COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

STATEMENT ON THE COT WORKSHOP ON TRANSGENERATIONAL EPIGENETICS

Introduction

1. As part of the COT's horizon scanning exercise in 2007, the issue of possible transgenerational effects due to epigenetic alterations was raised, a topic which had previously been discussed by the Committee on Mutagenicity in 2006. The COT agreed that transgenerational epigenetic inheritance could potentially mediate a wide range of toxicological effects and was an important area for future research.

2. In February 2008, a one-day workshop on transgenerational epigenetics was held, to enable the Committee to increase its awareness of current knowledge in this area, and to consider possible implications for chemical risk assessment. This report summarises information from the speakers' abstracts, presentations given at the workshop and subsequent discussions.

Epigenetics and the epigenetic code

3. In biology, "epigenetics" is concerned with alterations in phenotype due to changes in cellular properties that may be inherited, but do not represent a change in the DNA base sequence. From a developmental standpoint, it is associated with how a fertilised totipotent zygote progresses, via a series of developmental transformations and inductive processes, into a multicellular embryo and eventually an adult. From a molecular viewpoint it is concerned with how chemical modifications of DNA and histones alter gene function.

4. Epigenetic mechanisms regulate the setting up and maintenance of the two major forms of chromosomal structure; heterochromatin, where the DNA is tightly packed and genes are repressed, and euchromatin where genes are under active transcription. Mechanisms involved in epigenetic regulation of gene expression include DNA methylation and histone tail modifications, while small non-coding RNAs also play a role.

5. DNA methylation at the 5' position of cytosine, typically although not exclusively in CpG DNA sequences within regulatory regions of genes, is associated with gene repression *in vitro* and *in vivo*, while hypomethylation is generally associated with gene expression. Catalysed by DNA methyltransferases (DNMTs), DNA methylation can silence gene expression by directly interfering with the binding of a transcription factor to its recognition element, or indirectly by attracting methyl-

CpG-specific binding proteins such as MeCP2 (reviewed by Ptak and Petronis, 2008[1]). DNMT1 is a 'maintenance' methyltransferase which copies methylation patterns during DNA replication, while DNMT3a and DNMT3b are *de novo* methyltransferases, methylating DNA at previously unmethylated sites[1,2].

6. S-Adenosyl methionine (SAM) provides methyl groups for transfer, and is produced through the folate and methionine cycling pathways, using methionine, choline, folic acid and vitamin B12.

7. Histone modifications such as acetylation, methylation, phosphorylation and ubiquitylation regulate chromatin structure and hence gene expression. Active chromatin is generally characterised by overall hyperacetylation of histones and enrichment of histone H3 trimethylated at lysine 4 (H3K4Me3), di- or tri-methylated at lysine 36 (H3K36Me2/3) and dimethylated at lysine 79 (H3K79Me2), plus DNA hypomethylation. Methylation of Lys 36 and Lys 79 occurs at transcription units whereas Lys 4 is methylated at regulatory regions. Inactive chromatin is characterised by overall histone hypoacetylation, increased levels of H3 trimethylated at Lys 9 (H3K9Me3) and H4 trimethylated at Lys 20 (H4K20Me3), and DNA methylation (reviewed by Turner, 2007[3]). Further, histone modification enzymes can interact with DNMTs and target DNA methylation to chromatin that is already hypo-acetylated, thereby reinforcing gene silencing (reviewed by Meehan *et al.*, 2005[4]).

8. It has been proposed that histone tail modifications, together with DNA methylation, are part of an epigenetic code regulating chromatin structure and gene expression. Semiotics, the study of signs and symbols and their use or meaning, has been used as a guideline for defining the epigenetic code. A semiotic system consists of a sign, its meaning and the code used to interpret the sign. In the case of an epigenetic code the sign would be a combination of histone/DNA modifications and the meaning would be the initiation or termination of transcription at a specific time and cell type. Thus, the code comprises combinations of chromatin modifications that allow the transcriptional status of specific genes to be switched on or off in a particular cell type at a defined stage of development or differentiation[3].

