Epigenetic inheritance in the mouse

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Acquired epigenetic modifications, such as DNA methylation or stable chromatin structures, are not normally thought to be inherited through the germline to future generations in mammals [1,2]. Studies in the mouse have shown that specific manipulations of early embryos, such as nuclear transplantation, can result in altered patterns of gene expression and induce phenotypic alterations at later stages of development [3–5]. These effects are consistent with acquired epigenetic modifications that are somatically heritable, such as DNA methylation. Repression and DNA methylation of genes encoding major urinary proteins, repression of the gene encoding olfactory marker protein, and reduced body weight can be experimentally induced by nuclear transplantation in early embryos [4]. Strikingly, we now report that these acquired phenotypes are transmitted to most of the offspring of manipulated parent mice. This is the first demonstration of epigenetic inheritance of specific alterations of gene expression through the germline. These observations establish a mammalian model for transgenerational effects that are important for human health, and also raise the question of the evolutionary importance of epigenetic inheritance.

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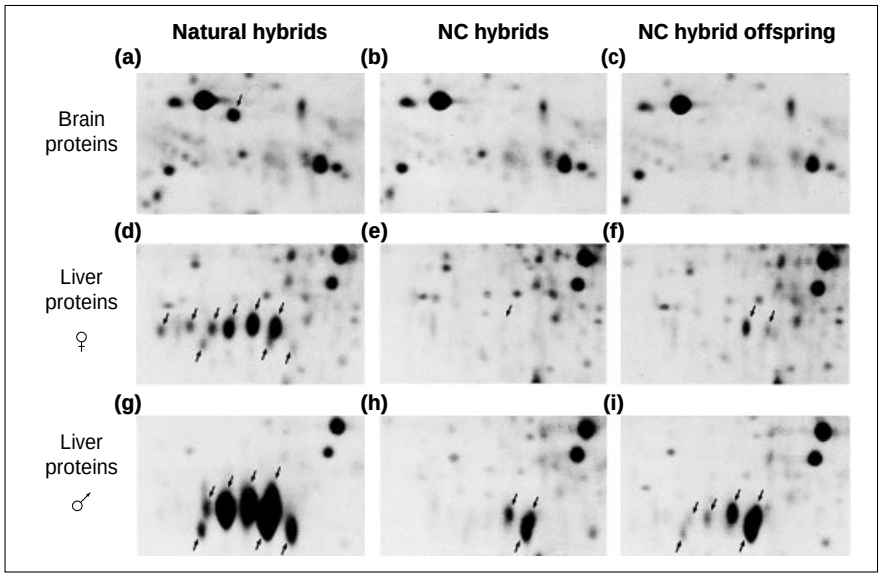
Results and discussion

In a previous study, we exposed mouse pronuclei at the one-cell stage to an altered cytoplasmic environment by transferring them to eggs of a different genotype. We observed that the resulting nucleocytoplasmic (NC) hybrids had an altered pattern of gene expression as revealed by two-dimensional electrophoresis of protein extracts from adult liver [4]. The major group of liver proteins to be repressed was identified by microsequencing as belonging to the major urinary protein (MUP) family; repression was at the level of transcription of group 1 *Mup* genes, and was associated with an increased level of DNA methylation [4]. We have now extended these studies to the examination of protein expression patterns in heart (data not shown) and brain (below). Comparison of NC hybrid animals with control animals revealed that there were a number of quantitative alterations of protein spots amongst thousands analyzed and one additional spot was almost completely repressed in the brains of NC hybrid animals (Fig. 1). This polypeptide was identified by microsequencing to be the olfactory marker protein (OMP). The combined results of MUP expression, *Mup* gene methylation, and OMP expression in NC hybrids and control hybrids is summarized in Table 1. Overall, 96% of NC hybrid animals had an altered phenotype.

