Maternal high-fat diet interacts with embryonic Cited2 genotype to reduce Pitx2c expression and enhance penetrance of left–right patterning defects

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Deficiency of the transcription factor Cited2 in mice results in cardiac malformation, adrenal agenesis, neural tube, placental defects and partially penetrant cardiopulmonary laterality defects resulting from an abnormal Nodal → Pitx2c pathway. Here we show that a maternal high-fat diet more than doubles the penetrance of laterality defects and, surprisingly, induces palatal clefting in Cited2-deficient embryos. Both maternal diet and Cited2 deletion reduce embryo weight and kidney and thymus volume. Expression profiling identified 40 embryonic transcripts including Pitx2 that were significantly affected by embryonic genotype-maternal diet interaction. We show that a high-fat diet reduces Pitx2c levels >2-fold in Cited2-deficient embryos. Taken together, these results define a novel interaction between maternal high-fat diet and embryonic Cited2 deficiency that affects Pitx2c expression and results in abnormal laterality. They suggest that appropriate modifications of maternal diet may prevent such defects in humans.

INTRODUCTION

Genetic evidence in mice and in humans indicates that the transcription factor Cited2 is essential for cardiac, adrenal, neural, lung, liver, lens and placental development, and for fetal haematopoiesis (1–9). On a mixed genetic background, cardiac malformations in mice lacking Cited2 are phenotypically heterogeneous, and include septal, outflow tract and aortic arch malformations (1,3,10,11). On a congenic C57BL/6J background, Cited2-deficient mice also exhibit partially penetrant left–right patterning defects characterized by right atrial and pulmonary isomerism, and abnormal ventricular topology characterized by sinistral looping (4,5).

Left–right patterning of the embryo arises as a result of Nodal-activated signalling in the left-lateral plate mesoderm (reviewed in 12). The morphogen Nodal is initially expressed in the node, and is asymmetrically transferred to the left-lateral plate mesoderm where it activates target genes such as Nodal, Lefty2 and Pitx2. Pitx2 encodes 3 isoforms (a, b, c) in mice, and Pitx2c dosage is critically important for establishing laterality, with deficiency resulting in right atrial and pulmonary isomerism (4,13). All three Pitx2 isoforms also have overlapping functions in craniofacial and branchial arch development (14). Deficiency of Nodal in the left-lateral plate mesoderm results in sinistral looping of the heart tube and abnormal ventricular topology (15). In Cited2-deficient embryos, Nodal is normally expressed in the node, but the expression of its target genes, Nodal, Lefty2 and Pitx2c, is deficient in the left-lateral plate mesoderm, subsequently causing the laterality defects observed in Cited2-deficient mice (4,5,16).

The increased penetrance of left–right patterning defects on a congenic C57BL6/3 as opposed to a mixed genetic background indicated the existence of genetic modifiers of the Cited2-deficient phenotype. Notably, Pitx2c deficiency was not apparent in the left-lateral plate mesoderm of mixed background mice, indicating that these genetic modifiers function by controlling Pitx2c expression (4). However, the partial penetrance of the laterality defect in Cited2-deficient
embryos, observed even on a congenic C57BL/6J background, also suggested a role for environmental modifiers.

Although the role of both genetic causes and factors that affect the intrauterine environment (such as maternal rubella and teratogen exposure) in human congenital heart disease is well established (reviewed in 17), the mechanisms by which environmental factors interact with genetic variants to cause birth defects is only beginning to be understood (reviewed in 18). A new and important source of environmental risk to the developing fetus is the growing epidemic of obesity in women of reproductive age (19,20). Epidemiologic studies indicate that maternal obesity is associated with an increased risk for cardiac, neural tube, cleft palate and other congenital defects (21). Maternal diabetes, a complication of obesity, is also a major risk factor for cardiac malformation, particularly of the types that are associated with defective left–right patterning (22–24).

High-fat, calorically dense diets are linked to the epidemic of human obesity (25,26), and also result in epigenetic modifications of embryonic chromatin (27,28). Based on the above observations, we hypothesized that a high-fat diet may interact with Cited2 deficiency to modulate the penetrance of the left–right patterning defect. Our results support this hypothesis, and show that maternal high-fat diet together with embryonic Cited2 deficiency significantly reduces the expression of the left-determining gene Pitx2, with a dramatic increase in the penetrance of left–right patterning defects, and the appearance of novel defects including cleft palate. These results demonstrate the existence of a hitherto unknown gene–environment interaction that has major consequences for patterning the developing embryo.

