Epigenetic reprogramming in mouse germ cells

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to my parents

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Zusammenfassung

Bei Säugerkeimzellen, Zygoten und Embryos in frühen Stadien kommt der epigenetischen Neuprogammierung eine außergewöhnlich wichtige Rolle in der Regulation der Genomfunktionen in entscheidenden Entwicklungsstadien zu. Die epigenetische Neuprogrammierung in Keimzellen löscht zuerst die Imprinting-Markierungen und Epi-Mutationen und stellt dann geschlechtsspezifische Markierungen (genomische Prägung) wieder her.

Die vorliegende Arbeit bezieht sich auf das Löschen epigenetischer Modifikationen in primordialen Mauskeimzellen (*primordial germ cells* (PGCs)) zwischen dem 10.5 bis 13.5 Tag nach der Befruchtung. Entgegen früheren Annahmen zeigen unsere Ergebnisse, daß primordiale Mauskeimzellen (PGCs) beim Eintritt in die embryonalen Keimdrüsen noch immer DNS Methylierungsmarker besitzen, die ähnlich dem Marker in somatischen Zellen sind. Kurz nach dem Eintritt in die Keimdrüsen werden die DNS Methylierungsmarker, die in Verbindung mit geprägten und nicht geprägten Genen stehen, gelöscht. Für die Mehrzahl der Gene beginnt die Löschung der Marker in männlichen und weiblichen Embryos gleichzeitig und ist innerhalb eines Entwicklungstages abgeschlossen. Diese Kinetik deutet auf einen aktiven Demethylierungsprozess hin, initiiert durch ein somatisches Signal, ausgehend von der embryonalen Keimdrüse. Der Zeitpunkt der Neuprogrammierung in den primordialen Keimzellen ist entscheidend, da er sicherstellt, daß Keimzellen beiden Geschlechts einen epigenetisch äquivalenten Status erhalten, bevor sie geschlechtsspezifisch ausdifferenzieren und anschließend neu elterlich geprägt werden.

Vollständiges Verständnis des Prozesses der Neuprogrammierung der Keimzellen ist nicht nur im Hinblick auf genomisches *Imprinting* wichtig, sondern auch für die Erforschung von Mechanismen für die Wiederherstellung von omnipotenten Zellen bei Klonierung und Stammzellenerhaltung.

Summary

Epigenetic reprogramming in mammalian germ cells, zygote and early embryos, plays a crucial role in regulating genome functions at critical stages of development. Germ line epigenetic reprogramming assures erasure of all the imprinting marks and epi-mutations and establishment of new sex-specific gametic imprints. The presented work focuses on the erasure of epigenetic modifications that occur in mouse primordial germ cells (PGCs) between day 10.5 to 13.5 post coitum (dpc).

Contrary to previous assumptions, our results show that as they enter the genital ridge the PGCs still possess DNA methylation marks comparable to those found in somatic cells. Shortly after the entry of PGCs into the gonadal anlagen the DNA methylation marks associated with imprinted and non-imprinted genes are erased. For most genes the erasure commences simultaneously in PGCs of both male and female embryos and is completed within only one day of development. The kinetics of this process indicates that is an active demethylation process initiated by a somatic signal emanating from the stroma of the genital ridge. The timing of reprogramming in PGCs is crucial since it ensures that germ cells of both sexes acquire an equivalent epigenetic state prior to the differentiation of the definitive male and female germ cells in which, new parental imprints are established subsequently.

Complete understanding of the germline reprogramming processes is important not only in the light of genomic imprinting but also for resolving other mechanisms connected with restoring cellular totipotency, such as cloning and stem cell derivation.

1 Introduction

1.1 Brief history of DNA methylation

The presence of the so-called "fifth base" in the DNA of eukaryotes (5-methylcytosine, 5-mC) was revealed already before the final proof that DNA constitutes the real carrier of genetic information. In 1948, while trying to detect amino acid contamination in nucleic acid samples, Hotchkiss found 5-mC by paper chromatographic method (Hotchkiss, 1948**)**. The result was shortly afterwards confirmed by Wyatt including the quantification of 5-mC contribution to the genome (Wyatt, 1951). In 1959 Kornberg suggested that 5-mC may be added onto DNA by post-replicative mechanism, implying for the first time the use of this base as a potential carrier of epigenetic information (Kornberg *et al.*, 1959). This hypothesis took; however, another nine years to be demonstrated experimentally (Billen, 1968; Lark, 1968).

Meanwhile, the first prokaryotic methyltransferases and restriction endonucleases had been identified and the role of DNA modification (5-mC and 6-mA) connected to the concept of bacterial restriction/modification genome defense system (Luria and Human, 1952; Bertani and Weigle, 1953; Arber and Dussoix, 1962; Srinivasin and Borek, 1964; Gold and Hurwitz, 1964).

The investigation of the eukaryotic DNA methylation proved to be far more difficult. The 5-mC content of DNA had been measured by different chromatographic methods (Sneider, 1972; Singer *et al.*, 1977; Culp *et al.*, 1970; Silber *et al.*, 1966) and mass spectroscopy (Gautier *et al.*, 1977). However, only further progress in recombinant DNA technology together with characterization of methylation sensitive restriction endonucleases permitted not only quantitative analysis of 5-mC content but also more importantly, analysis of its spatial distribution. Bird and Southern (Bird *et al.*, 1978) were the first to recognize the potential of these enzymes in combination with the Southern blotting technique to assess the methylation status of defined sites within specific gene regions. In the following decade, number of experiments employed this technique to connect the gene methylation with transcriptional

silencing (reviewed in Ehrlich *et al.*, 1981; Razin *et al.*, 1980; Felsenfeld *et al.*, 1982). Based on those results the two main models connecting methylation with gene expression have been postulated. The model of (a) direct transcriptional inhibition is based on the existence of transcription factors, which are sensitive to the presence of methylated cytosine(s) in their binding sites (such as AP-2 (Comb *et al.*, 1990), E2F (Kovesdi *et al.*, 1987), NF-κB (Bednarik *et al.*, 1991). Methylation within the regulatory sequences of a gene may thus prevent initiation of transcription. The model of (b) indirect inhibition is connected to proteins with the binding specificity for methylated DNA (for example MBD1 – methylated DNA binding protein (Zhang *et al.*, 1989), MeCP2 – methyl-CpG binding protein (Meehan *et al.*, 1989). Binding of those proteins not only makes the DNA inaccessible to transcription machinery, but as it was described rather recently, the proteins can directly interact with the histone deacetylase complexes (Feng *et al.*, 2001; Rountree *et al.*, 2000; Robertson *et al.*, 2000; Ng *et al.*, 1999, Nan *et al.*, 1998), thus bringing the chromatin into the inactive shape. The discovery of such protein factors and their interplay brought about focus on the machinery that "reads" and interprets the methylation mark inside the cell.

The growing knowledge concerning the biological significance of DNA methylation intensified the search for enzymatic activities responsible for the epigenetic marking of DNA. In late 1980s, at the time when the prokaryotic methyltransferases have been more or less thoroughly characterized (concerning both the protein structure and enzymatic functions – for review see (Noyer-Weidner *et al.*, 1993), only very little was still known about their eukaryotic counterparts. Although the existence of two distinct methyltransferase activities (the concept of maintenance and *de no*vo methyltransferase activity) had been predicted already in 1975 (Holliday *et al.*, 1975; Riggs, 1975); the first eukaryotic DNA methyltransferase (Dnmt1) was cloned from murine cells not earlier than in 1988 (Bestor, 1988; Bestor *et al.*, 1988). This enzyme has a 5- to 30-fold preference for hemimethylated DNA (Yoder *et al.*, 1997) and has therefore been assigned a role limited to the maintenance of methylation patterns (Lyko *et al.*, 1999). The residual level of DNA methylation found in Dnmt1 knock-out mouse embryos (Li *et al.*, 1992) confirmed the prediction, that this enzyme is not the only factor responsible for DNA methylation. It took, however, more than 10 years to clone methyltransferases (Dnmt3a, Dnmt3b – Okano *et al.*, 1999) with *de novo* methylation functions.

Whereas DNA methylation in prokaryotes is mainly involved in protecting the genome against the degrading nucleases (restriction/modification systems), and thus playing role in the host defense; the eukaryotic DNA methylation has evolutionary gained more complex function. Findings of the last decade show that in many eukaryotes cytosine methylation plays a pivotal role in the control of gene expression and in inactivation of transposable and repetitive elements (this genome protection function resembles the role of DNA methylation in prokaryotes) (reviewed in Yoder *et al.*, 1997). Additionally, this epigenetic modification is crucial for embryonic development of mammals regulating genomic imprinting, X inactivation and cell differentiation (Reik *et al.*, 2001a; Reik *et al.*, 2001b; Mlynarczyk *et al.*, 2000).

1.2 Genomic imprinting and its connection to DNA methylation

The term genomic imprinting mentioned throughout this thesis refers to a differential parent-of-origin dependent monoallelic expression of some genes.

The first indication that the two parental genomes contributing to the zygote are not functionally equivalent came in the early 1980s. The pronuclear transfer experiments performed by Surani and Solter demonstrated that both parental genomes are essential for normal embryonic development (McGrath *et al.*, 1984; Surani *et al.*, 1984). Embryos that contained two paternal genomes (androgenetic embryos) showed very poor embryonic development, whereas gynogenetic embryos (containing two maternal genomes) were deficient in developing extraembryonic tissues. In both instances, lethality occurred by mid-gestation. These results demonstrated that parental genomes play obviously complementary roles, involving differential (monoallelic) expression of essential genes in embryonic development.

In 1985 Cattanach and Kirk published their studies on mouse embryos containing uniparental duplications (uniparental disomies - UPDs) of sub-chromosomal regions (Cattanach *et al.*, 1985). The thorough study showed that some of the duplications resulted in embryonic lethality. Based on these results a chromosomal map was produced showing the regions, of which both parental copies are necessary for normal embryonic development. Ten such domains have been identified, located on mouse chromosomes 2, 6, 7, 11, 12 and 17. The map has been refined since, its current form is shown in Fig. 1.

Since then number of mouse and human genes that are differentially expressed depending on their parental origin (imprinted genes) have been identified (see Table 1). It is noteworthy that all the genes mapped so far are located within the regions depicted by Cattanach and Kirk. It seems to be an important feature of imprinted genes that they appear in clusters sharing probably the main regulatory elements (Paulsen *et al.*, 1998; Paulsen *et al.*, 2000; Engemann *et al.*, 2000).

Imprinted Loci	**Chr.**	**Chr. Region**	**Repressed parental allele; maternal / paternal**	**Name**
Nnat	2	distal 2	M	neuronatin
Gnas	2	distal 2	P	guanine nucleotide binding protein, alpha stimulating
Gnasxl	2	distal 2	M	guanine nucleotide binding protein, alpha stimulating, extra large
Nesp	2	distal 2	P	neuroendocrine secretory protein
Nespas	2	distal 2	M	neuroendocrine secretory protein antisense
Sgce	6	centromere to T77H (A3.2)	M	sarcoglycan, epsilon
Peg1/Mest	6	proximal 6 (distal to A3.2)	M	mesoderm specific transcript
Copg2	6	proximal 6 (distal to A3.2)	P	coatomer protein complex subunit gamma
Copg2as	6	proximal 6 (distal to A3.2)	M	antisense to Copg2
Mit1/lb9	6	proximal 6 (distal to A3.2)	M	mest linked imprinted transcript 1
Zim1	7	proximal 7	P	imprinted zinc-finger gene 1
Peg3/Pw1	7	proximal 7	M	paternally expressed gene 3
Usp29	7	proximal 7	M	ubiquitin specific processing protease 29
Zim3	7	proximal 7	P	Zinc Finger Gene 3 from Imprinted domain
Zpf264	7	proximal 7	M	Zinc Finger gene 264

Snrpn	7	central 7	M	small nuclear ribonucleoprotein polypeptide N
Snurf	7	central 7	M	*Snrpn* upstream reading frame
Pwcr1	7	central 7	M	Prader-Willi chromosome region 1
Magel2	7	central 7	M	Magel2
Ndn	7	central 7	M	necdin
Zfp127/Mkrn3	7	central 7	M	ring zinc-finger encoding gene
Zfp127as/Mkrn3as	7	central 7	M	ring zinc-finger encoding gene antisense
Frat3	7	central 7	M	Frequently rearranged in advanced T-cell lymphomas.
Ipw	7	central 7	M	imprinted in Prader-Willi Syndrome
Ube3a	7	central 7	P	E6-AP ubiquitin protein ligase 3A
Ube3aas	7	central 7	M	Ube3a antisense
Nap1l4	7	central 7	P	
H19	7	distal 7	P	H19 fetal liver mRNA
Igf2	7	distal 7	M	insulin-like growth factor 2
Igf2as	7	distal 7	M	insulin-like growth factor 2, antisense
Ins2	7	distal 7	M	insulin 2
Mash2	7	distal 7	P	mammalian achaete-scute homologue 2
Kvlqt1	7	distal 7	P	-
Kvlqt1-as	7	distal 7	M	Kvlqt1 antisense
Tapa1/Cd81	7	distal 7	P	Cd 81 antigen
$p57^{KIP2}$ / Cdkn1c	7	distal 7	P	cyclin-dependent kinase inhibitor 1C
Msuit	7	distal 7	P	mouse specific ubiquitously expressed imprinted transcript 1
Slc22l1 **Note Slc22l1 was formally known as Impt1, Itm, and Orctl2.**	7	distal 7	P	Solute carrier family 22 (organic cation transporter memter-1 like).
Ipl/Tssc3	7	distal 7	P	imprinted in placenta and liver (Tdag51?)
Tssc4	7	distal 7	P	
Obph1	7	distal 7	P	oxysterol-binding protein 1

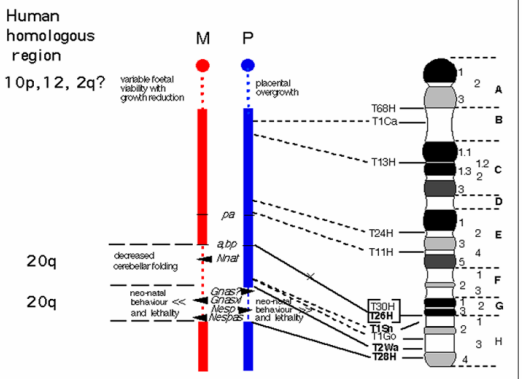
Rasgrf1	9	9	M	Ras protein specific guanine nucleotide-releasing factor 1
Zac1	10	10	M	Zinc finger DNA binding protein
Meg1/Grb10	11	proximal 11 (A1-A4)	P	growth factor receptor bound protein 10
U2af1- rs1	11	proximal 11 (A3.2-4)	M	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF), 35kDa, related sequence 1
Dlk	12	distal 12 (E-F)	M	delta like
Meg3/Gtl2	12	distal 12 (E-F)	P	gene trap locus 2
Htr2a	14	distal 14	P	5-hydroxytryptamine (serotonin) receptor 2 A
Slc22a2	17	proximal 17	P	Membrane spanning transporter protein
Slc22a3	17	proximal 17	P	Membrane spanning transporter protein
Igf2r	17	proximal 17	P	insulin-like growth factor 2 receptor
Igf2ras/Air	17	proximal 17	M	insulin-like growth factor 2 receptor antisense
Impact	18	proximal 18 (A2-B2)	M	Homology with yeast & bacterial protein family YCR59c/yigZ
Ins1	19	19	M	insulin 1

The following gene has now been shown not to be imprinted.

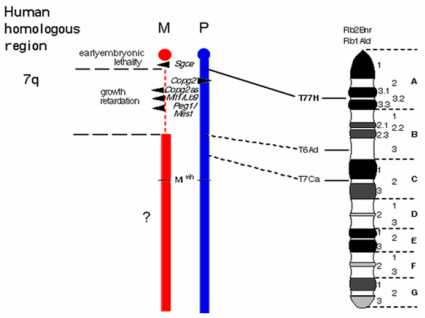
Mas	17	proximal 17	M	Mas proto-oncogene

Table 1: Imprinted genes

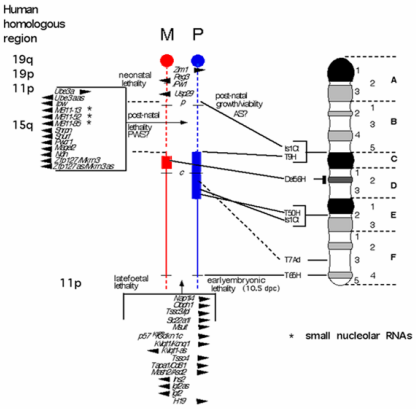
Chromosome 2



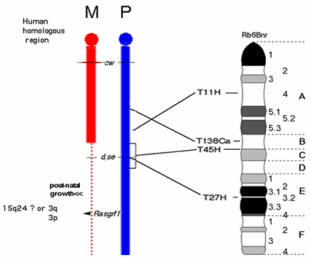
Chromosome 6



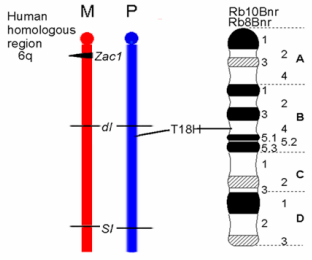
Chromosome 7



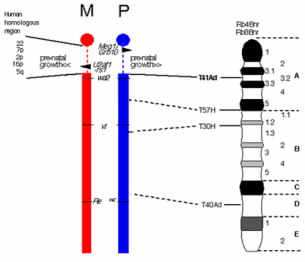
Chromosome 9

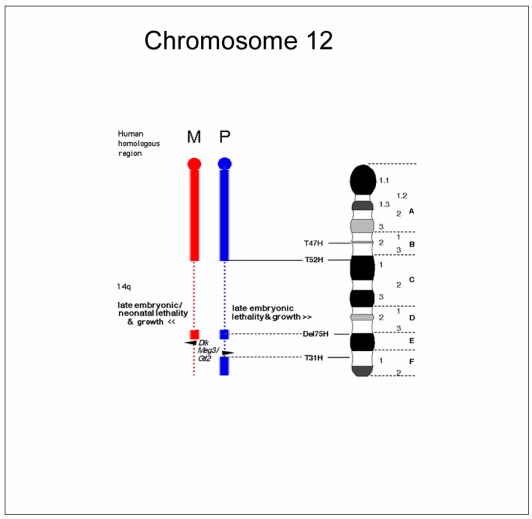


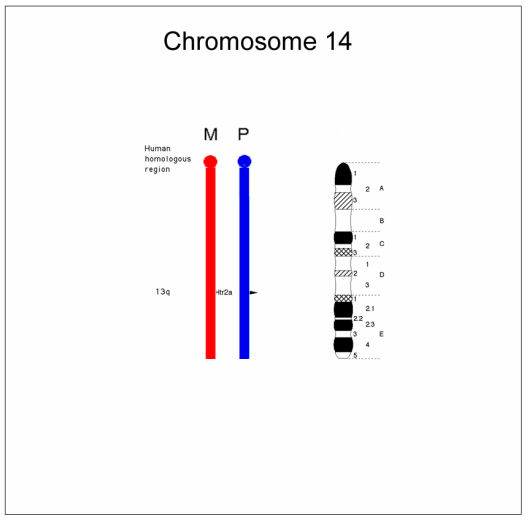
Chromosome 10

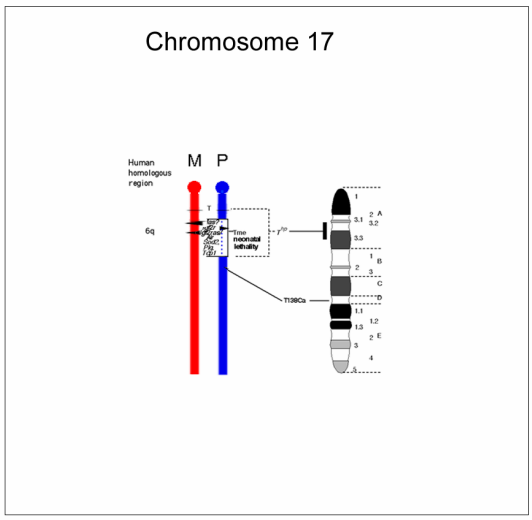


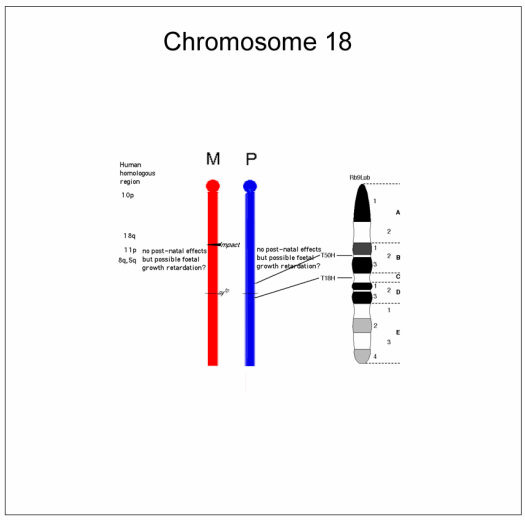
Chromosome 11











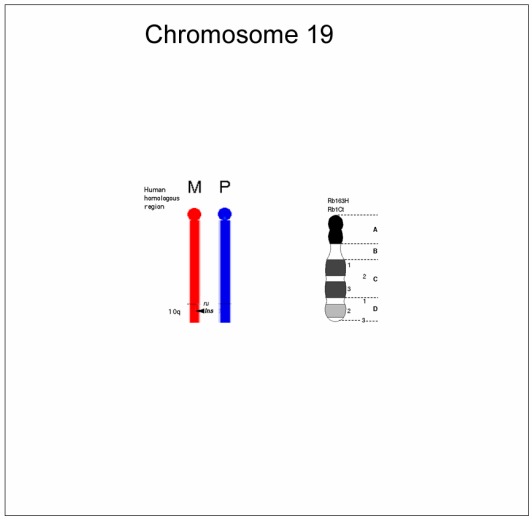


Fig.1: Physical maps of imprinted domains in the mouse chromosomes. (according to www.mgu.har.mrc.ac.uk/imprinting/)

For a paternal imprint to be established there must be a mechanism (marking) which distinguishes the DNA inherited from mother and father. Such a mark is presumably established in the germ line, when the two parental genomes are separated, is propagated by further post-fertilisation events and has to be erased and re-established again in the germ line (see Fig. 2). Possible candidates for such a mark include DNA methylation, or differences in chromatin structure, which may influence the accessibility of the region to imprinting factors.

Before any endogenous imprinted genes were identified, a number of transgenes were observed to be active only after passage through the germ line of one sex (reviewed in Reik *et al.*, 1990). Some of these were studied in detail and were found to carry different methylation patterns depending on their parental origin (Reik *et al.*, 1987; Sapienza *et al.*, 1987). These findings turned out to be real break-through to the field. Although cytosine methylation had long been proposed to fulfil all requirements for the postulated imprint (DNA methylation affects gene expression, is heritable and is reversible), only these discoveries of Reik and Sapienza finally unified the two fields in practice.

The later analysis revealed that all imprinted genes so far identified in mouse and human show regions that are differentially methylated in an allele-specific manner (differentially methylated regions - DMRs). These imprinting control regions are often complex with multiple functions acting to repress genes when methylated, or serving as boundary elements when unmethylated (Bell *et al.*, 2000; Hark *et al.*, 2000). Some DMRs also function as silencer elements when unmethylated (Constancia *et al.*, 2000), a function which is abolished when the DMR is methylated. In other cases, a DMR is associated with the expression of an antisense transcript whose expression in turn ensures repression of the upstream gene (Lyle *et al.*, 2000). In all the cases allele-specific methylation of DMRs ensures the monoallelic expression of imprinted genes.

1.3 The "life cycle" of imprinting in the mammalian development

The process of parental imprinting involves 4 distinct biological stages (steps) (see Fig. 2**)**. The imprints are established during gametogenesis (establishment), they

are maintained throughout the embryogenesis during the time when the rest of the genome undergoes de-methylation (maintenance) and are finally read in the somatic tissues of embryo and adult (reading). In the embryonic germ line, however, the imprints must be erased (erasure) and re-established according to the sex of individual (establishment), thus closing the "life circle" of imprint.

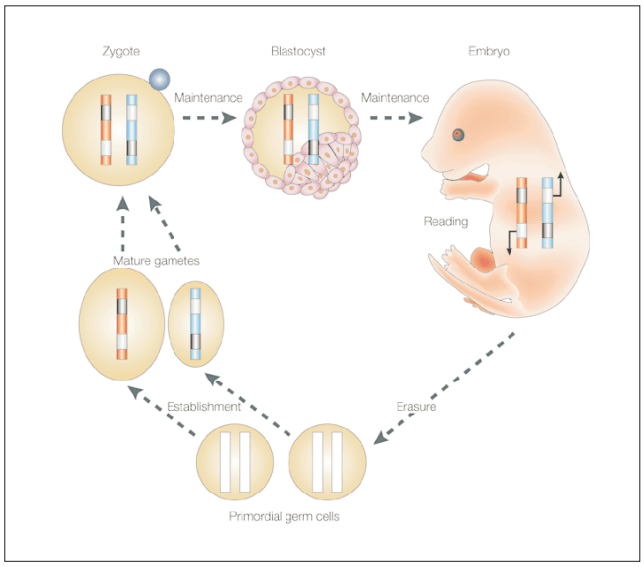


Fig. 2: "Life cycle" of imprinting in mammalian development
The figure shows the requirements genomic imprinting has to fulfill: parental specific marks present in gametes (red - maternal marks, blue - paternal marks) are combined in zygote and maintained through the waves of demethylation and *de novo* methylation to be finally read in the form of monoallelic expression in embryo. During the establishment of the germ line the imprinting marks have to be erased and re-established according to the sex of developing individual (according to Reik and Walter, 2001)

1.4 Imprinting and methylation changes during early embryogenesis

One of the basic criteria for the imprinting mark is that it is present in gametes. The differentially methylated regions (DMRs) of most of the imprinted genes carry the parental specific methylation in oocytes and sperms (Olek *et al.*, 1997; Tremblay *et al.*, 1997; El-Maarri *et al.*, 2001; Stoger *et al.*, 1993). The methylation mark might be not the only signal involved in recognising the paternal origin, though, as the exceptions to the rule have been identified. For example, the promoter of maternally methylated *Snrpn* gene was found to be methylated in mouse oocytes, but demethylated in human oocytes, despite of showing maternal methylation and imprinted monoallelic expression in embryonic as well as in somatic tissues of both species (El-Maarri *et al.*, 2001).

Just shortly after fertilisation, in the zygote, dramatic epigenetic changes occur. Studies at the level of the whole genome as well as the methylation analysis of

unique genes showed that prior to fusion of pronuclei, paternal genome undergoes overall (and most presumably active) demethylation (Oswald *et al.*, 2000; Mayer *et al.*, 2000**,** see Fig. 3). The demodification is probably connected to the remodelling of the paternal pronucleus accompanied by exchange of protamines for histones and commences before the onset of replication. It was well documented that some of the paternal methylation imprints (for example DMR2 of *Igf2* gene) do not withstand this demodification event (Oswald *et al.*, 2000), whereas others, as for example the upstream DMR of *H19* (Warnecke *et al.*, 1998) or *Ras Grf1* (Shibata *et al.*, 1998) seem to resist. Which mechanism keeps the memory of the erased paternal imprints in order to re-establish them later on during embryogenesis has still to be elucidated. Perhaps, only the imprints of secondary importance are erased, whereas the marks at the real imprinting centres of the whole cluster stay.

During the cleavage, following the early zygotic demethylation, the whole genome undergoes passive demethylation (see Fig. 3). The exceptions to the rule are DMRs of the imprinted genes, which are documented to keep their differential methylation (Brandeis *et al.*, 1993; Olek *et al.*, 1997). The pre-implantation demethylation is probably due to the exclusion of the maintenance methyltransferase (Dnmt1) from the nuclei of the early dividing embryo (Mertineit *et al.*, 1998; Howell *et al.*, 2001). However, in mouse eight-cell stage embryos, Dnmt1 is relocated back to the nucleus for just one replication cycle. The presence of this oocyte and early embryo form of Dnmt1 (Dnmt1o – Mertineit *et al.*, 1998) in the nucleus at that stage is apparently crucial for the maintenance of imprinted methylation - in a knockout of this Dnmt1 version the methylated allele of imprinted genes loses exactly 50% methylation (Howell *et al.*, 2001). It is plausible that other methyltransferases (perhaps Dnmt3a and Dnmt3b) are responsible for methylation of imprinted genes before and after the eight-cell stage when Dnmt1o is excluded from the nucleus into the cytoplasm.

Whereas the majority of CpG sites within the mammalian genome become demethylated prior to implantation, distinct sites connected to the imprinted genes are documented to undergo allele-specific *de novo* methylation (Oswald *et al.*, 2000; Brandeis *et al.*, 1993). *De novo* methylation thus reconstitutes the methylation marks lost by zygotic paternal demethylation (as in the case of Igf2 DMR2 – Oswald *et al.*, 2000) or establishes allele specific differential methylation (El-Maarri *et al.*, 2001) in regions which possibly carry gametic imprints of other type than methylation.

Around the time of implantation (blastocyst stage) a wave of global *de novo* methylation occurs (see Fig. 3). Non-imprinted genes as well as repetitive elements were found to gain methylation between 5.5 and 6.5 dpc (Kafri *et al.*, 1992; Monk *et al.*, 1987). The same process is probably responsible also for *de novo* methylation of retroviruses integrated into the DNA of pre-implantation mouse embryos (Jahner *et al.*, 1982). The wave of *de novo* methylation occurs mainly in the inner cell mass (ICM) cells of the expanded blastocyst and is proposed to be connected with the process of lineage decision and cell differentiation (Monk, 1995; Reik *et al.*, 2001a).

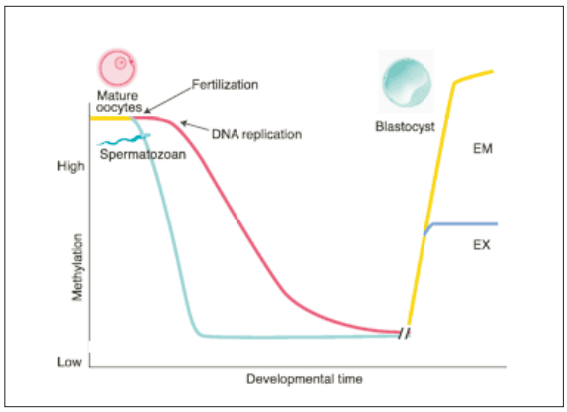


Fig. 3: Global changes of genomic methylation during early embryogenesis
Shortly after fertilisation the paternal genome (green) undergoes rapid and most probably active demethylation. To the contrary, the maternal genome (red) is demethylated by a slow passive mechanism during the first cleavages. Around the time of implantation (blastocyst stage) the wave of *de novo* methylation occurs (to a different extent in embryonic (EM) and extraembryonic (EX) tissues). (Adapted from Reik *et al*., 2001).

1.5 Germ line and its key role in the epigenetic "life cycle"

Whereas the cells of the future soma undergo described *de novo* methylation and differentiation, at the time when the work of this thesis was started it was still unclear what is the epigenetic origin of the germ lineage.

The germ cell lineage plays certainly a key role in the imprinting "cycle" (see Fig. 2). The germ cells are responsible for epigenetic resetting which not only deletes and re-establishes the imprinting marks according to the sex of the developing individual, but also is crucial to prevent the existing epimutations to be passed onto the next generation.

The origin and the epigenetic status of the germ cells have been extensively discussed over the years. Based on the limited number of experiments showing that

the DNA of primordial germ cells (embryonic precursors of the gametes) present in the developing genital ridges of the embryo is hypomethylated (Brandeis *et al.*, 1993; Kafri *et al.*, 1992), two main developmental models have been proposed (see Fig. 4) : (1) The germ cell lineage is derived early in the blastocyst prior to the wave of *de novo* methylation – i.e. the germ cells keep their undermethylated status to be re-programmed according to the sex later during the gametogenesis (see also Jaenisch, 1997). (2) The germ cells are derived in the blastocyst but undergo the *de novo* methylation process, the methylation is erased later on by germline specific mechanism and new methylation marks re-established subsequently in the gametogenesis.

