absence of genomic DNA contamination.. All PCR amplifications included a control lacking cDNA template to test for exogenous DNA contamination. Digestion by restriction enzymes was tested by adding fragments with a recognition site and a different size to individual digestion tubes or by including a sample homozygous for the digested allele.

8.1 Dlk1

Expression of *Dlk1* was examined using two SNPs located in the second and fifth exons. The polymorphism in exon 2 is located at nucleotide 2501 (GI:13365690). This SNP is recognized by *Dra*III in C57BL/6 DNA. The *Dra*III site is absent in the MOLF/Ei strain. The polymorphism in exon 5 is located at base 7821 and is not recognized by a restriction endonuclease. Thus, we used mismatch PCR-mediated site-directed mutagenesis (Volkova *et al.*, 1996) to introduce a *Dra*I recognition site in the C57BL/6 sequence. Transcripts were amplified using different combinations of primers (Table 8) and their parental origin was tested using one of the two RFLPs or by sequencing.

Table 7: Primers used in expression assays

Name	Sequence (5' to 3')	Position		
Dlk 1F	gtgcaaccctggctttcttcc	1022-1042	GI: 13365690	Exon 1
Dlk 1aF	gagaatcaggggtgtgctgt	2251-2270		Intron 1
Dlk 2F	tgtgacccccagtatggatt	2499-2518		Exon 2
Dlk 4F	aacaatggaacttgcgtgga	5600-5619		Exon 4
Dlk 4R	tgtgcaggagcattcgtact	5635-5654		Exon 4
Dlk 5R	tcaccagcctccttgttt*aa	7822-7841		Exon 5
DatR	ggaagctagaaagagcgccc	16275-16294	GI: 17426732	
Wars F	caagcatgcattttctggag	1231-1250	GI: 13097425	Exon 8
Wars R	ccttgtgccttcagagagga	1741-1760		Exon10
Yy1 F	accagctggttcatactgga	1070-1089	GI: 6678620	Exon 4
Yy1 R	cagcattactaagcatatccc	1697-1717		Exon 5
Vipr2 F	tgacctgctactgctggttg	72-91	GI: 6678574	Exon 1
Vipr2 R	cacagagatggctctcagca	558-577		Exon 4
Dio3 F	atcctcgactacgcacaagg	763-782	GI: 20859334	Exon 1
Dio3 R	tagccagggatggaactacg	1365-1384		Exon 1

Table 8: RT-PCR conditions used for *Dlk1*

Forward	Reverse	Fragment size	Annealing T°	[MgCl2]
Dlk 1F	Dlk4R	477	60°	1mM
Dlk1aF	Dlk4R	506	55°	1.5mM
Dlk1F	Dlk5R	1276	* 55°	1.5mM
Dlk2F	Dlk5R	1057	* 58°	1.5mM
Dlk4F	Dlk5R	849	55°	1.5mM
Dlk4F	DatR	---	* 60°	2mM

*PCR cycles were extended (95°C for 45 sec, annealing T° for 45 sec and 72 °C for 1.5 min

8.2 Dio3

To eliminate genomic DNA contamination, RNA samples were treated with *Deoxyribonuclease*I (Life Technologies, Burlington, ON, Canada) for 30 min at 37°C and 15 min at 65°C for inactivation prior to RT-PCR.

Reverse primers were labelled with $\gamma[^{32}P]$ ATP (Perkin Elmer, Boston, MA, USA), using T4 polynucleotide kinase (MBI Fermentas, Vilnius, Lithuania) and purified with ProbeQuant columns (Amersham Biosciences, Piscataway, NJ, USA) before standard PCR amplification. PCR fragments were digested with the restriction endonuclease *Taq*I and separated on denaturing 8% polyacrylamide gels.

8.3 Wars

Sequencing of PCR fragments amplified by WarsF and WarsR primers revealed 3 polymorphisms in exon 9. At nucleotide 1471, C57BL/6 had a T and MOLF/Ei had an C which creates a *Eco91*I restriction site in the MOLF/Ei sequence. Hence, RFLP analysis was used to distinguish *Wars* expression from different alleles.

8.4 Vipr2

Sequencing of *Vipr2* revealed 2 SNPs between C57BL/6 and BALB/c. Nucleotide 538 was an A in C57BL/6 and a G in BALB/c. The *Xmn*I enzyme recognizes a site in BALB/c alleles and creates specific band size for C57BL/6 and BALB/c alleles that were distinguishable in 2.5% agarose gel. Parental origin of *Vipr2* fragments was thus tested by RFLP analysis.

8.5 Yy1

Yy1 sequencing revealed two polymorphisms in the fifth and last exon. Nucleotide 1289 was an A in C57BL/6 and a G in MOLF/Ei. This SNP introduced a recognition site for *Ear*I restriction endonuclease in MOLF/Ei alleles. Hence, RFLP analysis was used to determine origin of *Yy1* transcripts amplified.

9. Cloning of PCR products

Dlk1 RT-PCR products were cloned using TA cloning kit (Invitrogen, Groningen, The Nederlands). PCR fragments were ligated in pCR2.1 vectors provided with the kit. These vectors contain an ampicillin resistance gene and a *LacZ* gene disrupted by PCR fragment integration. Colonies were selected on LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar) containing 100 μg/ml of ampicillin and covered with 40 μl of 40 mg/ml X-Gal and 40 μl of 100 mM IPTG. The presence of ampicillin eliminated cells that were not transformed and IPTG activated the *LacZ* gene in cells that integrated a self-annealed vector transforming X-Gal in a blue-colored by-product.

White colonies were chosen and clones were grown in 5 ml of LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) for one night. Cells were centrifuged and resuspended in lysis buffer (10mM Tris pH 8, 1mM EDTA, 15% weight/volume sucrose, 2mg/ml lysosyme, 0.2mg/ml Rnase and 0.1mg/ml BSA). After 10 minutes of incubation, the mixture was boiled for 2 minutes and cooled on ice. Samples were centrifuged to remove the bacterial material (including bacterial genomic DNA).