RESULTS

High-fat diet results in obesity and hyperglycaemia

We found that female C57BL6/J.Cited2+/− mice were significantly heavier and were mildly hyperglycaemic after 8 weeks on the high-fat diet, but without a significant difference in plasma insulin (Fig. 1, summary data in Supplementary Material, Table S1). We mated control diet and high-fat diet female C57BL6/J.Cited2+/− mice to male C57BL6/J.Cited2+/− mice and dissected out embryos at 15.5 days post coitum (dpc). There was no effect of the high-fat diet on average litter size (6.3 control, 6.5 high-fat diet), or on embryo Cited2+/−:Cited2+/−:Cited2−/− genotype ratios (control 52:87:43, high-fat 44:93:52).

Maternal high-fat diet increases penetrance of left–right patterning defects and cleft palate in Cited2−/− embryos

To determine the effect of maternal high-fat diet on the penetrance of left–right patterning in Cited2 deficiency, we examined embryos by magnetic resonance imaging (MRI, Fig. 2). In the high-fat group, a laterality defect (including cardiac right atrial isomerism or abnormal cardiac ventricular topology, right pulmonary isomerism or visceral situs inversus) occurred in 31 of 52 Cited2−/− embryos, compared with 10 of 43 Cited2−/− embryos in the control diet group (P = 0.0004, Fisher’s exact test, Supplementary Material, Table S2). These defects were not observed in wild-type embryos. Thus the penetrance of laterality defects in Cited2−/− embryos is more than doubled by a high-fat diet. Palatal clefting has previously not been reported in Cited2−/− embryos. We found, surprisingly, that the high-fat diet resulted in a dramatic increase in the frequency of cleft palate in Cited2−/− embryos (17 of 52 in the high-fat embryos compared with 2 of 43 in the control diet, P = 0.0006, Fisher’s exact test). Cleft palate was not observed in the wild-type or heterozygous embryos. Neural tube defects (exencephaly) occur at low frequency in Cited2−/− and have been occasionally reported in Cited2−/− embryos (1,2,10). Neural tube defects were observed in 10 of 52 high-fat diet and 13 of 43 control diet Cited2−/− embryos. These differences are not statistically significant (P = 0.24, Fisher’s exact test). They were not observed in the wild-type embryos. In the Cited2+/− group, neural tube defects were observed in 2 of 93 high-fat diet and none of the control diet embryos. These differences are again not statistically significant (P = 0.50, Fisher’s exact test).

Maternal high-fat diet increases severity of cardiac defects in Cited2−/− embryos

Previous observations indicate that cardiac defects are fully penetrant in Cited2−/− embryos but occur at low frequency in Cited2+/− embryos (16). We therefore explored the effect of maternal high-fat diet on cardiac malformations in Cited2+/− embryos. We found that the high-fat diet increased the frequency of cardiac malformations in Cited2+/− embryos, with 9 of 93 such malformations being present in the high-fat and 4 of 87 in the control diet group. Although these differences are not statistically significant (P = 0.25, Fisher’s exact test), the cardiac malformations observed between the two groups were qualitatively different. Only small ventricular septal defects were observed in Cited2+/− embryos from the control diet group, whereas in Cited2−/− embryos from the high-fat group we observed large ventricular septal defects, interrupted aortic arch, atrioventricular septal defect and double outlet right ventricle (Fig. 2 and data not shown). Cardiac defects were not observed in wild-type embryos in either diet group. As interrupted aortic arch has never been reported in Cited2−/− embryos, we determined the effect of maternal high-fat diet on aortic arch dimensions in Cited2−/− embryos. We found that the maternal high-fat diet resulted in transverse aortic arch hypoplasia—i.e. a significantly reduced volume and maximum diameter of the transverse aortic arch in Cited2+/− embryos (Fig. 2).