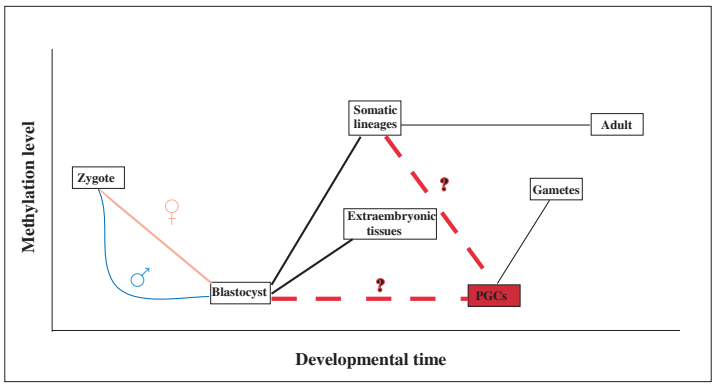


Fig. 4: "Epigenetic origin" of primordial germ cells
At the onset of this thesis there were two different hypothesis concerning the origin of the hypomethylated state of primordial germ cells : The germ cells could be derived from the blastocyst before the wave of *de novo* methylation or the germ lineage undergoes epigenetic changes similar to somatic (ev. extraembryonic) tissues, methylation is erased later by germ line specific mechanism.

1.6 Biological studies of primordial germ cells – origin, characteristics, development

1.6.1 Origin of primordial germ cells

Primordial germ cells (PGCs) are the earliest recognised precursors of gametes. During the early embryogenesis, the primordial germ cells are first detectable by their high level of tissue non-specific alkaline phosphatase activity midway through gastrulation at 7.2dpc. At that time they form a cluster of about 40-50 cells in the extraembryonic mesoderm at the base of the allantois (Ginsburg *et al.*,

1990; see Fig. 5). Initial experiments with mouse chimeras showed that PGCs are derived from the epiblast (Gardner, 1985). Later on, by following the fate of single epiblast cells injected with a lineage marker, Lawson and Hage (Lawson *et al.*, 1994) were able to locate the ancestral population as being among the most proximal epiblast cells, adjacent to the extraembryonic ectoderm. The xenotypic transplantation of epiblast cells revealed that even distal epiblast cells, which normally give rise to neuroectoderm, have the capacity to form PGCs when transplanted to the proximal region (Tam *et al.*, 1996). Additional experiments based on *ex vivo* cultivation of dissected epiblast cells confirmed the necessity of interaction with the extraembryonic tissues for the PGC formation. In the experimental set up, only the epiblast cells co-cultivated with adjacent extraembryonic tissues were able to form PGCs (Yoshimizu *et al.*, 2001). Thus, the location of the cells and their vicinity to the signal produced in the extraembryonic ectoderm is involved in the germ cell determination rather than any segregation of preformed cytoplasmatic determinants (as for example in *D. melanogaster*). The process of PGC determination is probably connected to the expression of bone morphogenetic protein-4 (Bmp4). The expression of this protein is confined to the extraembryonic extoderm prior to gastrulation; and moreover the Bmp4 homozygous null embryos fail to form PGCs (Lawson *et al.*, 1999).

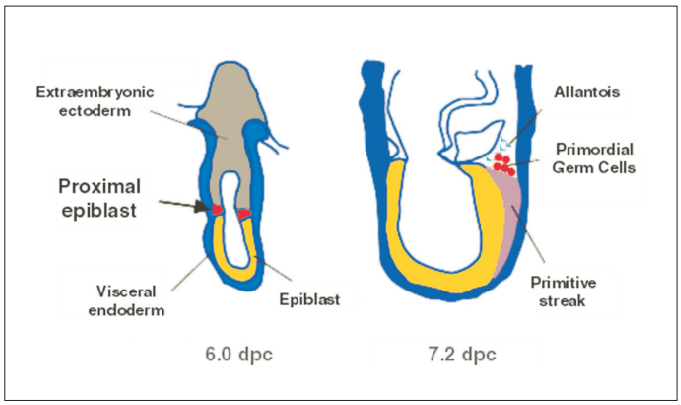


Fig. 5: Origin of primordial germ cells
PGCs are derived form the proximal epiblast; around 7.2 dpc PGCs are first detectable due to their high level of alkaline phosphatase activity.

The acquisition of germ cell status is accompanied by a marked reduction in proliferation rate (Lawson *et al.*, 1994). From an original doubling time of 7 hours the proliferation slows down to a doubling time of 16-17 hours, a rate which is maintained steadily for the next 6 days (Tam *et al.*, 1981). At about the same time the *Oct4* gene (responsible for repression of differentiation genes (Pesce *et al.*, 1998) switches to a transcript regulated by germ cell-specific distal enhancer (Scholer *et al.*, 1990; Yeom *et al.*, 1996).

1.6.2 Germ cells migration and colonisation of the embryonic genital ridges

Towards the end of gastrulation (around 8.0 dpc) the posterior visceral endoderm moves in to form the hindgut, carrying with it the germ cells from the cluster and distributing them along the length of the hindgut. It has been observed that PGCs do not have pseudopodia before they occur in hind gut endoderm (Tam *et al.*, 1981), suggesting that earlier PGCs do not undergo active migration but instead passively move with a morphogenetic expansion of embryonic tissues. Subsequently, around 9.5 dpc the PGCs emigrate from hind gut and move actively along the dorsal mesentery until they reach the genital ridge anlage (10.5 – 11.5 dpc) (see Fig. 6). The germ cells seem to emigrate independently, but soon afterwards they start to form extensive processes (up to 40 μm – Gomperts *et al.*, 1994) by which they are linked up to each other to form an extensive network. During migration PGCs express on their surface the Stage Specific Embryonic Antigen 1 (SSEA1, Fox *et al.*, 1981). Expression of this antigen, which is first evident at 9.5 dpc and is down regulated at about 12.5dpc, has been used as a PGC marker in several studies (Gomperts *et al.*, 1994; Garcia-Castro *et al.*, 1997).

The cellular and molecular basis of route finding during germ cell migration is poorly understood. The proliferation of migrating cells is dependent on the c-kit/stem cell factor signal transduction pathway, as the embryos homozygous for mutations in genes coding for either the receptor (*W*) of the ligand (*Steel*) are deficient in germ cells. It has been shown that PGCs interact with diverse extracellular matrix proteins on their way to the genital ridge. Among others, the interaction with laminin (Garcia-

Castro *et al.*, 1997) and B1 integrins (Anderson *et al.*, 1999) seems to be the most important for migration and colonisation of the genital ridges.

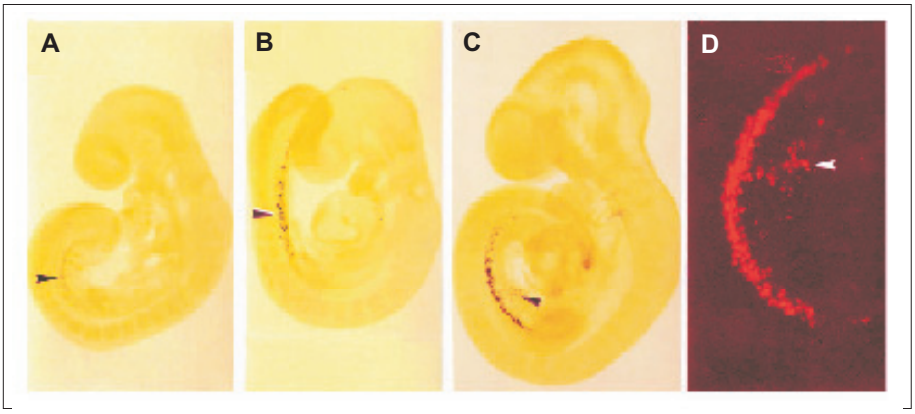


Fig. 5: PGC migration during the development of mouse embryo
Microscopic images show the migrating PGCs in 9.25 dpc (A), 9.5 dpc (B) and 10.5 dpc (C) mouse embryos. The migrating PGCs were visualised using the expression of *lacZ* under the control of germ line-specific *Oct4* promoter. D shows the dark field image of 10.5 dpc embryo. The arrows indicate the PGCs moving into the forming genital ridges. (Yeom *et a*l., 1996)

Germ cells in the mouse enter the genital ridge area between 10.5 and 11.5 dpc, at the time when the ridge is forming. By 11.5dpc a clear demarcation exists between genital ridge and mesonefros, making any subsequent PGC entry unlikely. Although the PGCs maintain their steady proliferation rate on entering the genital ridge, their morphology changes dramatically (Donovan *et al.*, 1986). The PGCs lose their ability to elongate, become round, less motile, and their ability to spread on the substrate declines. Within the genital ridges the dividing germ cells form clonal clumps of up to 32 cells that tend to go through mitosis synchronously (Pepling *et al.*, 1998).

1.6.3 Gametogenesis inside the developing gonads

The sex specific differences appear first at 12.5dpc when the genital ridges become morphologically distinct – differentiated Sertoli cells appear in male genital ridges, whereas in female embryos the supporting cells differentiate as granulosa cells (for review see McLaren, 2000). One day later (at 13.5dpc) the germ cells in male undergo mitotic arrest, whereas the female PGCs enter meiotic prophase and pass through leptotene, zygotene and pachytene before arresting in diplotene around the time of birth (Peters, 1970**,** see Fig. 7)

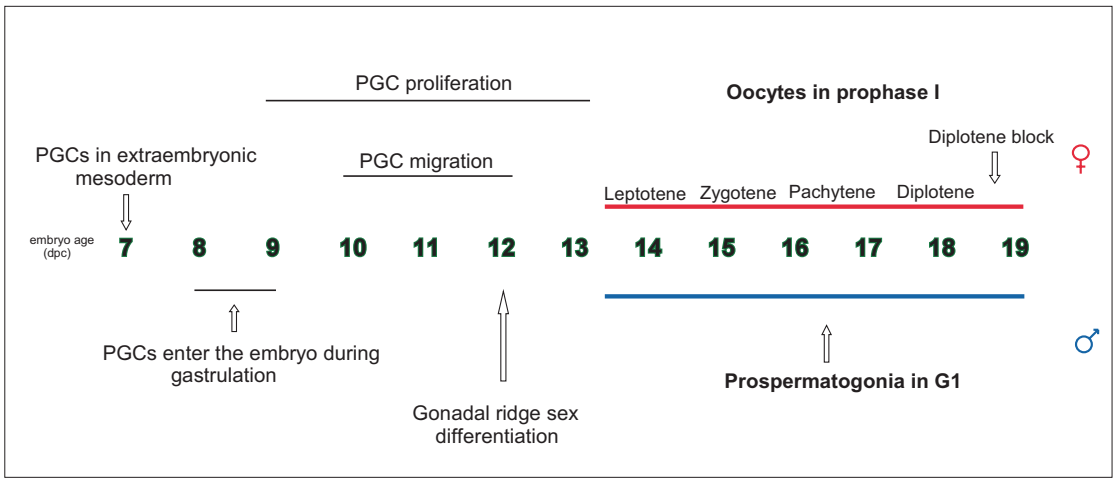


Fig. 7: Timing of germ cell development in the mouse embryo (for details see the text).

1.6.4 Brief summary of spermatogenesis

The male PGCs are mitotically arrested around 13.5dpc as T-prospermatogonia, in the G1 (G0) stage of the cell cycle (McLaren, 1984), not resuming mitosis until a week later, just after birth. The spermatogonia then proliferate rapidly, generating some progeny that retain the capacity to continue dividing indefinitely (as stem cell spermatogonia) and other progeny (maturing spermatogonia) that will, after a limited number of further normal division cycles,

enter meiosis. The first spermatogenic stages in mouse do not enter meiosis until at least a week after birth (McLaren *et al.*, 1997). After completion of second meiotic division the haploid spermatids are formed that differentiate into mature sperm (spermatozoa) (see Fig. 8).

1.6.5 Brief summary of oogenesis

Around 13.5dpc PGCs in female mice enter meiosis and arrest in diplotene of the first meiotic prophase around the time of birth (Constantini *et al.*, 1994). The arrested oocytes (15-20 μm in diameter) undergo a progressive growth – the volume increases about 200-fold as the diameter reaches 75-80 μm. At the time when the diameter reaches 60 μm, the oocytes become capable of re-entering the cell cycle but are maintained in meiotic arrest by the surrounding follicular cells. The final period of oogenesis does not proceed before the sexual maturity. In this hormonally controlled phase the oocytes are stimulated to resume the first meiotic cell cycle and undergo the first meiotic division before arresting at metaphase of meiosis II (see Fig. 8). The second meiotic division is then completed only after fertilisation.

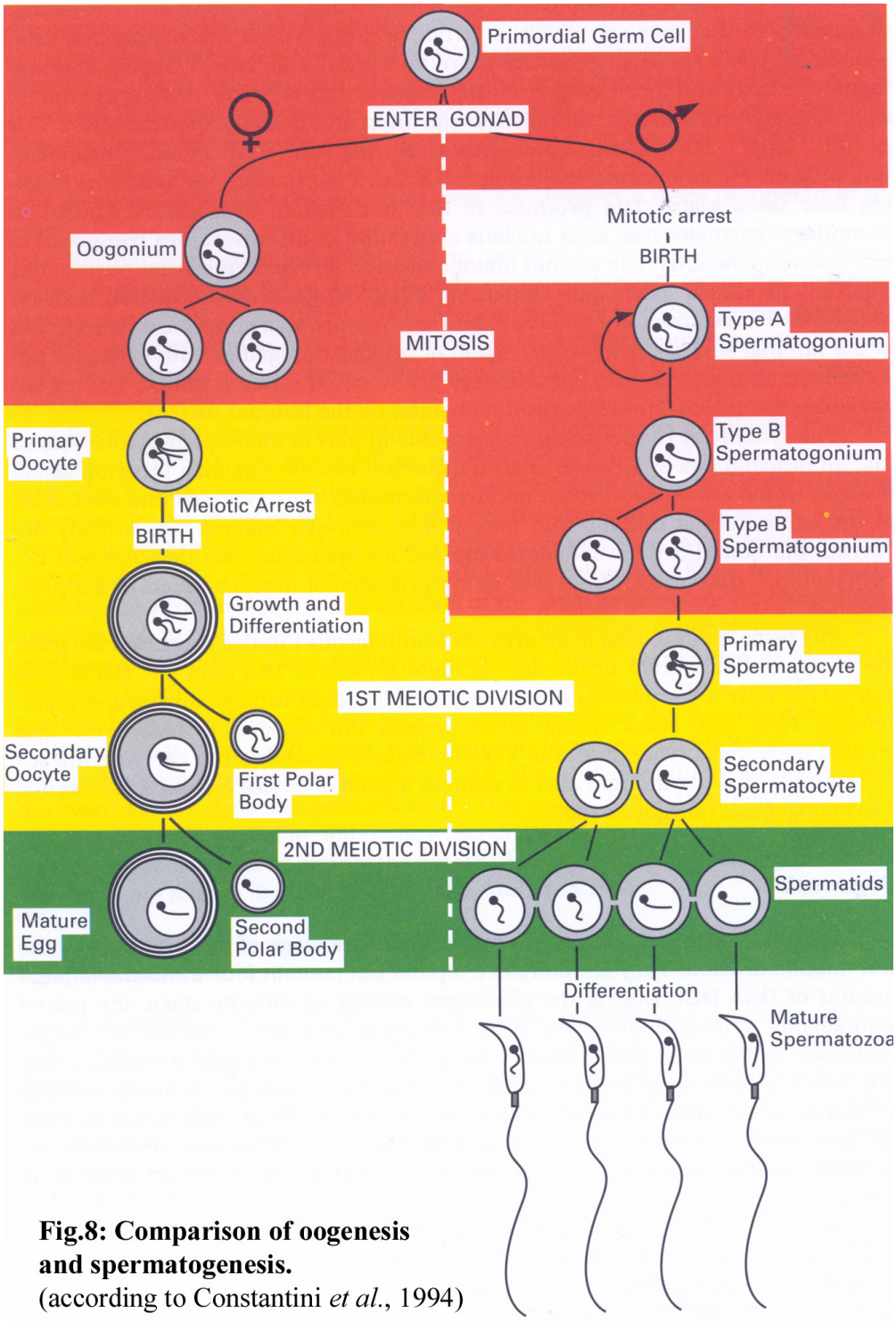


Fig.8: Comparison of oogenesis and spermatogenesis.
(according to Constantini *et al.*, 1994)

1.6.6 Germ cell development is connected with epigenetic reprogramming

The epigenetic changes occurring during the germ cell development have been an issue of great interest since the postulation of imprinting. The progress in the field has, however, been hindered for a long time by technical difficulties caused by the laborious germ cell isolation and disputable sample purity on one hand, on the other hand, new highly sensitive methods had to be developed for the analysis of very limited cell samples.

The first attempts to characterise the methylation status of early germ cells have not been published earlier than in late 1980s. Based on their previous observation of X chromosome re-activation in the female foetal germ cells (Monk *et al.*, 1981), Monk and colleagues predicted the presence of general epigenetic changes involving possibly also changes in DNA methylation. Later, by checking the general methylation status in PGCs isolated from genital ridges of different embryonic stages, the authors confirmed that the genome of 12.5 and 14.5 dpc primordial germ cells is hypomethylated in comparison with the embryonic somatic tissues (Monk *et al.*, 1987). The technique used in the study was, however, of a questionable sensitivity.

The observation of Monk *et al*. was further confirmed in early 1990s using more sophisticated methylation sensitive PCR assays (see chapter 1.7 Molecular techniques used for DNA methylation studies (Kafri *et al.*, 1992; Brandeis *et al.*, 1993). Investigation of the methylation status of number of restriction sites within well-characterised genes (imprinted as well as non-imprinted) revealed complete absence of methylation at all the tested sites in 12.5dpc and 13.5dpc (the earliest stages tested) primordial germ cells of either sex. The lost of imprinting in early PGCs was documented also at the level of transcription (Szabo *et al.*, 1995). In postmigratory PGCs purified from sexually still indifferent genital ridges, selected imprinted genes were shown to be expressed biallelically.

It is noteworthy that up to now all the studies describe PGCs at the stage when they are already free of imprints. Thus, it stays unclear whether the earlier stages of PGCs do posses established imprints, or whether they are derived earlier and escape the wave of *de novo* methylation.

More information about the epigenetic properties of primordial germ cells was brought about by the introduction of PGC-derived cell lines (embryonic germ (EG) cell lines). The first studies showed that EG cells have a similar epigenotype to PGCs

from which they are derived (Resnick *et al.*, 1992; Matsui *et al.*, 1992). In culture the EG cells keep their undifferentiated character, morphologically resembling embryonic stem cells (ES cells) and embryonal carcinoma cells. EG cells can contribute to most if not all the somatic tissues, as well as the germ line, and to this extent they are developmentally totipotent (Labosky *et al.*, 1994; Stewart *et al.*, 1994; Tada *et al.*, 1998). Both male and female 11.5-12.5 dpc EG cells undergo comparable epigenetic changes (Tada *et al.*, 1998) characterised by loss of imprinting of majority of the tested imprinted genes. The unique reprogramming activity of EG cells has been also shown in the EG-somatic cell fusion experiment (Tada *et al.*, 1997). It has still to be elucidated, however, whether the processes occurring in PGCs *in vivo* are indentical to those described for EG cells.

1.6.7 Re-establishment of genomic imprints

Whereas at the onset of the work of this thesis it was still unclear whether the initial reprogramming events in early developing gonads differ in male and female embryos, the establishment of new epigenetic modifications is undoubtedly sex-specific. Generally, more detailed knowledge is available on the establishment of new imprints during **spermatogenesis**. The fact is probably due to the easier preparation procedure and relative abundance of sperm samples. The immunostaining of developing mouse testis with anti mC antibodies showed that the euchromatic regions of germ pass from a demethylated to a strongly methylated status between 16 and 17 dpc (i.e. prenatally, before the onset of meiosis) (Coffigny *et al.*, 1999). Additional support for the idea, that the functional paternal imprint is established prior to meiosis came from the nuclear transfer experiments published independently by two groups in 1995 and 1998 (Kimura *et al.*, 1995; Ogura *et al.*, 1998). The mouse oocytes receiving nuclei from primary spermatocytes (see Fig. 8) developed normally, albeit at a low success rate. The pre-meiotic *de novo* methylation has been confirmed also at the level of a single gene: the bisulphite analysis of *H19* upstream DMR documented *de novo* methylation processes starting from 15.5 dpc on (Davis *et al.*, 2000 ; Ueda *et al.*, 2000). These experiments furthermore suggest that if methylation is indeed the imprinting mark, the paternal specific methylation pattern might be

established by the somatic form of Dnmt1, known to be present during this stage of spermatogenesis (see below).

The major step towards the understanding of imprint establishment during the **oogenesis** has been done by the work of Kono and colleagues (Kono *et al.*, 1996; Obata *et al.*, 1998). By generating parthenogenetic embryos with nuclei from non-growing and fully-grown oocytes, the authors determined that the development of these embryos was extended by 3 days compared to parthenogenetic embryos derived only from the fully-grown oocytes. This improved developmental potential was proposed to be caused by epigenetic inequivalence of the oocyte genomes of the two developmental stages. The expression of imprinted genes revealed that the non-growing oocyte had apparently not acquired the maternal identity yet, thus allowing expression of some of paternally expressed genes, which are normally maternally repressed (for example *Peg3*, *Peg1/Mest* and *Snrpn* – Obata *et al.*, 1998). Further experiments narrowed down the time window during the oocyte growth when the major epigenetic changes occur. The maternal genome is first competent to support development to term during the latter half of oocyte growth, at the time when oocyte becomes competent to enter metaphase of the first meiotic division (Bao *et al.*, 2000).

1.6.8 Factors possibly involved in the establishment of gametic imprinting

Dnmt1 was the first candidate suggested to be involved in the establishment of gametic imprints. The oocyte-specific Dnmt1 isoform (Dnmt1o) has been found to be present during the oocyte growth – ie. around the predicted time of imprint establishment. A spermatocyte-specific 5'exon, to the contrary, interferes with translation and prevents production of Dnmt1 during the crossing-over stage of male meiosis, thus protecting a preferred methylation target from aberrant modification (Mertineit *et al.*, 1998). The hypothesis was, however, disproved by the recent knockout of the oocyte-specific Dnmt1 isoform (Howell *et al.*, 2001). The normal establishment of imprints in oocytes deprived of Dnmt1o suggests presence of other DNA methyltransferases that are responsible for *de novo* methylation during oogenesis. Although Dnmt3a and Dnmt3b methyltransferases with the described *de*

novo methylation activities (Okano *et al.*, 1999) might be good candidates, further experiments are needed to elucidate their possible role in gametogenesis. The newest findings revealed another candidate likely to be involved in the establishment of imprinting methylation marks (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). Dnmt3L is expressed at the key stages of gametogenesis, the disruption of the gene, moreover, causes infertility in homozygous males and aberrant imprint establishment in oocytes of homozygous females. As Dnmt3L lacks the key catalytic domains characteristic for DNA cytosine methyltransferases, the protein is more likely to act as a regulator of imprint establishment rather than as a DNA methyltransferase.

1.7 Molecular techniques used for DNA methylation studies

Since the Hotchkiss'es discovery of 5mC number of techniques have been developed in order to perform DNA methylation analysis. In general, the techniques can be divided into two main groups: i) techniques for non-specific methylation analysis and ii) techniques for sequence-specific methylation analysis.

1.7.1 Non-specific methylation analysis

Large-scale genome-wide changes in cytosine methylation levels are probably best monitored by reverse-phase HPLC. This procedure is in principle the oldest available for methylation analysis (Kuo *et al.*, 1980; Christman, 1982; Gomes *et al.*, 1983). It relies on the quantitative hydrolysis of DNA using DNase I and nuclease P1 (Kuo *et al.*, 1980) or snake venom phosphodiesterase (Gomes *et al.*, 1983), followed by alkaline phosphatase treatment. The amount of material required for the analysis is relatively high (several μg of DNA); the technique is moreover rather demanding concerning technical optimisation (assuring the complete DNA degradation) and the sample purity.

Thin layer chromatography represents an alternative procedure for studying genome-wide methylation levels (Bestor *et al.*, 1984; Schmitt *et al.*, 1997). The DNA is initially cleaved with *MspI* restriction endonuclease (a recognition site CCGG is cleaved regardless of the methylation status; the method is based on the assumption that most of the vertebrate methylation occurs within CpG dinucleotides) and the internal cytosine labelled using [γ-^{32}P]ATP and polynucleotide kinase. The DNA is then hydrolysed to mononucleotides using nuclease P1, and separated on cellulose thin-layer chromatography plates. The relative intensity of the C to 5mC spots will show the proportion of *MspI* sites that are methylated in the genome. In theory, only C and 5mC should give spots in this experimental set-up; nevertheless additional signals corresponding to A, G and T are often observed probably due to random nicks in the DNA.

Also methyl accepting assay and a chloracetaldehyde reaction belong among the applicable though rather rarely used methods for methylation analysis. In the methyl accepting assay, *SssI* prokaryotic methyltransferase transfers the 3H labelled methyl groups of a methyl group donor (SAM – S-adenosyl-methionin) onto the isolated DNA (Schmitt *et al.*, 1997). The choracetaldehyde reaction couples the bisulphite modification (see below) with the subsequent chemical reaction of unconverted cytosines with chloracetaldehyde that yields an intensely fluorescent product (Oakeley *et al.*, 1999).

Last but not least, immunological methods have been applied for methylation studies. The detection of methylation using anti-mC antibodies has been described in many publications (Piyathilake *et al.*, 2000; Mayer *et al.*, 2000). The results (though very spectacular) are only qualitative, giving mainly the first impression about the overall methylation level.

1.7.2 Sequence-specific methylation analysis

Original methods to detect sequence specific genomic methylation were based on the digestion of DNA by methylation-sensitive restriction enzymes and subsequent Southern blot hybridization (Southern, 1975). Despite rather high amount of DNA

needed for such experiments (> 5 μg) and the possibility to investigate just the limited numbers of CpGs situated within suitable restriction sites, the method is still useful as the first indication of methylation in a specific region. To improve the sensitivity, the method was combined with PCR amplification (Singer-Sam *et al.*, 1990) and subsequent quantification of PCR products (Brandeis *et al.*, 1993; Kafri *et al.*, 1992). Although the use of PCR decreased the amount of template DNA necessary for the analysis, the whole procedure is highly demanding in terms of strictly standardized conditions of DNA preparation and PCR, since quantification is only possible within the exponential phase of amplification. Additionally, incomplete digestion of chromosomal DNA might be a frequent source of artifacts. Another disadvantage of such methods is that they provide data only about the average level of methylation; it is neither possible to discriminate between mosaic and even methylation patterns or to address hemi-methylation, which remains in general undetected.

The first information about the methylation of cytosine residues irrespective of their sequence context was obtained using a genomic sequencing protocol (Maxam *et al.*, 1980). This method identifies a position of 5-methylcytosine (5-MeC) in the genomic DNA as a site that is not cleaved by any of the Maxam and Gilbert sequencing reactions (Church *et al.*, 1984) and thus appears as a gap in a sequencing ladder. Although a detailed distribution of methylation in a given sequence can be analyzed by this method, it still requires relatively large amounts of genomic DNA and a certain level of experience in interpreting the sequencing results as bands of varying intensity and shadow bands may occur. An elegant combination of the chemical cleavage method with ligation mediated PCR (Pfeifer *et al.*, 1989) increases the sensitivity, but this modification makes the whole procedure rather laborious and technically challenging.

1.7.3 Bisulphite genomic sequencing

With a bisulphite genomic sequencing method (Clark *et al.*, 1994; Frommer *et al.*, 1992) a qualitatively and quantitatively new approach to methylation analysis has appeared. The bisulphite reaction leads to the conversion of cytosines into uracil

residues, which are recognized as thymines in subsequent PCR amplification and sequencing, whereas the modified cytosines do not react and are therefore detected as cytosines. Thus the method allows direct and positive determination of methylation sites in the genomic DNA, as only methylated cytosines are detected as cytosines. Products of PCR-amplified bisulphite-treated DNA can be used directly for sequencing (detection of average methylation status) or cloned and sequenced individually, when the information about the methylation pattern of single molecules is desired. Not only the methylation status of each single molecule but also the pattern of each DNA strand can be investigated, as the strands are no longer complementary following the bisulphite treatment and are amplified and sequenced separately.

Several modifications of the original bisulphite sequencing protocol improving the sensitivity and quality of the results have been published (Feil *et al.*, 1994; Olek *et al.*, 1997; Paulin *et al.*, 1998; Raizis *et al.*, 1995). In some cases a direct sequence analysis of the PCR products obtained may be desirable to estimate the average methylation at specific sites. For such direct quantification Gonzalgo and Jones (Gonzalgo *et al.*, 1997), proposed an elegant and simple procedure (Ms-SNuPE). A more sophisticated protocol for direct quantification of sequencing results is described by Paul *et al.* (Paul *et al.*, 1996).

The attributes of high sensitivity, the ability to detect single molecule methylation patterns as well as the possibility of addressing non-symmetrical methylation make bisulphite-based genomic sequencing the method of choice for a variety of applications.

1.8 The aim of the thesis

Epigenetic reprogramming in germ line has been one of the key questions in the field, since the discovery of imprinting (Surani *et al.*, 1984; McGrath *et al.*, 1984). However, at the time when the work on this thesis was being initiated only limited knowledge was still available about the underlying processes occurring in primordial germ cells. Whereas the majority of previous publications had concerned the onset of new, sex specific, imprints (Bao *et al.*, 2000; Davis *et al.*, 1999; Davis *et al.*, 2000; Kimura *et al.*, 1995; Trasler, 1998; Ueda *et al.*, 2000 etc), the experiments described in this work focused on the poor characterised process of demethylation / imprint erasure.

The main tasks that stood at the beginning of this work were thus:

1) The exact time definition of the main demodification changes that occur during the PGC development and the description of their kinetics.

2) The question of "epigenetic origin" of PGC. The knowledge about the methylation status of early PGCs was expected to bring more understanding concerning the epigenetic fate of PGCs after their formation around 7.2 dpc.

The main work of this thesis was therefore to describe the methylation status of primordial germ cells at different developmental stages. In order to do so, two different approaches were chosen: Single gene approach was based on detailed monitoring of the changes in methylation patterns of distinct imprinted and non-imprinted genes. Genome-wide approach exploited the method of immunohistochemical mC staining in order to characterise the global methylation changes.

2 Material & Methods

2.1 Material

2.1.1 Bacterial strains

Escherichia coli K12 DH5α	Hanahan, 1983; Bethesda Research laboratories, 1986
Escherichia coli K12 Sure	Stratagene, La Jolla, CA, USA
Escherichia coli K12 XL1-Blue	Bullock *et al*., 1987; Stratagene, La Jolla, CA, USA
Escherichia coli K12 Top10	Invitrogen, Leek, Netherlands
Escherichia coli K12 Invα F`	Invitrogen, Leek, Netherlands

2.1.2 Mouse strains

Primordial germ cells for the magnetic bead-based purification were obtained from the mouse embryos of outbred MF1 mouse strain.

The *Oct4-GFP* transgenic mice were prepared on a mixed F1-129 background. For the isolation of PGCs *Oct-GFP* males were mated with MF1 females. The mice harbour GFP/Oct4 fusion under the control of *Oct4* promoter region lacking the proximal enhancer (GOF18ΔPE - Yeom *et al.*, 1996, see Fig. 9). This deletion of proximal (but not of the distal) enhancer ensures the germ line specific transgene expression.