Individual clones were analyzed by RFLP analysis. Selected clones were sequenced to validate RFLP results after phenol/chloroform (1:1) purification and ethanol precipitation.

10. Sequencing

All the sequencing was done by the McGill University and Genome Quebec Innovation Centre (Montreal, Qc, Canada).

11. Electronic resources

Genotyping markers were selected in the Mouse Genome Database (http://www.informatics.jax.org). Genbank database (NCBI, http://www.ncbi.nih.gov) was used to obtain sequences for *Dlk1*, *Dat, Jag2, Tnfaip2, Vipr2*, *Wars* and *Yy1*. NCBI BLAST program was used to align sequences and to search for ESTs.

CHAPTER 3

RESULTS

1. Grandparental-origin effect on embryonic growth

To investigate the effect of grandparental origin on embryonic growth, we recorded fetal and placental weight of 15.5 d.p.c. and 18.5 d.p.c. mouse embryos (260 embryos for each age group). The embryos were genotyped for marker alleles closely linked to imprinted regions of distal chromosomes 2 (*D2Mit230*), 7 (*D7Mit174*) and 12 (*D12Nds2*).

Fetal weight average of fetuses inheriting each grandparental allele were calculated (Table 9). Krukal-Wallis tests were performed to determine if groups of fetuses inheriting alleles with different grandparental origin had significantly different fetal weight averages. The significance threshold needed to be adjusted for the total number of tests, i.e. 2 ages, 3 loci and 3 sex categories (males, females and both) for a total of 18 tests. Hence, assuming that all tests were independent, the threshold for significance was set to 0.05/18=0.0028 in order to have an overall 5% type 1 error rate (false positive).

For all groups, grandparental origin of inherited allele did not significantly affect fetal weight average (Table 9). However, analysis of the weights of 15.5 d.p.c. fetuses showed that males that inherited the grandpaternal distal chromosome 7 allele from their mother tended to weigh less than those that inherited the grandmaternal allele (p=0.012) and 15.5 d.p.c. fetuses that inherited the grandpaternal distal chromosome 2 allele from their mother tended to weigh more than those that inherited the grandmaternal allele (p=0.02). 18.5 d.p.c. fetuses that inherited the grandpaternal distal chromosome 12 allele from their mother tended to weigh more than those that inherited the grandmaternal allele (p=0.042) and this effect is more pronounced in female fetuses (p=0.023).

Table 9: Grandparental-origin effect on fetal weight: Weights of fetuses that inherited alleles of different grandparental origin from the mother in 3 imprinted regions

		Fetal weight at 15.5 d.p.c.						
		Grandmaternal allele			Grandpaternal allele			
		Mean	SE	P normal	Mean	SE	P normal	P mean
D2Mit230	Both sexes	404.4	4.3	0.141	413.8	4.8	0.376	0.020
	Females	403.1	6.0	0.194	407.9	5.1	0.966	0.132
	Males	406.1	6.3	0.269	419.4	7.9	0.390	0.071
D7Mit174	Both sexes	412.8	4.3	0.460	404.6	4.6	0.101	0.328
	Females	402.6	5.9	0.364	407.4	5.4	0.916	0.384
	Males	424.5	6.0	0.611	401.7	7.7	0.051	0.012
D12Nds2	Both sexes	405.4	4.7	0.500	411.5	4.3	0.104	0.498
	Females	403.7	5.9	0.522	406.4	5.4	0.656	0.500
	Males	407.3	7.6	0.234	416.9	6.6	0.095	0.886
		Fetal weight at 18.5 d.p.c.						
		Grandmaternal allele			Grandpaternal allele			
		Mean	SE	P normal	Mean	SE	P normal	P mean
D2Mit230	Both sexes	1235.0	9.1	0.052	1226.6	9.3	0.242	0.821
	Females	1228.7	14.0	0.417	1225.7	14.0	0.753	0.890
	Males	1233.2	12.0	0.594	1229.0	12.4	0.423	0.782
D7Mit174	Both sexes	1235.0	9.4	0.092	1225.5	8.9	0.164	0.525
	Females	1236.5	14.9	0.440	1219.0	12.9	0.540	0.320
	Males	1227.5	12.2	0.588	1231.7	12.4	0.447	0.866
D12Nds2	Both sexes	1217.0	8.8	0.130	1243.9	9.4	0.006	0.042
	Females	1206.8	13.0	0.645	1247.9	14.2	0.141	0.023
	Males	1225.8	12.0	0.320	1234.0	12.5	0.246	0.632

SE: Standard error of the mean. P normal: Probability that the weights follow a normal distribution, calculated with the Kolmogorov-Smirnov goodness-of-fit test. P mean: Probability that the means of the two groups are equal, calculated with the Kruskal-Wallace test. All P values are unajusted for multiple testing. Significance thresholds adjusted for multiple testing are Pmean $\leq$ 0.0028 and Pnormal $\leq$ 0.0014.

The grandparental origin of inherited alleles did not significantly affect placental weight (data not shown). Trends were not taken into account because of the difficulties to normalize the procedure to collect and weigh the placentas.

These differences in fetal weight averages could be due to major effects restricted to a small group of fetuses or to minor effects on a larger group or on all fetuses. We expected that grandparental-origin effects on fetal weight would be due to errors in the resetting of imprints which should affect only a proportion of fetuses. Therefore, the expectation was that the fetuses with incorrect imprints would increase one of the tails of the distribution of weights.

Thus, we tested if each distribution was significantly different from a normal distribution with the Kolmogorov-Smironov test. Again, the significance threshold had to be corrected for the multiple tests and was set to 0.0014. Fetal weight distribution of all groups was not significantly different from normal. However, fetal weight distribution for 18.5 d.p.c. fetuses that inherited the grandpaternal distal chromosome 12 allele tended to be different from the normal distribution (p=0.006). In contrast, the distribution of weights from fetuses that inherited the grandmaternal allele was not different from a normal distribution (p=0.13).