Figure 1



Sections from two-dimensional electrophoresis patterns [37] of mouse tissue proteins. Liver, heart (data not shown) and brain protein extracts from adult animals were separated by high resolution two-dimensional electrophoresis [37] and patterns were compared for experimental and control groups. In liver, ~10 000 spots were examined, in heart muscle 4 000, and in brain 8 448 (counted by laser densitometry and computer analysis). **(a–c)** Brain proteins: the arrowed spot is present in natural hybrids (C57BL/6 × DBA/2) (a), but completely absent in NC hybrids (BDB) (b) and in NC hybrid offspring (c). Sequencing of peptides from this brain protein (LIRPAES, VYRLDFIQQQ, VMYFLITFGEGVEP, ASVVFNQL; single-letter amino-acid code) identified it as OMP. **(d–i)** Liver proteins from (d–f) female and (g–i) male mice: the arrowed spots were previously identified by partial sequencing as MUPs, and are present in natural hybrids (C57BL/6 × DBA/2) (d,g), but substantially reduced in NC hybrids (BDB) (e,h) and in NC hybrid offspring (f,i). In addition to OMP and MUP shown in this figure, there were 11 spots in liver, 5 spots in heart and 12 spots in brain that showed quantitative differences between experimental and control groups.

Members of the MUP family of proteins are secreted into the urine and are thought to mediate sexual recognition by binding to pheromones [6] and can cause acceleration of the onset of puberty [7]. By contrast, OMP is expressed in the nasal mucosa and transported to the olfactory bulb [8], and is involved in presenting pheromone-binding molecules, such as MUP, to the olfactory system as an olfactory sensory neuron modulator. It is therefore intriguing that both proteins were affected by the altered epigenetic programming in the early embryo. Another phenotype that was noted was the growth deficiency of adult male NC hybrids (females were not tested) [4]. These results clearly show that aberrantly induced epigenetic events in the early embryo can have long-lasting effects on gene expression and the adult phenotype. It should be noted from Table 1 that a small proportion (5%) of control animals (embryos cultured from the one-cell to the two-cell stage and transferred) also showed these altered phenotypes. This finding may indicate that the extensive remodelling of chromatin which occurs upon fertilization may be involved in these phenotypic alterations.

Offspring from some of the NC hybrids also had altered phenotypes. We therefore designed a systematic breeding experiment to investigate MUP and OMP expression, and *Mup* methylation, in offspring of NC hybrid males backcrossed to C57BL/6 females. Transmission through the male germline was chosen because it is well-established that maternal constraints (such as disease or small size) can produce compromised offspring in the absence of other transmissible factors [9]. The results of this experiment are shown in Table 1. More than 50% of B_1 (first backcross generation) offspring showed reduced MUP and OMP expression, and increased *Mup* methylation (Figs 1,2); other quantitative alterations of protein spots (see Fig. 1 legend) were not consistently transmitted to B_1 animals. Furthermore, body weight of the progeny of NC hybrid males was also significantly reduced at postnatal day 35 (Fig. 3).

The difference in body weight between the experimental B_1 animals and the control B_1 animals appeared between days 14 and 15 of gestation (Fig. 3). In addition, significant mortality of experimental B_1 offspring occurred principally between birth and weaning age (day 21; B_1 experimental offspring rate of survival to weaning = 39%, n = 141 newborns from 29 litters; B_1 control offspring rate of survival to weaning = 92.3%, n = 142 newborns from 20 litters). Postnatal mortality in the original NC hybrid animals was less pronounced (NC hybrid rate of survival = 70%, n = 36). Hence, all of the phenotypes seen in the original NC hybrid animals can be transmitted to first-generation offspring through the male germline. Interestingly, some affected control animals that were tested also transmitted altered phenotypes to offspring (data not shown), suggesting that transmission is to some extent independent of the precise mechanism of induction of the altered phenotype.