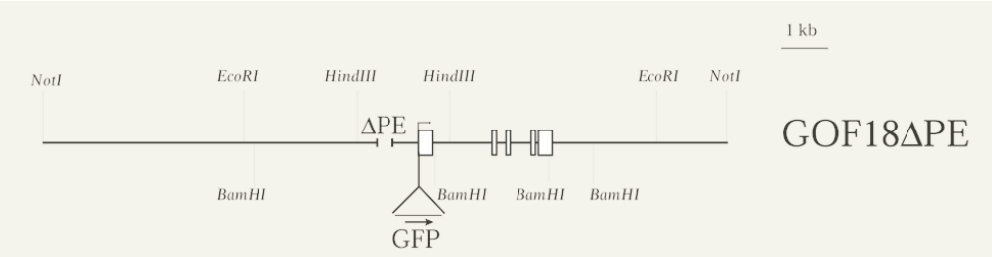


Fig.9: Scheme of the *Oct4/GFP* transgene.

2.1.3 Cloning vectors

pCR2.1, TA cloning kit	Invitrogen, Leek, Netherlands
pGEM, TA cloning kit	Promega, Mannheim

2.1.4 Enzymes

RNaseA	Boehringer Mannheim, Mannheim
ProteinaseK	Boehringer Mannheim, Mannheim
T4 DNA-ligase	Boehringer Mannheim, Mannheim, Promega , Mannheim
Taq-DNA-polymerase	Boehringer Mannheim, Mannheim
Trypsin	Biochrom, Berlin

Restriction endonucleases were purchased from either Boehringer Mahhneim (Mannheim), New England Biolabs (Beverly,MA,USA) or MBI Fermentas (Vilnius, Lituania).

2.1.5 Chemicals & other material

2.1.5.1 Chemicals

Acetic acid	Merck, Darmstad
Agarose	Biorad, Richmond, CA, USA
Sea-Plague Agarose	FMC BioProducts, Rockland, ME, USA
Ampicillin	Bayer, Leverkusen
Bacto-Agar	Difco, Detroit, USA
Bacto-Trypton	Difco, Detroit, USA
Bacto-yeast extract	Difco, Detroit, USA
5-Bromo-4-Chlorine-3-Indolyl-D--Galactoside (X-gal)	Roth, Karlsruhe
Boric Acid	Merck, Darmstadt
Bovine Serum Albumine	Merck, Darmstadt
Bromphenolblue	Merck, Darmstadt
Chlorophorm	Merck, Darmstadt
Deoxyribonucleotides (dNTPs)	Pharmacia, Uppsala, Schweden
4`,6-diamidino-2-phenylindole (DAPI)	Sigma, Deisenhofen
Dimethylsulfoxid	Merck, Darmstadt
Ethanol	Merck, Darmstadt
EDTA	Merck, Darmstadt
Ethidium Bromide 1%	Roth, Karlsruhe
Formamide (ultra-pure, deionised)	Sigma, Deisenhofen
Gelatine	Boehringer Mannheim, Mannheim
Glucose	Sigma, Deisenhofen
Glycerol	Merck, Darmstadt
Hydrochloric Acid (37%)	Merck, Darmstadt
Hydroquinone (1,4 Benzendiol)	Sigma, Deisenhofen
Isopropyl-thio-galactoside (IPTG)	Biomol, Hamburg
Kanamycin	Bayer, Leverkusen
Magnesium acetate	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt

Magnesium sulphate	Merck, Darmstadt
Methanol	Merck, Darmstadt
2-Mercaptoethanol	Merck, Darmstadt
Mineral oil (heavy white)	Sigma, Munich
Penicillin G	Sigma, Deisenhofen
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, Deisenhofen
Pepsin	Merck, Darmstadt
Poly-L-lysin	Sigma, Deisenhofen
Polyoxyethylensorbitan-Monolaurate (Tween 20)	Sigma, Deisenhofen
Potassium Chloride	Merck, Darmstadt
2-Propanol (Isopropanol)	Merck, Darmstadt
Streptomycin	Sigma, Deisenhofen
Sodium disulphite	Merck, Darmstadt
Sodium dodecylsuphate (SDS)	Sigma, Deisenhofen
Sodium citrate	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Tris (hydroxymethylaminomethan)	Merck, Darmstadt
Xylen-Cyanol FF	BioRad, Richmond, CA, USA

2.1.5.2 DNA-molecular weight markers

100bp DNA ladder	MBI Fermentas, Vilnius, Lituania
1kb DNA ladder	MBI Fermentas, Vilnius, Lituania
SPP1- Phage DNA digested with *EcoRI*	prepared in our laboratory

2.1.5.3 Kits

Expand Long Template PCR System	Boehringer Mannheim, Mannheim
ABI Prism Big Dye Sequencing Kit	Perkin Elmer Cetus, Norwalk, CT, USA
Jetsorb	Genomed, Bad Oeynhausen
Qiagen Plasmid Kit	Qiagen, Studio City, CA, USA

QiaExII	Qiagen, Studio City, CA, USA
TA-Cloning Kit	Invitrogen, Leek, Netherlands
pGEM I System (TA-cloning kit)	Promega, Mannheim
Alkaline Phosphatase (AP), Leukocyte (no.R86) Kit	Sigma Diagnostics, Deisenhofen
Dneasy™ Tissue Kit	Qiagen, Studio City, CA, USA

2.1.5.4 Other material

MiniMacs holder for magnetic bead-based cell sorting	Miltenyi BiotecGmbH, Gladbach
Dialysis filter, Type VS, 0,0025 μm	Milipore Corporation, Bedford, MA, USA
Sterile filter, 0,45 μm	Schleicher & Schüll, Dassel

2.1.5.5 Equipment

PCR Cycler PE 2400/9600	Perkin Elmer Cetus, Norwalk, CT, USA
PCR Trio-Thermoblock	Biometra, Göttingen
Master Cycler Gradient	Eppendorf, Hamburg
Incubator	Heraeus, Göttingen
Microscope	Leitz, Wetzlar
Binocular	Leitz, Wetzlar
Epifluorescence microscope equipped with the CCD camera (Photometrics CH250)	Zeiss, Jena

All other material and equipment were of today laboratory standard.

2.1.5.6 Bacterial media

LB-medium

10 g Bacto-tryptone

5 g Bacto-yeast extract

10 g NaCl

ad 1 l H_20, pH 7,0

SOB-medium

20 g Bacto-tryptone

5 g Bacto-yeast extract

0,5 g NaCl

ad 1 l H_20, pH 7,0

SOC-medium

SOB-medium substituted with 20 ml 1 M glucose.

For bacterial plates, 15 gr of bacto-agar was added per liter of medium

2.1.5.7 Buffers & solutions

Bisulphite solution

2,5 M metabisulphite

125 mM hydroquinone

Solution A (1x)

25 mM EDTA pH 8,0

75 mM NaCl

Solution B (1x)

10 mM EDTA pH 8,0

10 mM Tris-HCl pH 8,0

400 μg/ml Proteinase K

1 % (w/v) SDS

PBS buffer (1x)

2,5 mM KCl

136 mM NaCl

10 mM Natriumhydrogenphosphate

TE buffer (1x)

1mM EDTA

10 mM Tris-HCl pH 8,0

PCR buffer 10x (unless otherwise stated)	Loading dye for agarose gels (5x)
15 mM $MgCl_2$	0,2 % (w/v) Bromphenolblue
500 mM KCl	15 % (w/v) Ficoll Typ 400
100 mM Tris-HCl pH 8,3	40% (w/v) Glycerin
	0,2 % (w/v) Xylen-Cyanol

TBE buffer (1x)	TAE buffer (1x)
50mM Boric acid	40 mM Tris-acetate
1 mM EDTA pH 8,3	1 mM EDTA pH 8,3
40 mM Tris-base	

Unless otherwise stated, all the common laboratory solutions were prepared according to Sambrook *et al.*, 1989.

2.1.5.8 Media and chemicals for the germ cell preparation

Dulbecco's Modified Eagle's Medium (DMEM)	Sigma, Munich
Foetal calf serum (FCS)	Biochrom, Berlin
Penicillin/Streptomycin solution (100x)	Biochrom, Berlin
Non-essential amino acids	Sigma, Munich
Glutamine	Biochrom, Berlin
β-mercaptoethanol	Sigma, Munich
Sodium pyruvate	Sigma, Munich
PBS (phosphate buffered saline)	Biochrom, Berlin

2.1.6 Oligonucleotides

All primers are listed in 5' to 3' orientation.

2.1.6.1 Primers for the sex determination of early mouse embryos

(as described in Chuma *et al.*, 2001)

Ube1XF: TGG TCT GGA CCC AAA CGC TGT CCA CA
Ube1XR: GGC AGC AGC CAT CAC ATA ATC CAG ATG

2.1.6.2 Primers for the colony PCR

Colony PCR forward: GCT ATT ACG CCA GCT GGC GAA AGG GGG ATG TG
Colony PCR reverse: CCC CAG GCT TTA CAC TTT ATG CTT CCG GCT CG

2.1.6.3 Sequencing primers

Universal sequencing primer (M13 For-40): GTT TTC CCA GTC ACG ACG
Reverse sequencing primer (M13 Rev-28): AGG AAA CAG CTA TGA CCA T

2.1.6.4 Primers for the bisulphite analysis

In all the cases of the bisulphite PCR nested or semi-nested approach was chosen. The labelling of the primers is the following:

outer forward primer **F1**
outer reverse primer **R1**
inner forward primer **F2**
inner reverse primer **R2**

Primers for the analysis of ***Lit1*** gene(Acc# AJ271885):

F1: TAT TAT TTT GGT GTT GGT TAT ATC GGG TTA

R1: ATT TTT CTT CAA CAC CCT TCT TTT CCC T

F2: GGG TTA TAA AGT TTA GGG GTT TTT AGA TT

R2: AAA CTT TTC TAT TCA ACT TAA TTC CCA AC

Primers for the analysis of ***peg3*** gene (Acc# AF105262):

F1: TTT TTA GAT TTT GTT TGG GGG TTT TTA ATA

R1: AAT CCC TAT CAC CTA AAT AAC ATC CCT ACA

F2: TTG ATA ATA GTA GTT TGA TTG GTA GGG TGT

R2: ATC TAC AAC CTT ATC AAT TAC CCT TAA AAA.

Primers for the analysis of ***Igf2*** DMR2 (Acc# U71085):

(as described in Oswald *et al.*, 2000)

F1: AAC TAA AAT TAT CTA TCC TAT AAA AC

R1: TTG ATG GAT TTA TAT TGT AGA ATT AT

F2: GGA ATT CCC TAT AAA ACT TCC CAA ACA AAC CTT CAA A

R2: GGA ATT CCT GAT TTA TTG ATG GTT GTT GGA TAT TT

Primers for the analysis of ***H19*** upstream DMR (Acc#AF049091):

5' part:

(as described in Olek et al., 1997– the primer combination F9-R9)

F1: GGA ATT CCT ATA TGG GGA TGG GTG TTT AGA AGG GGA T

R1 (~R9): AAA AAC TAA CAT AAA CCC CTA ACC TCA TAA

F2 (~F9): AAG AAA AAG GTT GGT GAG AAA AAT AGA GAT

3' part:

(as described in Ueda *et al.*, 2000)

F1 (~BIS6T0B): AGG GAT TTA TAG GGG TGG TAA

R1 (~BIS7T0): AAA TAC ACA AAT ACC TAA TCC CT

R2 (~BIS7T2): CCT AAA ATA CTC AAA ACT TTA TCA C

Primers for the analysis of ***Snrpn*** DMR1(AH007008):

(as described in El-Maarri *et al.*, 2001)

F1 (~BI-MOSN-F1): AAA TTT GTG TGA TGT TTG TAA TTA TTT GGG

R1 (~BI-MOSN-R1): AAA ATC CAC AAA CCC AAC TAA CCT TCC

F2 (~BI-MOSN-F2): AAT TAT ATT TAT TAT TTT AGA TTG ATA GTG AT

R2 (~BI-MOSN-R2): TTT ACA AAT CAC TCC TCA AAA CCA A

Primers for the analysis of ***Snrpn*** DMR2:

F1: GTG TAA GTT TGG TAA AAT ATT AT

R1: AAT TAA AAA AAT AAA CCA ACA ATA ACA

F2: AAA AAA TAA ATT TCT TAT ACT ATA AAA C

Primers for the analysis of **α-actin** gene (Acc# M12347):

(as described in Oswald *et al.*, 2000)

F1: AAG TAG TGA TTT TTG GTT TAG TAT AGT

R1: ACT CAA TAA CTT TCT TTA CTA AAT CTC CAA A

F2: GGT TTT AGT TAT TTG GGT TAG GGT

R2: CCT ACT ACT CTA ACT CTA CCC TAA ATA

Primers for the analysis of ***mylC*** gene (Acc# X12972):

(as described in Oswald *et al.*, 2000)

F1: GTA TAA TAA ATT TGG ATA GGT AAA GGT TAG

R1: AAA CCT AAA ACA CTA ATC TTA AAA ATT TTA

F2: ATA TTA TAG TAG GGG TTG GAA TGA TTA AAG

R2: CCT ATT AAA CTA ATC TAA AAA ACA ATC CTC

Primers for the analysis of ***Xist*** promoter region (Acc# U50909):

F1: TGG TTT GTT TAA GTA GAA GAT ATA TTG

R1: AAA AAT CTT ACC AAA ACA TAT CAA AAC

F2: GTA TAG ATA GGT GTG TGA TTT AAT G

R2: TTT AAT ATA TTT TCT TAA ATA AAC C

2.2 Methods

2.2.1 Isolation of mouse primordial germ cells

The primordial germ cells from 11.5 dpc and 12.5 dpc mouse embryos were isolated using the immunoaffinity purification. For the selection the SSEA1 (Stage Specific Embryonic Antigen 1 – Fox *et al.*, 1981) antigen with characteristic germ line specific expression was used. The expression of this marker vanishes around 13.5dpc, thus, using the antibody-based procedure, we obtained only very few PGCs from 13.5dpc embryos suggesting that the obtained fraction was not representative. The samples of 13.5 dpc PGCs were obtained by FACS sorting of the cell suspension obtained from the genital ridges of the *Oct4/GFP* transgenic mice with germ line specific GFP expression (Yeom *et al.*, 1996). For the reason of simplicity, higher yield and purity also the samples of early post-migratory germ cells (10.5 dpc) were obtained using the same procedure.

2.2.1.1 Antibody based purification

The whole genital ridges (together with mesonephos) were isolated from 10.5-13.5 dpc mouse embryos and washed in PBS (day 0 refers to the day of a vaginal plug). The tissue was subsequently trypsinised (trypsin:EDTA solution, room temperature) in order to prepare a single cell suspension (clumps were removed by pipetting the suspension up and down several times). The trypsin was neutralised by adding MM medium and the cells recollected by centrifugation (5 minutes, 1 500 rpm). Afterwards, the sample was resuspended in 300 µl MM medium and incubated on a shaker with 50 µl of TG1 (mouse monoclonal anti SSEA1) antibody (Gomperts *et al.*, 1994) for 45 minutes at 4^{o}C. After changing the medium (addition of 200 µl of MM medium, centrifugation and resuspending in 300 µl of MM medium), the cells were incubated with 20 µl of anti-mouse secondary antibody coupled to magnetic beads (Miltenyi Biotec) for 20 minutes at 4^{o}C on a shaker. In the following step the cell suspension (volume increased to 500 µl) was loaded onto a column on the MiniMACS (Miltenyi Biotec) holder (pre-equilibrated with 500-1000 µl of MM medium) and the negative fraction was collected (this fraction was used as "somatic cells" in the control experiments – see Results). After two washing steps (500 µl of MM medium each), 500 µl of fresh MM medium was added on top of the column detached from the magnetic field and the fraction of the positive cells was collected with the help of a plunger. Finally, the positive cells were washed with PBS.

<u>MM medium:</u>

DMEM	9 ml
Penicillin/Streptomycin (100x)	100 µl
Sodium pyruvate	100 µl
Non-essential amino acids	100 µl
Glutamine	100 µl
β-mercaptoethanol	100 µl

2.2.1.2 GFP-based purification

The isolated genital ridges were trypsinised as described above. The trypsin was neutralised by adding MM medium. After centrifugation (5 minutes, 1 500 rpm) the cells were resuspended in PBS and FACS sorted on a MoFlo (Cytomation Bioinstruments GmbH, Freiburg im Breisgau).

2.2.2 Alkaline Phosphatase Staining

The purity of isolated primordial germ cells was checked using the alkaline phosphatase staining. (The germ cells are characterised by their high level of alkaline phosphatase activity – Ginsburg *et al.*, 1990**)**. The aliquot of the isolated germ cells was stained with Sigma Diagnostics Alkaline phosphatase (AP), Leukocyte (no. R86) kit. Shortly, the cells were incubated in staining solution (2,25ml ddH_2O, 50 μl sol.1 (sodium nitrate), 50 μl sol.2 (FRV alk.sol.), 50 μl sol.3 (naphtol AS-BI) in dark for 5-10 min and washed in PBS. Cells positive for the alkaline phosphatase activity are stained red.
The purity of isolated fraction always exceeded 95%.

2.2.3 Sex determination of embryos for the isolation of primordial germ cells

Determination of sex is simple in the case of 12.5 dpc and 13.5 dpc embryos. At this stage of mouse development the genital ridges of male and female show a distinct morphology (see Results Fig. 13**)**. Due to the indistinguishable morphology of genital ridges at earlier developmental stages the sex determination of 11.5 dpc embryos was based on the amplification of *Ube1* genes (Chuma *et al.*, 2001). (There are two *Ube1* genes in mice, *Ube1X* on the X chromosome and *Ube1Y* on the Y chromosome (Imai *et al.*, 1992**)**. The primers amplify fragments of both *Ube1X* and *Ube1Y*, but the PCR results in products of different size, due to several deleted regions between the two genes. Thus, two distinct bands are amplified from male

samples and a single band from female samples.) The procedure was the following: the embryos were decapitated, part of the head tissue boiled in a PCR cycler for 5 minutes and the supernatant used for the sex specific PCR reaction.

PCR conditions: concentration of Mg^{2+} 2mM
concentration of dNTPs 0.4mM

94 °C	1 min	
98 °C	15 sec	30x
66 °C	20 sec	
72 °C	1 min	

The PCR products were subsequently separated on a 2% agarose gel.

2.2.4 Isolation of chromosomal DNA from tissue samples

For isolation of high molecular weight chromosomal DNA from mouse embryonic tissues the DNEasy™ Tissue Kit (Qiagen) was used. Shortly, the small pieces of tissue were lysed in the ATL buffer in the presence of Proteinase K and incubated at 55°C for 3 hours. After short treatment with Rnase A (5 minutes at room temperature), the samples were mixed with the AL buffer, incubated at 70 °C for 10 minutes and precipitated with ethanol. The entire mixture was subsequently applied onto a DNEasy spin column and centrifuged. After extensive washing with buffers AW1 and AW2, the isolated chromosomal DNA was eluted using 100µl of elution buffer (AE) and stored at 4 °C.

2.2.5 Bisulphite based genomic sequencing

The method of bisulphite sequencing allows the determination of a methylation status of a known DNA sequence. The principle of the method is based on the reaction of single stranded DNA with sodium bisulphite resulting in the

conversion of cytosine residues into the uracil residues, while methylated cytosines stay unconverted (for details see 1.7 Molecular techniques used for DNA methylation studies). The bisulphite treated DNA is subsequently amplified in the PCR reaction, the PCR fragments cloned and sequenced.

The modification of a method published by Olek *et al.*, 1997 is routinely used in our laboratory (Hajkova *et al*., 2002). To enhance the complete separation of DNA strands, the digested DNA is denatured and embedded into a low melting point agarose. This step ensures the spatial separation of the DNA strands throughout all the following steps.

Two following variation of the procedure was used for the bisulphite treatment of PGCs.

2.2.5.1 The bisulphite treatment of single cell suspension

The isolated germ cells were dissolved in 3 µl of PBS and mixed with 8 µl of 2 % hot (80 ^{o}C) Sea Plaque (FMC) agarose. The cell/agarose mixture was overlaid with mineral oil and boiled in a water bath for 10 minutes. The samples were immediately transferred into ice and incubated for about 30 minutes to allow the agarose mixture to re-solidify (during the re-solidification an agarose bead containing the cells was formed at the bottom of each test tube). In the following step the agarose beads were overlaid with the 100 µl of lysis solution (solution A: solution B - 1:1, supplemented with 10 µl of Proteinase K (10mg/ml)) and incubated over-night at 50 ^{o}C. After several washes with 1 ml of 1x TE solution the agarose beads were equilibrated against the restriction buffer (2 x 15 minutes) and the embedded DNA digested with 20 U of restriction endonuclease over-night at 37 ^{o}C (for bisulphite analysis of most of the gene regions described in this thesis the EcoRI restriction endonuclease was used, the only exception was the distal part of the *H19* upstream DMR, in which case the PstI restriction endonuclease was used). Following the digestion the agarose beads were denatured with fresh 0,4 M NaOH (2 x 15 minutes), washed for 5 minutes with 0,1 M NaOH, overlaid with 500 µl of mineral oil and boiled for 5 minutes in a water bath. After boiling, the samples were immediately

transferred into ice and incubated for about 30 minutes to allow the DNA/agarose mixture to re-solidify. In the next step the agarose beads were overlaid with 500 µl of the bisulphite solution (2,5 M sodium metabisulphite, 125 mM hydroquinone) and incubated in darkness first 30 minutes on ice (the sulphonation step is faster at low temperature) and then for additional 3,5-4 hours at 50 ^{o}C (higher temperature is preferred for the deamination step). Following the incubation, the agarose beads were extensively washed with TE buffer (4 x 15 minutes with 1ml of 1 x TE buffer) and treated with fresh 0,4 M NaOH (2 x 20 minutes with 1ml of NaOH solution) (de-sulphonation step). Finally, the agarose beads were washed with TE buffer (2 x 20 minutes with 1ml of 1 x TE) and stored at 4 ^{o}C.

2.2.5.2 Bisulphite treatment of isolated chromosomal DNA

Approximately 700 µg of high-molecular -weight DNA was digested over-night in the volume of 21µl with 10U of restriction endonuclease at 37 ^{o}C. The restriction mix was subsequently denatured in the boiling water bath for 10 min. After edition of 2M NaOH into the final concentration of 0.3 M, the samples were incubated for additional 15 minutes at 50 ^{o}C to assure complete denaturation. The samples were then mixed with 2 volumes of hot 2% LMP agarose and 4-6 10 µl aliquots pipetted into the pre-chilled mineral oil overlaying 750 µl of bisulphite solution (see previous chapter). All the following steps were identical to the procedure described in the previous chapter.

2.2.6 Bisulphite PCR amplification

The bisulphite treatment was followed by the gene specific PCR amplification. In all the cases nested (or at least semi-nested) approach was chosen to ensure the highest specificity and sensitivity of the procedure.

Prior to setting up the PCR reaction the bisulphite treated beads were washed twice with dd H_2O (2 x 15 minutes with 1 ml of dd H_2O) in order to remove the traces of the TE buffer.

If not stated otherwise, the PCR was running in the following conditions:

1 x PCR buffer (15 mM $MgCl_2$ – for the composition see 2.1.5.7. Buffers & solutions), 200 µM dNTPs (each), 20 pmol primer (each), 4 U Taq polymerase. The reaction volumes were the following: 100 µl for the first PCR reaction; 50 µl for the second PCR reaction. One bisulphite treated agarose bead (10 µl) was used as a template for the first PCR, 1-3 µl of the first PCR product were used for setting up the second PCR reaction. All the PCR conditions were optimised using the gradient PCR cycler (Eppendorf) – the conditions with the highest annealing temperature still resulting in a PCR product were chosen for the experiments to ensure the specificity and high rate of bisulphite conversion.

Lit 1 amplification

Buffer: 10 x PCR buffer (15 mM $MgCl_2$)
Primers: F1, R1 (1st round)
F2, R2 (2nd round)

Conditions for bisulphite PCRs

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
59 °C	90 sec	30 x	58 °C	90 sec	30 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

peg3 amplification

Buffer: 10 x PCR buffer (15 mM $MgCl_2$)
Primers: F1, R1 (1st round)
F2, R2 (2nd round)

PCR conditions:

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
61 °C	90 sec	30 x	61 °C	90 sec	30 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

Igf2 DMR2 amplification

Buffer: 10 x PCR buffer (25 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F2, R2 (2nd round)

PCR conditions:

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
54 °C	90 sec	30 x	60 °C	90 sec	30 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

H19 amplification (3' part of the upstream DMR)

Buffer: Boehringer No. 2 (Long distance PCR kit, 22,5 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F1, R2 (2nd round)

PCR conditions:

1st round PCR			2nd roundPCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
57 °C	90 sec	30 x	57 °C	90 sec	30 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

H19 amplification (5' part of the upstream DMR)

Buffer: Boehringer No.2 (Long distance PCR kit, 22,5 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F2, R1 (2nd round)

PCR conditions:

1st round PCR			2nd roundPCR		
95 °C	5 min		95 °C	5 min	
95°C	45 sec		95 °C	45 sec	
65°C	1 min 15 sec	30 x	62 °C	1 min 15 sec	30 x
72 °C	1 min 45 sec		72 °C	1 min 45 sec	
72 °C	10 min		72 °C	10 min	

Snrpn DMR1 amplification

Buffer: 10 x PCR buffer (15 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F2, R2 (2nd round)

PCR conditions:

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
°C	90 sec	30 x	°C	90 sec	30 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

Snrpn DMR2 amplification

Buffer: 10 x PCR buffer (15 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F2, R1 (2nd round)

PCR conditions:

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
59 °C	90 sec	30 x	57 °C	90 sec	30 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

Xist amplification

Buffer: 10 x PCR buffer (15 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F2, R2 (2nd round)

PCR conditions:

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
61 °C	90 sec	35 x	59 °C	90 sec	35 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

α-actin amplification

Buffer: 10 x PCR buffer (15 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F2, R2 (2nd round)

PCR conditions:

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
57 °C	2 min	5 x	55 °C	2 min	5 x
72 °C	3 min		72 °C	3 min	
95 °C	30 sec		95 °C	30 sec	
57 °C	2 min	25 x	55 °C	2 min	25 x
72 °C	90 sec		72 °C	90 sec	
72 °C	5 min		72 °C	5 min	

mylC amplification

Buffer: 10 x PCR buffer (15 mM $MgCl_2$)

Primers: F1, R1 (1st round)

F2, R2 (2nd round)

PCR conditions:

1st round PCR			2nd round PCR		
95 °C	5 min		95 °C	5 min	
95 °C	1 min		95 °C	1 min	
53 °C	90 sec	30 x	52 °C	90 sec	30 x
72 °C	90 sec		72 °C	90 sec	
72 °C	10 min		72 °C	10 min	

2.2.7 Isolation of a DNA fragment from the agarose gel

The products of the bisulphite PCR were separated on the 1 % agarose gel (run in TBE buffer); the specific PCR products were cut out using a sterile scalpel. The DNA was subsequently extracted using the QiaEx II kit (Qiagen). Briefly, the agarose was dissolved in QX1 buffer at 50 °C in the presence of glass milk, the mixture was centrifuged and the glass milk pellet containing bound DNA washed twice with PE buffer. The pellet was subsequently air-dried for 15 minutes at RT and the bound DNA eluted with 20 µl of dd H_2O. The rests of glass milk inhibiting the following ligation were removed by short additional centrifugation.

2.2.8 Cloning

In all the PCR experiments the unmodified Taq polymerase was used. Since this enzyme is prone to non-template addition of adenine nucleotide(s) to the 3'-terminus of newly synthesised DNA strands a very simple cloning procedure based on those "sticky ends" could be used. The purified PCR fragments were ligated using the TA cloning kit (Promega) into a pGEM based plasmid vector with T-overhangs on both 3'- termini. (In the original experiments the PCR fragments were cloned into a pBluescript based pCR 2.1 TA cloning system (Invitrogen). This procedure, however, resulted several times in a bias towards the cloning of unconverted molecules. The

system was exchanged for the pGEM-based vectors, in which case the bias has never been observed).

Depending on the yield of the PCR reaction, 3-5 µl of purified PCR product were added to the ligation mix (all the components provided by the kit) and incubated for 1 hour at room temperature (fast ligation scheme using the 2 x ligation buffer). 5 µl of this ligation reaction were subsequently transformed into commercially available ultra-competent *E.coli* cells (Top10 or INVαF' strains, both Invitrogen) or to competent *E.coli Sure* cells prepared in our laboratory (see the following chapter).

The transformation into competent *E.coli* cells was performed using the heat shock protocol. Briefly, the aliquot of frozen competent cells was thawed on ice, mixed with 5 µl of the ligation mix and incubated on ice for additional 30 minutes. The incubation was followed by heat shock performed at 42 ^{o}C for exactly 90 seconds. After a short (1-2 minutes) incubation on ice 250 µl of pre-warmed (37 ^{o}C) SOC medium were added and the cell suspension rigorously shaken for 1 hour at 37 ^{o}C. Finally, the transformed cells were plated on LB agar plates supplemented with ampicillin as a selection marker, IPTG (20 µl per plate) and X-gal (50 µl per plate) for the blue/white screening of transformed colonies (the insertion of a fragment into the cloning vector results in the disruption of the *lacZ* ORF, thus the colonies containing the insert appear white in the screening procedure). Plates were incubated at 37 ^{o}C overnight.

2.2.9 Preparation of competent *E.coli Sure* cells

30 ml of bacterial LB-medium was inoculated with 300 µl of the over-night *E.coli* culture and incubated until it reached the cell density of OD_{560}=0,4. All the following steps were carried on strictly on ice. The *E.coli* cells were centrifuged for 10 min at 6000 g, re-suspended in 15 ml of 50 mM $CaCl_2$ and incubated on ice for 15 min. The centrifugation (10 min, 6000 g) was repeated and the pellet re-suspended in 3 ml of the $CaCl_2$ solution. After the addition of glycerol (to the final concentration 15 %, (v/v)), the 100 µl aliquotes of the cell suspension were shock-frozen in liquid nitrogen and stored at –80^{o}C.

The transformation efficiency was estimated by transformation of 1ng of a control pUC19 plasmid and determined as a number of transformed colonies per 1 μg of plasmid DNA.

2.2.10 Detection of positive clones by colony PCR

The experience of our laboratory showed that some of the bisulphite fragments were unstable in bacterial host. For this reason the transformed colonies were always checked for the presence of the insert of the correct size by colony PCR
The procedure was the following: the PCR mix was pipetted into the wells of 96-well MTP. The white transformed colonies were picked by the autoclaved toothpicks and dipped one by one into the wells of MTP. The same toothpick was used to inoculate the field in a grid of a replica agarose plate (colonies growing on this plate could be used when it was necessary to repeat the sequencing procedure).

Colony PCR mix: (for 25 reactions - 30 μl each)

10 x PCR buffer (15 mM $MgCl_2$)	60 μl
dNTPs (10 mM)	60 μl
primers * (forward + reverse,10μM each)	12 + 12 μl
Taq (4 U / μl)	6 μl
formamide (ultra-pure)	12 μl
dd H_2O	438 μl

* . The primers for the colony PCR anneal in the polylinker of the cloning vector, outside the annealing positions for the M13 universal and reverse sequencing primers.

Conditions for the colony PCR:

95°C	5 min	
95 °C	10 sec	35 x
65 °C	30 sec	
72 °C	1 min	
72 °C	5 min	

5 µl of the colony PCR products were loaded onto a 1 % agarose gel in order to check the size of the product. The rests of the PCR reactions belonging to the clones with the correct-size PCR product were frozen and handed over to the sequencing facility of the institute. (Routinely 15-20 clones were sequenced per each transformation.)

2.2.11 Sequencing and the sequence evaluation

The colony PCR products were purified using the fully automated magnetic bead system of our sequencing facility. The purified DNA was subsequently used as a template for the Big Dye sequencing procedure and sequenced on the ABI 377 automatic sequencer.

The sequences were checked individually for their quality using Chromas (Microsoft Windows based) software or using the GCG 10.0 Wisconsin Package operating on a Unix interface. With the help of this Unix-based software the sequences of the parallel clones were piled-up and the information about the methylation status of the tested DNA region converted manually into the dot diagrams presented throughout this thesis.