2. Expression of *Dlk1*

Dlk1 expression was analyzed in 11.5 d.p.c. embryos from two reciprocal crosses between MOLF/Ei and C57BL/6 strains (Figure 6). Embryos that inherited a MOLF/Ei *Dlk1* allele from their mother and a C57BL/6 *Dlk1* allele from their father are referred to as MB, whereas the embryos that inherited C57BL/6 *Dlk1* allele from their mother and a MOLF/Ei *Dlk1* allele from their father are referred to as BM.

Amplification of exons 1 to 4 of *Dlk1* revealed that *Dlk1* imprinting was maintained in BM embryos but *Dlk1* was biallelically expressed in all studied embryos and placentas from the MB cross (Figure 7). Quantification revealed that the expression from the paternal allele represented 50-87% of total *Dlk1* mRNA in MB embryos and 100% of total *Dlk1* mRNA in BM embryos. In contrast, most of the placentas from MB embryos had biallelic expression of *Meg3/Gtl2* but only 30% of MB embryos expressed the paternal allele to some extent (Croteau *et al.*, 2003).

This unusual asymmetry of expression between two reciprocal crosses could be explained by combination of monoallelic strain-specific expression from a non-imprinted promoter and parental-specific expression from another imprinted promoter (Figure 8a). It is also possible that imprinted expression is limited to certain tissue types or organs (Figure 8b).

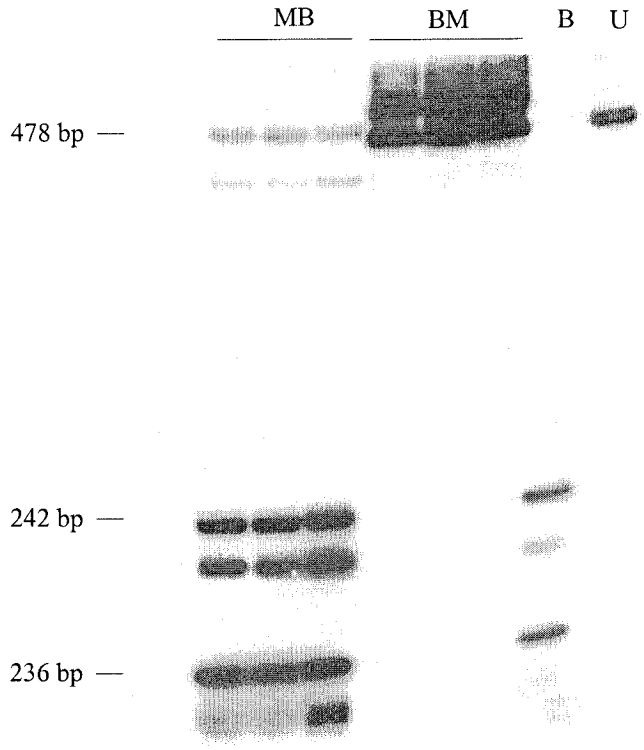

Figure 7: RFLP analysis of RT-PCR products amplified with primers in exon 1 and 4 of *Dlk1* in MB 11.5 d.p.c. embryos (MB), in BM 11.5 d.p.c. embryos (BM) and in C57BL/6 homozygous embryos (B). Products were digested with the *Dra*III enzyme and an undigested PCR product is also shown (U). Products from C57BL/6 allele are represented by 236 bp and 242 bp products and product from MOLF/Ei allele remain undigested and are represented by 478 bp bands.

