THE ROYAL SOCIETY

Published online 7 July 2003

Epigenetic asymmetry in the mammalian zygote and early embryo: relationship to lineage commitment?

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Epigenetic asymmetry between parental genomes and embryonic lineages exists at the earliest stages of mammalian development. The maternal genome in the zygote is highly methylated in both its DNA and its histones and most imprinted genes have maternal germline methylation imprints. The paternal genome is rapidly remodelled with protamine removal, addition of acetylated histones, and rapid demethylation of DNA before replication. A minority of imprinted genes have paternal germline methylation imprints. Methylation and chromatin reprogramming continues during cleavage divisions, but at the blastocyst stage lineage commitment to inner cell mass (ICM) or trophectoderm (TE) fate is accompanied by a dramatic increase in DNA and histone methylation, predominantly in the ICM. This may set up major epigenetic differences between embryonic and extraembryonic tissues, including in X-chromosome inactivation and perhaps imprinting. Maintaining epigenetic asymmetry appears important for development as asymmetry is lost in cloned embryos, most of which have developmental defects, and in particular an imbalance between extraembryonic and embryonic tissue development.

Keywords: epigenetic; DNA methylation; chromatin modifications; mammalian embryo; lineage

1. INTRODUCTION

Historically, the earliest indication of epigenetic asymmetry between parental genomes in mammals comes from the studies of X-chromosome inactivation and imprinting. X-chromosome inactivation in extraembryonic tissues of some eutherian mammals is non-random with imprinted inactivation of the paternal X chromosome (Takagi & Sasaki 1975; West *et al.* 1977). Imprinting of the parental genomes during gametogenesis leads to different potentials of the maternal and paternal genomes, respectively, in the development of the embryonic and extraembryonic tissues (Barton *et al.* 1984; McGrath & Solter 1984). This is attributed to the expression of imprinted genes (currently approximately 60 have been discovered in the mouse genome) that have important roles in the development, growth and function of embryonic and extraembryonic tissues (Brannan & Bartolomei 1999; Ferguson-Smith & Surani 2001; Sleutels & Barlow 2002; Reik *et al.* 2003). Imprinted gene expression is controlled by DMRs in or near the genes that receive their parent-specific methylation in the parental germlines (Brannan & Bartolomei 1999; Ferguson-Smith & Surani 2001; Reik & Walter 2001*a*; Sleutels & Barlow 2002). It has been noted that although there are approximately equal numbers of maternally and paternally expressed imprinted genes (http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html), most DMRs are methylated in oocytes, and only very few are in sperm (Reik & Walter 2001*b*; Wilkins & Haig 2002). This is an indication of epigenetic asymmetry that leads to 'indirect' and often complex ways of gene silencing in imprinting, involving antisense RNAs, insulators and silencers (Reik & Walter 2001*b*; Wilkins & Haig 2002). However, this asymmetry between gametes may be confined to DMRs in imprinted genes and thus only applies to a very small fraction of the genome.

There is also epigenetic asymmetry between the extraembryonic tissues (arising in large parts from the TE in the blastocyst and forming the placenta in eutherian mammals) and embryonic tissues (arising exclusively from the ICM). X-chromosome inactivation is random in embryonic but imprinted in extraembryonic tissues. In general, the levels of DNA methylation are much lower in extraembryonic tissues than in embryonic ones (Chapman *et al.* 1984). Other epigenetic marking systems, such as histone methylation and polycomb-dependent silencing, may also have markedly different distributions and functions in embryonic compared with extraembryonic tissues (Wang *et al.* 2001; Mak *et al.* 2002). Epigenetic asymmetrics in the mammalian embryo are shown schematically in figure 1.