2.2.12 mC immunofluorescent staining

The isolated germ cells on poly-L-lysin coated slides (Sigma) were swollen in 1 % Na citrate hypotonic solution for 5 min. Freshly prepared fixative acetic alcohol (Methanol: glacial acetic acid 3:1) was dropped directly onto the cells and slowly air dried in a humid chamber. The cell preparations were treated with 100 µg/ml RNase A in 2x SSC at 37 °C for 60 min and with 0.01 % pepsin in 10 mM HCl at 37 °C for 10 min, and then dehydrated in an ethanol series (70, 85 and 100 %). The slides were denatured in 70 % formamide, 2x SSC for 1 min at 80 °C and then dehydrated in an ice-cold ethanol series. After brief air-drying, the slides were first incubated with blocking solution (3 % BSA, 0.1 % Tween 20, 4x SSC) in a Coplin jar for 30 min and then with mouse anti-mC antibody (hybridoma supernatant, a kind gift of A. Niveleau), diluted 1:50 with PBS, in a humidified incubator at 37 °C for 30 min. The slides were then washed in PBS three times for 10 min each and incubated for 30 min with fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgG (Dianova) appropriately diluted with PBS. After three further washes with PBS, the preparations were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in 2x SSC for 5 min. The slides were mounted in 90 % glycerol, 0.1 M Tris-HCl, pH 8.0 and 2.3 % 1,4-diazobicyclo-2,2,2-octane. Images were taken with a Zeiss epifluorescence microscope equipped with a thermoelectronically cooled charge-coupled device camera (Photometrics CH250).

3 Results

The epigenetic reprogramming taking place in primordial germ cells has been the object of number of previous experiments. Whereas the majority of publications concerned the onset of new, sex specific, imprints, our interest was focused on the poor characterised process of demethylation / imprint erasure. Based on the published data describing the hypomethylation and the absence of imprints in the primordial germ cells at 12.5-13.5 dpc (Kafri *et al.*, 1992), we decided to focus our analysis mainly on the primordial germ cells of the earlier developmental stages (11.5 and 12.5 dpc), the stages where we expected the process of imprint erasure to take place.

3.1 Purification of primordial germ cells

The samples of 11.5 dpc and 12.5 dpc primordial germ cells were isolated from the genital ridges using the immunoaffinity purification in combination with the magnetic bead sorting system (see Material and Methods). Shortly, whole genital ridges of mouse embryos were trypsinised in order to produce a single cell suspension. Primordial germ cells were subsequently isolated using monoclonal TG1 antibody (Gomperts *et al.*, 1994) in combination with the secondary anti-mouse antibody coupled to magnetic beads (MiniMacs sorting system, Miltenyi Biotec – see Fig. 10). TG1 antibody recognise the germ line specific SSEA1 antigen (SSEA1 – Stage Specific Embryonic Antigen 1 - is a trisaccharide of the form galactose [β1-4]N-acetylglucosamine[α1-3]fucose , Fox *et al.*, 1981).

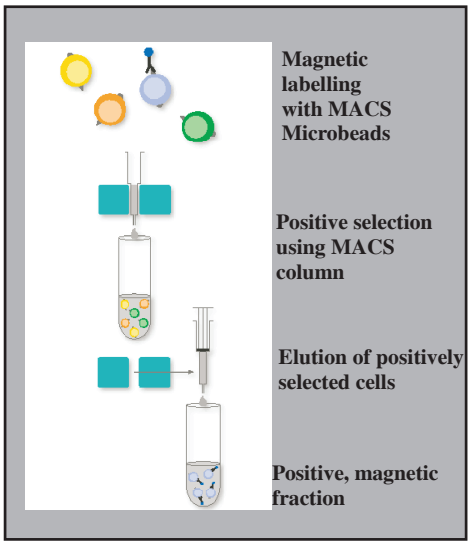


Fig.10: Scheme of the magnetic bead-based cell separation

Whereas the samples of 11.5 dpc and 12.5 dpc primordial germ cells were collected using the immunoaffinity purification, the method did not approve to be useful in the case of 13.5 dpc cells. The expression of the SSEA1 antigen, which is the target for the TG1 antibody, diminishes after 12.5 dpc; thus it loses the properties of a suitable selection marker. To isolate the primordial germ cells of later as well as earlier stages of development, we took the advantage of a *Oct4-GFP* transgenic mouse with the germ line specific GFP expression (see Fig. 11), which was created at Wellcome/CRC Institute, Cambridge, UK (for details see Material and methods).

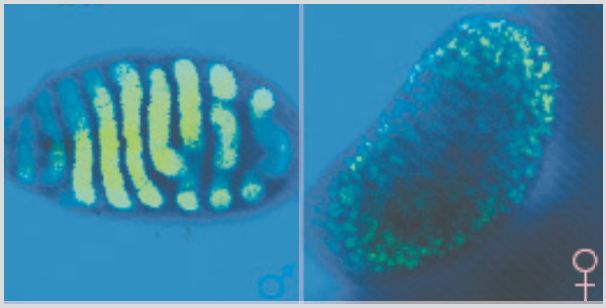


Fig.11: GFP expression in 13.5 dpc gonads of Oct4-GFP transgenic embryos
Note the sexual dimorphism of embryonic gonads at this developmental stage.

Since PGCs are characterised by high levels of alkaline phosphatase activity (Ginsburg *et al.*, 1990) each of our PGC preparations was checked for the purity using the alkaline phosphatase staining (see Fig. 12**,** and Material and Methods). The purity of the samples always exceeded 95%.

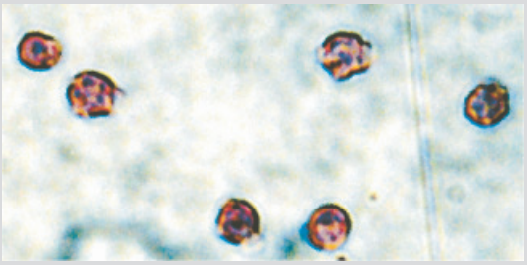


Fig.12: Single cell suspension of MiniMacs sorted PGCs (12.5 dpc) stained for tissue non-specific alkaline phosphatase.
The positive cells are stained in brown.

The genital ridges of the 12.5 dpc embryos show sex specific morphology (see Fig. 13), thus making the sex determination of PGC samples simple. However, at 11.5 dpc the genital ridges of female and male are still indistinguishable. As a consequence, in majority of our experiments the 11.5 dpc germ cells were used as a mixed gender population. In limited number of control experiments the sex of collected 11.5 dpc samples was determined by the PCR approach (amplification of *Ube1* genes, *Ube1X* and *Ube1Y* are located on X and Y chromosomes, respectively - for details see Material and Methods).

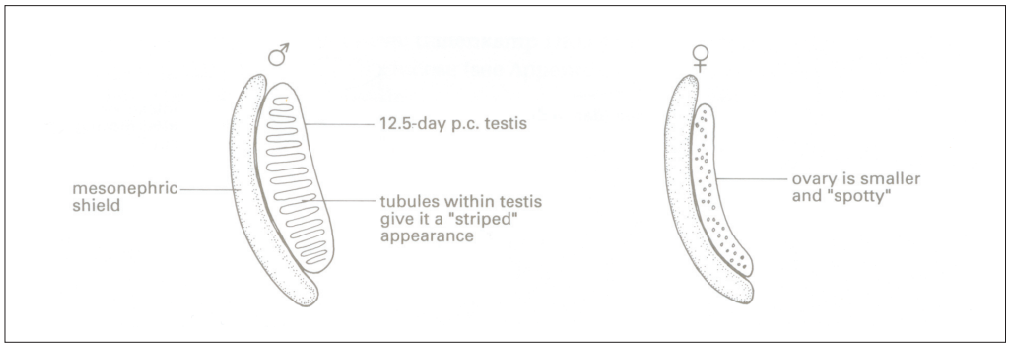


Fig.13: Sex specific morphology of the mouse embryonic genital ridges.
(Hogan *et al*., 1994)

3.2 Methylation analysis using the bisulphite genomic sequencing

To assess the methylation status of primordial germ cells of different developmental stages we took the advantage of bisulphite genomic sequencing (see 1.7 Molecular techniques used for DNA methylation studies and Hajkova *et al*., 2002). The method is based on the chemical reaction of single stranded DNA with sodium bisulphite under acidic pH and following desulphonation in highly alkalic conditions (see Fig. 14). Consequently, the single stranded cytosin residues are converted through several reaction intermediates into uracil residues, whereas 5'methyl-cytosin residues remain unconverted. The bisulphite modified DNA strands are in the following steps amplified (PCR), cloned and sequenced. In the PCR reaction the uracil residues (originally non-methylated cytosines) are amplified as thymines, i.e. the cytosines found in the final sequence correspond to the positions of methylated cytosines in original DNA molecules (see Fig. 15). Among advantages of this method belong: a) low requirements for the amount of starting material (a crucial parameter when working with embryonic material - as few as several dozens cells are sufficient for the analysis); b) the possibility to analyse all the cytosine residues within the region defined by the PCR primers (to the contrary, using the methods based on methyl-sensitive restriction it is possible to analyse only a limited number of CpG sites) and c) the possibility to reveal the pattern of single DNA molecules, which is important when studying the dynamic processes such as imprint establishment or erasure.

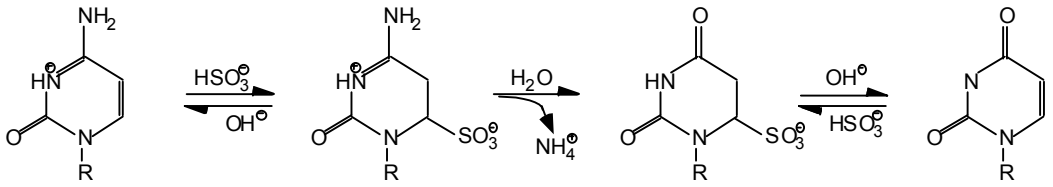


Fig.14 The reaction of single stranded cytosine residues with sodium bisulphite
Chemistry of the reaction steps: I) sulphonation at the position C6 of cytosine, II) irreversible hydrolytic deamination at the position C4 generating 6-sulphonate-uracil and III) subsequent desulphonation under alkaline conditions. Note that methylation at the position C5 impedes sulphonation at the C6 position (step 1).

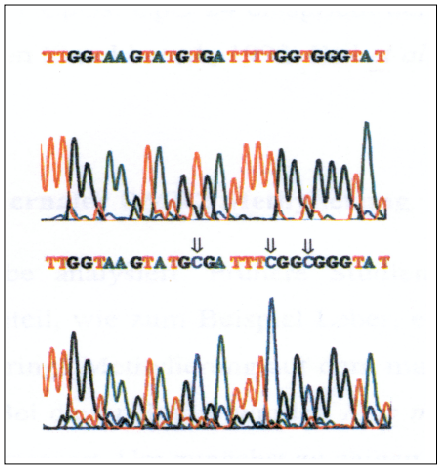


Fig.14: The examples of bisulphite treated sequences. The example of a non-methylated clone is shown above (cytosines are converted to thymines). The example of a methylated clone is given in the lower part of the figure (methylated cytosines resisted the conversion).

3.3 Methylation status of imprinted genes

In order to investigate the reprogramming (especially the erasure) of imprints we decided to follow the methylation status of well described **d**ifferentially **m**ethylated **r**egions (DMRs; sequences carrying sex specific methylation) connected to the imprinted genes. The DMRs of the following imprinted genes were included into our analysis: ***peg3, lit1, Snrpn*** (DMR1) as examples of maternally methylated regions; and ***igf2, h19,*** and ***Snrpn*** (DMR2) representing paternally methylated sequences (see Table 2)

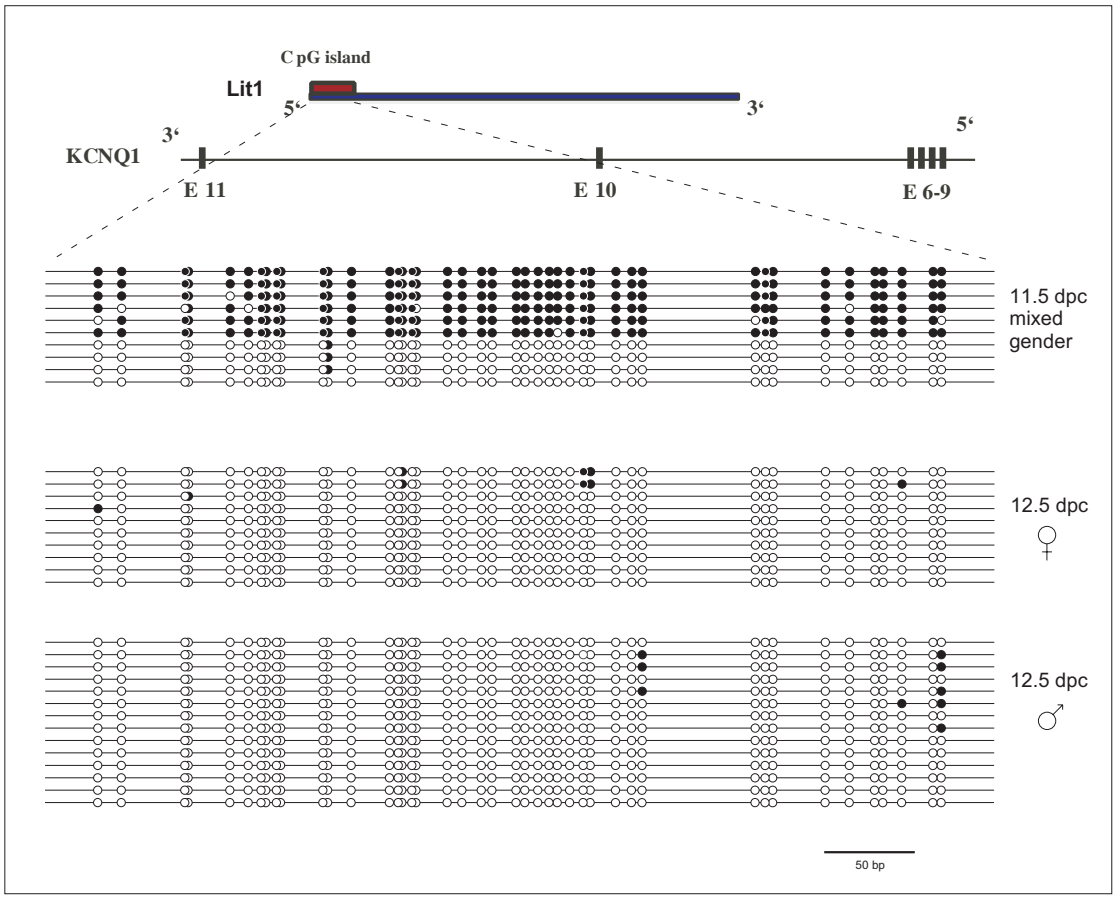
Gene	Tested region	Location	Methylation mark
Snrpn	DMR1	promoter + 1st exon	maternal
	DMR2	intron 8	paternal
Peg3	CpG island	1st exon	maternal
Lit1	CpG island	promoter	maternal
Igf2	DMR2	exon 5-6	paternal
H19	upstream DMR	5' part (Olek *et al.*, 1997).	paternal
		3' part (Ueda *et al.*, 2001)	paternal
Xist	promoter		*
mylC	promoter		non imprinted
α-actin	promoter		non imprinted

Table 2: **Schematic overview of the regions tested in the bisulphite analysis**
* *Xist* gene shows an imprinted character only during early stages of embryogenesis; random monoallelic *Xist* expression is characteristic for somatic cells .

3.3.1 Methylation changes in *lit1* CpG island

Lit1 (**l**ong QT **i**ntronic **t**ranscript **1,** GenBank acc.# AJ271885, mouse distal chromosome 7) was originally identified as an antisense orientation transcript within the imprinted *KVLQT1* gene (Lee *et al.*, 1999). The *lit1* itself shows also imprinted character, the transcription being active only on a paternal chromosome. The expression of both imprinted genes seems to be regulated from a pronounced *lit1* CpG island located within the intron 10 of *KVLQT1*, which was proposed to function as an additional imprinting centre in the Beckwith-Wiedemann cluster (Maher *et al.*, 2000; Smilinich *et al.*, 1999; Engemann *et al.*, 2000).

The *lit1* CpG island carries the germline methylation mark; the region was shown to be heavily methylated in oocytes, whereas it appears to be non-methylated in sperm (Engemann *et al.*, 2000). Our bisulphite analysis was directed to the 3'part of the CpG island; the amplified part of the CpG island (524bp) contains 43 CpG sites (Fig. 16).



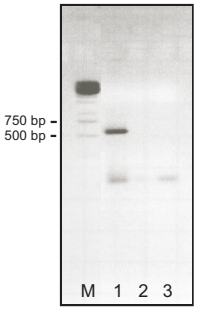


Fig. 16: Bisulphite analysis of *Lit1* CpG island
The upper part of the figure shows the results of the bisulphite analysis. Each line and circle represent a unique clone and single CpG position, respectively. The filled circles stand for the methylated CpGs, the open circles represent non-methylated positions. The product of bisulphite specific PCR is shown on the gel left: **M** - 1kb ladder (Fermentas), **1**- PGC sample, **2** - water control for bisulphite treatment, **3** - water control for PCR amplification.

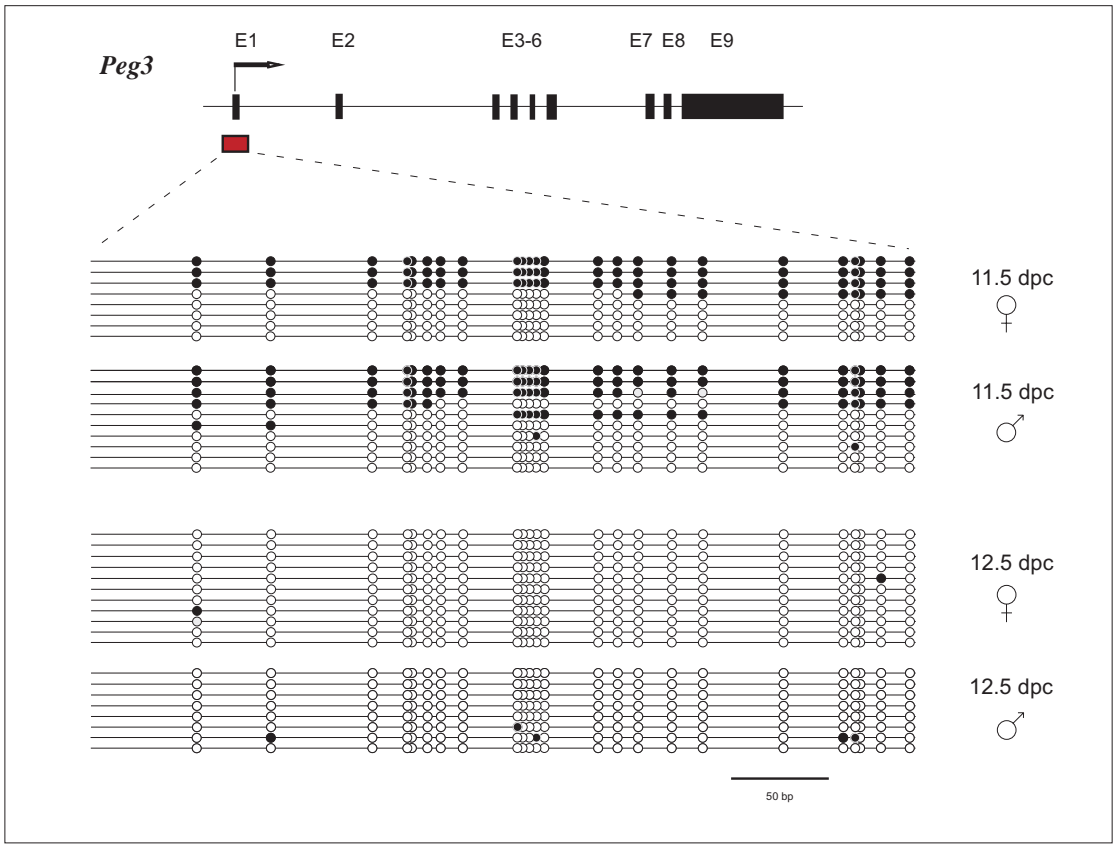
The mixed gender 11.5 dpc primordial germ cells display approximately 1:1 distribution of completely methylated vs. completely non-methylated clones which corresponds to the pattern seen in zygote and ES cells (Engemann *et al.*, 2000) and expected in somatic cells. Thus, at 11.5 dpc the PGCs still maintain the methylation imprint. However, at 12.5 dpc only completely unmethylated clones are present in samples of both genders (the data represent the combination of two independent bisulphite treatments and several PCR amplifications). These results might bring about the following conclusions: a) PGCs contain normal imprint at 11.5 dpc which

excludes the theory that the precursors for the germ cells are separated early during the embryogenesis and never gain somatic type of methylation; b) reprogramming of the imprint does not seem to be a gradual process of imprint loss and reestablishment, but it might be initiated as a rapid and complete erasure of a full somatic type of imprint.

3.3.2 Methylation changes in *peg3* gene

Another example of a maternally imprinted gene is *Peg3*. *Peg3* (**p**aternally **e**xpressed **g**ene 3, GenBank acc.# AF105262; mouse proximal chromosome 7) encodes a zinc finger protein that is expressed only from the paternal allele in embryos and adult brain (Kuroiwa *et al.*, 1996; Li *et al.*, 2000). The protein has been shown to be involved in TNF-NFkappaB signal transduction pathway (Relaix *et al.*, 1998) and in regulation of maternal behaviour (Li *et al.*, 1999). The gene consists of nine exons spanning 26kb; the 5`region is rich in repeated sequences and contains a CpG island. This region has been proven to carry a differentially methylated mark: the gene is preferentially methylated on the inactive maternal allele, as shown by comparing embryos with paternal and maternal duplication of proximal chromosome 7 (Li *et al.*, 2000).

Our methylation analysis was focused on this CpG rich region; namely the 1st exon, which is a part of the above-mentioned CpG island. The amplified region is 422bp long and contains 24 CpG dinucleotides (Fig. 17).



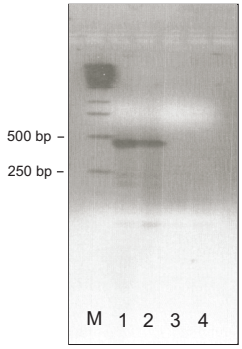


Fig. 17: Bisulphite analysis of *Peg3* gene.
The results of the bisulphite analysis including the scheme of the genomic organisation of *Peg3* gene are depicted in the upper part of the figure. Each line represents a unique bisulphite clone. Filled and open circles represent methylated and non-methylated CpG dinucleotides, respectively. The gel with the products of bisulphite PCR is shown left: **M** - 1kb marker (MBI Fermentas), **1,2** - examples of PGC samples, **3** - water control for the bisulphite treatment, **4** - water control for PCR amplification.

The mixed gender samples of 11.5 dpc primordial germ cells were used in the previous experiments. To exclude the possibility that there is a sex specific difference at the onset of epigenetic reprogramming we isolated the PGCs separately from the individual 11.5 dpc embryos, that were later sexed using a PCR approach (see Material and Methods). The presented bisulphite data argue that there is no difference in the timing of major demodification event in female and male germlines.

The 11.5 dpc samples show methylation of a distribution explainable by the presence of a normal imprint (maternal allele methylated, paternal allele not methylated; i.e. methylated vs. non-methylated clones in a 1:1 ratio). However, only one day later in the development the same tested region appears to be completely demethylated (at least two independent bisulphite treatments for sample type) in PGCs of both female and male.

The results on the 11.5 dpc samples uncover an interesting phenomenon: not all the methylated DNA molecules appear to be methylated completely (the normal bisulphite pattern observed in somatic cells consists of either completely methylated or completely non-methylated DNA strands). Interestingly, the partially methylated DNA strands do not show the stochastic type of methylation, but rather patchy pattern of modification. This observation might shed more light on kinetics (or possible mechanism) of the demethylation process; the responsible enzymatic machinery is likely to work in a processive manner. Additionally, the presence of partially demethylated DNA strands ad 11.5 dpc suggests, that 11.5 dpc might be the critical time point when the reprogramming starts.

3.3.3 Methylation changes in *Igf2* gene

Lit1 and *Peg3* represent the imprinted genes carrying maternal methylation imprint, to gain more complex insight into the problematic of imprint reprogramming we focused on the paternally methylated genes in the following experiments.

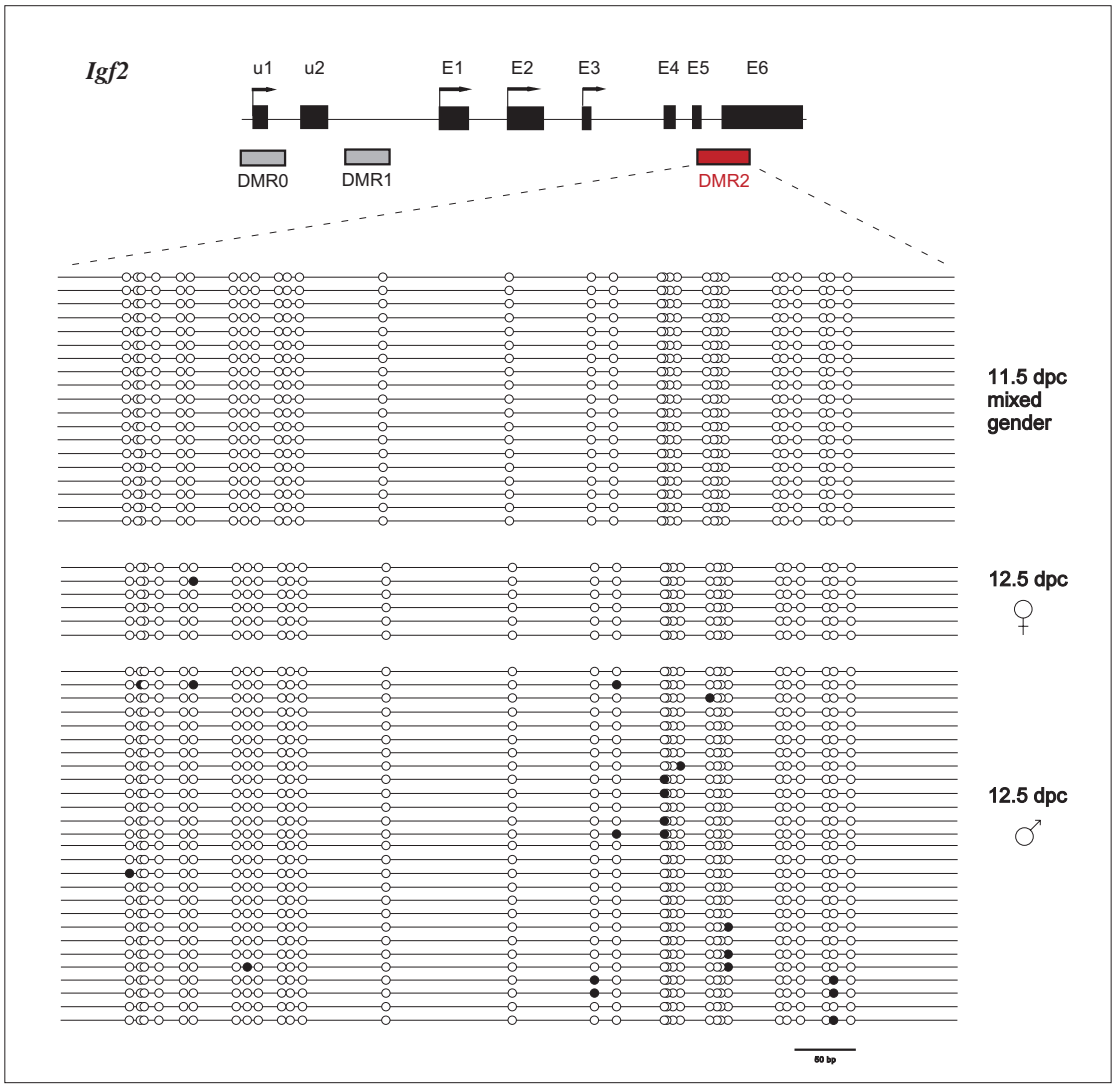
Insulin-like **g**rowth **f**actor **2** (GenBank acc.# U71085, mouse distal chromosome 7) is a typical candidate of an imprinted gene characterised by paternal methylation. *Igf2* begins to be transcribed shortly after implantation first in extraembryonic tissues, and then throughout mesodermal and endodermal tissues in postimplantation embryos (Lee *et al.*, 1990). The gene encodes a single polypeptide involved in signalling through the IGF/INS pathway, thus having implications for embryonic growth (foetuses lacking IGF-II are growth retarded (DeChiara *et al.*,

1991), overepression causes an increase of size at birth (Sun *et al.*, 1997**;** Eggenschwiler *et al.*, 1997).

Imprinting of the *Igf2* gene has been the subject for number of publications. Using the knock-out technology it was shown that the transcription of the gene is active only on the paternal allele (DeChiara *et al.*, 1991). There have been three differentially methylated regions identified within the gene; methylation of those regions is tightly connected with *Igf2* expression. DMR0 (positioned upstream of placenta specific promoter 1) shows differential methylation only in placenta, where the maternal allele is preferentially methylated (Moore *et al.*, 1997). DMR1 (located upstream from the promoter 1) is not differentially methylated in germ cells, but becomes so soon after fertilisation (Sasaki *et al.*, 1992**;** Shemer *et al.*, 1996). Preferentially methylated is the paternal transcriptionally active allele. The best-characterised DMR is the DMR2, which lies in 3'part of the coding region. DMR2 is differentially methylated in germ cells, and loses its methylation in early preimplantation embryo, which then becomes re-established later on (Oswald *et al.*, 2000; Feil *et al.*, 1994).

The decision to choose DMR2 from the above mentioned *Igf2* DMRs for our analysis was based on the following facts: a) DMR2 displays considerable parent-of-origin specific methylation. (As we were not able to distinguish maternal and paternal alleles in our experimental set-up, we had to focus on regions with pronounced difference in sex specific methylation.) b) Our laboratory described in detail the methylation dynamics of DMR2 during embryogenesis (Oswald *et al.*, 2000; Hajkova - unpublished data). Thus there was a number of comparative data available giving us the idea about the level of methylation to expect in somatic cells of embryo.

The amplified part of DMR2 is 731bp long and spans 30 CpG sites (Fig. 18).



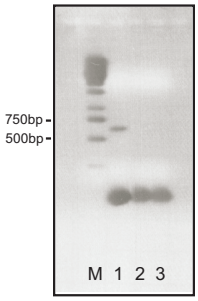


Fig 18: Bisulphite analysis of *Igf2* DMR2
The results of several bisulphite treatments are depicted above. Each line represents a unique bisulphite clone (open and filled circles correspond to non-methylated and methylated CpGs, respectively). Despite the number of bisulphite experiments, it was not possible to detect any methylation in 11.5dpc PGC samples (for more see the text). The photography left shows the gel with the products of bisulphite PCR amplification: **M** - 1kb ladder (MBI Fermentas), **1** - PGC sample, **2** - water control for the bisulphite treatment, **3** - water control for the PCR amplification.

Despite performing 3 independent bisulphite treatments we were not able to detect any methylation in 11.5 dpc PGCs. (Each separate line in a figure represents a unique clone based on single nucleotide polymorphism concerning the unconverted cytosines.) This result was rather unexpected as we know, that the methylation imprint is present in this region already in the earlier stages of embryogenesis (see

Fig. 19). Absence of methylation is also surprising in the light of the previous experiments: *lit1* and *peg3* (as well as other genes – see the following chapters) keep the methylation imprints in primordial germ cells up to 12.5 dpc. Thus the primordial germ cells pass during the development a stage when they posses normal methylation imprints present in somatic cells. This taken together suggests that the DMR2 gets probably demethylated earlier during the PGC development.