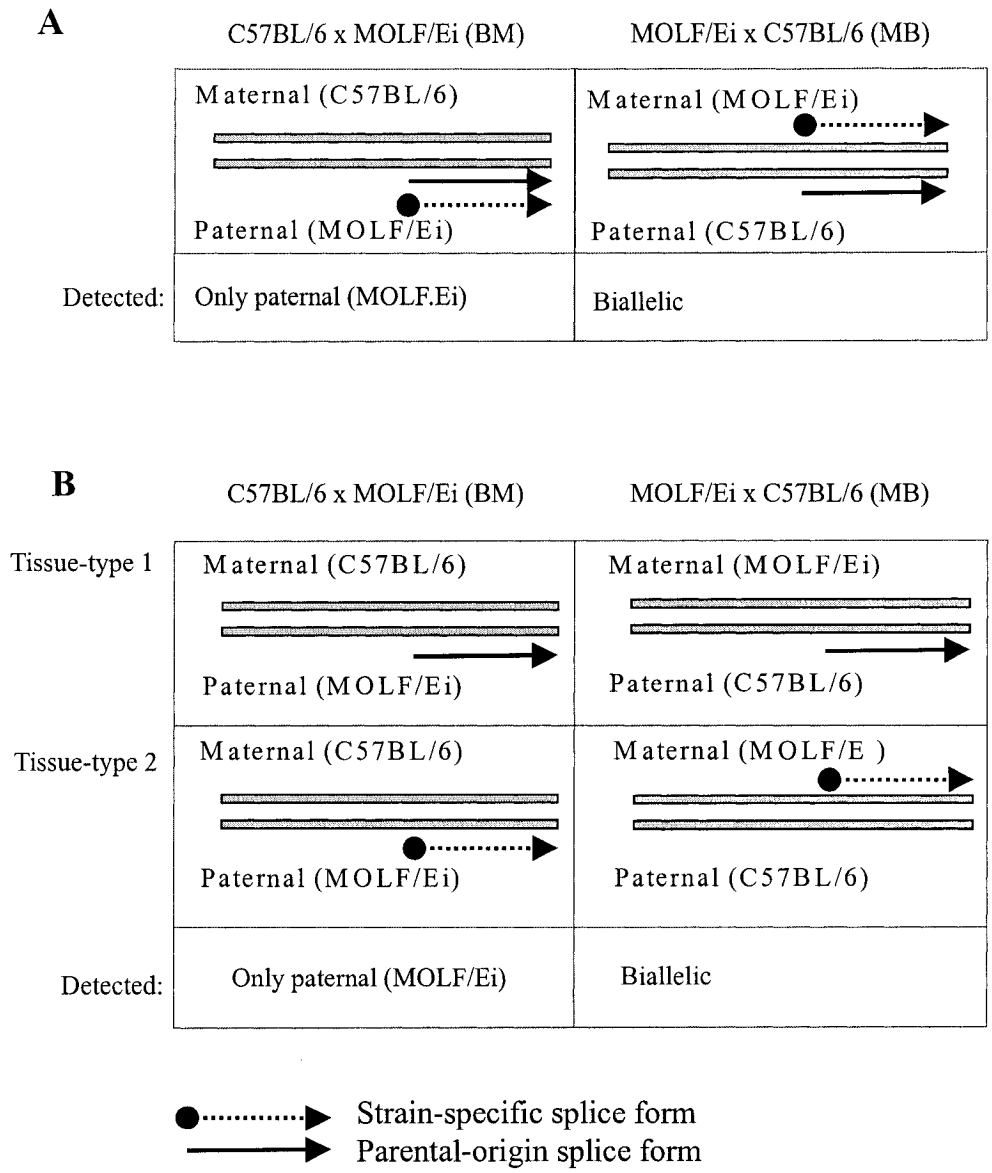

Figure 8: Schematic representation of our hypothesis. In both situations, imprinted expression would be detected in BM animals and biallelic expression in MB animals. **A)** *Dlk1* expression consists of an imprinted transcript and a strain-specific transcript. Assuming that the imprinted transcript is expressed exclusively from the paternal allele (solid arrow) and the strain-specific splice-form is present only on the MOLF/Ei allele and is expressed independently of its parental origin (discontinuous arrow), only MOLF/Ei products would be detected in BM animals and products from both alleles would be detected in MB animals. **B)** Alternative hypothesis: *Dlk1* is normally imprinted in tissue-type 1 and the MOLF/Ei allele is preferentially expressed in tissue-type 2.

We investigated the possibility that different splice-forms of *Dlk1* transcribed from unidentified promoters have different parental bias in expression. *Dlk1* has 2 major types of transcripts with different sizes resulting from alternative splicing of exon 5 (Smas *et al.*, 1994) (Figure 9). To verify that expression of both forms is parental-origin dependent and to rule out the possibility of incomplete digestion with *Dra*III, we cloned individual PCR products and determined their size and parental origin by RFLP analysis or sequencing. All cloned products analyzed corresponded to either the long form containing the full length of exon 5 or the short form missing 220 base pairs in exon 5 (Figure 9). Among the cloned *Dlk1* RT-PCR products derived from BM embryos, we found 10 corresponding to long transcripts and 15 corresponding to short transcripts and all were derived from the paternal allele as expected. In contrast, among the cloned *Dlk1* transcripts from MB embryos, we found a total 20 clones corresponding to long paternal transcripts, 12 to long maternal transcripts, 14 to short paternal transcripts and 2 to short maternal transcripts (Table 10). These results demonstrated that there was no difference in imprinting between the long and the short *Dlk1* splice-forms.

Table 10 : Parental origin of Dlk1 RT-PCR products cloned

	PCR primers (see table 7)	Splice form	MOLF/Ei from the mother and C57BL/6 from the father (MB)		C57BL/6 from the mother and MOLF/Ei from the father (BM)	
			Paternal	Maternal	Paternal	Maternal
A	Total	Long	20	12	10	0
		Short	14	2	15	0
B	Dlk1F - Dlk5R	Long	12	1	7	0
		Short	4	0	7	0
	Dlk2F - Dlk5R	Long	8	4	Not tested	Not tested
		Short	4	0	Not tested	Not tested
	Dlk4F - Dlk5R	Long	0	7	3	0
		Short	6	2	8	0

Number of clones obtained for each form of Dlk1 using different sets of primers and each parental origin. A) Total of amplified transcripts of each form. B) Amplified transcripts separated by primers used
(This table is borrowed from Croteau *et al.*, 2003, table 2)