9. The epi-genotype is dynamic and responsive to environmental signals, and it has been proposed that the influence of environment and genotype on the phenotype of an organism may in part be mediated indirectly via the epi-genotype.

10. Epigenetic alterations that arise during the lifetime of an organism are proposed to result from both stochastic processes and systematic environmental influences. Epigenetic marks are in constant flux and the maintenance methyltransferase DNMT1 has been estimated to have a 5% error rate, compounding this flux (reviewed by Whitelaw and Whitelaw, 2006[5]). Environmental influences include dietary factors and maternal behaviour. For example, decreased maternal grooming in rats has been correlated with reduced DNA methylation and histone acetylation within the glucocorticoid receptor gene proximal regulatory unit in the hippocampus, and induction of altered stress responses in later life. Infusion of a histone deacetylase inhibitor reversed these effects[6]. Dietary supplementation with methyl donors has also been shown to induce epigenetic alterations in animal models[7].

11. Epigenetics has been suggested to be involved in a range of complex diseases that arise from a combination of heritable and environmental factors, including cancer, metabolic syndrome, anxiety and depression, schizophrenia and bipolar disorder (reviewed by van Vliet *et al.*, 2007[2]).

Evidence for transgenerational epigenetic inheritance – animal studies

12. While transmission of acquired epigenetic changes to subsequent generations has been well documented in plants[8], in mice epigenetic reprogramming is associated with a global decrease in methylation levels at two developmental periods, during gametogenesis and during early embryogenesis followed by *de novo* methylation [9], suggesting that acquired epigenetic changes should not be inherited. However, there is robust evidence that epigenetic information can be inherited across generations in mammals (see below and Chong *et al.*, 2007[10]).

13. Epigenetic inheritance has been clearly demonstrated through to the F1 generation in two mouse models; Agouti viable yellow (A^{vy}) and Axin-fused ($Axin^{Fu}$).

14. The A^{vy} allele contains an intracisternal A particle (IAP) retrotransposon upstream of the *agouti* gene, which encodes a signalling protein that causes hair follicle pigment production to switch from eumelanin, which is black, to phaelomelanin which is yellow. When the IAP in the A^{vy} allele is unmethylated, a promoter drives ectopic *agouti* expression resulting in a yellow coat colour, and these mice have a predisposition for development of obesity and diabetes. When the IAP is methylated, *agouti* expression is not induced, mice have a brown coat and are phenotypically normal. Genetically identical mice heterozygous for the A^{vy} and *a* (the *nonagouti* allele that does not produce functional agouti protein) alleles display a wide range of coat colour phenotypes from yellow to mottled to brown ('pseudoagouti'), depending on the level of methylation at the A^{vy} IAP. The A^{vy} alleles with differing levels of methylation are referred to as 'epialleles'[11,12,13].

15. Dams with a hypomethylated A^{vy} allele (yellow coat phenotype) produce yellow and mottled offspring but no pseudoagouti (hypermethylated) offspring, while pseudoagouti dams produce 20% pseudoagouti offspring[11].

16. Axin regulates embryonic axis formation in vertebrates, and the *axin-fused* ($Axin^{Fu}$) allele is a dominant gain of function allele that has an IAP retrotransposon inserted. The $Axin^{Fu}$ phenotype is a kinked tail, but in some mice the tails appear completely normal. The phenotype has been shown to be correlated with methylation at the IAP long terminal repeat (LTR). The IAP is heavily methylated in mice without the tail kink, while in mice with a kinked tail the region is relatively hypomethylated. $Axin^{Fu}$ transgenerational epigenetic inheritance has been shown to occur with both maternal and paternal transmission[14].

17. Epigenetic inheritance at the A^{vy} and $Axin^{Fu}$ alleles appears to be influenced by the genetic background of the mouse strain in which it is present. A^{vy} in the C57BL/6J strain displays transgenerational epigenetic inheritance after maternal transmission only[11], whereas $Axin^{Fu}$ displays inheritance after maternal and paternal transmission in the 129RrRk/J strain[14]. However, cross-over studies have shown that inheritance of A^{vy} via paternal transmission does occur when C57 males are crossed

with 129 females, while paternal *Axin*^{Fu} transmission does not occur when 129 males are crossed with C57 females[14].