2. EPIGENETIC ASYMMETRY IN THE ZYGOTE

The first indication of large-scale epigenetic asymmetry in the zygote was obtained from studies that examined DNA methylation using an antibody against 5 methyl cytosine or bisulphite sequencing of individual genes, including repeated gene families. A dramatic loss of DNA methylation occurs a few hours after fertilization, in different sequences, including single copy genes and Line 1 repeats (Mayer *et al.* 2000; Oswald *et al.* 2000; Barton *et al.* 2001; Dean *et al.* 2001; Santos *et al.* 2002; Shi & Haaf

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One contribution of 14 to a Discussion Meeting Issue 'Epigenesis versus preformation during mammalian development'.

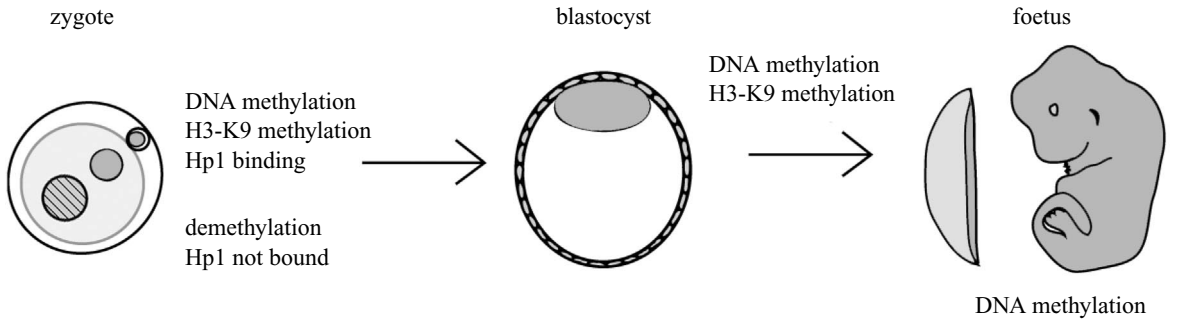

Figure 1. Epigenetic asymmetry in the mammalian embryo. In the zygote, the maternal genome is highly methylated in its DNA and its histones, and the heterochromatin protein HP1 is bound. The paternal genome (dashed) lacks histone methylation and loses DNA methylation rapidly after fertilization. In the blastocyst, an increase in DNA and histone methylation occurs such that the ICM (dark grey) is hypermethylated compared with the TE (light grey). In foetal tissues there are high levels of DNA methylation (pink) but these are much lower in the placenta (grey).

2002; Lane *et al.* 2003). Some sequences, however, such as the DMR of the imprinted gene H19 and the intracisternal A particle family of repeat elements are resistant to demethylation (Olek & Walter 1997; Lane *et al.* 2003). However, a complete catalogue of sequences in the sperm DNA that are methylated and which become or do not become demethylated at fertilization is not yet available. Because this demethylation event occurs before DNA replication, it is thought that active demethylation is involved. The biochemical properties of this active demethylation are unknown; direct demethylation of the cytosine base has been proposed to be carried out by the protein MBD2 (Battacharya *et al.* 1999), but a role for MBD2 in zygotic demethylation is not supported by the observation that demethylation occurs normally in MBD2 knockout zygotes (Santos *et al.* 2002). Other mechanisms proposed from *in vitro* studies include deamination of methylated cytosine followed by mismatch repair, removal of the methylated base by a glycosylase and repair, or nucleotide excision repair (Cedar & Verdine 1999). These mechanisms all need to be investigated in detail; as yet, there is no evidence for large-scale repair processes in the zygote which could be involved in demethylation. Whether the zygotic demethylation is mechanistically related to that occurring in primordial germ cells (between E11.5 and 12.5) is also not known (Hajkova *et al.* 2002; Lee *et al.* 2002). An important difference between the two is that imprinted genes become demethylated in primordial germ cells but not in the zygote. This may reflect differences in the enzymatic process or in the epigenetic marks on the genes (see below).