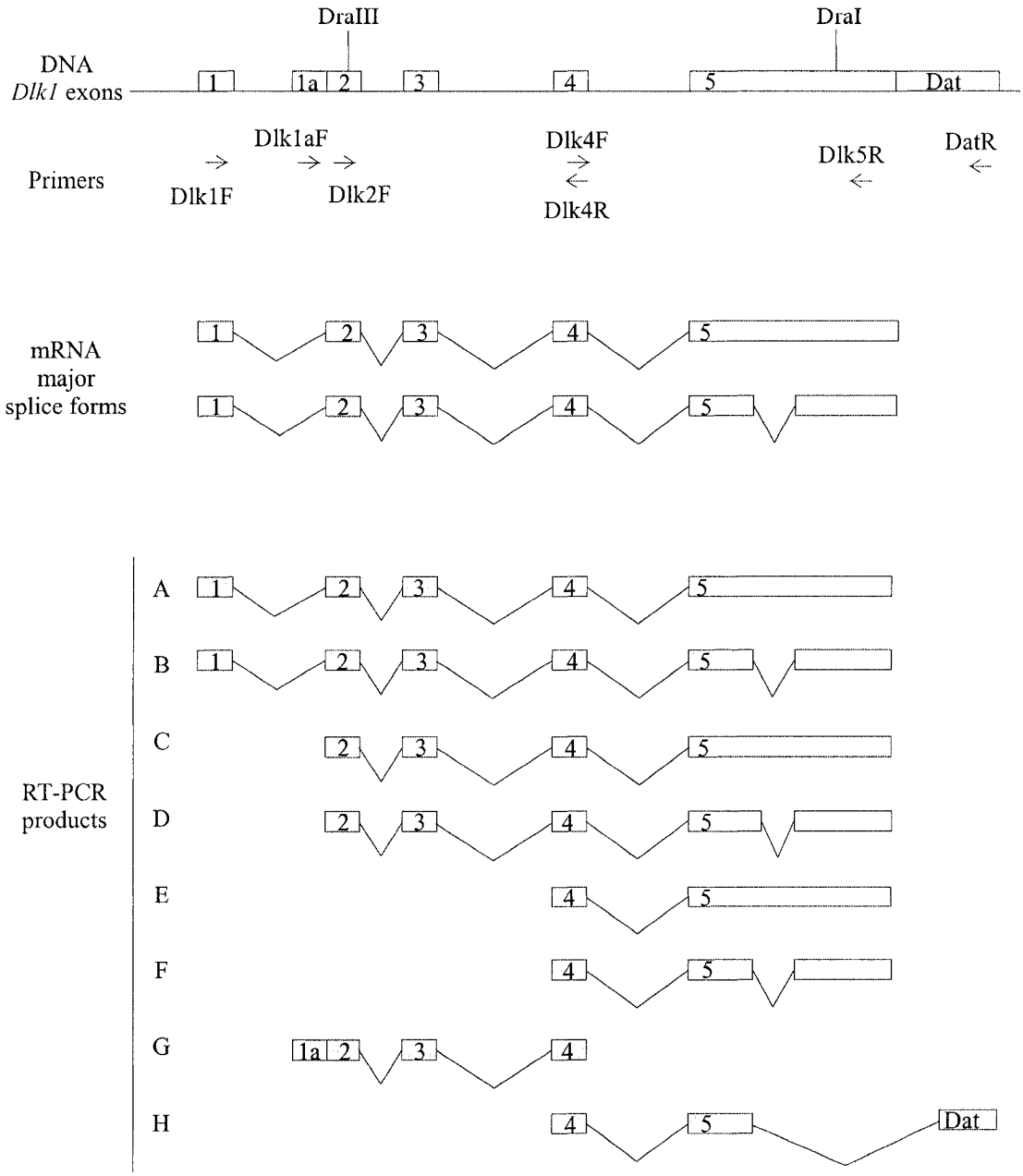

Figure 9: *Dlk1* gene organization. Exons are represented as boxes and restriction sites polymorphic between C57BL/6 and MOLF/Ei strains are indicated. Primers used throughout the study are indicated by arrows. Major mRNA forms of *Dlk1* and RT-PCR products amplified and cloned are illustrated.

However, when the cloned products were examined according to the primers used, we observed that no paternal long transcripts were amplified by primers in exons 4 and 5 while it was the most common form when using primers in exons 1 and 5 or 2 and 5 (Table 10). This finding suggested the presence of an additional *Dlk1* form and possibly of an unknown promoter. This new *Dlk1* mRNA would be paternally expressed and not amplified by the primer in exon 4 or maternally expressed and not amplified by the primers in exons 1 and 2.

We investigated expression of Dlk1 associated transcripts *(Dat)*. These transcripts are expressed from the region downstream of *Dlk1* and probably present extended forms of *Dlk1* (Paulsen *et al.*, 2001). We succeeded in amplifying a PCR product with primers in exon 4 of *Dlk1* and in the *Dat* region. Sequencing of this product revealed that it is composed of exon 4, the first portion of exon 5 and 265 bases in the *Dat* region (Figure 9 product H). We could not test the allelic origin of this transcript because we did not find a polymorphism.

We searched mouse expressed sequence tag (EST) database for the presence of different *Dlk1* splice-forms. We found the sequences of two cDNAs isolated from mouse embryos starting in intron 1 (GI 28224588 and 1744491). We investigated expression of this transcript using primers in intron 1 (or exon 1a) and exon 4 (figure 9 product G). We amplified this transcript in MB and BM embryos. Expression analysis revealed that it is expressed mainly from the paternal allele, hence it is not responsible for the maternal expression that we detected (Figure 10).

To test the hypothesis that *Dlk1* parental-origin dependent expression is restricted to specific tissues, we examined *Dlk1* expression in different adult tissues. We found that *Dlk1* expression in brains displays different levels of parental bias between individuals. In contrast, *Dlk1* expression was exclusively paternal in muscles. Parental origin of *Dlk1* exons 1 to 4 amplified from brains of 11 animals and muscles of 10 animals was determined by sequencing. We found as expected that all muscles analyzed expressed only the paternal allele. We found variable level of maternal expression in 3 out of 11 brain samples tested.

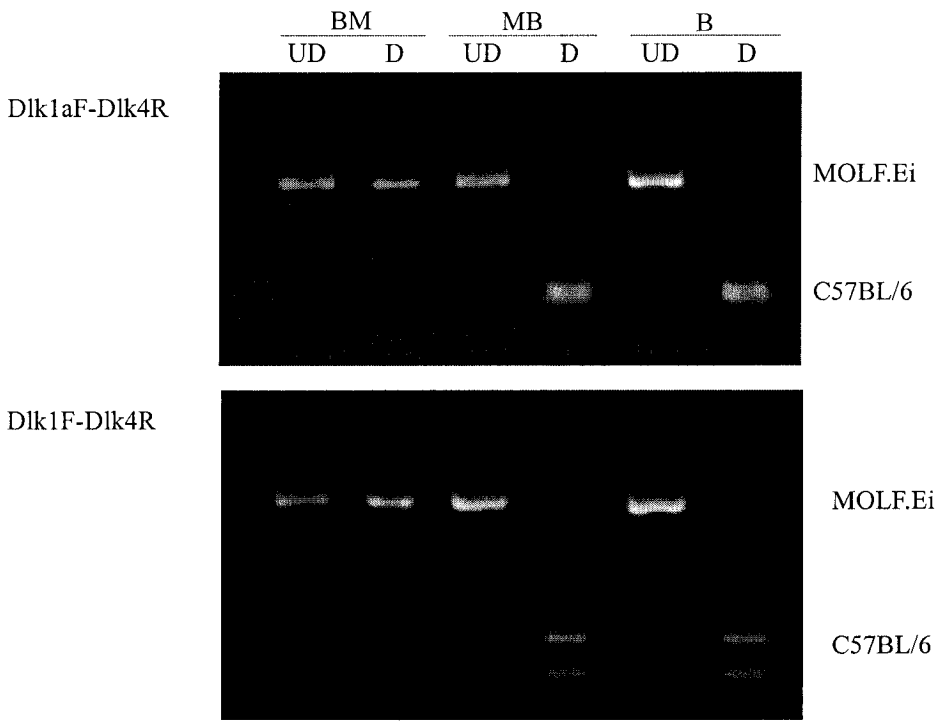

Figure 10: Imprinting assay of *Dlk1* products amplified with primer in intron 1(Dlk1aF) or primer in exon 1(Dlk1F) in a BM 11.5 embryo (BM), a MB 11.5 embryo (MB) and a C57BL/6 control embryo (B). Samples (D) were digested with the *Dra*III enzyme which recognizes a site in C57BL/6 strain only. Samples (UD) were not digested.
(This figure is borrowed from Croteau *et al.*, 2003, figure 3)