18. This finding suggests that the epigenetic 'mark' is reprogrammed by the C57 egg but not by the 129 egg. The factor that causes this reprogramming is unknown, but could potentially be any protein that influences reprogramming in early embryogenesis. Genetic variation in such factors would also be expected to influence epigenetic inheritance at other loci. As such events are known to have strain-specific liabilities it is probable that species-specific confounders also exist.

19. In the case of environmental factors that affect the epigenome, confirmation of transgenerational inheritance requires observation of effects in at least the F3 generation. This is because when an F0 pregnant female is exposed to an environmental agent, the F1 generation embryo and the germline of the F2 generation are also directly exposed.

20. Environmental factors have been shown to influence the epigenetic code in the F1 generation in several animal studies. Dietary supplementation of pregnant female *A*^{vy} mice with methyl donors and co-factors resulted in a darker average coat colour and systemic *A*^{vy} hypermethylation in their *A*^{vy}*/a* offspring[7]. However, a follow-up study indicated that the effect did not accumulate across the F2 and F3 generations, suggesting that the diet-induced *A*^{vy} hypermethylation is not inherited transgenerationally through the female[15].

21. In sheep, administration of a hypomethylating diet containing low levels of vitamin B12 and methionine from 8 weeks preceding to 6 days following conception resulted in a range of effects in adult offspring. Effects observed included increased weight, altered body composition (increased fat and reduced muscle), altered immune responses to antigenic challenge, immune resistance and elevated blood pressure[16]. Analysis of CpG islands in fetal liver indicated that methyl-deficient animals had alterations in methylation status at a number of loci compared with controls.

22. The only reported example of transgenerational transmission of effects on the epigenetic code elicited by an environmental agent in animals is that of the anti-androgen vinclozolin. Intraperitoneal (i.p.) exposure of pregnant F344 rats to vinclozolin during the time of fetal sex determination (embryonic days 8-14) has been shown to result in a range of adverse effects in male offspring of the F1-F4 generations, transmitted through the male germ line. The effects observed include spermatogenic defects, and in adults, male infertility, prostate disease, kidney disease, immune system abnormalities, hypercholesterolemia and an increased rate of tumour development[17,18,19].

23. The frequencies of these abnormalities ranged from 20-90%, suggesting that mutations in the DNA sequence are not likely to be responsible. The frequency of germ line DNA sequence mutations, even with ionising radiation, has been estimated as being normally less than 0.01% and ranging from only 1-5% for hot-spot mutations in the F0-F2 generations (reviewed by Jirtle and Skinner, 2007[20]). It was therefore suggested that an epigenetic mechanism may be involved, and in support of this several genes and other DNA sequences in the sperm of vinclozolin-treated animals

were identified that had altered methylation patterns in the F1-F3 generations[17,21]. In initial reports the anti-androgen methoxychlor was also found to promote male infertility and decreased spermatogenic capacity in the F1 and F2 generations, although effects on DNA methylation with this compound have not been reported.

24. Recently, additional effects have been reported in the offspring of vinclozolin-treated rats. Females showed a mate preference for males from an unexposed lineage over those of the vinclozolin lineage, whereas males exhibited no such preference for female type[22]. These findings led the authors to hypothesise that transgenerational epigenetic inheritance may represent an 'unappreciated force in sexual selection'. Effects in F1-F3 female progeny have also been reported, including uterine haemorrhage and/or anaemia late in pregnancy, glomerular abnormalities and a statistically non-significant increased incidence of tumours compared with controls[23]. While the effects in males were transmitted through the male germline, both male and female parents had to be of vinclozolin-exposed lines for female offspring to show the pregnancy disease phenotype.