Table 1

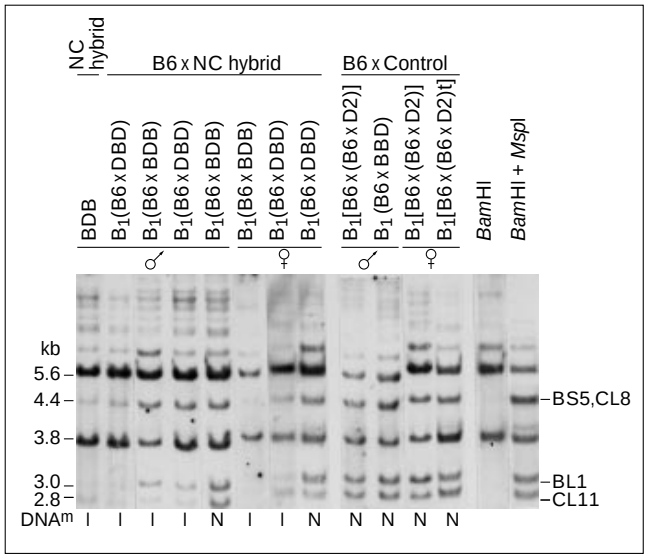
Protein expression and DNA methylation of *Mup* genes in liver, and protein expression of *Omp* genes in brain of adult NC hybrids and control mice, and mice obtained by backcrossing NC hybrid males with C57BL/6 females.

	Number of animals investigated							
	OMP protein		MUP protein*		*Mup* methylation*		Total number of animals†	
Mice	R	N	R‡	N	I§	N	A	N
NC hybrids:							27	1
BDB	10	0	16	0	18	0		
DBD	1	1	5	1	8	1		
Control hybrids:							3	60
BBD	0	5	0	8	0	11		
F_1 (D2 × B6)t	0	3	0	12	0	13		
F_1 (B6 × D2)t	2	8	2	8	2	15		
F_1 (D2 × B6)	0	2	0	8	0	4		
F_1 (B6 × D2)	–	–	0	7	0	4		
Parental strains:							0	65
D2	0	10	0	12	0	7		
B6	0	20	0	18	0	9		
Backcrosses:								
B6 x NC hybrids:							33¶	10¶
B_1 (B6 × BDB)	14	1¥	8	7	10	8		
B_1 (B6 × DBD)	16	3¥	17	8	17	8		
B6 x control hybrids:							0	44#
B_1 (B6 × BBD)	0	11	0	11	0	11		
B_1 [B6 × (B6 × D2)t]	–	–	0	9	0	9		
B_1 [B6 × (D2 × B6)]	0	8	0	8	0	8		
B_1 [B6 × (B6 × D2)]	0	16	0	16	0	16		

All animals were 12–15 weeks old at analysis. *These data include some from a previous paper for the hybrid but not the backcross experiments [4]. †The figures indicate the total number of animals analyzed, but not all animals were analyzed for all three phenotypes. ‡As far as investigated, in all cases in which MUP expression was repressed in an animal, OMP expression was also repressed in that animal. §As far as investigated, in all cases in which *Mup* methylation was increased in an animal, MUP expression was repressed in that animal. ¥These four animals were normal for both MUP and OMP expression. ¶Nine litters from five different NC hybrids. #12 litters from 7 different hybrids. Abbreviations: R, repressed; N, normal; I, increased; A, affected. The generation of NC (BDB and DBD) hybrids and control hybrids has been described [4]. Control hybrids are defined as BBD; embryo transferred (t; usually isolated at one-cell stage, cultured to two-cell stage, and transferred into day 1 recipients); and natural. Briefly, B stands for the C57BL/6 genotype and D for the DBA/2 genotype. The first letter denotes the origin of the egg cytoplasm, the second one the female pronucleus, and the third one the male pronucleus: BDB, therefore, is a reconstituted egg with a C57BL/6 type cytoplasm, DBA/2 female pronucleus, and C57BL/6 male pronucleus. The phenotypes of OMP and MUP expression of all animals in this table were determined by two-dimensional electrophoresis (see Fig. 1). DNA methylation of *Mup* was determined by methylation-sensitive restriction enzymes and Southern blotting [4] (see Fig. 2). Blots were scanned and 'increased' and 'normal' methylation patterns were defined from the quantitative methylation indices as explained in Fig. 2. Crosses are indicated in the order female × male.