3. Expression of *Dio3*

We examined *Dio3* expression in BM and MB 11.5 d.p.c. embryos. In BM embryos, we found 78% of *Dio3* expression coming from the paternal allele (Table 11). This was consistent with the results of Chen-En Tsai and colleagues (2002) who found that paternal expression represented 84% of total *Dio3* expression in 15.5 d.p.c. embryos. In contrast, only 53 % of *Dio3* expression was from the paternal allele in MB embryos, suggesting that similarly to *Dlk1*, *Dio3* imprinting was lost in this cross.

We also investigated *Dio3* expression in adult brains because we found LOI of *Dlk1* in this tissue. Our results suggest that *Dio3* is not imprinted in this organ (Table 11).

4. Expression of *Vipr2*

We examined *Vipr2* expression in 15.5 d.p.c. embryos and placentas. (C57BL/6 x BALB/c) F1 females were mated to C57BL/6 males. Offspring heterozygous for the *D12Nds2* marker were used to assay *Vipr2* imprinting. We observed that both maternal and paternal alleles were expressed in all embryos (n=5) and placentas (n=2) (Figure 11). This suggests that *Vipr2* gene is not imprinted in 15.5 d.p.c. embryos and placentas.

5. Expression of *Yy1*

We investigated *Yy1* expression in embryos, placenta and different adult organs. In all tissues, we observed biallelic expression (Figure 12). Furthermore, we did not find any bias in transcription of individual alleles by quantification.

6. Expression of *Wars*

We evaluated *Wars* expression in embryos, placentas and different adult organs. Again, we found biallelic expression in every sample (Figure 13). Quantification of individual bands revealed that MOLF/Ei mRNA was approximately 4 times more abundant than the C57BL/6 mRNA in the embryos, independently of the parental origin (Table 12).

Table 11: Quantification of *Dio3* expression

Types of samples and cross	Number of samples tested	Paternal allele expression (% of total)
MB 11.5 d.p.c. embryos	8	53
BM 11.5 d.p.c. embryos	6	78
MB adult brain	3	51
BM adult brain	2	48

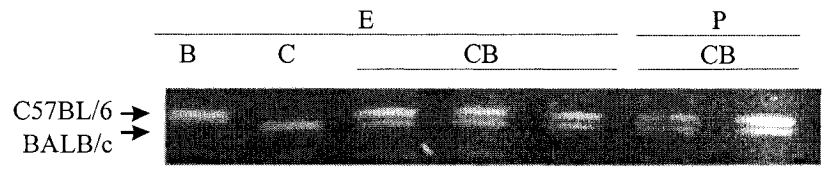

Figure 11: Imprinting assay for *Vipr2* gene expression in embryos (E) and placentas (P) of C57BL/6 (B) and BALB/c (C) control animals and heterozygote offspring of backcrosses between (C57BL/6 x BALB/c) females and C57BL/6 males (CB). Samples were digested with *Xmn*I which recognizes the BALB/c genotype only.

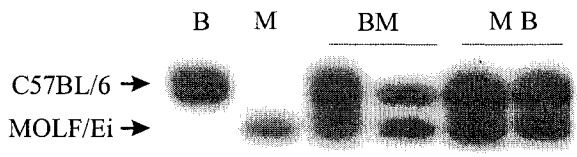

Figure 12: Imprinting assay for *Yy1* gene expression in C57BL/6 and MOLF/Ei control embryos (B and M respectively) and in 11.5 d.p.c. embryos from BM and MB crosses. Samples are digested with *Ear*I which recognizes the MOLF/Ei sequence only.

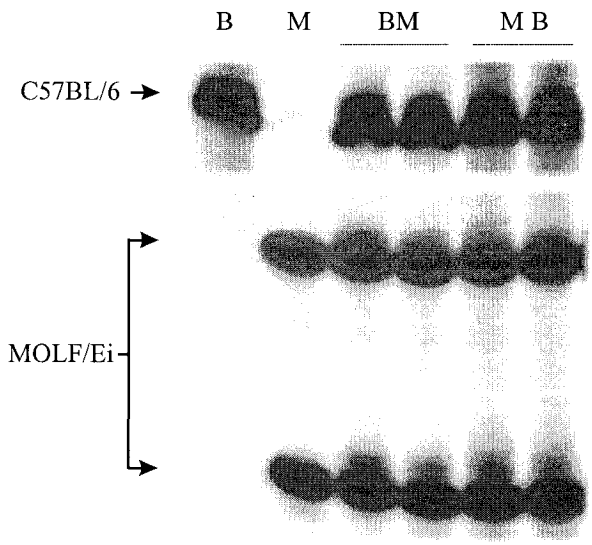

Figure 13: Imprinting assay for *Wars* gene expression in C57BL/6 and MOLF/Ei control embryos (B and M respectively) and in 11.5 d.p.c. embryos from BM and MB crosses. Samples are digested with *Taq*I which recognizes the MOLF/Ei sequence only.