25. The above findings have yet to be reproduced by other laboratories or for other chemical exposures. While the effects described occurred following i.p. administration of high doses (100 mg/kg) of vinclozolin, an abstract presented at the Society of Toxicology annual meeting in 2007 reported no adverse transgenerational effects on sperm number, morphology and motility in F1-F3 generations following oral administration, also at 100 mg/kg b.w., to pregnant rats[24]. However, this study was conducted in a different rat strain (Wistar) from the earlier studies (F344), and the genetic background may have had an influence on the findings. An abstract presented at the 2007 meeting of the International Congress of Toxicology by Kawabe *et al.*[25] reported no effects on spermatogenesis in the F1 generation of Crl:CD(SD) rats, and no alterations in DNA methylation in testes and sperm in the F0, F1 and F2 generations, following i.p. administration of vinclozolin (100 mg/kg b.w.), procymidone (100 mg/kg b.w. i.p.) and flutamide (10 mg/kg b.w. i.p.) on gestational days 8-15.

26. The nature of the inherited epigenetic 'mark' that might mediate transgenerational effects is unclear. There is some evidence that IAPs are largely resistant to demethylation during gametogenesis and early development,[26] but a study in A^{vy} mice showed that methylation at the IAP is cleared during early embyogenesis and then reset[15]. This suggests that histone modifications or RNA-mediated mechanisms may play a role. To date, it is uncertain whether genetic elements other than IAPs, such as promoters, enhancers or other retro-elements may also be sites of epigenetic inheritance. IAPs are transposon units rather than part of the 'normal' genetic code and it is unclear whether effects seen at these sites would also occur in native genomic DNA.

27. RNA may also play a role in mediating transgenerational epigenetic effects. In a recent study, genotypically wild type offspring of heterozygous $Kit^{tm1Alf/+}$ mice were found to display the white-spotted phenotype characteristic of the heterozygous animals[27]. The spermatoza of these paramutated offspring contained unusual amounts of RNA. Furthermore, the white-spotted phenotype could be reproduced in non-paramutated wild type offspring by microinjection of sperm RNA from $Kit^{tm1Alf/+}$ heterozygotes, or microRNAs that target *Kit* mRNA, into fertilised eggs.

Evidence for transgenerational epigenetic inheritance – human studies

28. Conclusive evidence of epigenetic inheritance in humans is currently lacking. A study of a family affected by hereditary nonpolyposis colorectal cancer (HNPCC) reported a germline allele-specific hypermethylation of the DNA mismatch repair (MMR) *MSH2* gene, without evidence of DNA mismatch repair mutation, in three successive generations[28]. Several family members with this methylation developed colorectal cancer or other HNPCC-related cancers. While it has been suggested that this is an example of transgenerational epigenetic inheritance, it has also been argued that the 'epimutation' could be the result of an underlying modifying genetic mutation that causes the hypermethylation of the *MSH2* gene to be re-established in each generation[10]. It is therefore uncertain whether the epigenetic pattern is causative of the observed predisposition to cancer, or consequential of some other underlying factor.

29. Suter *et al.*[29] reported two individuals with soma-wide, allele specific and mosaic hypermethylation of the MMR gene *MLH1*. Both individuals lacked evidence of mutation in any MMR genes, but had multiple primary tumours showing deficiency in MMR and met the clinical criteria for HNPCC. The epimutation was detected in the spermatozoa of one of the individuals, suggesting a germline defect and potential for transmission to children. However, recent reanalysis of the spermatozoa sample with more sophisticated techniques has indicated that the *MLH1* methylation detected was most likely derived from residual somatic DNA in the sample, rather than present in male germ cells[30].

30. Epigenetic inheritance has also been proposed as a possible mechanism underlying transgenerational responses to smoking and nutrition observed in human populations. Analysis of individuals recruited into the Avon Longitudinal Study of Parents and Children (ALSPAC) indicated that early paternal onset of smoking was associated with greater body mass index at age 9 in sons, but not in daughers[31]. A trend for lower gestation length with earlier paternal smoking was also observed, in boys but not girls.