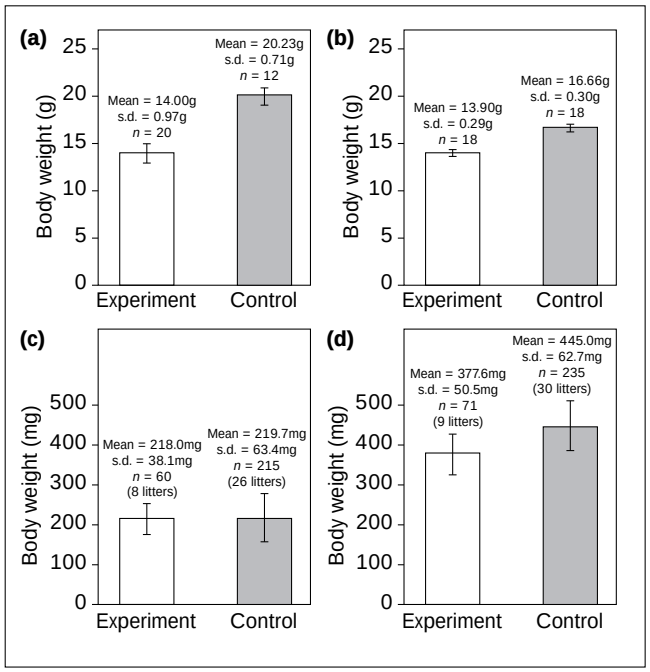
To our knowledge, this is the first male-lineage transgenerational effect in the mouse for which specific alterations in gene expression have been reported. Examples of epigenetic inheritance have been found in yeast [10–12], filamentous fungi [13], plants [14–17] and *Drosophila* [18], but in mammals the only other well-established observations appear to be the epigenetic inheritance of transgene methylation patterns [19–23], and the epigenetic inheritance of altered states of the *fused* mutation in the mouse [24]. It is interesting to note that, at least in one of the transgenic examples, methylation increases stepwise from generation to generation and thus leads to progressively more 'severe' phenotypes [22].

Figure 2



Methylation patterns of *Mup* genes obtained from B_1 mice of the backcrosses B6 × DBD and B6 × BDB, from an NC hybrid male (BDB), and from controls. Males and females were investigated. Note that there are sex-specific differences in methylation of some *Mup* genes [4], which is why we show both male and female methylation patterns. DNA samples from adult liver (10 μg) were digested with *Bam*HI and *Msp*I or the methylation-sensitive restriction enzyme *Hpa*II, as recommended by the supplier (NBL). Digested samples were electrophoresed through 0.8–1% agarose gels and blots were hybridized and washed at high stringency [38]. The inserts of the probe BS655 [39] were digoxigenin-labelled by random priming [40]. The detection of digoxigenin-labelled nucleic acids by enzyme immunoassay with luminescence on nylon membranes was performed following the standard procedure described in the manual of the DIG luminescence detection kit (Boehringer-Mannheim, Germany) with some modifications: the detection procedure was modified by doubling the hybridization volume and the number of blocking, washing and incubation steps. The organization of group 1 *Mup* genes, restriction enzyme sites and the probe used have been described elsewhere [4]. *Bam*HI–*Msp*I fragments diagnostic for the *Mup* genes BS5, CL8, BL1 and CL11 are indicated on the right, and DNA marker sizes on the left. Abbreviations: DNA^m, DNA methylation; N, normal; I, increased. The mouse types are explained in the legend to Table 1. All gels were scanned and methylation indices were calculated from the ratio of uncut (methylated) fragment to cut (unmethylated) fragment. The indices were: CL11 (3.8 kb → 2.6 kb); BL1 (3.8 kb → 3.0 kb); BS5, CL8 (5.6 kb → 4.4 kb). For each gel, ratios of these indices were calculated between NC hybrid animals and controls. Because BS5, CL8 and BL1 show sex-specific methylation differences [4], the CL11 index was used to define 'normal' and 'increased' methylation. The control group had a mean of 1 and a maximum of 1.5. Animals in the experimental group were described as having 'normal' methylation if they fell into this range, and as having 'increased' methylation starting from an index of 2 and ranging up to 10.