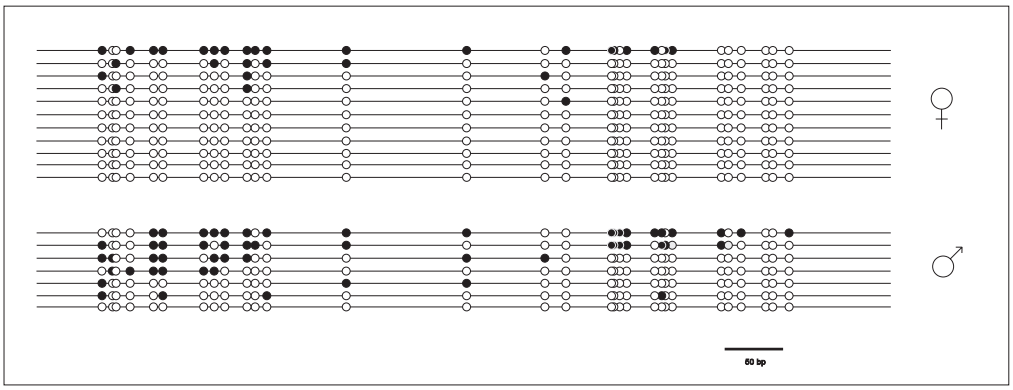


Fig. 19: Allele specific methylation of the *Igf2* DMR2 in the embryonic tissues of 8.5 dpc embryos.
The figure shows the results of the bisulphite analysis: individual lines represent unique bisulphite clones, methylated CpGs are shown as filled circles, non-methylated are represented by open circles. The alleles were distinguished based on the sequence polymorphism. Note the presence of the allele specific methylation already at this early embryonic stage.

The bisulphite treatments performed on 12.5 dpc samples of both genders revealed no methylation in the tested DMR either. This is in agreement with the previous results showing demethylated status of the tested genes at 12.5 dpc.

The possibility that we were not able to detect methylation in the tested region because of the methodical problems (bias in bisulphite-PCR, bias in cloning etc.) was excluded by the fact, that identical primers, PCR and cloning conditions were previously tested for the bias and used in number of experiments performed in our laboratory (Oswald *et al.*, 2000; Hajkova – unpublished results).

The methylation changes occurring in *Igf2* DMR2 differ from the changes detected in previous experiments on *peg3* and *lit1*. The demethylation step obviously occurs earlier, so that the DMR region stays unmodified throughout the time window

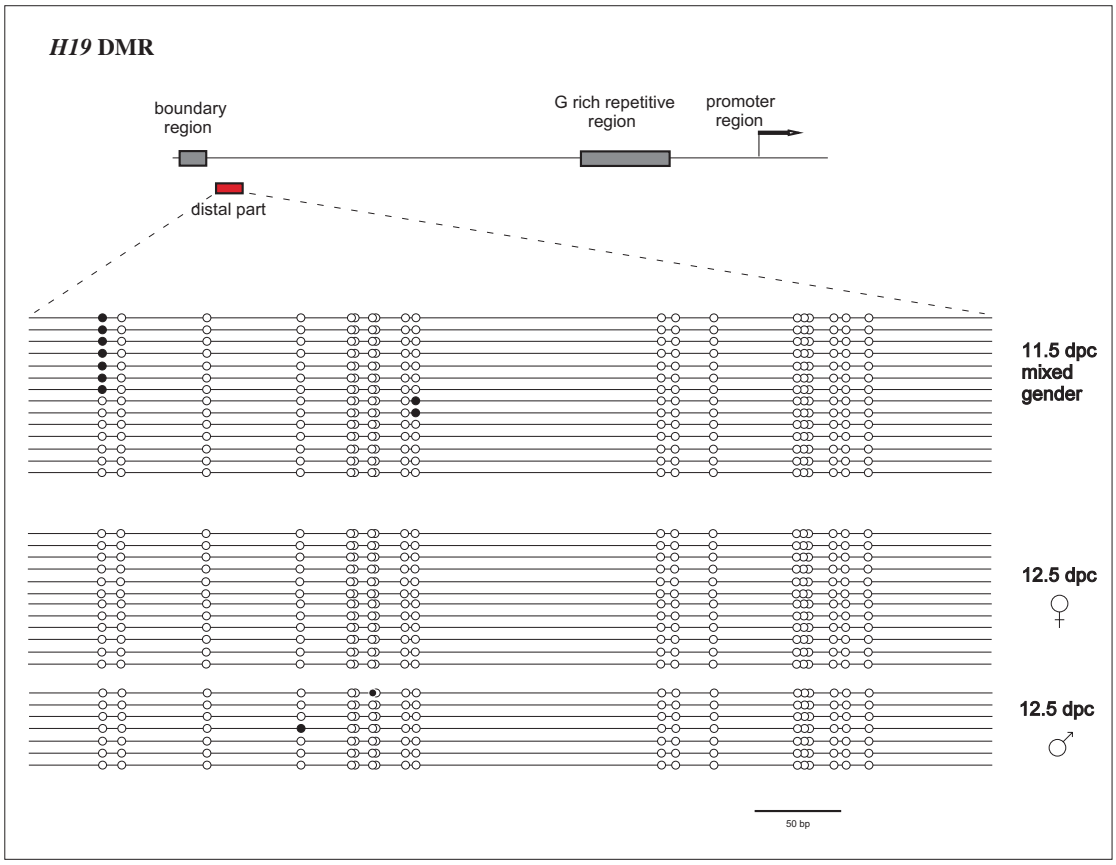
tested. The demethylated status at 12.5 dpc is, however, in agreement with the above-described experiments showing that the imprints of PGCs are erased at 12.5 dpc.

3.3.4 Methylation changes in *H19* gene

The next example of a widely studied imprinted gene with paternal methylation is *H19* (GenBank acc.# AF049091**,** mouse distal chromosome 7). This gene which encodes RNA of unknown function is highly expressed from the maternal allele in embryonic tissues of endodermal and mesodermal origin (Brannan *et al.*, 1990**;** Poirier *et al.*, 1991). The mechanism of *H19* imprinting is thought to involve paternal-specific methylation of the 5`flank of the gene (Bartolomei *et al.*, 1993**;** Ferguson-Smith *et al.*, 1993). As required for a gametic imprinting mark, methylation is found in sperm but not in oocytes and is maintained throughout embryogenesis in all tissues (Olek *et al.*, 1997; Tremblay *et al.*, 1995**;** Tremblay *et al.*, 1997).

First we decided to analyse the proximal (5'part) of the *H19* DMR (see Fig. 20). The analysed region corresponds to the part of the DMR amplified by (Olek *et al.*, 1997) using F9R9 primer combination. The sequence was shown to carry methylation imprint in gametes (sperm completely methylated, oocytes non-methylated) as well as in somatic cells of the embryo.

The amplified fragment is 553bp long and comprises 19 CpG sites.



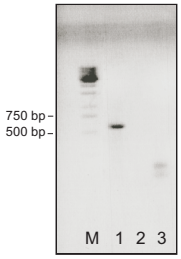


Fig. 20: Bisulphite analysis of the distal part of upstream *H19* DMR.
The results of the bisulphite analysis are depicted above. The lines represent unique clones, filled circles stand for methylated CpG positions, the open circles represent non-methylated CpGs. The gel photograph (left) shows the product of the bisulphite PCR amplification. **M** - 1kb ladder (MBI Fermentas), **1** - PGC sample, **2** - water control for the bisulphite treatment, **3** - water control for the PCR amplification.

The bisulphite analysis of the *H19* DMR was technically challenging. Despite extensive optimisation of PCR condition, the rate of successful amplification appeared to be low with frequent appearance of multiple bands. Additional problems appeared during the cloning procedure - the number of obtained clones was low; additionally, some clones turned out to be not completely converted. It is interesting to note, that incomplete bisulphite conversion (patchy pattern) was observed solely when analysing the *H19* DMR.

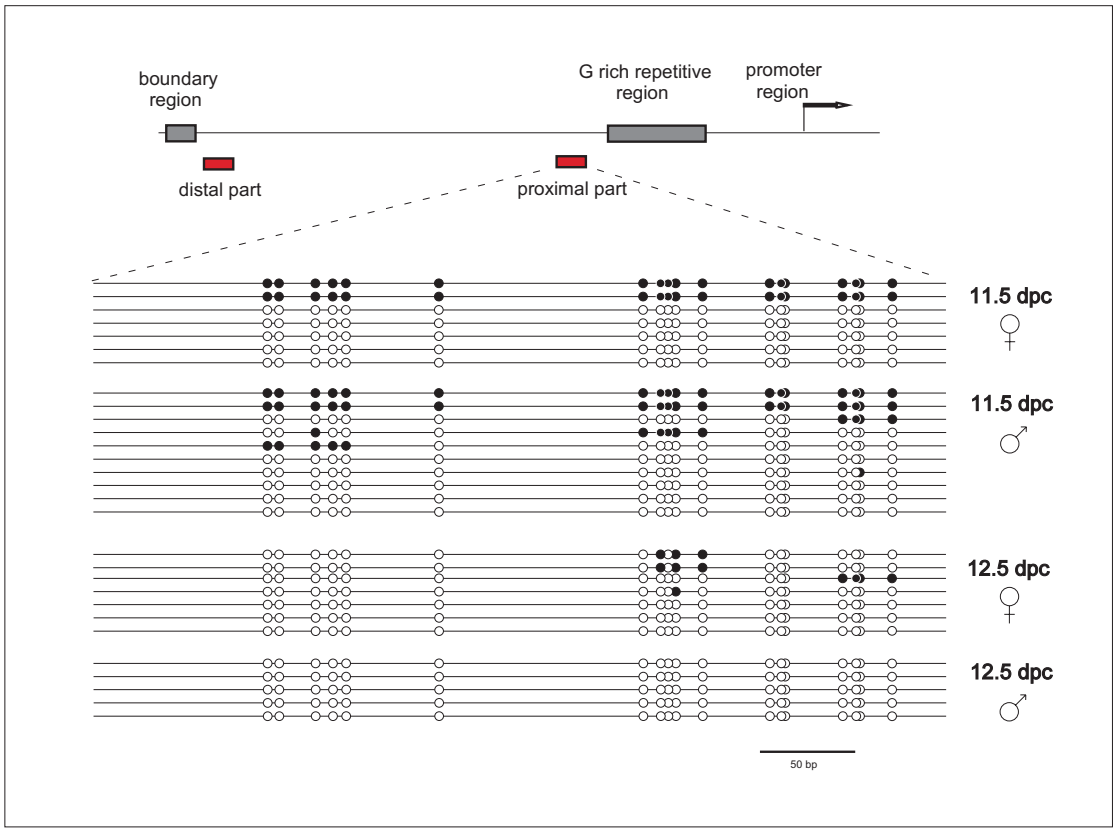
Analysis of methylation pattern of upstream *H19* DMR revealed pattern similar to that of *Igf2* DMR2. The bisulphite experiments were carried out several

times, nevertheless, there was no methylation imprint detectable in the samples of 11.5 dpc neither 12.5 dpc PGCs (the group of 11.5 dpc bisulphite clones showing the same single methylated site at 5'end indicate a possible problem of clonality in one of the bisulphite treatments). The obtained results are raising the following questions: a) the paternally methylated imprinted genes might behave differently (i.e. the imprint erasure of those genes occurs earlier ev. faster) b) the phenomena could be specifically connected to the *H19*/*Igf2* locus (the genes are located within the same chromosomal region 80 kb apart from each other) c) our bisulphite analysis might not target the real imprinting mark; the regions we investigated could represent the regions of secondary level in "imprinting hierarchy", which could eventually display less of the imprint maintenance.

Recently a new investigation on the methylation status of H19 DMR in primordial germ cells was published by (Ueda *et al.*, 2000). Using a methylation sensitive PCR the authors identified 2 CpG positions where the methylation seems to be present still (or already?) at 13.5 dpc (the earliest time point included into the analysis). According to the analysis the *HhaI* sites #5 and #7 show significant methylation, whereas the rest of the tested region seems to be non-methylated. The results were subsequently confirmed by bisulphite analysis on the region surrounding the *HhaI* site #7. In male 13.5 dpc germ cells the region shows about 30% methylation. This observation might suggest that the real "imprinting centre" lies in the vicinity of the described *HhaI* sites.

The region analysed by (Ueda *et al.*, 2000) does not, overlap with the region analysed in our study, but is located more downstream in direction to the H19 promoter (see Fig. 21).

Our analysis of this downstream region (445bp, 18 CpG sites) revealed the following:



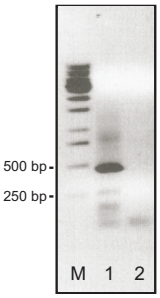


Fig. 21: Bisulphite analysis of the proximal part of *H19* upstream DMR.
The results of the bisulphite treatments are depicted above. Each line represents a unique bisulphite clone, the filled circles stand for the methylated CpG positions, open circles represent the non-methylated CpGs. The example of a result of the bisulphite PCR amplification is shown left. **M** - 1kb ladder (MBI Fermentas), **1** - PGC sample, **2**- water control for the bisulphite treatment.

For our analysis of epigenetic reprogramming it was important to know if there is any difference in the initial processes taking place in female and male PGCs. As it was discussed already before, all the 12.5 dpc samples used in our experiments were sex sorted. The determination of sex is more laborious in the case of 11.5 dpc genital ridges (for details concerning the sexing procedure see Material and Methods). As a consequence, the sexed samples were used only in limited number of control experiments. In the case of downstream part of *H19* DMR (as in the case of *peg3* gene) we carried out the bisulphite experiments on sexed 11.5 dpc samples. As

obvious from the result, also in this case we could not detect any significant difference between the 11.5 dpc primordial germ cells of female and male.

The bisulphite treatments of 11.5 dpc samples revealed fully methylated as well as completely non-methylated clones. The presence of methylated clones indicates that the methylation imprint is probably still maintained at this time point (the uneven distribution of methylated vs. non-methylated clones is possibly due to the low number of obtained clones or the bias in the bisulphite PCR). Similarly to *peg3* 11.5 dpc results, some of the clones show patchy pattern of methylation. As discussed above (see 3.1.7 Methylation changes in *peg3*) this finding supports the idea, that 11.5 dpc is the critical time point in the scenario of germ cell reprogramming.

To the contrary, the tested region showed no methylation in the 12.5 dpc samples, or only the remnants of it (see Fig. 21). This finding corresponds to the results of previous experiments: the imprints are maintained up to 11.5 dpc and diminish at 12.5 dpc.

The results of the downstream *H19* DMR analysis are interesting in the light of previous experiments performed on the upstream part of the DMR. Taken together with the observation of (Ueda *et al.*, 2000) the data indicate that the sequence in the vicinity of *HhaI* site #7 might function as an imprinting "core" element. Interestingly, the same part of the *H19* DMR is conserved between human, mouse and rat (Frevel *et al.*, 1999) and was shown to contain CTCF binding site (see Fig. 22) (Bell *et al.*, 2000**;** Hark *et al.*, 2000), which is involved in the regulation of whole *Igf2*/*H19* cluster (methylation of the site blocks the binding of CTCF thus allowing the enhancers to access the Igf2 promoter – see Fig. 22 and Fig. 23). Thus the region tested in our bisulphite experiments may serve as a primary imprint for the whole Igf2/H19 cluster (which might also explain the absence of methylation imprint in the upstream part of *H19* DMR and in *Igf2* DMR2 at 11.5 dpc)

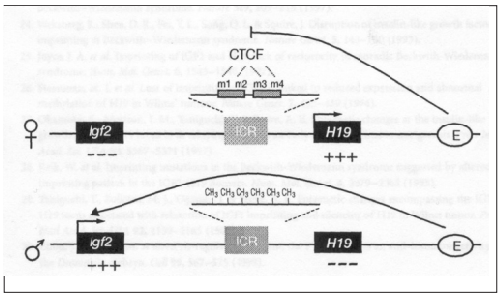


Fig. 22: Proposed model of CTCF function (taken from Bell *et al.* 2000). On the maternally inherited chromosome CTCF binds to unmethylated CTCF binding sites (two in each of the nuclease hypersensitive regions - shaded boxes). The resulting insulator prevents activation of the maternal *Igf2* allele by the *H19* enhancer. On the paternally inherited chromosome the CTCF sites are methylated, thus preventing CTCF binding. Absence of insulator activity enables activation of *Igf2* by the *H19* enhancer. (+/- corresponds to the transcriptional activity).

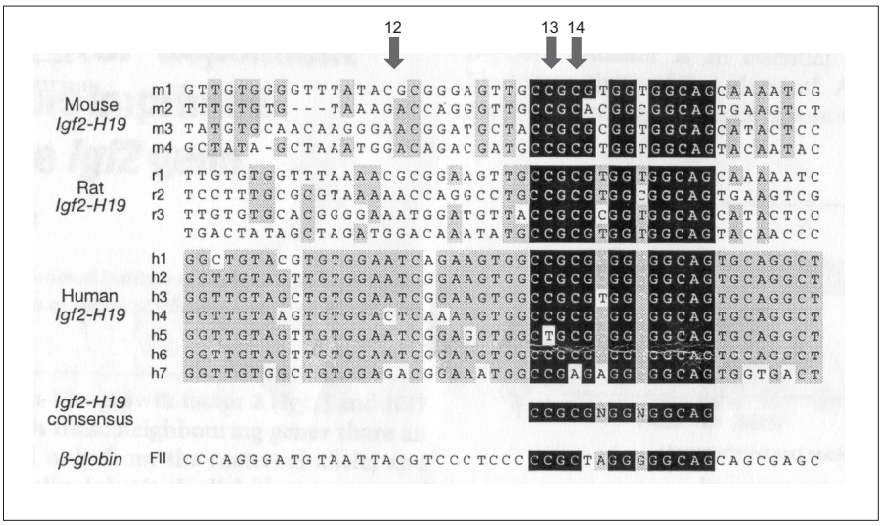


Fig. 23: Conserved CTCF sites within the *H19* DMR (taken from Bell *et al.*,2000). Comparison of the sequence of the mouse, rat and human *H19* DMR regions shows the conservation of the CTCF binding site (for comparison see the b-globin CTCF binding site). Species-specific identities are shown in grey, cross-species sequence conservation is depicted in black. The sequence of the mouse strand used in our experiments corresponds to m4. The arrows indicate the CpGs included into our bisulphite analysis (the number corresponds to the position of CpG in the bisulphite dot diagram). Note that the methylation detected in 12.5dpc samples was located explicitly outside the labeled CpG sites.

3.3.5 Methylation changes in *Snrpn* gene

Whereas the previous chapters concerned the reprogramming of imprinted genes carrying either a maternal (*lit1*, *peg3*) or a paternal (*Igf2, H19*) methylation

mark, the following paragraphs deal with a unique example of a gene carrying both parental imprints.

Snrpn (**s**mall **n**uclear **r**ibonucleoprotein **p**olypeptide **N**, mouse central chromosome 7, GenBank acc.# AF063659) is an imprinted gene situated in the centre of the chromosomal domain involved in two neurogenetic disorders, Prader-Willi syndrome (PWS) of Angelman syndrome (AS), respectively. The gene is paternally expressed (Leff *et al.*, 1992; Glenn *et al.*, 1996) primarily in brain and heart (Gerrelli *et al.*, 1991), coding for a protein (Smn) that is thought to be involved in splicing (Steitz *et al.*, 1988).

The Snrpn gene was included into our analysis because of its unique imprinting features: the promoter and the 1st exon were shown to be maternally methylated, whereas the 3'end of the gene is paternally modified (Shemer *et al.*, 1997). Thus analysing the reprogramming process in both regions, we were able to perform a direct comparison of behaviour of a maternal and a paternal imprint within the same chromosomal locus.

3.3.5.1 Snrpn *DMR1*

First we analysed the maternal imprint in the 5'part of the *Snrpn* gene:

Maternal methylation at the 5'end of the gene (DMR1) spans from the promoter region over the 1st exon to the 1st intron (Shemer *et al.*, 1997; El-Maarri *et al.*, 2001) and was shown to correlate inversely with the *Snrpn* expression (the gene is expressed from the paternal non-methylated allele). The imprint is present in gametes: the region being completely methylated in oocytes, whereas non-methylated in sperm; as well as in embryonic and adult tissues (Shemer *et al.*, 1997; El-Maarri *et al.*, 2001).

The following results were obtained when analysing the region described in (El-Maarri *et al.*, 2001); the amplified sequence lies within the *Snrpn* exon 1 region, is 289bp long and contains 14 CpG positions (Fig. 24).

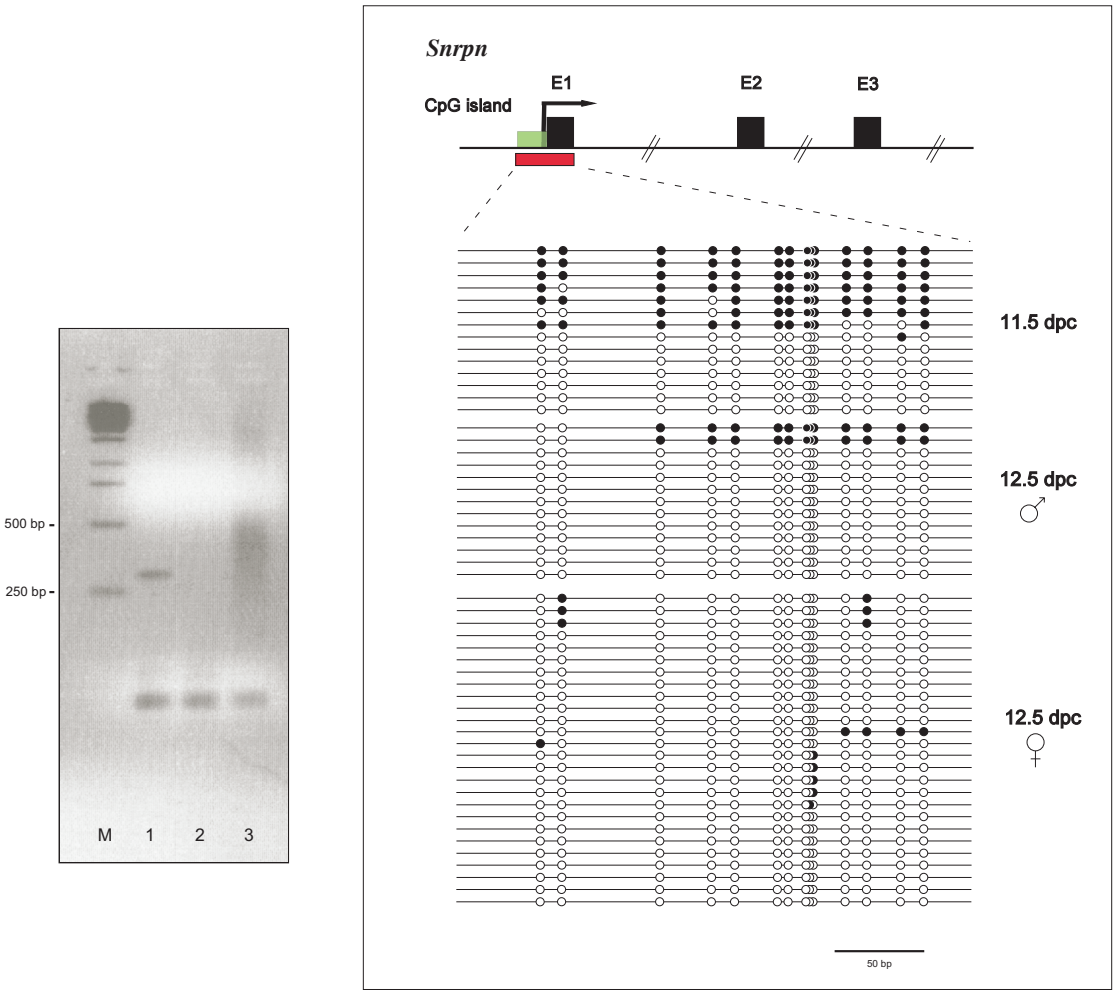


Fig. 24: Bisulphite analysis of *Snrpn* DMR1.

The results of the bisulphite treatment are shown right Individual line represent unique bisulphite clones, the filled and open circles stand for the methylated and non-methylated CpG positions, respectively. The result of one of the bisulphite PCRs is shown in the upper right part of the figure. **M** - 1kb ladder (MBI Fermentas), **1** - PGC sample, **2** - water control for the bisulphite treatment, **3** - water control for the bisulphite PCR.

The results of the *Snrpn* DMR1 bisulphite analysis document the presence of methylated clones in 11.5 dpc primordial germ cells. The distribution of methylated vs. non-methylated clones (approximately 1:1) corresponds to the pattern observed in zygote and somatic cells (El-Maarri, unpublished results) and is characteristic for a DMR of an imprinted gene. As obvious from the experiments, between 11.5 and 12.5 dpc the *Snrpn* DMR1 methylation pattern changes from the fully established imprint to a completely erased status (as observed with other genes in previous experiments). The continuous methylation was detected only in one of the 12.5 dpc female clones and in two of the male 12.5 dpc clones (see Fig. 24).

Similarly to the phenomena observed in *peg3* and in the downstream part of the *H19* DMR, there is a tendency to detect a patchy methylation rather than a random one. Such an observation could be explained by a processive action of the demethylation machinery. The sporadic occurrence of patchy methylated clones in 12.5 dpc samples (as well as the presence of such clones in 11.5 dpc samples when analysing other genes) brings about not only the hint of the character of possible demodification mechanism; it also defines a time window of its action.

3.3.5.2 Snrpn *DMR2*

The second region analysed within the *Snrpn* gene is a preferentially paternally methylated DMR2 situated at the 3'end of the gene (see Fig. 25). The region has been identified by restriction mapping of the P1 clone containing the complete mouse *Snrpn* gene (Shemer *et al.*, 1997). The complete genomic sequence of the gene has, however, not been published yet. In order to perform the bisulphite analysis in the DMR2 region, the clones had to be fished out from the database of the Sanger Centre Mouse Sequencing Project and of the Celera Corp. based on the homology with the Snrpn cDNA. Those clones were subsequently aligned into a contig with the help of the Lasergene - MegAlign software. Using this approach it was possible to reconstitute the part of the genomic *Snrpn* sequence spanning from exon 5 to exon 10. The following sequence analysis revealed that this part of the *Snrpn* gene is very poor in respect to CpG dinucleotides. The only fragment with slightly higher density of CpG sites (5 CpG positions within approximately 400bp) is localised within the intron 8 as a part of a *Line1* repetitive element. Being the only suitable candidate, this part of the genomic sequence was chosen as a target for our bisulphite analysis.

The amplified fragment (578bp) contains 5 CpG positions (Fig. 26).

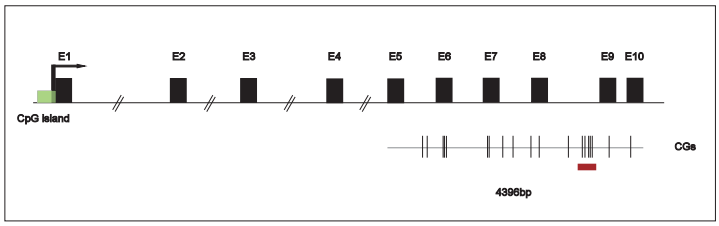
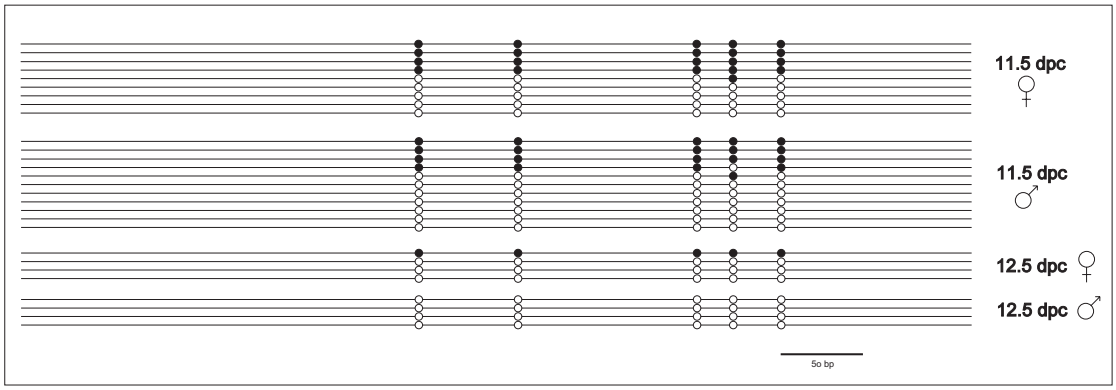


Fig.25: Genomic organisation of mouse *Snrpn* gene.
The barrows represent the *Snrpn* exons 1-10. CpG positions are shown as vertical lines, the region analysed by bisulphite sequencing is depicted in red.



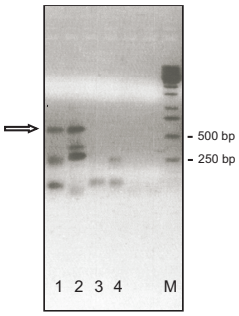


Fig. 26: Bisulphite analysis of *Snrpn* DMR2.
The results of the bisulphite treatments are depicted in the upper part of the figure. Each line represents a unique bisulphite clone, methylated CpG positions are represented by filled circles; non-methylated CpG by open circles. The example of the bisulphite PCR amplification is shown left: **M** - 1kb ladder (MBI Fermentas), **1,2** - PGC samples, **3** - water control for the bisulphite treatment, **4** - water control for the bisulphite PCR. The unspecific PCR products frequently appeared despite extensive optimization of the PCR conditions. The primers for the bisulphite PCR were designed outside the Line 1 repetitive element; nevertheless, the fact that the target for the amplification was a repetitive element might explain the observed low specificity of amplification.

Bisulphite analysis performed on 11.5 dpc samples documents that also the *Snrpn* DMR2 is still differentially methylated at this stage of PGC development. The samples of both genders revealed the presence of methylated and non-methylated clones in approx. ratio 1:1, which characterises the imprinted pattern. It should be pointed out, that the *Snrpn* DMR2 was the 3rd control region, where the 11.5 dpc primordial germ cells of female and male were analysed separately (for the other examples: peg3 and the downstream part of *H19* DMR see the previous chapters; for the details concerning the sexing procedure see Material and Methods). As even in this case we could not detect any difference between the 11.5 dpc germ cells of either

sex, we accepted as proven that at the initial stage of PGCs reprogramming the cells of both genders follow the identical scenario.

Due to technical problems connected with the *Snrpn* DMR2 amplification and cloning (caused possibly by the fact that the tested region was a part of a repetitive element) the number of obtained bisulphite clones was very low. Despite number of trials we were not able to obtain any specific PCR fragment from the male 12.5 dpc sample; in the case of female 12.5 dpc cells the analysis yielded only 4 specific clones.

At the same time when the experiments described in this thesis were carried out, the parallel investigation of the reprogramming of repetitive elements in the same PGC samples was being performed in the co-operation with the group of Dr. W. Reik in the Babraham Institute, Babraham, UK. The methylation analysis of *Line1* and *IAP* repetitive elements showed that those sequences undergo gradual loss of modification rather than a fast demethylation event (N.Lane – unpublished observation, Hajkova *et.al* - submitted). In the light of those findings the presence of a fully methylated clone in female 12.5 dpc cells may indicate, that the tested DMR as being a part of a *Line1* repeat follows the scenario of repetitive elements, i.e. the gradual loss of methylation.

The imprinted status of the *Snrpn* DMR2 has been up to now elucidated solely on the basis of the methylation-sensitive restriction analysis of 4 *HhaI* sites (Shemer *et al.*, 1997). Thus, our experiments represent the first detailed bisulphite based methylation study of the region. The data confirm the existence of a differential methylation within the region, which was in the samples of PGCs more pronounced than in the original report – Shemer *et al.*, 1997 described the presence of partial methylation on a maternal allele and complete methylation on a paternal allele.

3.4 Methylation status of non-imprinted single copy genes

In the previously described experiments we elucidated the methylation changes occurring within the differentially methylated regions of imprinted genes. Though approximately 40 examples have been identified up to date, the imprinted genes still represent a very specialised part of the genome. In order to gain more complex understanding about the PGC reprogramming, the examples of non-imprinted single copy genes had to be included into our analysis.