31. Sex-specific transgenerational effects in a cohort of individuals born in Överkalix, Sweden in 1890, 1905 and 1920 have also been reported. The paternal grandfather's food supply, estimated from historical data on harvests and food prices, was linked to the mortality risk ratio (RR) of grandsons, while the paternal grandmothers' food supply was associated with granddaughters' mortality RR[31,32]. No associations were found between paternal grandfathers' diet and granddaughters' mortality RRs, or between paternal grandmothers' and grandsons' mortality. Analysis suggested that particular periods of exposure were critical. Poor grandfather's or grandmother's food supply during the slow growth period before puberty (8-12 years) was associated with reduced mortality RRs for grandsons and granddaughters, respectively while good food supply at this time was associated with higher mortality RRs. The paternal grandmothers' food supply from when she was a fetus to age 4 years had the opposite effect, with good or poor supply correlating with lower or higher mortality RRs, respectively.

32. Transgenerational effects associated with exposure to betel nuts have been reported in both humans and animals. In CD1 mice, paternal exposure to betel nut

was associated with an increased risk of hyperglycaemia in non-betel fed F1 offspring[33], while an epidemiological study in Taiwan reported paternal betel nut chewing was associated with an increased risk of early onset of metabolic syndrome in offspring[34].

33. The mechanisms responsible for these possible sex-line specific transmissions have not yet been identified, but it has been postulated that they may be mediated by a signal, possibly epigenetic, carried on the X and Y chromosomes. It is hypothesised that the non-recombining region of the Y chromosome can transmit environmentally-induced epigenetic states or reversible DNA changes to subsequent generations. Father to son and paternal grandfather to grandson effects could be mediated by the Y chromosome, while the X chromosome passed by a woman to her son can only be passed to her granddaughters, not grandsons[31]. However, evidence demonstrating a role for epigenetics in mediating these effects is currently lacking.

Implications for risk assessment

34. The possibility that environmental exposures during pregnancy or in the neonatal period could result in epigenetic alterations that lead to adverse effects in the F1 generation or even beyond is gaining attention. The hypothesis is being tested by the new, emerging field of investigation known as Environmental Epigenomics.

35. Given that this field is in its infancy and the analytical techniques used to assess epigenetic effects are still evolving, it has been suggested that it is premature to conclude that epigenetic evaluations should be incorporated into chemical risk assessment at this time[35]. There are a number of questions which need to be addressed. Questions raised at the workshop include:

- Do we know enough about the available animal models, such as A^{vy} and $Axin^{Fu}$, in order to properly interpret the data they generate? For example, transposable elements such as IAPs are expected to be methylated, and it is uncertain whether there is something unusual about the Agouti and Axin model IAPs that allows them to be hypomethylated.

- How do we assess whether an epigenetic change is adverse?

- Epigenetic mechanisms include DNA methylation, histone alterations and effects of non-coding RNAs and it is uncertain which of these mechanisms play a role in transgenerational epigenetic inheritance. Is there a need to evaluate all three of these parameters? It is also important to consider what technique(s) would best be employed.

- It is important to consider normal epigenetic variability, from individual to individual and over time. There are also species differences in relative epigenetic stability. For example, stability of methylation in c-myc in the liver and resistance to X chromosome reactivation during aging is greater in humans than in mice[36,37].

- In addition to the agouti and axin models, are there other endpoints, such as imprinted genes, that should be evaluated? What model compounds should be used?

- The finding that maternal grooming behaviour can have epigenetic effects (see para 18) indicates that it is important to take parental behaviour into consideration.

36. There are also questions as to whether current regulatory toxicity testing would be sufficient to detect transgenerational epigenetic effects on phenotype. Comparison of the data generated from regulatory studies for developmental, reproductive and endocrine toxicity of vinclozolin with those generated in the studies on its transgenerational effects suggests that the regulatory tests would adequately detect the effects of vinclozolin on the androgen receptor, fertility, reproductive organ development, male genitalia and anogenital distance. However, multi-generation assays for reproductive toxicity would not predict that treatment of the F0 generation only could produce testicular abnormalities up to the F4 generation, as dosing is continuous across generations in these studies (NB: these observations are being questioned, see para 25 above). In addition, the adult onset increase in prostate, kidney and immune system lesions detected in the transgenerational studies is unlikely to be picked up by regulatory reproductive studies, as these are generally terminated after weaning or mating. Alternative strategies to detect the potential for such effects without requiring testing up to the F3 or F4 generations may need to be developed.