In most species the sperm genome is complexed with highly basic protamines, and has a minority of the chromatin packaged in histones (Perreault 1992). Protamines are very rapidly replaced by acetylated histones on fertilization (Adenot *et al.* 1997; Santos *et al.* 2002), and these histones are not methylated (at H3 K4, K9, K27; Arney *et al.* 2002; Cowell *et al.* 2002; F. Santos, A. Peters, T. Jenuwein, W. Reik and W. Dean, unpublished data). Towards the very late 1-cell stage, however, paternal chromosomes begin to show some histone methylation, indicating that the oocyte probably contains histone methyltransferases, but the precise kinetics of the acquisition of histone methylation are not known (Cowell *et al.* 2002; F. Santos, A. Peters, T. Jenuwein, W. Reik and W. Dean, unpublished data). In dramatic contrast, the maternal pronucleus is highly methylated in both its DNA and H3 residues (Mayer *et al.* 2000; Barton *et al.* 2001; Dean *et al.* 2001; Arney *et al.* 2002; Santos *et al.* 2002; Cowell *et al.* 2002; figure 2), by DNA (Bourc'his *et al.* 2001*a*; Hata *et al.* 2002) and presumably histone methyltransferases acting during oogenesis. If there is DNA demethylase activity in the oocyte cytoplasm (which is suggested from the observation of some loss of methylation in cloned 1-cell embryos; Dean *et al.* 2001), the maternal genome may be protected from it by the high levels of histone methylation. Alternatively, the paternal genome may be actively targeted for demethylation. The comparison of the chromatin structure of paternally derived sequences that do or do not undergo demethylation will be important, and may give important clues about the mechanism of demethylation. In addition, *de novo* methylation occurring in the female pronucleus shortly after fertilization may further reinforce asymmetry (Oswald *et al.* 2000; Arney *et al.* 2002).

In the mammalian zygote, there is therefore a general (genome-wide) epigenetic asymmetry, with more heterochromatic (repressive) chromatin structures in the maternal genome and more euchromatic (permissive) structures in the paternal one. How long general differences persist and how extensive they are later on is not known. However, it is possible that epigenetic differences between the genomes are not restricted to DMRs in imprinted genes and the X chromosomes, but are more widespread. This would provide an explanation for the high frequency of parental effects observed with various transgenes in the mouse, which, in most cases, results in hypermethylation with maternal transmission and hypomethylation with paternal transmission of the transgenes (Surani *et al.* 1988; Chaillet 1994).

Demethylation of the paternal genome appears to be conserved in eutherian mammals (with the possible exception of the sheep, though this needs to be established more fully; Dean *et al.* 2001; Wilmut *et al.* 2002) but is not observed in *Xenopus* or zebrafish (Macleod *et al.* 1999; Stancheva *et al.* 2002). This phylogenetic distribution led to the suggestion that paternal demethylation has a role in imprinting, acting as a weapon for the maternal genome (present in the oocyte) against paternal germline methylation, which confers increased expression of some patern-