Maternal high-fat diet and genotype affects other aspects of embryonic development

Previous reports have indicated abnormalities in placental and adrenal development in Cited2 deficiency, and in nephronic epithelia formation (1,7,29). Examination of Cited2+/− embryos by MRI also indicated that the thymus appeared to be small. We therefore explored the effects of high-fat diet and Cited2 deficiency on these aspects of embryonic growth using volumes measured from MRI data sets which were corrected for embryo weight (30). The summary data are shown in Supplementary Material, Table S3. Using two-factor ANOVA
(for diet and genotype), we found that both genotype and diet significantly affected embryo and organ development (Fig. 3), but without a significant genotype–diet interaction. Specifically, we found that embryo weight, and the kidney and thymus volumes (corrected for embryo weight) were significantly reduced by loss of Cited2 and by high-fat diet. In addition, loss of Cited2 led to reduction in placental weight and adrenal hypoplasia, consistent with previous observations (1,7).

Maternal high-fat diet and genotype affects embryonic gene expression
To begin to determine the molecular basis of the diet and genotype effects, we used expression profiling. We compared three Cited2+/+ and three Cited2−/− littermate embryos at 8.5 dpc from each of the two dietary groups, using probes that were detected in at least one sample. The high-fat diet resulted in 117 of 23 104 detected probes being significantly dysregulated by loss of Cited2 (P < 0.01). In comparison, the control diet resulted in 32 of 23 499 detected probes being significantly dysregulated by loss of Cited2 (P < 0.01). These results indicate that the high-fat diet results in a substantially greater level of gene disregulation in embryos lacking Cited2 (117/23 104 versus 32/23 499, P = 0.0001, χ² test). Of the 117 probes dysregulated in Cited2−/− embryos from the high-fat group, 17 (associated with 13 genes) are involved in cardiac development (Supplementary Material, Table S4). In comparison, of the 32 probes dysregulated in Cited2−/− embryos from the control diet group, only one cardiac developmental gene (Cited2 itself) is dysregulated. The high-fat diet alone did not affect expression of known cardiac developmental genes.

Maternal high-fat diet and genotype affects embryonic Pitx2c expression
We next used a two-factor ANOVA to identify statistically significant differences in transcript expression which result from the interaction of embryonic genotype with maternal high-fat diet. We identified 34 probes (27 transcripts) that showed a main effect of genotype, 30 probes (29 transcripts) that showed a main effect of maternal diet and 41 probes (40 transcripts) that showed significant interaction between maternal diet and genotype (Supplementary Material, Table S5, P < 0.01 and fold change >1.5). The most significant transcript showing an interaction effect was Pitx2c (Illumina probe 1820746, P = 0.0007) and a second Pitx2c probe ranked 7th (Illumina probe 7320338, P = 0.002). Both probes showed a substantial reduction in Pitx2c expression in Cited2−/−

Figure 1. Effect of maternal high-fat diet on maternal weight, fasting glucose and insulin. (A) Experimental design. C57BL6/J Cited2+/+ females were weaned at 3 weeks, and at 6 weeks (day 0) were switched from chow to either a control diet (CD) or to a high-fat diet (HFD). Timed matings with C57BL6/J Cited2+/+ males were started at day 56, and embryos for MRI collected at 15.5 dpc, or for microarray and quantitative reverse-transcriptase–polymerase chain reaction (QRT–PCR) at 8.5 dpc. (B–D) Weight, fasting glucose and insulin by diet (CD or HFD) and time-point day 0 or day 56. Data are shown as mean ± standard error. The number of observations is given in Supplementary Material, Table S1. P is the probability of a type 1 error. NS = not significant.
embryos from mothers fed with high-fat diet compared with control diet. In Cited2+/− embryos, Pitx2 expression for both diets was comparable with Cited2+/+ control diet. Both probes in the above experiment target the common 3′- untranslated region of Pitx2a, b and c isoforms. The left-determining isoform Pitx2c, however, uses an alternative promoter and an asymmetric enhancer (31). We therefore performed quantitative PCR using isoform-specific probes to determine whether there was a specific change in Pitx2c expression. We found that while the high-fat diet did not affect Pitx2c expression in the Cited2+/+ embryos, it resulted in a significant (2.2-fold, P = 0.0051, t-test) reduction in Pitx2c in Cited2−/− embryos compared with Cited2−/− control diet embryos (Fig. 4A and B), consistent with the microarray result.