Figure 3



Growth deficiency of NC hybrid offspring. The histograms show body weights (mean ± standard deviation) of B_1 male mice (**a**) and B_1 female mice (**b**) on postnatal day 35 of the B6 × DBD and B6 × BDB backcross (Experimental), and B6 × BBD controls (Control); and of B_1 embryos and B_1 controls on embryonic days 14 (**c**) and 15 (**d**). The differences in weight in (a,b) were significant by Welch-test ($p < 0.01$). The difference between experimental and control B_1 mice continued to be significant ($p < 0.05$) on days 40 and 60 in the male group, but not in the female group. Variances pooled between male and female groups were significantly higher in the experimental than the control group ($p < 0.05$ on day 35), suggesting that the differences between the experimental and control groups could be due to the presence of normal-sized and significantly smaller animals in the former group. A bimodal distribution, however, could not be fitted to the data. (c,d) The difference in body weight between the experimental and control B1 groups apparently arises between embryonic days 14 and 15 (variance analysis $p < 0.001$).

The very high incidence of the phenotypic changes seen here in NC hybrids would appear to exclude mutations at the DNA level from being responsible for the transmission of altered phenotypes to offspring. Rather, some form of epigenetic inheritance is likely to be responsible. The mechanism of this epigenetic inheritance is unknown at present. The only mechanism for epigenetic inheritance described so far is that of DNA methylation of transgenes. The simplest model would therefore be that epimutations — heritable alterations of gene expression that are not based on sequence change [25] — in the form of altered methylation patterns, in for example the *Mup* genes, are transmitted through male gametogenesis. B_1 offspring from NC hybrids were therefore examined for linkage of the aberrant *Mup* phenotype (repression of expression) to the *Mup* locus on chromosome 4, but no evidence for linkage was obtained (data not shown). However, assuming that epimutations at specific loci are responsible for the inheritance patterns observed, and that their frequency of reversion is low, a systematic mapping experiment could be carried out in the backcross or in other appropriate crosses. It should be emphasized that such a systematic study should be done in parallel in a specific pathogen-free environment. Our experiments were carried out in an open facility and, therefore, involvement of pathogens in the phenotypes described in this study cannot be excluded. Preliminary results suggest that altered phenotypes can be transmitted further from the B_1 to the B_2 generation, but a systematic study has not yet been completed.

The precise mechanism underlying our observations remains to be elucidated. However, it is clear that gametic [26] and early embryonic programming effects [27–32] and transgenerational effects [33] in mammals are not infrequent. Early embryonic programming effects are increasingly

being recognized as important determinants of human and animal health [27–30]. For example, long-term effects of *in vitro* fertilization in the human [30], embryo culture and embryo cloning by nuclear transplantation in cattle and sheep (the 'large calf syndrome') [28], and embryo freezing [29] may be due to early epigenetic events. Our observations raise the question of whether some of these influences could actually have a long-term impact by being transmitted to future generations. Several of the established transgenerational effects are also important for human health [33–36]; there is, for example, the intriguing observation that women who were themselves exposed, *in utero*, to the Dutch famine then had offspring with reduced birthweights [34]. It is expected that the model for epigenetic inheritance described here will provide insights into these effects.

If epigenetic inheritance indeed exists, what is its evolutionary significance? The extent of its effects will depend on the number of genetic loci in the genome that can be modified epigenetically, and on the stability of the modifications. Whether 'epimutations' have any adaptive significance also remains to be established. It should be emphasized that this type of inheritance is rooted firmly in Darwinian selection, with selection possibly both for the modified locus and for the genes that control epigenetic modifications.

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