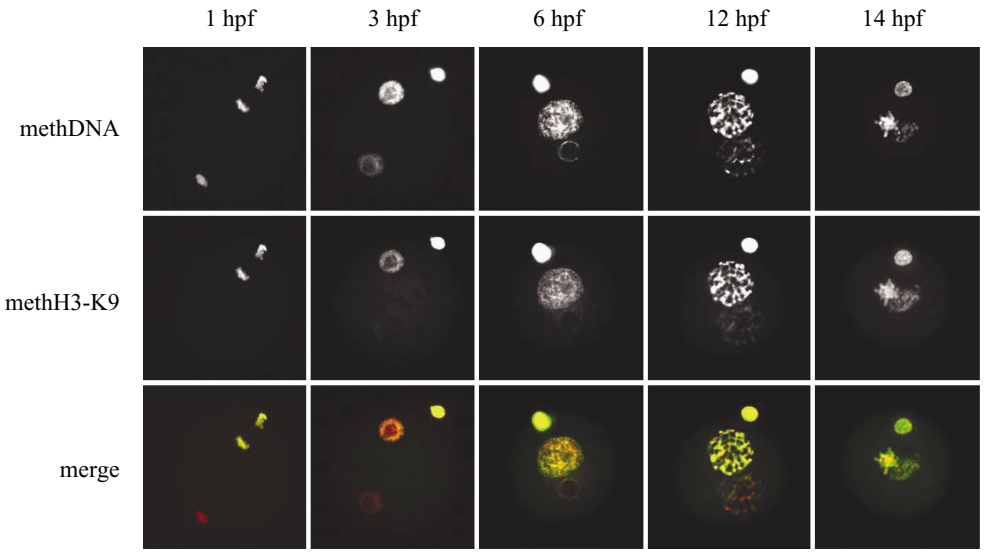

Figure 2. Asymmetric distribution of methylated H3-K9 and DNA methylation during the first cell cycle. Fertilized oocytes were produced from IVF and collected at the specified hours post-fertilization (hpf), fixed and processed for double labelling to assess the patterns of both DNA and histone H3-K9 methylation at these stages. Double labelling of methylated H3-K9 (anti-α-4x-methH3-K9) together with DNA methylation indicate that there is an asymmetric distribution of H3-K9 methylation in the female half of the recently fertilized oocyte. Although DNA methylation is evident at 1 hpf in both the maternal and paternal components, only the female is positive for the methylated H3-K9. By 6 hpf, the paternal DNA has become demethylated and the chromatin remains negative for H3-K9 methylation, whereas the maternal is unchanged. This pattern is maintained throughout the first cell cycle even after replication (12 hpf). After syngamy the chromosomes condense before aligning at the metaphase plate (14 hpf). The residual paternal DNA methylation, located primarily at the centromeres, becomes evident as chromosomes condense. Maternal chromosomes are readily identified owing to the intensity of methylation signal.

ally expressed growth-promoting genes (Bell & Felsenfeld 2000; Constancia *et al.* 2000; Hark *et al.* 2000; Murrell *et al.* 2001; Lopes *et al.* 2003). It would thus be in the 'interest' of the maternal genome to counteract the effect of these genes by demethylation of the sperm genome in the zygote (Reik & Walter 2001*b*). This scenario has been mathematically modelled and it has been demonstrated that such 'modifiers' (e.g. a demethylase in the oocyte) of imprinting would indeed have a selective advantage that could lead to their fixation (Wilkins & Haig 2002), and that it could explain the relative paucity of paternal germline methylation imprints. Another view of the rapid demethylation of the sperm genome is that it is needed for its rapid transcriptional activation.

3. EPIGENETIC ASYMMETRY IN THE BLASTOCYST AND AFTER IMPLANTATION

Reprogramming of these epigenetic patterns in the zygote occurs during cleavage stage development up to the morula and blastocyst stage. DNA demethylation (largely of the maternal genome) takes place because DNA is replicated while the maintenance methyltransferase Dnmt1 is sequestered in the cytoplasm, though its presence in the nucleus at the 8-cell stage is apparently required for the maintenance of methylation imprints in DMRs (Howell *et al.* 2001). Methylation reaches a low point in the morula-stage embryo. Interestingly, at the blastocyst stage, two epigenetically distinct cell populations have been established. TE cells remain fairly low in both DNA and histone methylation although there is an increase from the morula stage. A striking increase occurs in the ICM in which both DNA and H3K9 methylation levels are greatly increased (Santos *et al.* 2002, 2003); *de novo* methylation has obviously begun and it is likely that there is a mechanistic link between histone and DNA methylation as observed in other organisms (Tamaru & Selker 2001; Fuks *et al.* 2003; Jacobs *et al.* 2002). It is also likely that these increases are caused by *de novo* methyltransferases Dnmt3a and b and by various histone methyltransferases including, perhaps, suvar3(9), G9a and the polycomb group enzymes. This increase in repressive epigenetic marks may be necessary for further lineage restriction and differentiation during embryonic development. Why the extraembryonic tissues are largely spared from imposing repressive epigenetic marks is not clear since differentiation is also occurring. However, a simple explanation could be that the placenta is a short-lived tissue in which the long-term stability of differentiation is not important. Consistent with this possibility is the observation that methylation levels are higher in bovine TE than in mouse, and the bovine placenta is longer lived than that of the mouse (Dean *et al.* 2001; Santos *et al.* 2003).