3.4.1 Methylation changes in *α-actin* gene

The skeletal α-actin (GenBank acc.# M12347) is an example of a single copy gene characterised by tissue specific expression. The gene is not transcribed in the early undifferentiated embryo, expression corresponds with the appearance of differentiated muscle tissue following implantation (Sassoon *et al.*, 1988; Taylor *et al.*, 1990).

To assess the methylation changes occurring within this gene we analysed the 5` region covering the 3`part of the promoter and proximal part of the 1st exon. Our choice was based on the following facts: a) 5'region of the gene contains a CpG island, which makes it a suitable candidate for a bisulphite analysis b) this region has been used in the previously published studies describing the methylation changes occurring during the embryogenesis Warnecke *et al.*, 1999;Oswald *et al.*, 2000), thus we were provided with satisfactory amount of comparative data.

The analysed region is 266bp long and comprises 11 CpG dinucleotides (Fig. 27).

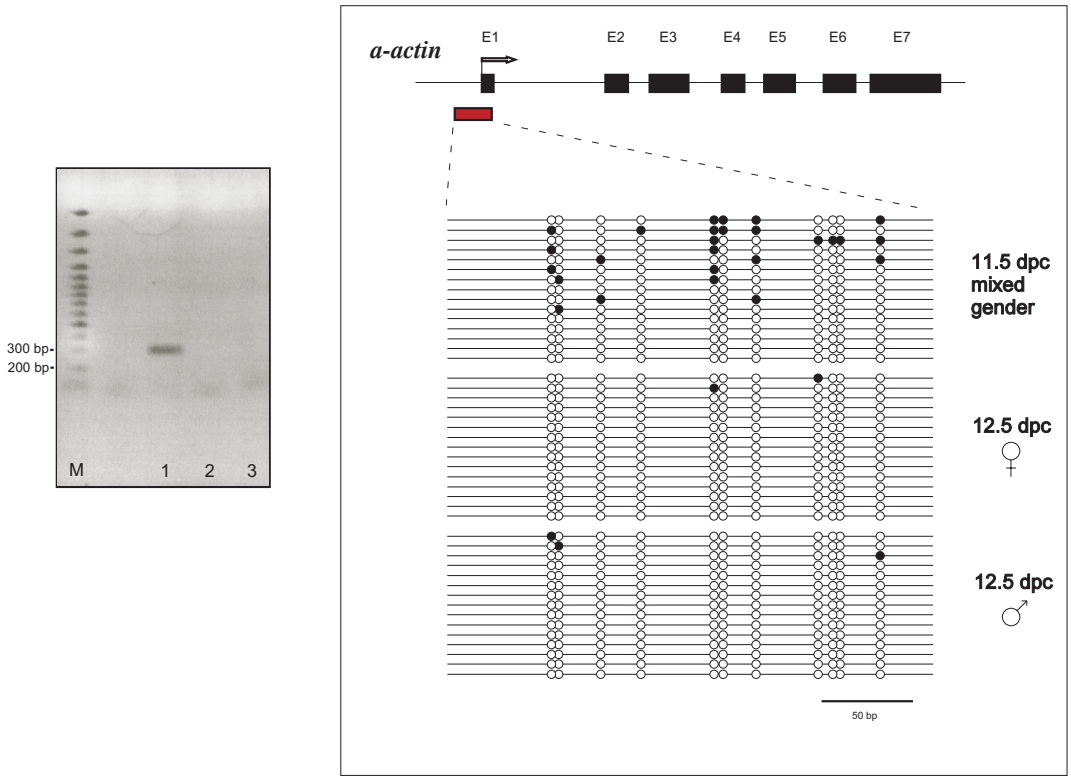


Fig. 27: Bisulphite analysis of *a-actin*.

The right part of the figure shows genomic organisation of *a-actin* gene (the position of the region chosen for the bisulphite analysis is shown in red) and the results of bisulphite treatments Individual lines represent unique bisulphite clones. Methylated and non-methylated CpG positions are represented by filled and open circles, respectively. The example result of the bisulphite PCR is shown left **M** - 100 bp ladder (MBI Fermentas), **1** - PGC sample, **2** - water control for the bisulphite treatment, **3** - water control for the bisulphite PCR.

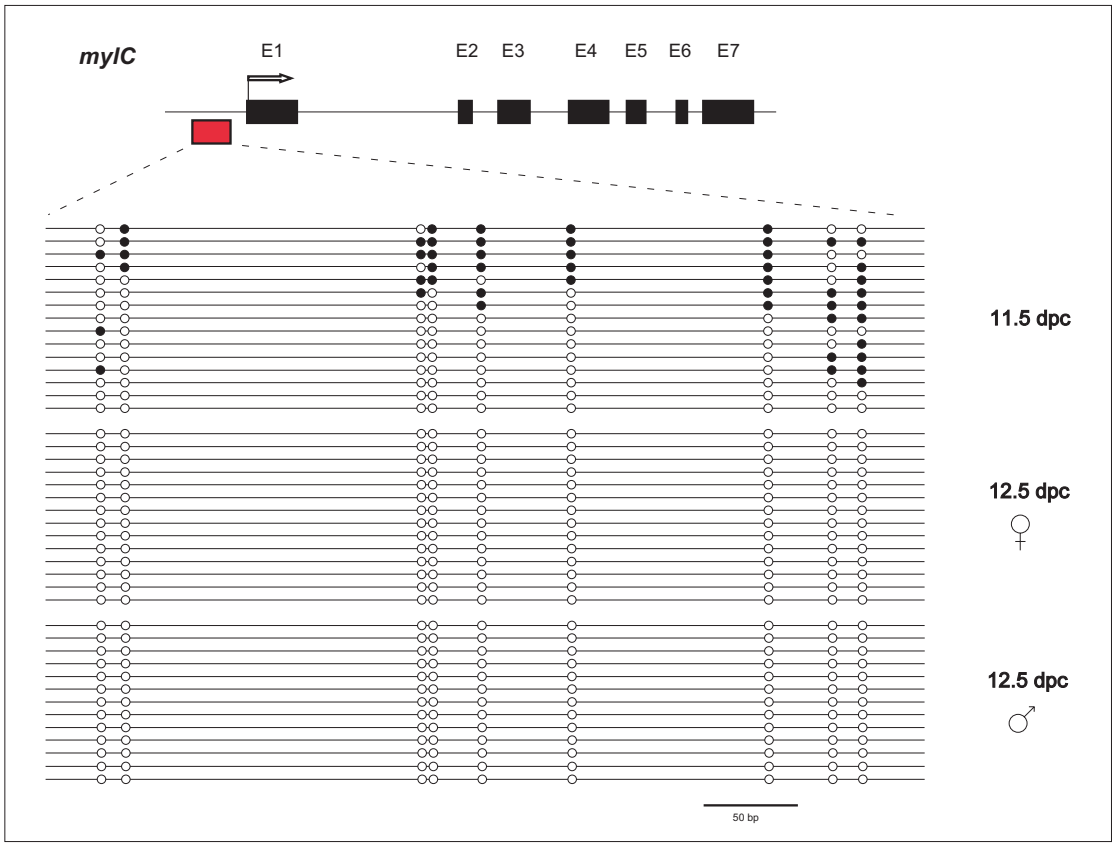
As obvious from the dot diagrams the 5`region of α-actin is still methylated at 11.5 dpc (mixed gender samples). The level of methylation at distinct CpG sites varies between 7.1 and 42.9%, which corresponds to the level found in adult tissues (0-30% in heart and skeletal muscle) expressing the gene (Warnecke *et al.*, 1999). Similarly to the behaviour of imprinted genes, the region appears to be completely non-methylated in both female and male 12.5 dpc PGCs (the results based on two independent bisulphite treatments and several amplifications).

3.4.2 Methylation changes in *mylC* gene

As the second candidate non-imprinted gene we chose the alkaline **my**osin **l**ight **c**hain (GenBank acc.# X12972). Similarly to the α–actin the MylC belongs

among the tissue specific genes, expression limited to ventricular myocardium and slow skeletal muscle (Barton *et al.*, 1985). Methylation features of the gene have been extensively studied (Walsh *et al.*, 1999, Oswald, 2000 #2) – the 5`region being found heavily methylated in oocytes and sperm and partially methylated in somatic tissues.

To follow the methylation changes we analysed 9 CpG sites positioned within the promoter region (Fig. 28).



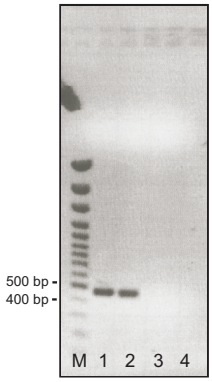


Fig. 28: Bisulphite analysis of *mylC*.
The results of the bisulphite treatments are shown in the upper part of the figure.including the schematic drawing of the *mylC* genomic orgainisation. The region analysed by the bisulphite genomic sequencing is depicted as a red box. Each line represents a unique bisulphite clone. Methylated and non-methylated CpG positions are represented by filled and open circles, respectively. The figure left shows the result of the bisulphite PCR. **M** - 100bp ladder (MBI Fermentas), **1,2** - PGC sample, **3** - water control for the bisulphite treatment, **4** - water control for the bisulphite PCR.

The methylation changes of the *mylC* promoter region follow the scenario observed with the other genes. The 11.5 dpc samples show at the tested CpG sites methylation between 20 and 66.7 %, which is in the range found in somatic tissues (Walsh *et al.*, 1999). After 11.5 dpc the methylation level decreases dramatically (similar to behaviour of other tested genes); there was no methylated site detected in the 12.5 dpc primordial germ cells of either sex.

The control experiments carried out on the non-imprinted genes confirmed the previous results obtained on imprinted genes. The promoter regions of *α-actin* and *mylC* carry at 11.5 dpc the methylation comparable to that detected in somatic tissues. In the 12.5 dpc PGC samples, both regions, however, appeared to be completely non-methylated. These findings are in full agreement with the observation made on the imprinted genes - The majority of the tested regions (the only exceptions were *Igf2* DMR2 and upstream part of the *H19* DMR) appeared to be methylated in 11.5 dpc primordial germ cells. Whereas all the tested regions were found completely non-methylated in 12.5 dpc PGCs of both genders.

These findings thus implicate a presence of a phenomenon, which is not related solely to the imprinted genes, but concerns the whole genome of the primordial germ cells. This reprogramming starts in the PGCs of both genders around 11.5 dpc, the main erasure step being finished at 12.5 dpc i.e. within only 24 hours. Our data also exclude the hypothesis of a gradual change from the maternal to the paternal (or vice versa) imprinting pattern. The reprogramming obviously happens in two distinct steps – the initial imprint erasure and the following imprint re-establishment, where only the latter is sex specific.

3.5 Methylation status of the 13.5 dpc primordial germ cells

After their entry into the genital ridges around 10.5 dpc, the primordial germ cells keep on slowing down the life cycle until 13.5 dpc, when the female PGCs enter the meiotic and the male PGCs the mitotic arrest, respectively (Tam *et al.*, 1981; McLaren, 2000). The results presented in previous chapters of this thesis describe in detail the methylation changes taking place in PGCs of 11.5 dpc and 12.5 dpc embryos, i.e. at stages, when the PGCs still undergo proliferation. However, to gain a complete overview of the process of methylation erasure it was necessary to follow the PGC development beyond the critical time up to the point of the sex-specific differentiation.

The 13.5 dpc PGCs were isolated from the *Oct4-GFP* transgenic embryos (see Material and Methods). The FACS sorting of the cell suspension prepared from the GFP positive 13.5 dpc genital ridges revealed surprisingly high variability in the size of positive cells (see Fig. 29a and 29b). As mentioned above, the primordial germ cells are supposed to undergo meiotic/mitotic arrest at 13.5 dpc. The germ cells of the genital ridges are, however, not synchronised, so at 13.5 dpc the "arrested" germ cells represent still only a fraction of the cell population. As we believed that those cells might be characterised by a different cell size, we decided to separate the GFP positive cells into two distinct fractions: the fraction of "large" cells and the fraction of "small" cells. Those fractions were treated in the subsequent experiments separately.

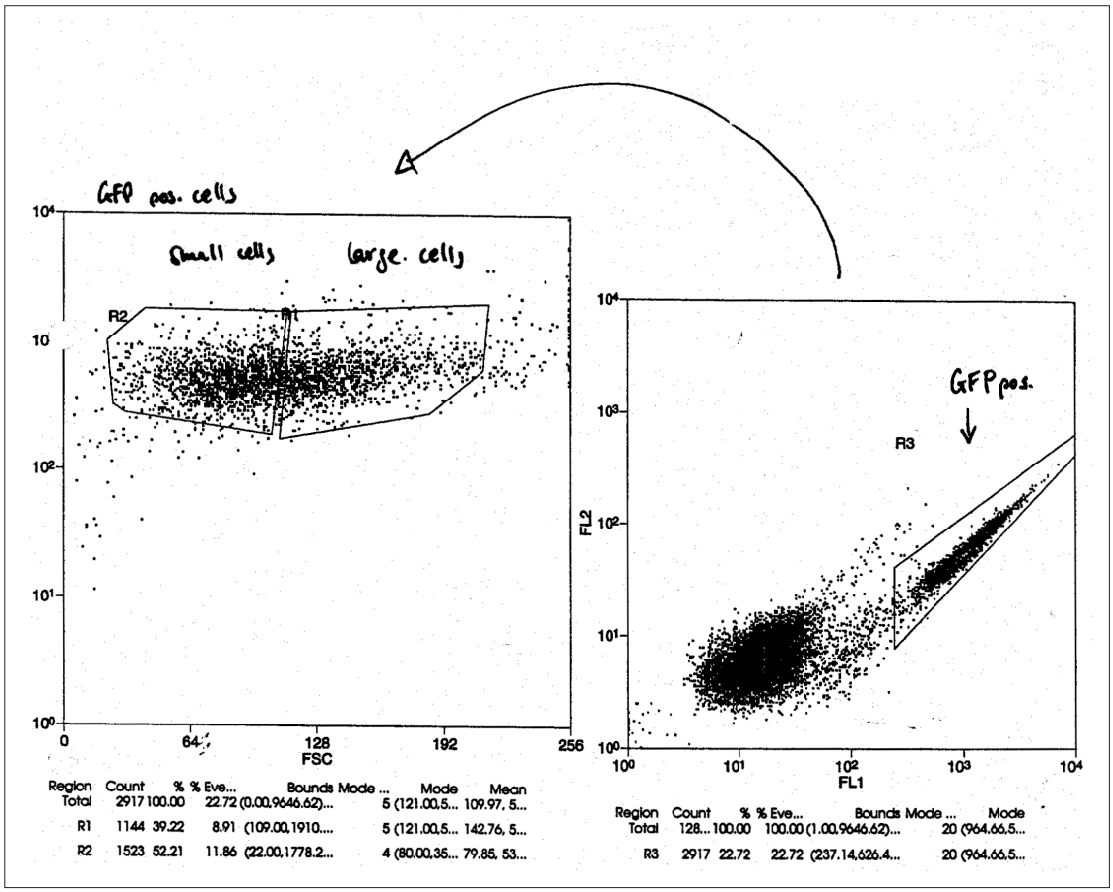


Fig. 29a: FACS sorting of the 13.5 dpc primordial germ cells (female samples).

The isolated genital ridges of 13.5 dp embryos were trypsinised to yield single cell suspension. The cells were subsequently used for FACS sorting- first based on the GFP positive signal (right). Note that the variability in GFP expression is rather low. The positive cells, however, appear to vary in their size (see left part of the figure). To examine the possible differences (for more see the text) the cell size was chosen as the second sorting parameter.

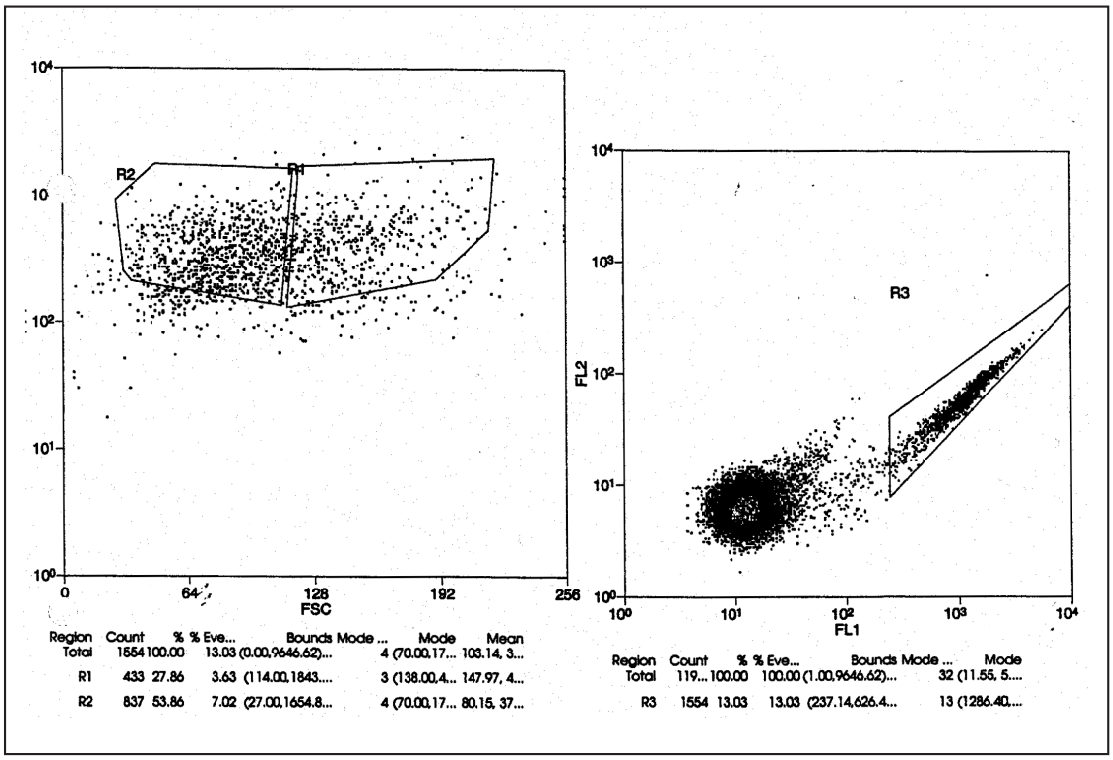
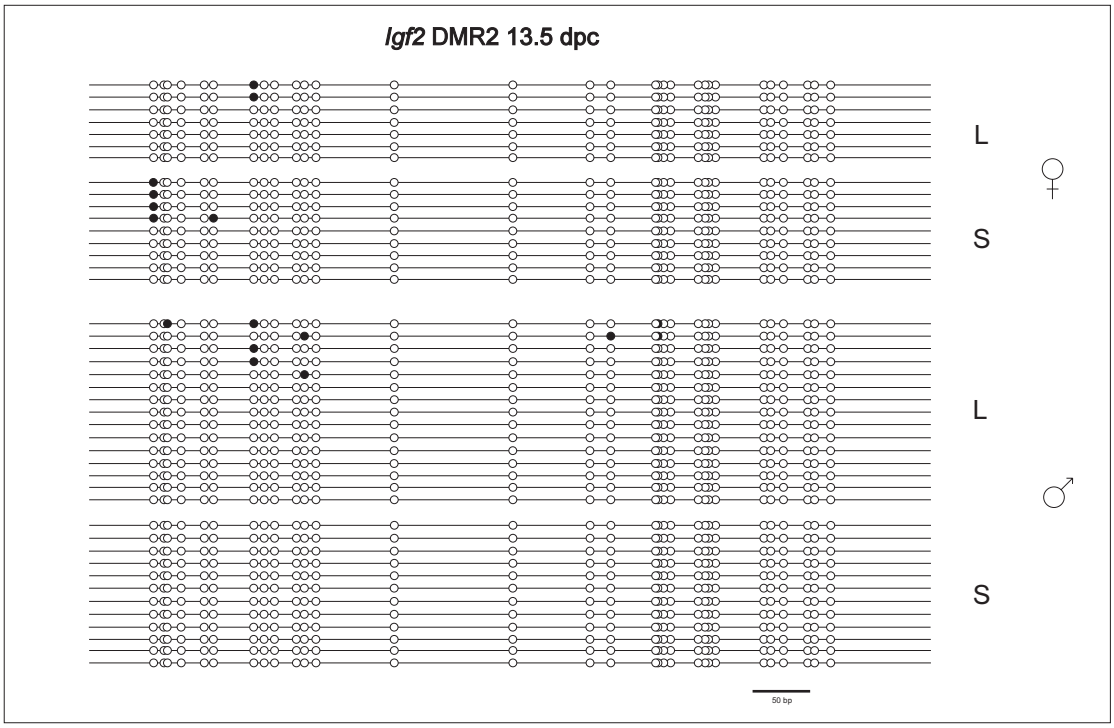


Fig. 29b: FACS sorting of the 13.5dpc primordial germ cells (male samples).
Single cell suspension of 13.5dpc embnryonic genital ridges was FACS sorted according to the GFP signal and cell size (for details see the previous figure). The results of the FACS sorting procedure did not reveal any significant differences between male and female 13.5dpc primordial germ cells.

The methylation analysis of 13.5 dpc germ cells brought the following results:



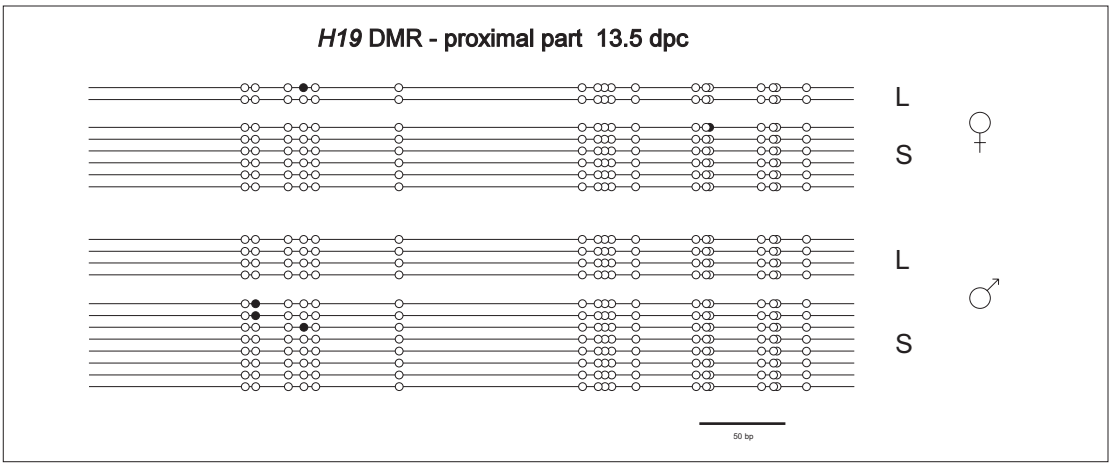


Fig. 30a: Bisulphite analysis of the 13.5 dpc primordial germ cells.
The figure shows the results of bisulphite analysis of the *Igf2* DMR2 and the proximal part of the upstream *H19* DMR. In both tested regions no methylation is detectable neither in female nor in male PGCs. As there was no significant difference between the fractions of large (**L**) and small (**S**) cells the dot diagrams for the other genes are shown as a summary (see fig. Xb).

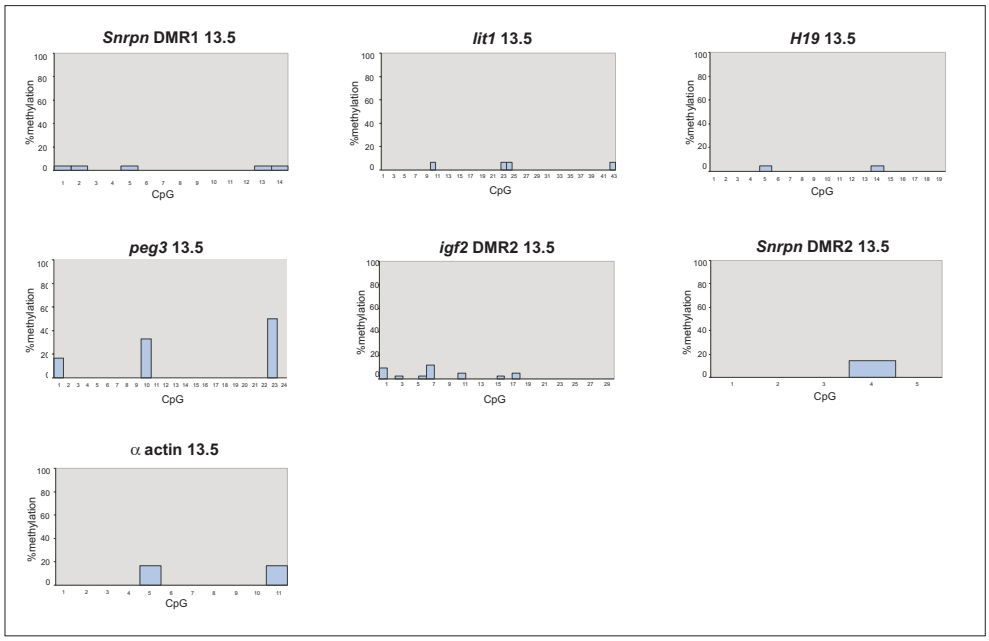


Fig. 30b: Bisulphite analysis of the 13.5 dpc primordial germ cells.
The figure shows the overview of the results of the bisulphite treatments. The graphs combine the results of 13.5 dpc germ cells of both genders and both isolated fractions of large and small cells. Higher methylation levels shown in the case of *peg3* and *a-actin* are likely to be caused by very low numbers of unique bisulphite clones.

As shown by the results of bisulphite analysis, all the tested DMR regions were found non-methylated in 13.5 dpc primordial germ cells. These results are in agreement with the observation published by Kafri *et al.*, 1992 and Brandeis *et al.*, 1993. In their report Kafri and colleagues used the methylation sensitive PCR to describe the demethylated status of non-imprinted *ApoAI* and *globin* genes in 13.5 dpc germ cells. The same method was used in the study of Brandeis *et al*. to assess the methylation status of the imprinted genes (*Igf2*, *Igf2r* and *H19* included into the analysis). The data of both indicate the complete absence of methylation in the tested regions in 13.5 dpc germ cells.

Recently, two independent investigations of the methylation in the H19 upstream region have been published (Ueda *et al.*, 2000; Davis *et al.*, 1999). Although both authors described the presence of low level of methylation in the 3' part of the H19 DMR, we were not able to confirm the observation. Different results might have been caused by cross-contamination of PGC samples with somatic cells (interestingly, in Davis *et al*. the male 13.5 dpc samples showing higher level of methylation were of

lower purity - 93 and 89% versus 96% for female samples), or possibly by using the different mouse strain.

It should be noted that we did not detect any difference between the fractions of "large" and "small" cells neither between the cells of different gender. Taken together, the data implicate that the primordial germ cells after the initial step of imprint erasure do not change their methylation status upon their entry into the mitotic/meiotic arrest.

3.6 Methylation analysis of 10.5 dpc primordial germ cells

Having analysed the methylation status of primordial germ cells between 11.5 and 13.5 dpc, our interest turned towards the earlier stages of PGC development. Our previous analysis showed that the vast majority of the DMRs carries the methylation imprint still at 11.5 dpc. The DMR2 of Igf2 gene appeared to be one of the exceptions being apparently non-methylated at that stage. Based on the bisulphite analysis performed on the 8.5 dpc embryonic cells (for details see 3.1.3 Methylation changes in *Igf2*) it is probable that the DMR2 methylation imprint is present at the earlier stages of PGC development and is subsequently erased earlier than the methylation marks of other DMRs. Such an option opens, though, a general question concerning the existence and extent of imprints in migrating and early post-migratory primordial germ cells.

The hints to the answer might be found in the earlier report of (Tam *et al.*, 1994). The authors are describing in detail the process and timing of X chromosome inactivation (and re-activation) taking place in early migratory and post-migratory PGCs. According to their investigation the X inactivation occurs in most of the PGCs during the time of migration through mesentery (i.e. before entering the genital ridge). It is very likely that the parental imprints inherited from the germ line also become properly established at this time, if not earlier. However, up to now, no data supporting this hypothesis has been published.

We took the advantage of our model system (the transgenic mouse with the germ line specific *GFP* expression) and isolated the early post-migratory germ cells from 10.5 dpc old mouse embryos to investigate their imprinting status.

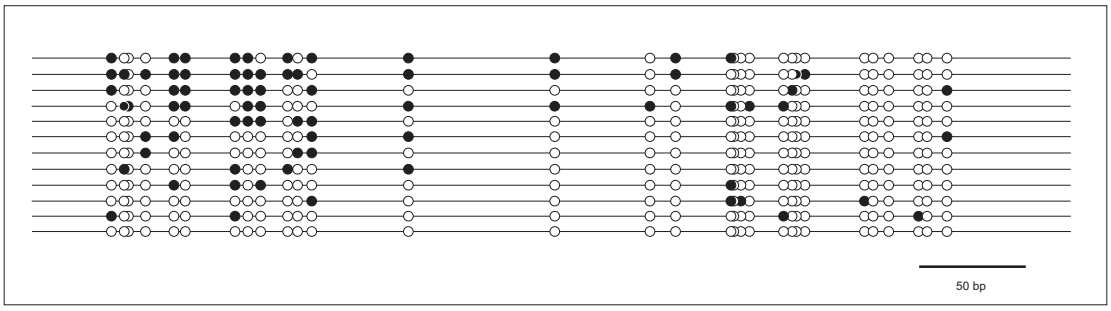


Fig. 31: Bisulhite analysis of *Igf2* DMR2 in 10.5 dpc primordial germ cells.
The methylated CpG positions are represented by filled circles, the non-methylated CpGs by open circles.

The bisulphite analysis of early post-migratory germ cells (at 10.5 dpc the PGCs have just reached the genital ridge) confirmed the presence of methylation in the *Igf2* DMR2 region (Fig.31). As we were not able to distinguish paternal alleles in our system and the methylation in this region shows never a “black-white” pattern; it is difficult to make any statements concerning the extent of imprint at this stage. However, the more pronounced methylation in the 5’ region of DMR2 corresponds to the pattern seen in early embryos (8.5 dpc).

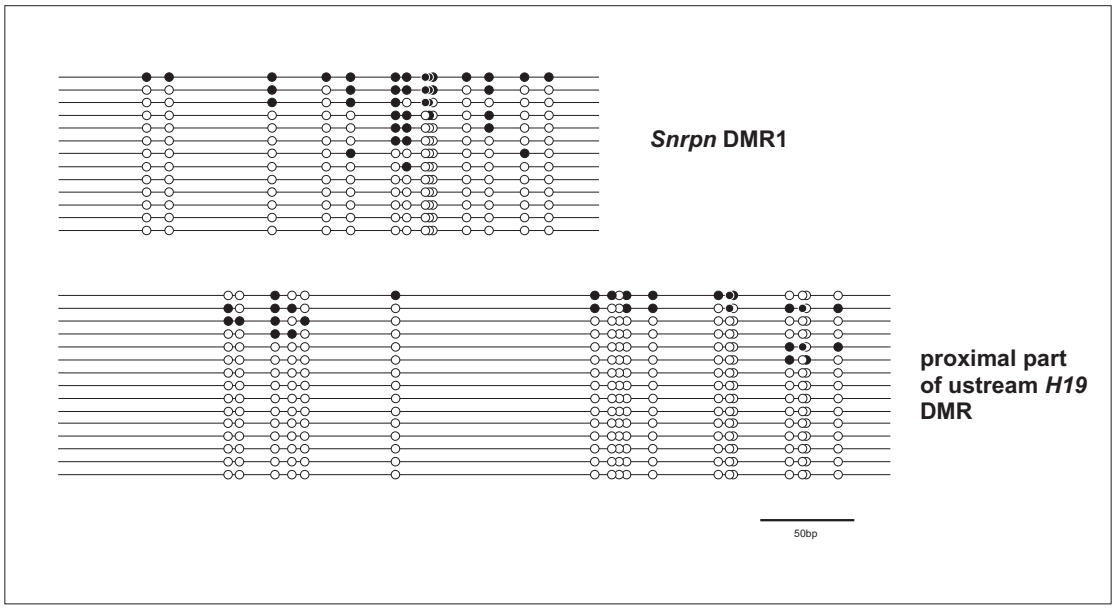


Fig.32: Bisulphite analysis of 10.5dpc primordial germ cells.
Methylated CpG are shown as filled circles, non-methylated CpGs as open circles. Each line represents a unique bisulphite clone.