Table 12: Quantification of *Wars* expression

Types of samples and cross	Number of samples tested	Paternal allele expression (% of total)
BM 11.5 embryos	2	80
MB 11.5 embryos	2	22
BM 11.5 placenta	2	73
MB 11.5 placenta	1	14
BM brain	2	72
MB brain	3	35

CHAPTER 4

DISCUSSION

1. Failure to reset imprints and grandparental-origin effect on embryonic growth

Low birth weight is an important indicator of susceptibility to chronic disease, such as type 2 diabetes, hypertension and renal failure (Barker, 1992a; Barker, 1992b; Barker *et al.*, 1993), and mortality among new-borns (Leon, 1991). Low birth weight may result from low growth rate or impaired placental function. There is solid evidence that imprinting and embryonic growth are linked. Initially, abnormal pre- and post-natal growth rates were observed in mice and humans with UPD of several imprinted regions (Tables 3 and 5). Furthermore, experiments in mice with targeted mutations of imprinted genes demonstrated that several imprinted genes influence growth while others are essential for formation and proper functioning of the placenta (Table 3). These observations led Moore and Haig (1991) to propose that imprinting evolved because of parental conflict over offspring growth and maternal resources usage. By means of genomic imprinting, males and females can achieve their different reproductive aims: males favour larger offspring with better chance of survival while females promote the development of smaller offspring that will not be detrimental towards their littermates as well as her future reproduction performance (Moore and Haig, 1991).

Further investigations are needed to understand the relevance of imprinting and the expression of abnormally imprinted genes for embryonic growth and placental development in the absence of genetic mutations. Lindsay and colleagues (2002) searched for parent-of-origin effects on birth weight in humans. They performed a genome-wide linkage analysis to map genes that affect birth weight. They found significant evidence of linkage between paternally derived chromosome 11p alleles and birth weight (Lindsay *et al.*, 2002).

In this study, we used an original approach and examined grandparental origin of alleles and their effect on embryonic growth. We proposed that erasure of imprints during gametogenesis stochastically fails in a proportion of gametes, in which grandparental

imprints remain unchanged. Hence, a proportion of grandpaternal alleles transmitted by a female would remain paternally marked. This would result in abnormal dosage of affected imprinted gene products in a proportion of embryos that inherited the grandpaternal allele from their mother. Consequences would depend upon the function of affected genes; therefore offspring that inherited incorrectly reset imprints in chromosomal regions harbouring imprinted genes regulating embryonic growth would differ is size from their littermates. Thus, we hypothesized that a group of fetuses that inherited an incorrectly imprinted grandpaternal allele from their mother in these regions would be larger or smaller than their littermates.

We chose to investigate grandparental-origin effects of alleles inherited in the distal portion of chromosomes 2, 7 and 12. We chose chromosomes 2 and 12 because mice with UPD of these regions have fetal weight different from their wild type littermates. We also chose to examine the distal region of chromosome 7 because of the presence of the growth factor *Igf2*. However, phenotypic effects associated with UPD are only suggestive of imprinted genes involved in growth regulation residing in a particular chromosomal region. These mice have abnormal dosage of all imprinted genes in the UPD region. In contrast, errors in imprint resetting can affect a variable number of imprinted genes and produce different phenotypic effects. Also, errors in resetting the imprints in the distal region of chromosome 7 could lead to abnormal expression of *Igf2* and of several other imprinted genes located nearby, including *Ascl2* and *Tssc3* which affect placental development and could therefore influence embryonic growth.

We observed that grandparental origin of alleles inherited in the three studied regions tends to influence mean fetal weight. Also, grandparental origin of the allele inherited for the distal region of chromosome 12 tends to influence the weight distribution. We grouped fetuses by age and gender and performed several tests. The results of these tests are not statistically significant after we applied the Bonferroni adjustment for multiple tests. However, the observed tendencies create a solid basis for validation experiments. More powerful statistical analysis could be done because the variance and effect of imprinting errors could be predicted. Corrections for multiple testing could be avoided by genotyping one locus in each group of fetuses generated specifically for this test (i.e.

D2Mit230 in 15.5 fetuses, *D7Mit174* in 15.5 d.p.c. males and *D12Nds2* in 18.5 d.p.c fetuses).

2. Distal chromosome 12 imprinted genes and embryo loss

Embryos that inherit the grandpaternal allele in distal chromosome 12 from their mother are less likely to survive as demonstrated by the grandparental-origin dependent TRD found in this region (Croteau *et al.*, 2002). This suggests that imprinting marks in one or more genes essential for embryonic survival are not equally well established or maintained amongst individual embryos. In this study, we conducted a search for candidate genes located in the distal region of chromosome 12 that are responsible for this embryonic loss. Such candidate genes should be imprinted, located within the TRD region and be essential for embryonic survival.

The known imprinted genes in this region are: *Meg3/Gtl2*, *Dlk1*, *Dio3*, RNA imprinted and accumulated in the nucleus (*Rian*) and Small nucleolar RNAs (*snoRNAs*). Here, we investigated *Dlk1* and *Dio3* expression. Imprinting of *Meg3/Gtl2* gene was investigated in a parallel study (Croteau *et al.*, 2003).

We found that imprinting of *Dlk1* and *Dio3* is maintained in BM (C57BL/6 x MOLF/Ei) embryos but is lost in embryos from the reciprocal cross MB (MOLF/Ei x C57BL/6). All MB embryos expressed *Dlk1* and *Dio3* biallelically. Hence, instead of imprinting errors in a group of embryos, we found strain and parental-origin specific loss of imprinting.

Do *Dio3* and *Dlk1* have a role in the grandparental-origin lethal effect observed? The TRD was found in crosses between C57BL/6 and BALB/c mice, while *Dlk1* and *Dio3* imprinting assays were performed in crosses between C57BL/6 and MOLF/Ei mice. C57BL/6 and BALB/c are *Mus musculus domesticus* strains while MOLF/Ei is a *Mus musculus molossinus* strain. It is possible that imprinting relaxation in *Dio3* and *Dlk1* is specific for crosses between strains of different subspecies and hence would not be found in crosses between C57BL/6 and BALB/c. Due to the lack of polymorphisms

between C57BL/6 and BALB/c, we could not test expression of *Dlk1* and *Dio3* in these crosses.

We examined the average litter size in BM and MB crosses (Croteau *et al.*, 2003). We found that although MB embryos display LOI of *Dlk1* and *Dio3*, litter sizes of BM and MB crosses are similar. This suggests that LOI of *Dlk1* and *Dio3* does not affect embryonic survival.