4. EPIGENETIC ASYMMETRY AND LINEAGE COMMITMENT

An important question arising from the observation of epigenetic asymmetry in the blastocyst is whether this is caused by differentiation during blastulation, or whether, perhaps more interestingly, epigenetic asymmetry precedes lineage allocation, and is ultimately traceable to the asymmetry in the zygote. It is possible that epigenetic marks, such as DNA methylation, which initially persist on maternal chromosomes, become diluted as cells divide and the methylated (or rather hemimethylated) alleles of

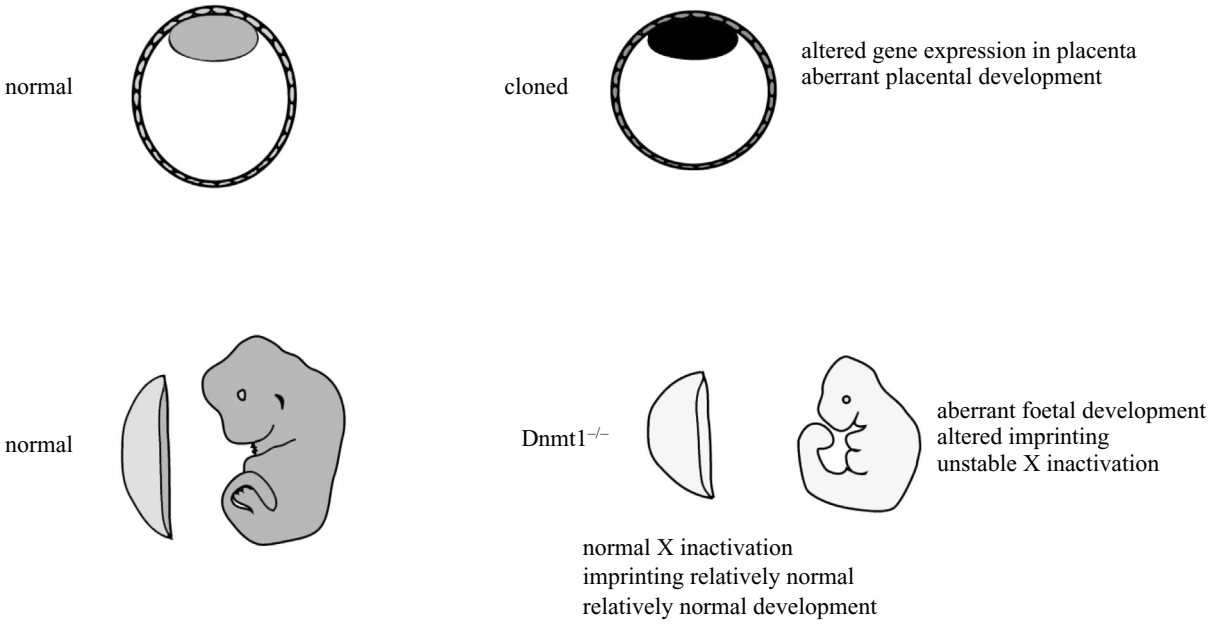

Figure 3. Altered epigenetic asymmetry in the mammalian embryo. The normal blastocyst shows asymmetry with high levels of DNA and histone methylation in the ICM (dark grey), whereas cloned blastocysts (with somatic cell nuclei) have lost the asymmetry and have elevated levels of DNA and histone methylation in ICM and TE (black). The normal foetus has high levels of DNA methylation (dark grey) but these are much lower in the placenta (light grey). In mutants that have lost the activity of the maintenance methyltransferase Dnmt1, methylation levels are reduced in the foetus (white, but much less reduced in the placenta). Foetal development and epigenetic gene regulation is more severely affected than that of the placenta.

any specific locus find themselves allocated to two blastomeres in the morula (Dean & Ferguson-Smith 2001; Dean *et al.* 2003). Thus, if there are specific loci that are sensitive to this methylation and determine cell fate, these two cells may acquire a different cell fate. It is interesting to note that the founder population of the ICM in the morula has been estimated to be 1–2 cells. However, if there is segregation of epigenetic marks it still needs to trigger widespread *de novo* methylation in the descendants of the founder cells.