DISCUSSION

Gene–environment interactions are said to occur when there is a phenotypic variation for a given genotype in different environments (32). The role of gene–environment interaction in determining phenotypes in the developing mammalian...
embryo is only beginning to be understood, and has major significance in understanding and preventing birth defects (18). The results presented here identify a hitherto unknown gene–environment interaction between a genetic deficiency in Cited2 and a maternal high-fat diet that affects Pitx2c expression, and strikingly enhances penetrance of embryonic left–right patterning and palatal defects. Palatal clefting has not previously been reported as a phenotype occurring in Cited2-deficiency, but was revealed through the use of an environmental stressor—a maternal high-fat diet—emphasizing the importance of assessing knockout phenotypes under different environmental challenges. Notably palatal clefting is an important consequence of maternal obesity (21). It is also striking that these effects on penetrance are specific to left–right patterning and palatal defects. Palatal clefting has not previously been reported as a phenotype occurring in Cited2-deficiency, but was revealed through the use of an environmental stressor—a maternal high-fat diet—emphasizing the importance of assessing knockout phenotypes under different environmental challenges. Notably palatal clefting is an important consequence of maternal obesity (21). It is also striking that these effects on penetrance are specific to left–right patterning and palatal clefting: we observed no significant change in penetrance of neural tube closure defects or adrenal hypoplasia in Cited2-deficient mice. This specificity is noteworthy as previous experiments have reported that maternal folic acid supplementation will reduce the penetrance of neural tube defects in Cited2-deficient mice (2).

Consistent with previous reports we found that Cited2 deficiency reduces embryo and placental weight, and kidney and adrenal volumes (1,7,29). We also found that placental weight positively correlated with embryo weight, and that this correlation was observed in all three genotypes. We also found that Cited2 deficiency reduces thymus volume. A high-fat maternal diet led to significant reductions in embryo weight, kidney and thymus volumes across all genotypes. As all organ volumes were corrected for embryo weight, we interpret these organ volume reductions as specific effects of diet and genotype. We did not detect any significant genotype—maternal diet interactions that affected embryo or placental weight, or organ volumes.

Our data indicate that the molecular mechanism underlying the enhanced penetrance of left–right-patterning defects and possibly cleft palate in Cited2-deficient embryos from the high-fat group lies at least in part in the reduced expression of Pitx2c. Downregulation of Pitx2, specifically in the high-fat maternal diet Cited2-deficient embryos, was identified in an unbiased whole-embryo transcriptional-profiling experiment, and quantitative PCR showed that high-fat diet reduces the Pitx2c isoform in Cited2-deficient embryos. Pitx2c is necessary for normal cardiopulmonary laterality, and aortic arch development (4,33–35). The mechanism of palatal clefting and Pitx2c’s involvement or otherwise is not understood and will require further study.

While previous studies indicate that Cited2 is necessary for Pitx2c transcription and is present at the Pitx2c promoter in vivo (4), the experiments reported here do not clarify whether Pitx2c transcription or transcript stability, or its
spatial extent and expression through different developmental stages is affected by the high-fat diet, and further studies exploring the possible mechanisms are necessary. However, as Cited2 functions to recruit EP300 and CREBBP histone acetyl transferases to chromatin, and as high-fat maternal diet results in epigenetic modification of embryonic chromatin (27), an attractive model is that the loss of Cited2 and high-fat diet interacts to reduce Pitx2c transcription by adversely affecting chromatin structure. This hypothesis could be addressed in future work.

The experimental design of this study was chosen to mimic the high-fat calorically dense diet that is linked to the epidemic of human obesity (25,26). The high-fat diet used in our study achieves a high calorific value by replacing starch with lard as a nutritional source. Previous studies using a similar diet in C57BL6/J mice show that it results in weight gain, glucose intolerance and fasting hyperinsulinaemia (36). A limitation of the experimental design of the present study is that we do not yet understand the relative importance of the exposure of the embryo to maternal hyperglycaemia or to high maternal dietary fat versus maternal obesity per se. Another limitation is that the role of individual dietary components (such as the ratio of saturated to unsaturated fat, or the source of fat) is not understood. One possibility is that Cited2 mutant embryos show exaggerated sensitivity to hyperglycaemia. Supporting this idea it is known that non-obese diabetic embryos at 8.5 dpc on control and on high-fat diets (CD-wt and HFD-wt). Data show mean ± standard error of Ro values, and individual data points are shown. P is the probability of a type I error. NS, not significant.