3. Distal chromosome 12 imprinting instability

On the basis of the effect of the grandparental origin of distal chromosome 12 alleles on embryonic survival and/or fetal growth, we suggested that imprinting of genes in this region is not equally well reset or maintained in every individual. Here and in Croteau *et al.*, 2003, we found strain-specific and parent-specific spontaneous LOI of three genes on distal chromosome 12, confirming that imprinting of this region is unstable.

To determine if this effect is due to *cis* or *trans*-acting factors, congenic mice with a MOLF/Ei distal chromosome 12 allele in a C57BL/6 background were generated. To produce congenic mice, MOLF/Ei mice were backcrossed to C57BL/6 mice for 10 generations. The genome of the resulting congenic mice is composed of more than 99.9% of C57BL/6 sequence although they have a MOLF/Ei distal chromosome 12 allele. Offspring of congenic females homozygous for the MOLF/Ei allele in distal chromosome 12 and C57BL/6 males display LOI of *Meg3/Glt2* and *Dlk1* similarly to MB embryos (Croteau *et al.*, 2003 and data not showed). This suggest that LOI observed in MB embryos is due to *cis*-acting factors or to factors encoded by genes on this chromosomal region.

Schmidt *et al.* showed that the *Dlk1* gene is normally imprinted in embryos from crosses between *Mus musculus castaneus* and C57BL/6 (Schmidt *et al.*, 2000) and Yevtodiyenko et al. showed that *Dio3* is imprinted in embryos from crosses between congenic mice with a *Mus musculus castaneus* distal chromosome 12 allele in C57BL/6 background and C57BL/6 mice (Yevtodiyenko *et al.*, 2002). These facts imply that LOI of *Dlk1* and *Dio3* genes in MB embryos is caused by the MOLF/Ei sequence in the distal region of chromosome 12.

This study presents an example of strain dependent spontaneous loss of imprinting of the endogenous genes *Dlk1* and *Dio3*. LOI in this chromosomal region appears to depend on the strain and parental origin of the allele. There are other examples of strain-specific LOI involving interspecific crosses between mouse strains: (1) Offspring from 129/SvEv females and CAST/Ei males display monoallelic maternal expression of the *Kcnq1* gene (distal part of chromosome 7) in embryos and most tissues except adult brain. In contrast, offspring from the reciprocal cross (CAST/Ei mother and 129/SvEv father) express only the maternal *Kcnq1* allele in 6.5 d.p.c. embryos and then both parental alleles in all organs of 12.5 d.p.c. embryos and adults (Jiang *et al.*, 1998b). Thus, it appears that imprinting maintenance or recognition fail in postimplantation embryos from one cross. (2) Imprinting of Coatomer protein complex, subunit gamma 2, antisense 2 (*Copg2as2*) (proximal part of chromosome 6) and *Cdkn1c* (distal part of chromosome 7) was shown to be lost in heart, lung and muscle of F1 hybrids from C57BL/6 females and KJR/Msf males (a *Mus musculus molossinus* strain) and maintained in embryos from the reciprocal cross (Lee *et al.*, 2000b; Park and Chung, 2001).

The asymmetric LOI between two reciprocal mouse crosses is intriguing. It was suggested that this phenomenon is due to interspecific incompatibility between imprinting factors (Jiang *et al.*, 1998b; Vrana *et al.*, 2000). However, the mechanisms behind these LOI remain unclear.

Little is known about how distal chromosome 12 imprinted genes are regulated. Paternal methylation of a DMR located between *Dlk1* and *Meg3/Gtl2* is inherited from sperm and is thought to be the parental mark for this region (Takada *et al.*, 2002). After fertilization, the paternal allele of another DMR located in the *Dlk1* gene becomes partially methylated and the promoter of the *Meg3/Gtl2* gene also becomes methylated on the paternal silenced allele (Takada *et al.*, 2002). However, factors and sequences important for imprinting regulation of this region could be identified by comparing MOLF/Ei and C57BL/6 distal chromosome 12 sequences.

Overall, our findings demonstrated that interspecific crosses between C57BL/6 and MOLF/Ei mouse strains represent a useful tool to study factors influencing imprinting

variations. We demonstrated that imprinting of the mouse distal chromosome 12 region is unstable and may lead to embryonic lethality and/or abnormal growth. Investigations of the interactions between imprinting elements of different mouse strains could lead to better understanding of how imprinting is influenced by polymorphisms among human populations.

CHAPTER 5

CONCLUSIONS

The goal of this study was to investigate spontaneous imprinting errors in mouse embryos. We used two different approaches. Firstly, we investigated grandparental-origin effects in 3 chromosomal regions containing imprinted genes with a role in embryonic growth regulation. We hypothesized that grandparental origin would affect embryonic weight if errors in resetting the imprints occur. We did not detect statistically significant effect. However, we found trends for grandparental effects in the three tested chromosomal regions. It would be necessary to replicate this experiment to confirm if the observed effects are real or not.

Secondly, we investigated expression of individual imprinted genes on distal chromosome 12. Both the results of our previous study (Croteau *et al.* 2002) and our preliminary data on the grandparental-origin effect on fetal growth suggest that imprinting of one or more genes of this region is not equally well established or maintained in all embryos (Croteau *et al.*, 2002). Failure to reset imprints in this region may lead to embryonic lethality and/or embryo overgrowth. We aimed to identify genes responsible for this effect.

We observed loss of imprinting of *Dlk1*, *Meg3/Gtl2* and *Dio3* genes in (MOLF/Ei x C57BL/6) embryos and normal imprinted expression in embryos from the reciprocal cross (C57BL/6 x MOLF/Ei). This asymmetric imprinting relaxation suggests an interaction between strain-specific and parent-of-origin specific factors in these crosses. The expression data confirm that imprinting of *Meg3/Gtl2*, *Dlk1* and *Dio3* is not stable. However, the biallelic expression of these genes does not seem to be responsible for the fetal loss that we have previously reported (Croteau *et al.*, 2002).

CHAPTER 6

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APPENDIX 1

COMPLIANCE CERTIFICATES

1. Approved animal use protocol
2. Radioactivity permit