As noted above, extraembryonic lineages also remain undermethylated later in postimplantation development (Chapman *et al.* 1984; Rossant *et al.* 1986). Are there specific differences in epigenetic marking systems and their function between the two compartments? The features of X-chromosome inactivation, imprinting and cloning suggest that this is indeed the case. At least in the mouse, X-chromosome inactivation in extraembryonic tissues is imprinted with paternal chromosome inactivation. Although Xist is necessary for imprinted inactivation (Lee 2001), no germline methylation imprint has been detected on Xist that would explain the imprinting. Indeed, the polycomb proteins eed and enx (the latter being a histone methyltransferase) coat the inactive X chromosome in trophoblast stem cells (Mak *et al.* 2002), and maintenance of X-chromosome inactivation in extraembryonic tissues requires the eed product (Wang *et al.* 2001), although it is not yet known how these gene products affect X-chromosome inactivation in embryonic tissues. Nevertheless, enx coating of the inactive X chromosome in extraembryonic tissues is consistent with the inactive X chromosome having high levels of H3K27. It is therefore possible that the paternal X chromosome receives a polycomb imprint with subsequent K27 methylation at fertilization, which leads to inactivation in extraembryonic tissues. This would be consistent with the observation of an epigenetic marking process of the paternal genome at fertilization that is independent of DNA methylation (Pickard *et al.* 2001). Strikingly, postzygotic deficiency in Dnmt1 leads to demethylation of Xist (on the maternal chromosome) but not to altered X-chromosome inactivation in extraembryonic tissues (Sado *et al.* 2000), whereas X-chromosome inactivation is affected in embryonic tissues in Dnmt1-mice.

As far as imprinting in extraembryonic tissues is concerned, there are also some interesting differences in comparison with embryonic tissues. There are an increasing number of imprinted genes that are exclusively imprinted in the placenta and not in embryonic tissues (Engemann *et al.* 2000; Zwart *et al.* 2001; Yatsuki *et al.* 2002; Clark *et al.* 2002). Most of these imprinted genes are maternally expressed and do not possess differential methylation (T. Mukai, K. Mitsuya, W. Dean and W. Reik, unpublished data). Interestingly, paternal repression of some of these genes (Mash2, p57Kip2) is relieved, to some extent, in the Eed mutant, although it is not yet known whether this partial loss of repression is limited to extraembryonic tissues (Mager *et al.* 2003). Significantly, we find in Dnmt1-mice that imprinting of several genes in the distal chromosome 7 cluster is not affected in extraembryonic tissues but is affected in embryonic ones (K. Mitsuya, W. Dean and W. Reik, unpublished data), thus extending earlier findings on the Mash2 gene (Tanaka *et al.* 1999). Indeed, at the simplest level, development of extraembryonic tissues is largely unaffected by the Dnmt1 mutation, whereas embryonic development is severely retarded and abnormal, attesting to the relative lack of importance of DNA methylation in extraembryonic tissues.

In addition to DNA methylation mutants, epigenetic asymmetry in the mammalian embryo is also disturbed, in the opposite direction, by cloning. Briefly, cloned embryos are severely deficient in epigenetic reprogramming of the somatic epigenetic marks, characterized by high levels of DNA and histone methylation (Dean *et al.* 2001; Kang *et al.* 2001, 2002; Bourc'his *et al.* 2001*b*; Santos *et al.* 2003). Therefore, cloned preimplantation embryos have aberrantly high levels of these epigenetic marks and in particular, blastocysts retain very high levels of methylation in the TE (Dean *et al.* 2001; Santos *et al.* 2003). We have also detected an association between relatively normal (i.e. asymmetric) patterns of epigenetic marks in these embryos and their developmental potential (Santos *et al.* 2003). Intriguingly, the most consistent developmental abnormality of cloned embryos are malformations of the placentae; X-chromosome inactivation is deficient more frequently in cloned placentae than in embryos (Xue *et al.* 2002), and gene expression is more generally deregulated (silenced!) in cloned placentae than in embryos (Inoue *et al.* 2002). Therefore, this situation seems to represent the opposite of Dnmt1 deficiency; whereas hypomethylation affects embryonic epigenetic regulation and development far more than the extraembryonic one, hypermethylation affects predominantly epigenetic regulation and development of extraembryonic tissues (figure 3).