Due to the very limited amount of 10.5 dpc samples it was not possible to perform the analysis on all the previously described DMRs. The next two tested regions, *Snrpn* and the downstream part of *H19* DMR, both confirmed the presence of methylation in DMRs of imprinted genes at this early stage of PGC development (Fig. 32). However, the pattern observed identically in both regions at 10.5 dpc is not identical with the fully established imprinting pattern seen in PGCs only one day later. The methylation analysis of 11.5 dpc primordial germ cells revealed the presence of completely methylated and completely non-methylated clones ("black-white" pattern) whereas there were only completely non-methylated and partially methylated molecules, present in 10.5 dpc samples. Thus, although some methylation imprint is present in primordial germ cells at the time when they reach the genital ridge, the imprint is obviously not complete (or not so pronounced as observed in somatic cells in later stages of embryogenesis). This fact might, though, be not germ line specific, as the somatic type of imprints is probably only being established at this stage of embryogenesis.

3.7 Methylation status of the somatic cells forming the stroma of the genital ridge

The previously described experiments analysed in detail the reprogramming of primordial germ cells after their colonisation of the genital ridge. The results showed that the PGCs do posses imprints at the time when they reach the genital ridge, the imprints are maintained up to 11.5 dpc and get subsequently erased within only one day before 12.5 dpc. This undermethylated status is kept up to 13.5 dpc. It is believed that the whole reprogramming process is strictly germ line specific to ensure the proper erasure of parent-of-origin specific imprints and possible epimutations and to enable the setting of new, sex specific, modifications. To pronounce the unique character of the germ lineage in comparison with the surrounding cells we decided to assay the cells of the genital ridge stroma for their imprinting/methylation status (Fig. 33).

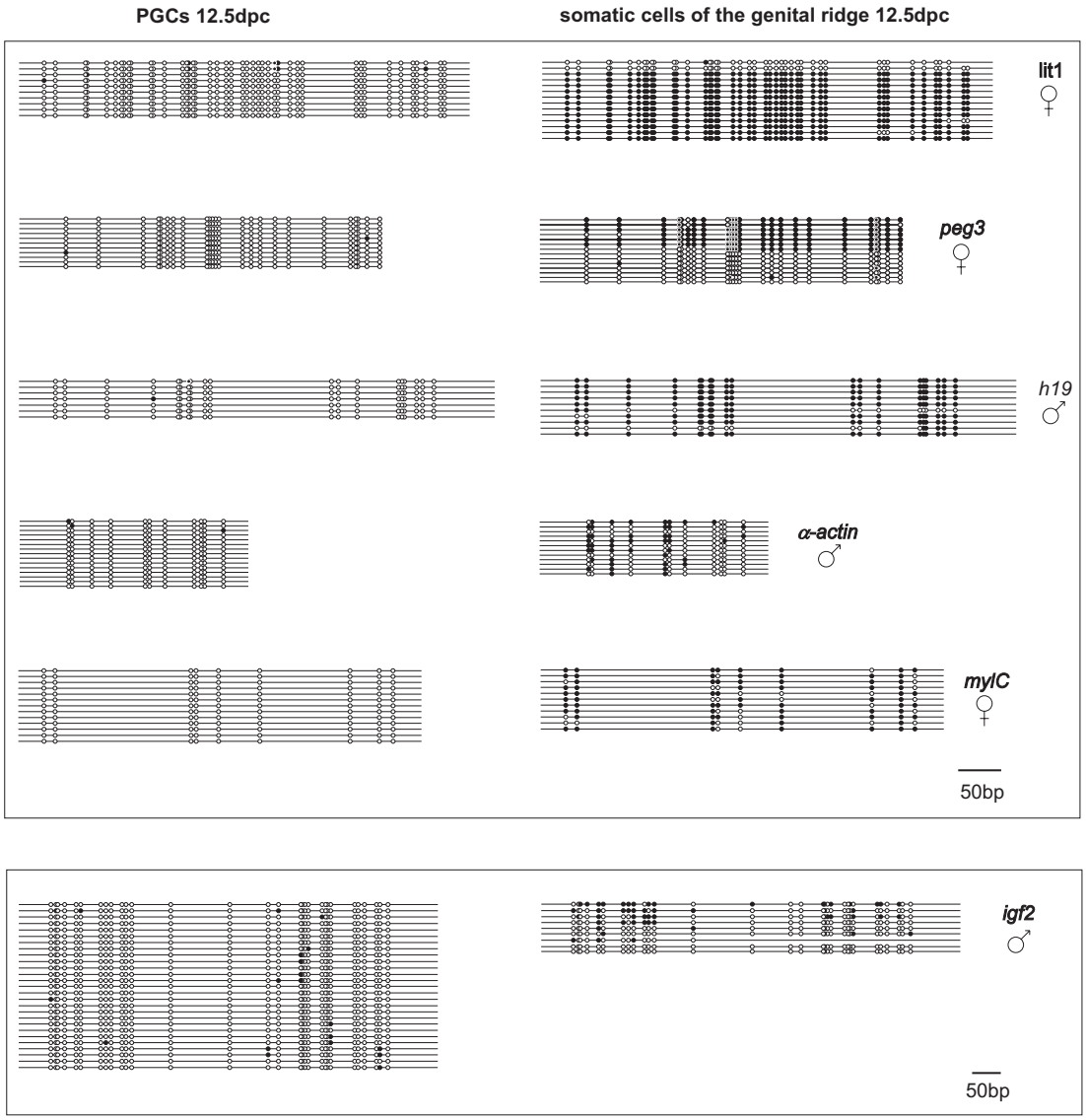


Fig.33: Methylation pattern of 12.5dpc somatic and primordial germ cells - comparison.
The figure shows the results of bisulphite treatments carried on the somatic cells of the genital ridges compared to the results obtained on the primordial germ cells. The somatic cells were obtained as a flow-through fraction from the immunoaffinity purification procedure of PGCs (for details see Materials and Methods).

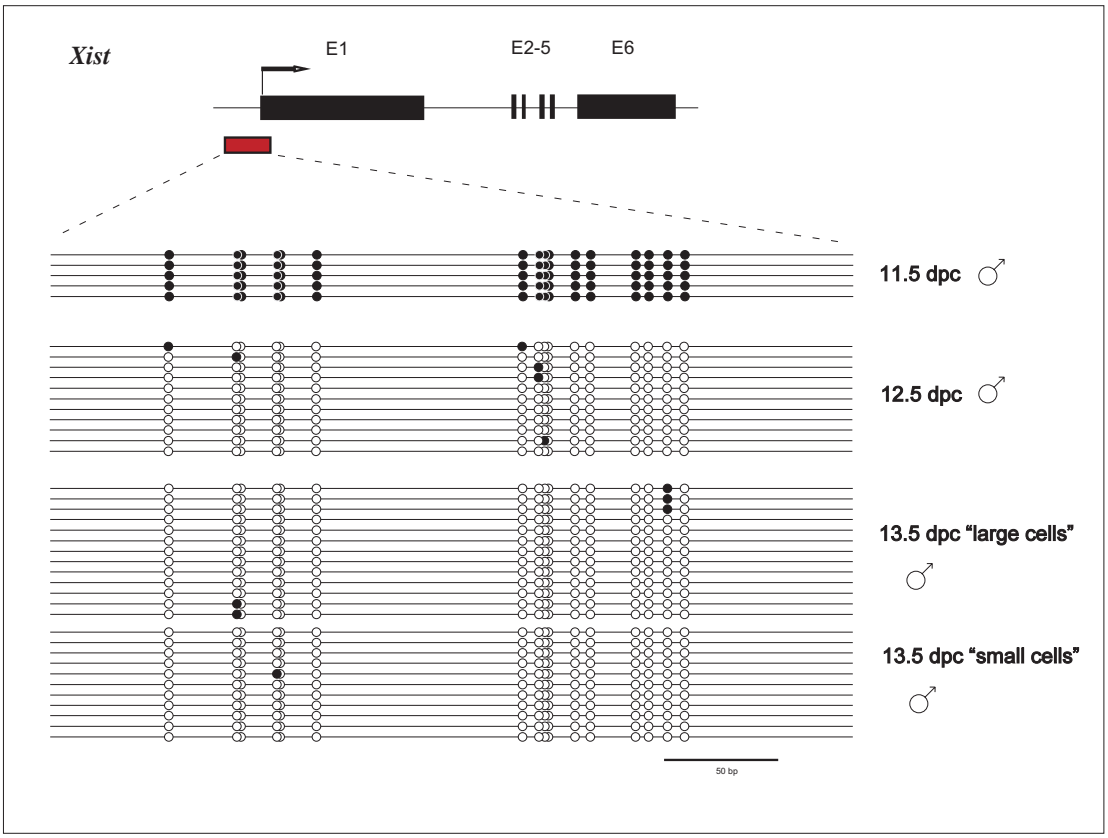
Despite the dramatic changes taking place in the primordial germ cells, the surrounding somatic cells maintain their somatic methylation status. In the tested 12.5 dpc somatic cells we detected the presence of imprints as well as methylation in the promoters of tissue specific non-imprinted genes. The unequal distribution of methylated vs. non-methylated clones in the case of *lit1* and *H19* could be explained by the fact, that those results are based on a single bisulphite treatment. More bisulphite analyses would be necessary to elucidate whether this uneven distribution is real.

3.8 Methylation changes in the promoter of the *Xist* gene, correlation to the X chromosome reactivation

The mouse *Xist* gene is expressed exclusively from the inactive X chromosome and is apparently involved in the initiation of X inactivation (reviewed in Mlynarczyk *et al.*, 2000). It has been well documented that the *Xist* expression is regulated by methylation in the promoter region. In somatic tissues, the 5'end of the silent *Xist* allele on the active X is known to be fully methylated whereas the expressed allele on the inactive X is unmethylated (Norris *et al.*, 1994**;** Allaman-Pillet *et al.*, 1998).

The inactivation of the X chromosome occurs during early embryogenesis in a developmentally regulated manner (Monk *et al.*, 1979). The inactivation occurs first in the extraembryonic trophectoderm and primitive endoderm lineages. This early inactivation is non-random, with exclusive inactivation of the paternal X chromosome (Takagi *et al.*, 1975; West *et al.*, 1977). In the embryonic lineage the random X inactivation occurs around the time of gastrulation. It is known that the random X inactivation occurs during the migration phase of primordial germ cells, which coincides with the time of X inactivation in somatic tissues (Tam *et al.*, 1994). However, following the entry of PGCs into the genital ridge the inactive X chromosome is re-activated in the majority of the PGCs by 13.5 dpc (Monk *et al.*, 1981; Tam *et al.*, 1994).

It should be noted, that in this case the reactivation of the X chromosome, which is normally connected with the methylation of the *Xist* promoter, occurs at the time when the rest of the genome is apparently undermethylated (see our previous results). To elucidate the methylation processes connected to the X chromosome reactivation we carried out the methylation analysis of the *Xist* promoter (Fig. 34).



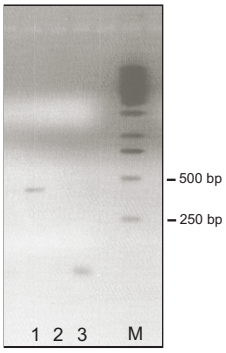


Fig. 34: Bisulphite analysis of *Xist* promoter region in male PGCs.
The figure shows the results of bisulphite treatments. The methylated and non-methylated CpG positions are shown as filled and open circles, respectively. The fractions of large and small cells were obtained using FACS sorting (for details see the text). Example of the bisulphite PCR result is shown left. **M** - 1kb ladder (MBI Fermentas), **1** - PGC sample, **2** - water control for the bisulphite treatment, **3** - water control for the bisulphite PCR.

In the case of *Xist* promoter region we focused our interest on the male primordial germ cells. Cells of male individuals contain a single X chromosome that is maintained active, i.e. the *Xist* gene is not expressed and its promoter region is methylated. According to our results, this is the case for the 11.5 dpc male PGC, where all the clones obtained in our bisulphite analysis indicate full methylation. Similarly to other single copy genes the *Xist* undergoes full demethylation before 12.5 dpc and is found to be demethylated still at 13.5 dpc. As in the case of the other tested regions we did not detect any difference between the fraction of "small" and "large"

13.5 dpc primordial germ cells. Thus, the *Xist* gene in male PGCs follows completely the scenario valid for other single copy genes.

Interestingly, the single X chromosome of male germ cells is reported to be active until the onset of meiosis when short period of X inactivation (accompanied by *Xist* expression) occurs (McCarrey *et al.*, 1992a; McCarrey *et al.*, 1992c; McCarrey *et al.*, 1992b). This implicates that at 13.5 dpc the X chromosome is still active, i.e. *Xist* not expressed despite the non-methylated status of the promoter region.

Similarly, female 13.5 dpc primordial germ cells (see Fig. 35) with both X chromosomes re-activated (Monk *et al.*, 1981; Tam *et al.*, 1994) revealed absence of methylation in the *Xist* promoter region. Thus there must be another mechanism that keeps the *Xist* gene silent in the absence of DNA methylation. The existence of such a mechanism has been already proposed by McDonald *et al*. The authors documented the absence of methylation in the *Xist* promoter region during early mouse embryogenesis, i.e. at the time when the Xist expression is supposed to be imprinted.

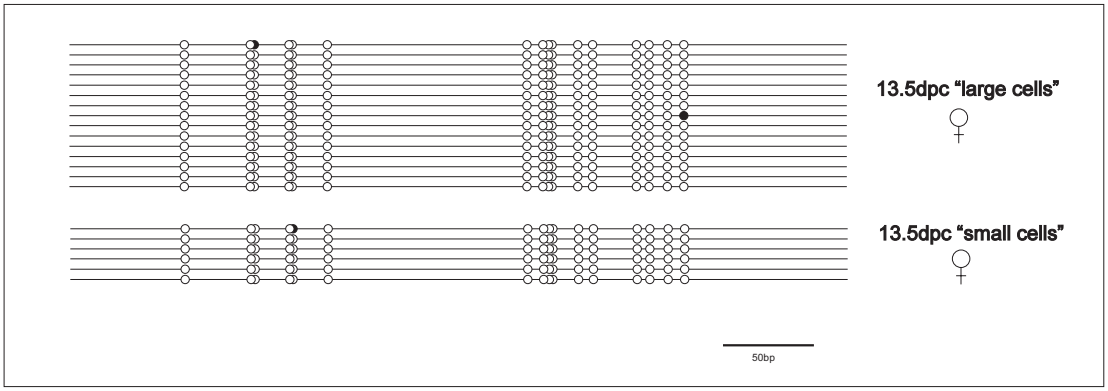


Fig.35: Bisulphite analysis of *Xist* promoter in 13.5dpc female PGCs .
Methylated CpG positions are shown as filled circles, non-methylated are represented by open circles.The "large" and "small" cells represent the fractions obtained using the FACS sorting (for details see the text).

3.9 Global approach to the methylation changes occurring during the development of primordial germ cells – immunofluorescent mC staining

The method of immunofluorescent mC staining (Mayer *et al.*, 2000) is based on labelling the denatured DNA of interphase nuclei with anti mC antibodies (Coffigny *et al.*, 1999). In our experimental system the technique was used to compare the data obtained from the bisulphite analysis ("single gene" approach) with the global ("whole genome") approach.

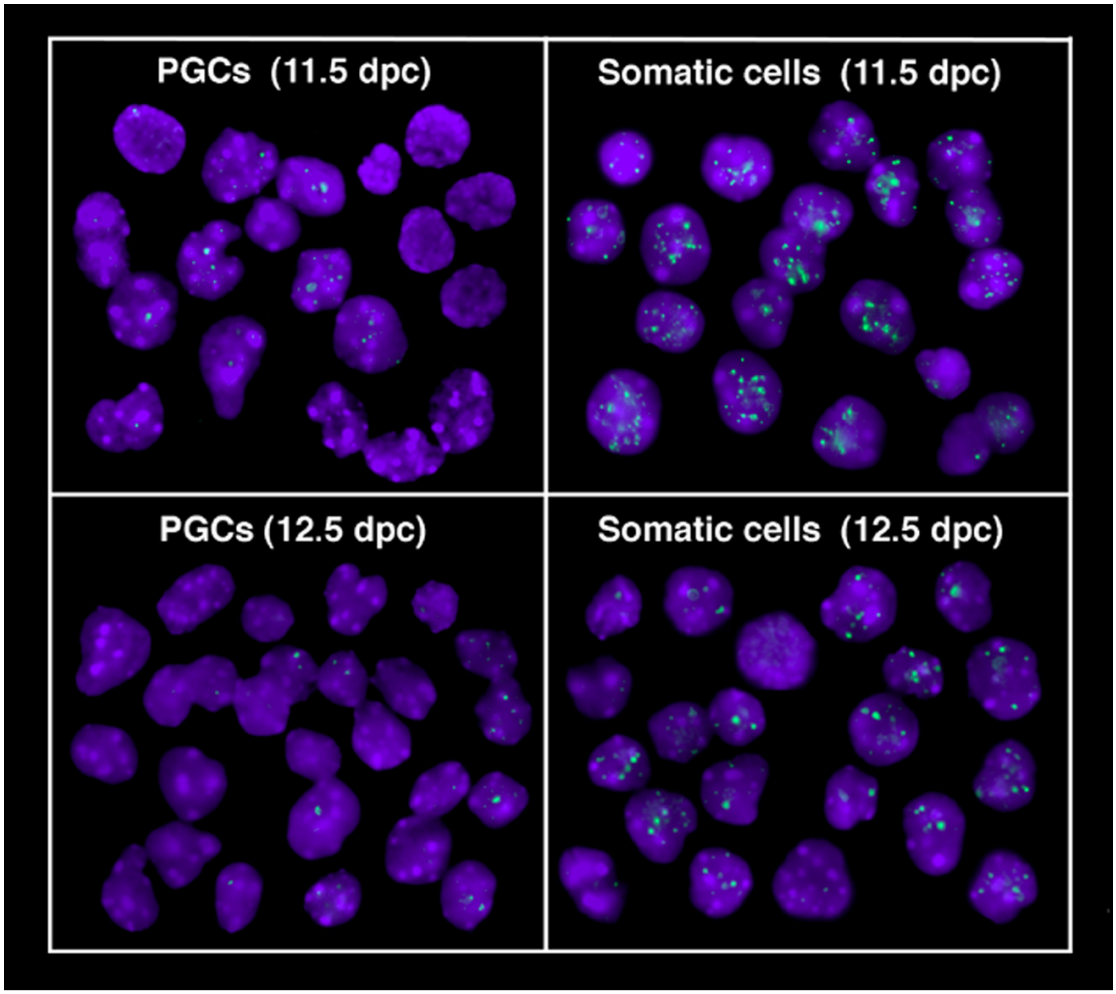


Fig.36: Immunohistochemical staining of mC.
The figure shows nuclei (stained by DAPI in blue) of PGCs and stage-matched somatic cells. Foci stained in green (FITC) indicate presence of mC.

	Female germ line		Male germ line	
Stage	PGCs	Somatic cells	PGCs	Somatic cells
11.5 dpc	67%	95%	77%	90%
12.5 dpc	67%	88%	74%	93%
13.5 dpc	45%	88%	56%	96%

Table 3: Percentage of nuclei with brightly staining MeC foci and speckles
On average 100 cells were analysed for each experiment.

At the first sight there is an obvious difference between the staining pattern of the germ cells and their somatic counterparts (see Fig. 36). Somatic cells of genital ridge stroma show in all the tested stages presence of brightly stained foci. The positive foci are seen only in a fraction of primordial germ cells, the foci are apparently less prominent as those seen in somatic cells. Figure 36 Table 3 show that there is a clear tendency of demethylation observed in primordial germ cells between 11.5 and 13.5 dpc, whereas the numbers of positively stained somatic cells remain constant.

However, at 12.5 and 13.5 dpc we could still detect primordial germ cells with positively stained foci despite the results of the bisulphite analysis documenting complete demethylation of the genes. Thus the results of the staining indicate a protracted demethylation process rather than a rapid demethylation. These results may correspond to the observation on methylation of repetitive elements (N. Lane – unpublished results). Repetitive elements undergo during the PGC reprogramming slow and incomplete demethylation, with remarkable percentage of repeats being still methylated at 13.5 dpc.

It is difficult to judge about the nature of the target for the mC antibody staining. The stained foci do apparently not correspond to the heterochromatin as the

heterochromatic regions overlap with the areas of brighter DAPI staining. Whether the repetitive elements are the target for the mC immunofluorescent staining remains speculative.

4 Discussion

4.1 Epigenetic reprogramming in the germ line

Germ line has unique features compared to other stem cell lineages. As a source of genetic material for the next generation, the primordial germ cell population is set aside very early during embryogenesis, constantly slowing the proliferation rate (presumably to minimise the risk of mutations) and kept totipotent (Lawson *et al.*, 1994; Tam *et al.*, 1981). The role of the germ line is, however, not solely a transfer of genetic material; the postulation of genomic imprinting predicted that the gametes carry also epigenetic information that is sex-specific and necessary for the normal development of the embryo (Surani *et al.*, 1984, McGrath *et al.*, 1984).

The predicted scenario of genetic imprinting requires the presence of epigenetic parent-of-origin specific marks, which are present in gametes and are maintained through cell divisions into an adult organism. . It is apparent that in the germ line these sex specific marks have to be erased and newly established according to the sex of the developing individual. Such germline epigenetic reprogramming had been predicted but at the onset of this thesis there was still only scarce experimental evidence available.

The aim of this thesis was thus to describe the epigenetic changes that occur in the developing germ line. To do so, we focused on monitoring the changes in DNA methylation, which was shown to function as an imprinting mark (reviewed in Reik *et al.*, 2001). For that purpose mouse primordial germ cells (PGCs), where the major epigenetic changes were expected to take place, were collected from embryos of different developmental stages (10.5 – 13.5 dpc) and subjected to the bisulphite analysis. The methylation analysis was predominantly focused on well-characterised imprinted genes; for the reason of simple evaluation of methylation changes and of differences between parental alleles the bisulphite analysis was targeted at the DMRs with clear bimodal parent-of-origin methylation pattern. The selection of imprinted genes included examples of both maternally (*Peg3*, *Snrpn* DMR1, *Lit1*) and paternally

(*Igf2* DMR2, *H19* upstream DMR, *Snrpn* DMR2) methylated genes. The kinetics of reprogramming (methylation/demethylation) processes was additionally monitored by methylation changes occurring in *Xist* promoter, as one of the markers for X re-activation. Last but not least, the observed methylation changes were compared to the changes occurring within non-imprinted genes (*mylC*, *α-actin*) to address to question of ubiquitous character of the reprogramming process.

4.2 Imprinted and non-imprinted genes undergo in PGCs fast and complete demethylation

The genome-wide methylation changes occurring during the PGC development were first described in 1987 by Monk and colleagues (Monk *et al.*, 1987). The authors described the hypomethylated state of the genomic DNA isolated from 12.5 dpc and 14.5 dpc PGCs. The methylation changes were further confirmed also at the level of single genes: the tested genes were found hypomethylated and free of imprints in the PGCs isolated from 12.5 dpc and 13.5 dpc mouse embryos (Brandeis *et al.*, 1993; Kafri *et al.*, 1992). All the previously published observations, however, concerned PGCs at the stages where the cells were already devoid of methylation imprints. As our main interest was to describe the hypothesised demethylation event we focused on the earlier developmental stages of PGCs, where we expected the methylation imprints to be still present.

The results of the performed bisulphite analysis are summarised in the Fig. 37.

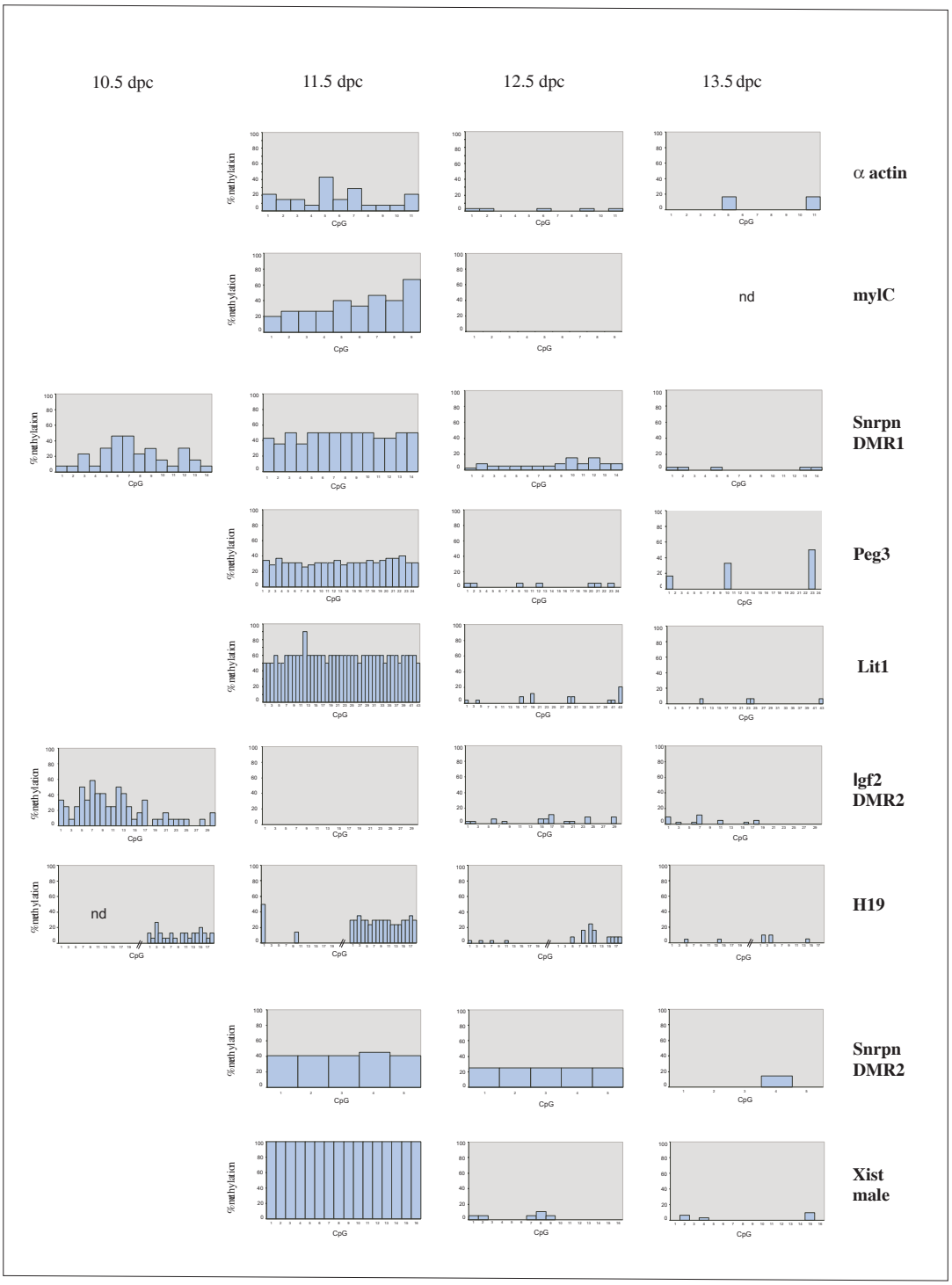


Fig. 37: Summary of the bisulphite analysis performed on primordial germ cells.
The graphs show relative methylation at the distinct CpG positions within the analyzed regions (for details see chapter Results). Note that the H19 graph combines the methylation analysis of the proximal (right) and the distal (left) part of the H19 upstream DMR.

4.2.1 Reprograming of maternally methylated imprinted genes

Using the bisulphite approach we found that all the tested maternally methylated DMRs (*Snrpn* DMR1, *Peg3*, and *Lit1*) undergo in primordial germ cells identical changes of their methylation patterns (see Fig. 37). In the PGCs isolated from 11.5 dpc mouse embryos the maternally methylated DMRs were found to be methylated in about 50% of sequenced clones, which, as we assume, indicate a presence of an imprinting mark. This assumption is based on the following: 1) Similar results were obtained when working with the somatic tissue samples in experimental set-ups, where allele discrimination was possible. Moreover, in 11.5 dpc PGCs likewise to somatic samples mainly completely methylated or completely non-methylated clones were detected, which is a typical feature when analysing the imprinted DMR methylation. 2) The primers and the conditions for the bisulphite PCR had been previously intensively tested in our laboratory in order to uncover possible bias in our experimental procedure.

To the contrary, none or only sporadic methylation was detected in the samples of 12.5 dpc and 13.5 dpc PGCs. Elevation of methylation levels at some CpG positions within *peg3* gene was presumably caused by low number of obtained unique clones.

4.2.2 Reprogramming of paternally methylated imprinted genes

Similarly to maternally methylated regions discussed above, we found the tested paternally methylated DMRs (*Igf2* DMR2 and *H19* DMR) non-methylated in PGCs of 12.5 dpc and 13.5 dpc mouse embryos. The absence of methylation in the 3'part of *H19* DMR was unexpected as it was in an obvious disagreement with the recently published observation of Ueda and colleagues (Ueda *et al.*, 2000). The authors described *de novo* methylation occurring in this particular part of the H19 DMR from 13.5 dpc on. As in both analysis the same primers and PCR conditions were used for the bisulphite PCR, the discrepancy might be explained by the use of a different mouse strain (C57Bl/6 vs. outbred MF1 strain used in our experiments), or perhaps by a different time-scheme of sample collection. Considering the results of our methylation analysis it is apparent, that the major methylation changes can occur

in PGCs within several hours. The different time schedule of the hormone induction, fertilisation and finally the PGC collection might thus account for a different result. It should be also noted that using a very sensitive bisulphite approach the purity of the isolated PGCs is a key factor. Any (even very low) contamination with the somatic cells of the embryonic gonads might lead to the observed low levels of methylation. It is thus very important to mention that all our samples (regardless whether MACS or FACS sorted) were checked additionally for purity using an alkaline phosphatase staining. The purity of collected samples exceeded always 95%. Such a purity-control check was, however, not mentioned in the work of Ueda *et al* (Ueda *et al.*, 2000).

Contrary to the described maternally methylated DMRs, the paternally methylated DMRs did not show a uniform methylation patter at 11.5 dpc (see Fig. 37). In the samples of 11.5 dpc PGCs only the 3' part of *H19* DMR appeared to be methylated, whereas both the 5' part of *H19* DMR and the DMR2 of *Igf2* revealed no methylation. The results obtained on the 10.5 dpc samples showed that at least *Igf2* DMR2 was methylated in the earlier stages of PGCs. As our bisulphite analysis of earlier stages of PGCs failed in the case of the 5'part of *H19* DMR, it can still be hypothesised, that the region is not methylated up to 13.5 dpc

Our finding that the Igf2 DMR2 undegoes demethylation earlier (between 10.5 dpc and 11.5 dpc) could indicate that the "core" imprinting centre in the *Igf2-H19* locus is located in the 3' part of the *H19* DMR. This region thus behaves in the same manner as the tested maternally methylated DMRs (see above), whereas the 5' part of *H19* DMR and the DMR2 of *Igf2* act as "second level" DMRs reacting on the demethylation events faster. Prediction of such a DMR hierarchy in the *H19-Igf2* genomic locus is supported also by the response of the DMRs during the zygotic demethylation (see following chapters). Whereas the DMR2 of *Igf2* was documented to undergo complete demethylation (Oswald *et al.*, 2000), the DMR of *H19* (or at least some part of it) keeps its methylated status (Warnecke *et al.*, 1998). Additional evidence comes from the mouse knockout experiments: the deletion of the *H19* DMR influences the methylation of the *Igf2* DMR2, but to the contrary, the absence of the *Igf2* DMR2 does not have any effect on the H19 DMR methylation (S.Lopes – manuscript in preparation).