Discussions

37. Discussion at the workshop predominantly centred on the implications of the data presented for risk assessment. These discussions are summarised below.

38. Opinions vary on the most appropriate approach for assessing the potential for transgenerational epigenetic inheritance following chemical exposures. Focussing on one or two well characterised imprinted genes involved in transcription has been proposed, but there is no evidence that such genes are those most likely to be modified by environmental exposures, or that epigenetic alterations at these genes will have a significant functional impact.

39. Results from the A^{vy} and $Axin^{Fu}$ animal models should be treated with caution, particularly given the reported strain differences in transmission of epigenetic states.

40. An alternative approach may be to use techniques that measure effects on the whole epigenome to search for candidate genes or modifications on which to focus. However, while a broad approach may be useful, it is important to consider the possibility for ‘epi-phenomena’ – epigenetic changes can occur that have no effect on phenotype. For example, much of the bulk histone that can be analysed will be non-coding and it is possible to get massive changes in bulk histone acetylation without much change in gene expression. It is therefore critical to establish what regions of

the epigenome are important. It will also be important to consider the lessons that can be learnt from experience with other 'omic' technologies.

41. Confirming whether transgenerational epigenetic inheritance occurs in humans will be extremely difficult and will require complementary studies in model organisms. A suggested approach is first to identify an environmental insult or influence resulting in an alteration in phenotype; then determine the location and nature of an epigenetic change and establish a link between epigenotype and phenotype. It will also be necessary to rule out other genetic factors or a familial effect such as constant exposure to an environmental factor. The need to study through to the F3 generation was reiterated, as effects in the F1 or F2 generations could be due to *in utero* exposure. A potential problem with this approach is that most differences in phenotype will not be inherited.

42. In addition to identifying critical regions of the epigenome to assess and applying the appropriate technique(s) for analysis, it is important to gain an understanding of the background variation at such regions, both inter- and intra-species. Similarly, there is a need to gain an understanding of background variation that can arise with time; for example, it has been demonstrated that that epigenetic differences between monozygotic twins increase over a lifetime[38].

43. There is some evidence of epigenetic effects in tumour suppressor genes associated with cancer, although it is unclear whether such changes are causal. It was suggested that it may be useful to investigate whether there are environmental causes for these changes.

44. Presuming that transgenerational epigenetic effects can be shown to occur, it was proposed that it will be important to identify whether such effects should be viewed as being thresholded or not. Experiments to define the shape of the dose-response would be necessary in order to determine this.

45. Overall, it is clear that a stronger science base is required before evaluation of epigenetic status can be included in regulatory risk assessment.

COT Conclusions

46. There is reasonable evidence that epigenetic changes associated with environmental exposures during development can result in adverse effects. Such effects might be detected in the F1 and F2 generations by standard regulatory toxicity testing.

47. Transgenerational epigenetic inheritance of effects in the F3 generation and beyond would also be of potential relevance to risk assessment. If epigenetic inheritance does occur, it is possible that this could lead to an accumulation in risk across generations. In addition, such epigenetic changes could be developed as biomarkers of effect.

48. However, the science is not yet developed and therefore assessment of transgenerational epigenetic inheritance cannot be incorporated in regulatory risk assessment at present.

49. It is still unclear whether transmission of environmentally acquired epigenetic changes across generations occurs in humans and if so, what mechanisms of epigenetic modification are important,

50. Priorities for future research include assessment of whether important examples of epigenetic inheritance seen in animals also occur in humans. In addition, it may be useful to investigate aberrant phenotypes in humans which might possibly have a transgenerational, epigenetic basis. It is feasible to undertake genome wide profiling to ascertain if changes in DNA methylation patterns underlie environmentally acquired epigenetic changes that occur in experimental models and perhaps human populations.

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