5. CONCLUSIONS

Epigenetic asymmetry involving DNA and histone modification is present in the mammalian zygote and in the early embryo where it marks the earliest lineage decision, that between extraembryonic and embryonic lineages. Whether there is a mechanistic relationship between zygotic and blastocyst asymmetry is an intriguing question, and one that is important to address experimentally. Upsetting epigenetic asymmetry has detrimental consequences for differentiation and development, from the zygote (Shi & Haaf 2002) and the blastocyst (Dean *et al.* 2001; Santos *et al.* 2003) to later stages of development (Inoue *et al.* 2002).

The authors' work is funded by BBSRC and MRC. H.M. is a C. J. Martin postdoctoral fellow.

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Discussion

R. G. Edwards (*Reproductive BioMedicine Online, Dry Drayton, Cambridge, UK*).We are being taught in our human studies that you have to avoid serum in culture medium in the early embryo because this could lead to imprinting changes with abnormal birth weights or other epigenetic syndromes. How do you know that in the mouse or other animal models the imprinting patterns are as they are *in vivo* when you are studying all your embryos *in vitro*. What precautions do you take to make sure that the observed imprinting patterns are not due to the culture medium rather than normal embryogenesis and could you comment on the relevance of this to work on human embryos *in vitro*?

W. Reik. Some epigenetic marks in mouse embryos have been found to be influenced by *in vitro* culture conditions. So it is important to carry out careful comparisons between *in vitro* cultured embryos and *in vivo* ones; this may enable you to define more precisely the external factors that influence epigenetic marks, and this in turn may enable you to improve culture conditions for human preimplantation embryos.

R. G. Edwards. If you wanted to test a preimplantation embryo for epigenetic abnormalities by removing one blastomere, and leaving the remaining embryo viable, could this procedure itself lead to epigenetic changes?

W. Reik. We do not know this. In principle, any additional manipulation could increase the risk of epigenetic errors but the individual factors cannot be assessed precisely at present.

J. P. Renard (*Laboratoire de Biologie du Developpement et Biotechnologie, INRA, Paris, France*). You show in your talk that epigenetic marking depends on donor cells, and in your nuclear transfer experiments this is correlated with developmental potential. Have you ever tried to pre-treat these donor cells with 5 azacytidine or TSA to see if the difference persists?

W. Reik. We have not done these experiments. They are interesting experiments to do, and I would think that epigenetic marks in clones would be altered as a consequence. I doubt, though, that these treatments would cause radical improvements in cloning efficiency; they are so unspecific that they will probably cause both beneficial and detrimental epigenetic changes. For example, demethylation with 5 azacytidine will likely lead to altered imprinting patterns, which might affect development adversely.

B. Hendrich (*ISCR, University of Edinburgh, Edinburgh, UK*). Why do embryos show parallel patterns of mutually exclusive H3 modifications such as acetylation and methylation?

W. Reik. The embryos can show parallel patterns of histone acetylation and methylation at the same residue (H3-K9) because we are looking at genome-wide patterns. The bulk of the K9 methylation will be found in constitutive and perhaps facultative heterochromatin, whereas the bulk of K9 acetylation will probably be in euchromatic regions. So you can have parallel changes of modifications which on a single histone H3 are of course exclusive, by having these modifications mark different regions of the genome.

GLOSSARY

DMR: differentially methylated region
ICM: inner cell mass
TE: trophectoderm