4.2.3 DMR2 of Snrpn undergoes protracted demethylation

A special example of paternally methylated DMR is the DMR2 of *Snrpn*. As the only available information concerning the region was based solely on the mapping with restriction endonucleases (Shemer *et al.*, 1997) it was first necessary to perform a detailed sequence analysis. This surprisingly revealed that the region is CG poor and, moreover, the highest density of CpGs is associated with a part of repetitive (Line1) element.

Concerning the CpG density the DMR2 of *Snrpn* represent certainly a unique example among up-to-now characterised DMRs. Whereas typical DMRs have the features of CpG islands or are spanning clusters of CpG, the *Snrpn* DMR2 comprises 19 CpGs over more than 4,3 kb of genomic sequence. It is disputable whether the density of DNA methylation is sufficient to carry the epigenetic information in this region, or whether (presumably) the imprinting mark is formed by a combination of different types of epigenetic mechanisms (i.e. histone acetylation, histone methylation etc.). Such a possibility has to be, though, yet experimentally elucidated.

As our results represented the first bisulphite analysis of this region, it was important to determine, whether the sequence outside the previously tested restriction sites displays differential methylation. Bisulphite analysis performed on the 11.5 dpc PGCs manifested that the region is fully methylated in about 50% of sequenced clones, that is obviously in agreement with the presence of expected imprint. Still, it is necessary to point out, that the set-up of our experiments did not allow distinguishing the alleles with regard to their parental origin.

The bisulphite analysis of the 12.5 dpc and 13.5 primordial germ cells revealed that the *Snrpn* DMR2 undergoes protracted demethylation reaching the demethylated state at 13.5 dpc in a gradual manner. Such a behaviour contrasts with demethylation kinetics observed in other DMRs (both paternally and maternally methylated) and resembles more the behaviour of repetitive elements (see later). Such a finding raises the questions about the methylation profile of the rest of the *Snrpn* DMR2. Slow demethylation of the rest of the DMR would suggest that the features of the whole DMR are directed by the integrated repetitive element. To the contrary, it could be also imagined that the integration event happened after the region had gained its properties as a DMR element. The integrated repetitive element thus subsequently gained the imprinted properties, but is by some cellular machineries still recognised as

being a repetitive element. It should be noted, that the gain of imprinting following the retrotransposition of genes into the imprinted region has been already described (Chai *et al.*, 2001). It is remarkable that imprinted genes are often found to be associated with repetitive elements. It is, however, questionable, whether the imprinted status of the particular genomic region is connected with the presence of repetitive elements (repetitive elements might be responsible for certain regional chromatin configuration), or whether the specific chromatin properties typical for DMRs enable easy and efficient transposition events. Further investigation is, however, needed to resolve the biological background of this phenomenon.

4.2.4 Non-imprinted genes follow the same demethylation scheme as imprinted genes

Except of imprinted genes, two examples of single copy non-imprinted genes (*α-actin*, *mylC*) were included into the bisulphite analysis in order to clarify the specificity of the demethylation process. Identically to the imprinted genes we found the investigated non-imprinted regions methylated in 11.5 dpc PGCs with the methylation levels similar to those observed in somatic tissues (Warnecke *et al.*, 1999; Walsh *et al.*, 1999). Furthermore, also in the non-imprinted regions the complete demethylation occurred between 11.5 dpc and 12.5 dpc (see Fig. 37).

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4.3 Biological aspects of the germline demethylation

The presented comprehensive data document a presence of a widespread demethylation mechanism affecting in the same manner imprinted genes (regardless of the origin of their methylation marks) as well as non-imprinted genes. It should be pointed out that the data represent the first solid experimental evidence concerning the mechanism, which had been previously hypothesised as an essential part of the predicted scenario of imprinting, but up to now not experimentally documented.

The results of our experiments show that at 10.5 dpc –11.5 dpc at the time when they enter the developing genital anlagen the PGCs contain high levels of methylation. Shortly afterwards – between 11.5 dpc and 12.5 dpc – the majority of the tested genes undergo fast and complete demethylation. This is in complete agreement with the hypomethylated state of 12.5 dpc and 13.5 dpc PGCs previously described by Kafri *et al.* and Brandeis *et al.* (Kafri *et al.*, 1992; Brandeis *et al.*, 1993) and the documented biallelic expression of imprinted genes at this stage of PGC development (Szabo *et al.*, 1995). The demethylation affects both the imprinted genes regardless of the origin of their imprinting methylation mark as well as non-imprinted genes. Moreover, our data clearly demonstrate that the methylation erasure proceeds identically in the primordial germ cells of female and male. The epigenetic resetting commences in the not yet sexually differentiated gonads and is probably the only time during the development when the germ cells of either sex are equivalent and free of any epigenetic imprint. This epigenetic "zero baseline" is apparently the starting point for the subsequent sexual differentiation (starting around 13.5 dpc) and the later initiation of new gamete specific imprints (see Fig. 38).

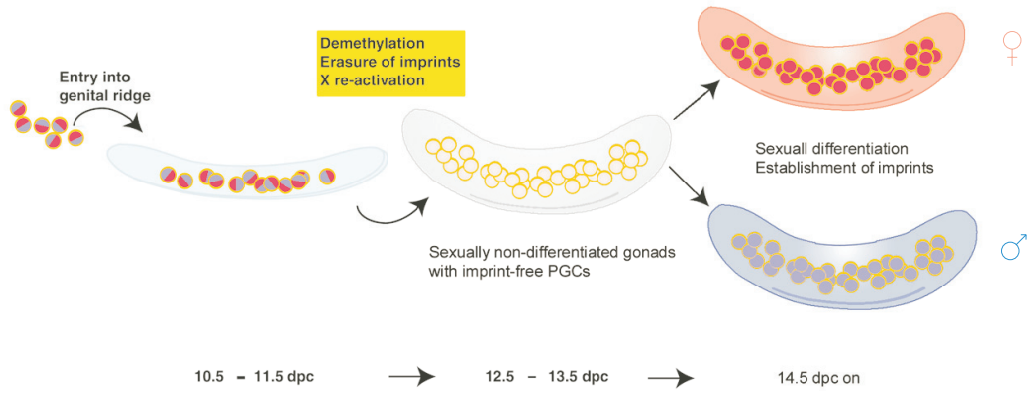

Fig. 38: Epigenetic reprogramming in the germ line.
As the migrating PGCs colonise the genital ridges they possess methylation imprinting marks (shown in blue and red). Shortly afterwards the reprogramming commences in still bipotential gonads perhaps as a reaction to a somatic signal emanating from the stroma of a genital ridge; the imprints are erased and the inactive X chromosome in PGCs of female re-activated. Sex specific methylation imprints are established later in sexually fully differentiated gonads (male and female gonads shown in blue and red, respectively).

An important feature of the germ line demethylation is that the imprinting methylation marks are erased completely (such a statement is, however, not valid for

repetitive elements as discussed in the following chapters). This finding is important in the light of the work recently published by Davis *et al* (Davis *et al.*, 1999; Davis *et al.*, 2000). The authors described the differences between establishment of the *H19* imprinting mark on the allele of a maternal and a paternal origin during spermatogenesis, speculating that the *H19* methylation mark persists (at least partially) on the allele of the paternal origin. Our results describing complete loss of imprints in 12.5 dpc and 13.5 dpc PGCs do not justify such a hypothesis. The faster (or perhaps easier) remethylation of the *H19* paternal allele could be caused by the persisting differential chromatin structure or modification (histone acetylation, histone methylation etc.) Hence, it has to be stressed out that the data of this thesis concern solely the erasure of methylation epigenetic marks, whereas the destiny of other types of epigenetic marks (for example the histone modifications) remains speculative.

Another important characteristic of the demethylation process is its tissue specificity. The strict restriction of the demethylation to the germ cells was proven by the results of methylation analysis performed on the stage-matched somatic cells of the genital ridge. Whereas the 12.5 dpc PGCs were completely devoid of methylation imprints, the corresponding cells of the genital ridge stroma kept somatic methylation pattern. The reprogramming ability is thus an intrinsic feature of the germ line.

In connection with the methylation analysis many questions have been raised concerning the occurrence of unusual methylation patterns. The presence of asymmetrically methylated sites has been described to occur in the imprinted *H19-Igf2* locus (Vu *et al.*, 2000) or in the systems over-expressing Dnmt3a methyltransferase (Lyko *et al.*, 1999; Ramsahoye *et al.*, 2000; Lin *et al.*, 2002**)**. The presence of such a methylation pattern has, however, never been detected in our PGC methylation studies. Due to the mechanism of the bisulphite conversion (the DNA strands are no longer complementary following the bisulphite treatment) only one DNA strand is usually subjected to the methylation analysis. Similarly, in all our experiments only one DNA strand was analysed for its methylation status. It could be thus speculated that the observed methylation changes are strand specific and the demethylation connected perhaps to the ongoing replication that leaves the newly synthesised DNA strand devoid of methylation. Such a scenario is, however, difficult to imagine, as in some cases the methylation pattern of the upper DNA strand (*H19, Peg3, Xist, Snrpn, α-actin*) was analysed whereas in other cases the analysis was

focused on the lower DNA strand (*mylC, Igf2*). To finally exclude such a possibility the methylation status of both DNA strands will have to be investigated for some regions.

4.4 PGCs *in vivo* differ from the PGC-derived EG cell lines

Due to technical difficulties connected with the isolation of primordial germ cells in an amount sufficient for the methylation analysis, many previously published experiments used PGC-derived EG (embryonic germ) cell lines as an experimental model for PGCs. The EG lines were shown to keep the undifferentiated morphology similar to ES (embryonic stem) cells and to be able to contribute to all types of tissues including the germ line when used to produce mouse chimeras (Labosky *et al.*, 1994; Stewart *et al.*, 1994; Tada *et al.*, 1998). The genome of EG cell lines derived from 11.5 dpc and 12.5 dpc PGCs was shown to be grossly hypomethylated and devoid of methylation imprints (Kato *et al.*, 1999; Tada *et al.*, 1998).

In the light of our findings it is intriguing that the genome of EG cell lines derived from 11.5 dpc PGCs is completely demethylated whereas the DNA of PGCs isolated from the 11.5 dpc mouse embryos contains still methylation imprints. The epigenotype of the 11.5 dpc derived EG cell lines is thus similar to the 12.5 dpc PGCs, rather than to 11.5 dpc PGCs. This discrepancy is supported furthermore by the recent observation of Durcova-Hills *et al.* (Durcova-Hills *et al.*, 2001). The authors describe the EG cell lines derived from migrating PGCs of 9.5 dpc mouse embryos. Also these EG cell lines are devoid of methylation imprints. It seems that the PGCs are already "programmed" at the time point of the isolation to commence the demethylation, with which they proceed once they are transplanted into the cell culture. Alternatively, the demethylation could can occur during the cultivation as a response to the cellular signals provided by the co-cultivated somatic cells.

4.5 A global character of genomic demethylation is confirmed by the pattern of anti-mC staining

A further support for the genome-wide character of the demethylation occurring in the primordial germ cells was given by the results of immunohistochemical staining using the anti-mC antibody. Whereas multiple distinct positively stained speckles were characteristic for the nuclei of control stage-matched somatic cells of embryonic gonads, the speckles appeared only sporadically in the nuclei of primordial germ cells. Furthermore, the number of positively stained PGCs declined between 11.5 dpc and 13.5 dpc. It is remarkable that, the main change in the number of positively stained cells appears between 12.5 dpc and 13.5 dpc, whereas the single copy genes undergo the demethylation earlier - between 11.5 dpc and 12.5 dpc. Moreover, even among the 13.5 dpc PGCs we found the cells with positively stained foci. This delayed and incomplete disappearance of the positive signal could be explained by the residual methylation of repetitive elements, which were shown to undergo protracted and incomplete demethylation (N.Lane – unpublished data, Hajkova *et al.* – submitted). It is also possible that the sporadically appearing positively stained cells are due to a low somatic cell contamination of our PGC samples (although the purity of the PGC samples always exceeded 95%). It is remarkable that the staining pattern did overlap neither with centromeric regions nor with the DAPI staining suggesting that the DNA methylation is not focused to the heterochromatic DNA. Such a distribution could be easily explained assuming that the anti-mC staining is targeted to the repetitive elements.

4.6 Germ cells do not escape from the wave of *de novo* methylation in the gastrula stage

The previously published observations documented that the genome of 12.5 dpc and 13.5 dpc PGCs is grossly hypomethylated (Kafri *et al.*, 1992; Brandeis *et al.*, 1993; Monk *et al.*, 1987). Since this was more or less the only knowledge available the methylation status of PGCs of earlier developmental stages was only speculated. Based on that several theories appeared: One of the possible explanations for the low

levels of methylation found in post-migratory PGCs was that the founder population of germ cells does not undergo the pre-implantation wave of *de novo* methylation (Jaenisch, 1997; Monk *et al.*, 1987). Alternative scenario suggested that the founder PGCs undergo the de novo methylation event similarly to the somatic lineage, the PGCs get demethylated subsequently by a germline specific mechanism (Monk *et al.*, 1987) (see Fig. 39).

Our experiments clearly show, that up to 11.5 dpc primordial germ cells contain high levels of methylation including fully established methylation imprinting marks. Such a finding strongly suggests that the founder population of PGCs is indeed subjected to the pre-implantation *de novo* methylation processes (see Fig. 39). However, it has to be still experimentally elucidated whether the discussed *de novo* methylation proceeds identically in the developing germ line and in the somatic lineage.

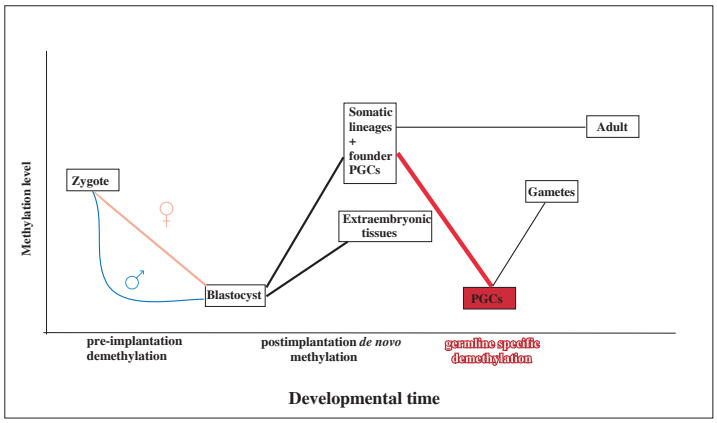

Fig. 39: Dynamics of DNA methylation in mouse germ line.
Following the pre-implantation genome wide demethylation the somatic lineages together with the founder population of PGCs undergo a wave of post-implantation *de novo* methylation. The methylation is erased in the differentiating PGCs after their entry into the gonads by a germ line specific mechanism. The new methylation imprints are established subsequently in the process of gamete maturation.

4.7 Single copy genes and repetitive elements follow different demethylation kinetics

Contrary to the single copy genes (both imprinted and non-imprinted), which undergo fast and complete demethylation between 11.5 dpc and 12.5 dpc, the demethylation of repetitive elements is in PGCs (11.5 dpc – 13.5 dpc) prolonged and

incomplete (see Fig. 40). Such a conclusion is the result of the comprehensive methylation analysis of two classes of mouse repetitive elements (*Line1* and *IAPs*) performed in the parallel to the work of this thesis in the Babraham Institute (Babraham, UK, N.Lane unpublished data).

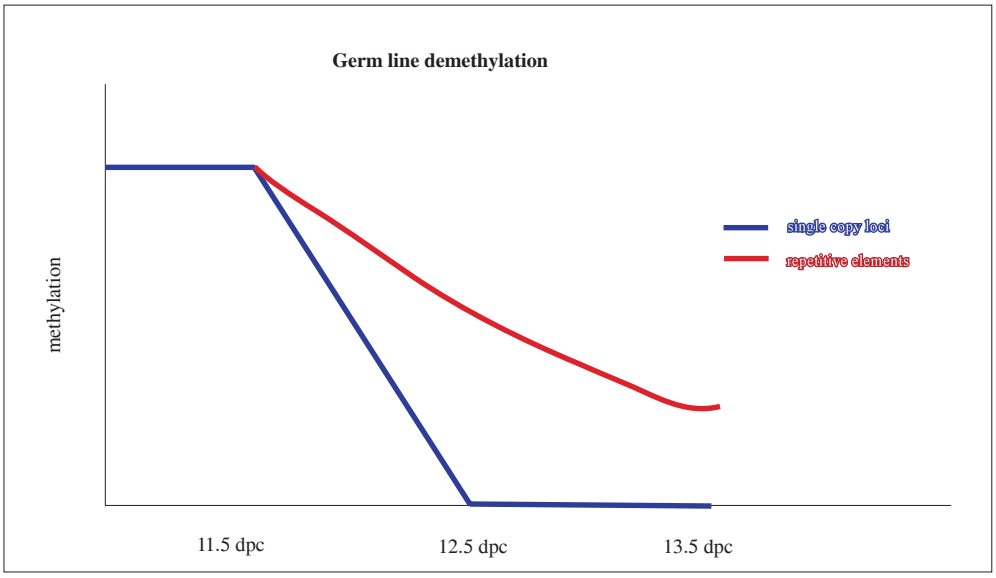

Fig. 40: Comparison of the demethylation kinetics in the germ line: single copy versus repetitive sequences.
Single copy loci undergo fast and complete demethylation in PGCs between 11.5 dpc and 12.5 dpc. To the contra

The effect of the genomic localisation and the chromatin accessibility of the particular repetitive element could possibly explain the fact that not all of the repetitive elements undergo demethylation at the same time point. Such an explanation is, however, not very likely, as we did not observe any variability while investigating the single copy genes (assuming that the tested genes were not by coincidence located in the loci of the same chromosomal features).

A more plausible scenario could be to imagine the situation in PGCs as the dynamic process of the demethylation and *de novo* methylation. Whereas the demethylation machinery would work in a genome–wide non-specific manner, the *de novo* methylation processes might be specifically targeted to the repetitive sequences. Such *de novo* methylation mechanism could be potentially induced by the expression of the demethylated repetitive sequences (the inhibitory effect of the DNA

methylation on the expression of repetitive elements has been well documented (Walsh *et al.*, 1998) in a process similar to PTGS (post-transcriptional gene silencing – for review see Cogoni *et al.*, 2000). Alternatively, the specificity of *de novo* methylation could be determined by the substrate specificity of the acting DNA methyltransferase. It should be noted, that the newly described knock-out of *Dnmt3L* (a protein with high similarity to Dnmt3a and Dnmt3b methyltransferases - Bourc'his *et al.*, 2001) affects solely methylation of single copy genes suggesting that the single copy and repetitive sequences are methylated by distinct cellular machineries.

Another possible scenario is that the fast demethylation targets only single copy loci and the loss of methylation in the tested repetitive sequences is due to the on-going replication in the absence of the *de novo* methyltransferases. As revealed by the immunostaining experiments Dnmt3a is absent in PGCs and Dnmt3b is excluded from the nucleus showing cytoplasmic localisation. Contrary to the *de novo* methyltransferases, high level of the maintenance Dnmt1 methyltransferase was detected in PGC nuclei (S. Erhardt – unpublished data, Hajkova *et al*. – submitted). However, as documented by recently published results, the presence of all the three eukaryotic methyltransferases (Dnmt1, Dnmt3a and Dnmt3b) is necessary in order to maintain the methylation of repetitive elements (Liang *et al.*, 2002).

The presence of the mechanism, which maintains (at least partially) the methylated and silenced status of the repetitive elements is crucial from the evolutionary point of view to protect the integrity of the genetic information in the germ line from the deleterious effects of retroelements and other transposable sequences. Moreover the incomplete erasure of methylation in repetitive elements could be seen an enhancing force for a evolutionary variability of the species. Such an example is given by a mouse *agouti* locus, where the activity of the neighbouring gene is influenced by the methylation of the integrated transposable element. (Morgan *et al.*, 1999;Wolff *et al.*, 1998). The incomplete loss of methylation within this repetitive element in the germ line yields in the high variability of the *agouti* expression in the progeny.

4.8 *Xist* expression is silenced in post-migratory PGCs by a mechanism distinct to DNA methylation

Mammals compensate for different dose of X-linked-genes in male (XY) and female (XX) diploid cells by inactivating all but one X chromosome in each cell (for review see Mlynarczyk *et al.*, 2000). Although the mechanism of the X chromosome inactivation is not yet completely understood, the initiation of the process is known to be connected with the expression of the non-coding *Xist* (X inactivation specific transcript) RNA. In the last decade several scientific reports suggested methylation of the Xist promoter as the key regulator in the X inactivation (Allaman-Pillet *et al.*, 1998; Norris *et al.*, 1994). The role of methylation in the X inactivation was, however, undermined by the finding that the process occurs in the early embryogenesis apparently in the absence of promoter methylation (McDonald *et al.*, 1998).

Reactivation of the inactive X chromosome in the primordial germ is considered as the marker for the germ line reprogramming processes (McLaren *et al.*, 1997; Monk *et al.*, 1981). The reactivation occurs between 11.5 dpc and 13.5 dpc, i.e. after PGCs colonised the genital ridges and is probably triggered by a somatic signal from the stroma of the genital ridge (Tam *et al.*, 1994). While following the fate of methylation in the primordial germ cells after their entry into the genital anlagen it was of a high interest to investigate also the methylation status of the *Xist* promoter. For the reason of simplicity we followed the methylation pattern of the *Xist* promoter in male PGCs that contain a single active X chromosome with transcriptionally silent and hence presumably methylated *Xist* gene. The *Xist* promoter appeared to be no exception to the other tested single copy genes – the promoter sustains complete demethylation between 11.5 dpc and 12.5 dpc. This loss of promoter methylation seems to be surprising since the X chromosome has been reported to remain active in the maturing PGCs (Nesterova *et al.*, 2002). Furthermore, recent evidence shows that despite demethylation of the *Xist* promoter in PGCs documented by our results, *Xist* transcript decreases progressively and is extinguished in most PGCs by 13.5 dpc (Nesterova *et al.*, 2002). It seems, therefore, that the *Xist* of the active X chromosome is in PGCs (similarly to the early embryogenesis) transcriptionally silenced by a mechanism distinct to promoter methylation.

4.9 Is germline demethylation an active process?

Two general models have been postulated for the mechanism of demethylation (see Fig. 41). Passive demethylation is caused by replication proceeding in the absence of the maintenance activity represented in mammalian cells by Dnmt1 (DNA methyltransferase 1). As a consequence, methylated DNA strands are gradually diluted with the increasing number of replications. To the contrary, active demethylation requires an enzymatic activity. It has been proposed that such a demethylation involves either a glycosylase and repair activity (Jost *et al.*, 2001; Zhu *et al.*, 2001; Zhu *et al.*, 2000**)** or a nucleotide excision and replacement activity (Weiss *et al.*, 1996). Recently, however, the existence of novel "demethylase" was reported (Bhattacharya *et al.*, 1999; Cervoni *et al.*, 1999). The described enzyme is predicted to transform methylated cytosines to cytosines by direct removal of a methyl group and to work in a processive manner. The nature of this enzyme and exact activity have to be, though, still elucidated as some of the results appeared to be apparently not reproducible .

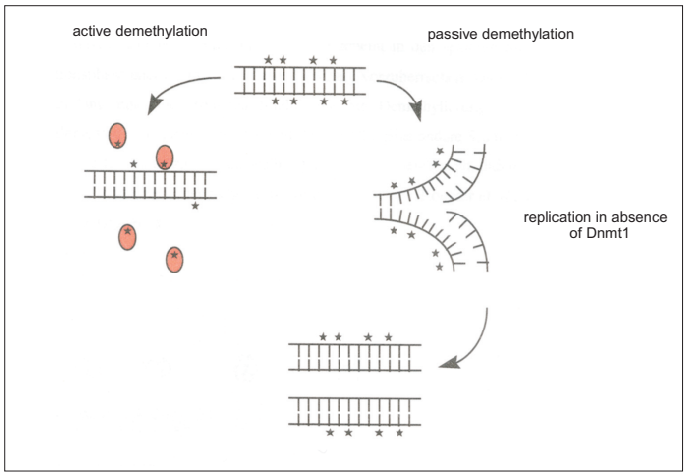

Fig. 41: Comparison of active and passive demethylation.
Passive demethylation occurs as a consequence of replication proceeding in the absence of maintenance methylation (provided by Dnmt1). Contrary to passive demethylation, active demethylation is replication independent and requires an enzymatic activity,

Interestingly, the demethylation process occurring in primordial germ cells exhibit all the features of active demethylation. First of all, the demethylation of single copy loci is completed within only one day (between 11.5 and 12.5 dpc) and possibly even faster. Considering the replication time of PGCs at that developmental stage (16-17 hours (Tam *et al.*, 1981), it is obvious that the demethylation occurs within one replication cycle. Second, the immunofluorescent staining of the primordial germ cells of the corresponding developmental stages manifested high level of Dnmt1 expression as well as the nuclear localisation of the enzyme, which suggests that the demethylation occurs in the presence of Dnmt1 (S. Erhard – unpublished data, Hajkova *et al*. - submitted).

Similarly to PGCs, also PGC-derived EG cells possess strong demethylation activity – when fused to a somatic cell, they can cause the demethylation of the somatic nucleus (Tada *et al.*, 1997). In the light of our findings it is presumable that those dominant reprogramming activities are associated with the same active demethylation process. It is imaginable that similar demethylation (reprogramming) activities are a feature common to all pluripotent cells. However, this is not the case, as no similar activities were found in ES cells (Tada *et al.*, 2001). The demethylation activity thus seems to be a striking feature of primordial germ cells and their derivatives – EG cells.

Zygotic versus germline demethylation

The demethylation observed in primordial germ cells is not the only example of the described active demethylation - a similar process has been documented to occur in a zygote just several hours after fertilisation (Mayer *et al.*, 2000; Oswald *et al.*, 2000). The zygotic demethylation, similarly to the germline demethylation, commences in absence of DNA replication, the process is, however, restricted only to the paternal pronucleus and probably linked to chromatin remodelling of protamine-packed sperm DNA (Barton *et al.*, 2001). Additionally, whereas several single copy sequences as well as *IAPs* and *Line1* elements have been described to be affected by the zygotic demethylation, some regions (for example - H19 DMR, Warnecke *et al.*,

1998) apparently withstand the process. This is in a striking contrast to the germ line demethylation, which affects all the tested genomic regions.

In the light of the latest findings connecting the DNA methylation with the methylation status of histones (Tamaru *et al.*, 2001) it is possible that the germline specific demethylation is also connected with the global chromatin changes (though probably of a different character then those occurring during the zygotic demethylation). Such a possibility has to be yet experimentally elucidated.

4.10 A somatic signal rather than an intrinsic clock triggers the germ line demethylation

The previous experiments with the EG cell lines suggested that the reprogramming activities are intrinsic features of PGCs and happen precociously. The EG cell lines derived from migratory (9.5 dpc) and early post-migratory (11.5 dpc) PGCs are heavily hypomethylated and avoid of imprints (Tada *et al.*, 1998; Durcova-Hills *et al.*, 2001).

Contrarily, the work of Tam *et al*. (Tam *et al.*, 1994) clearly described that in order to re-activate the inactivated X chromosome the PGCs have to enter the genital ridge. Such a finding thus strongly argues for the presence of a somatic signal emanating from the stroma of a developing genital ridge. Also our experiments support this notion: at the time when the PGCs reach the genital ridge (10.5-11.5 dpc) they still posses methylation patterns comparable to the somatic cells, however, only shortly afterwards (12.5 dpc) the PGCs appear to be completely demethylated (see Fig. 38). Such kinetics could be explained by a somatic signal triggering the whole process. It is also possible, that the primordial germ cells "sensitive" to such a signal have to develop separately (i.e. outside the genital ridge) to reach the genital anlagen (and to be reprogrammed) just in time before the sexual differentiation of the gonads. Hence, it might be that the migration of germ cells and epigenetic reprogramming are phenomena that are evolutionary connected.

It should be noted, that during the EG cell lines derivation the PGCs are cultivated in a mixture with the somatic cells of the genital ridge stroma. The

hypomethylation observed in EG cells could thus be induced by a signal emanating from those somatic cells or by a factor connected to tissue culture conditions.

Taking into account that the germline reprogramming is a typical example of a developmental process restoring cellular totipotency, the understanding of the nature and the action of the signal triggering this process might shed more light also on the processes such as cloning and derivation of stem cells.

4.11 Evolutionary aspects of germline demethylation

The germline demethylation assures complete erasure of imprints and of most of "non-imprinted" methylation. Such a mechanism has presumably two major functions: a) it is crucial in order to prevent mutations to be spread through the generation b) it is vital for the function of gametic imprinting. It is, however, difficult to argue, which function appeared primarily during the evolution.

From the evolutionary point of view, there might be an interesting connection between the zygotic and the germline demethylations (both processes were compared in the previous chapter). The zygotic demethylation was described in mouse and bovine (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Dean *et al.*, 2001). Similar process was, however, not found in Xenopus (Stancheva *et al.*, 2002) and neither early Zebrafish embryos display any methylation dynamics (Macleod *et al.*, 1999). Hence it seems that this demethylation process might be limited to mammals (i.e. the species with gametic imprints). It is particularly interesting, that similar active demethylation events have been recently described in the flowering plants showing phenomena analogous to genomic imprinting (Spielman *et al.*, 2001). The demethylation occurs during the first 5 hours of seed germination prior to DNA replication and thus likely in an active manner (comparable to zygotic demethylation?) (Zluvova *et al.*, 2001). This finding thus implies that the demethylation processes might not be limited to the mammals, but might rather be a feature of organisms, which evolved gamete-specific epigenetic differences.

More experimental work is nevertheless needed to uncover whether the germline demethylation occurs always in connection with the zygotic demethylation and evolved thus possibly with genomic imprinting; or whether it has appeared independently during the evolution as the germline mechanism preventing accumulation of mutations.

4.12 On the nature of paternal and maternal imprints

Since the discovery of genomic imprinting it has been speculated what is the real nature of the imprinting mark. The scientific reports of the last decade brought rather vast evidence that imprinting is connected with the appearance of so called differentially methylated regions (DMRs) (for review see Reik *et al.*, 2001). Parent-of-origin specific methylation detected in those regions is believed to be responsible for monoalellic expression and hence to act as the imprinting mark.

A question that is not widely discussed is the difference between paternal and maternal imprinting marks. Whereas it might be generally believed that the parental imprinting marks do not differ, the thorough analysis of the published results might indicate otherwise:

1) The re-establishment of the paternal methylation marks seems to be generally easier: The EG cell lines that are devoid of methylation imprints restore paternal but not the maternal methylation marks in mouse chimeras. Similarly, the Dnmt1 -/- cells can reconstitute the paternal methylation imprinting marks after the introduction of the Dnmt1 expressing construct. However, much higher overexpression of Dnmt1 is needed in order to restore the maternal methylation marks (Biniszkiewicz *et al.*, 2002; Zhan Jun – unpublished results).
2) As documented in the case of imprinted human *Snrpn* gene the methylation is not absolutely necessary for the maternal imprinting mark – the promoter of *Snrpn* is not methylated in human oocytes, the maternal methylation mark is established and propagated postzygotically (El-Maarri *et al.*, 2001). It might be thus speculated that the methylation of the maternal allele is directed by some other signal - for example chromatin modification.

Based on the above-mentioned observation it is possible to speculate that the maternal imprinting mark is more complex than the paternal one. Maternal imprint might be created on several distinct levels – one certainly including DNA methylation, the other(s) exploiting different possibilities of chromatin modifications. It should be noted, that two of the maternally methylated DMRs (*Snrpn* and *U2af1-rs1*) have been recently described to be associated with the differential histone acetylation (Gregory *et al.*, 2001). Reconstitution of such a complex maternal mark would be then clearly more complicated, which would explain the observation mentioned above.

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Publications

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