**Figure 4.** Effect of diet on Pitx2c expression. (A) Quantitative reverse transcriptase–polymerase chain reaction (QRT–PCR) for Pitx2c mRNA in Cited2+/+ embryos at 8.5 dpc on control and on high-fat diets (CD-wt and HFD-wt). Data show mean ± standard error of Ro values, and individual data points are shown. P is the probability of a type I error. NS, not significant. (B) QRT–PCR for Pitx2c mRNA in Cited2−/− embryos at 8.5 dpc on control and on high-fat diets (CD-null and HFD-null).

Other left–right-patterning genes where mutation is associated with congenital heart disease include ZIC3 (OMIM:300265), DNAH11 (OMIM:603339), DNAH5 (OMIM:603335), LEFTY2 (OMIM:601877) and ACVR2B (OMIM:602730). We hypothesize that a high-fat maternal diet may interact with variants in these genes to increase the penetrance of congenital heart disease. This can be experimentally tested in appropriate mouse models.

Taken together, our results indicate that a high-fat diet interacts with Cited2 deficiency to affect Pitx2c expression, left–right patterning and palatal development. These results unequivocally show that the observed variation in penetrance of these defects is dependent, at least in part, on environmental factors. They demonstrate the existence of a hitherto unknown gene–environment interaction that has major consequences for patterning the developing mammalian embryo. They provide a potential mechanism for the increased risk of cardiovascular and palatal defects observed with human maternal obesity (21), and suggest that appropriate modifications of maternal diet may prevent such defects in humans.

**MATERIALS AND METHODS**

**Diets**

Custom formulated high-fat and control diets were obtained from Special Diets Services, Witham, Essex UK (Supplementary Material, Table S6). The proportion of nutrients (%) in control versus high-fat diets was: fat 5.4/40; protein 20.3/20.3; ash 3.5/3.5; fibre 3.5/3.5, nitrogen-free extract (NFE) 62/28; sugar 34/27; starch 26/0. Trace minerals and vitamins were identical between the two groups apart from total vitamin D and E, which were higher in the high-fat diet as they are present in animal fat.

**Mice**

Congenic C57BL6/J.Cited2+/− mice (backcrossed >9 generations to C57BL6/J) have been described previously (4), and were maintained by breeding to C57BL6/J mice obtained from Harlan Laboratories (Bicester, UK). Mice were genotyped as described using allele-specific polymerase chain reaction (1). Female C57BL6/J.Cited2+/− were fed with Beekay Rat and Mouse Diet (BK002P) between weaning and 6 weeks, and then were randomly assigned to specially formulated high-fat or control diets, which were provided ad libitum. Both groups were housed in identical specific-pathogen-free conditions, with a 12-h light dark cycle. At baseline and after 8 weeks on the diet, the mice were weighed and a 6-h fasting blood sample was collected. Blood glucose was measured using an Accu-check Glucometer (Roche Diagnostics, UK) and plasma insulin using a microinsulin ELISA kit for mouse (Mercodia, Sweden), following the manufacturer’s instructions. All animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

**Embryos**

Following 8 weeks on diet, C57BL6/J.Cited2+/− female mice were mated with C57BL6/J.Cited2+/− males, and the diet
continued through pregnancy. For MRI, embryos were harvested at 15.5 dpc and imaged as described previously (30). For microarray and qPCR analysis, embryos were harvested at 8.5 dpc and flash frozen in liquid nitrogen. Three pairs of littermate Cited2+/+ and Cited2−/− embryos were studied in each diet group, and all Cited2+/+ embryos were stage matched based on somite number. Pitx2c mutant embryos (Pitx2c-Δneo) have been described previously (4,35).

MRI analysis
All volumes were measured by segmentation analysis using Amira 3.1 (Visage, Berlin) software as described previously (30). The volume of the transverse aortic arch was measured from the right brachiocephalic artery to the insertion of the ductus arteriosus immediately after the origin of the left subclavian artery. Linear measurements of the narrowest segment of the left fourth aortic arch (between the right brachiocephalic and left common carotid arteries) were made using the measurement tool in Amira. All measurements were corrected for embryo weight.

Microarray
Amplification of RNA was performed using the Illumina TotalPrep RNA Amplification kit. Microarray was performed and analysed using the ‘whole-genome’ MouseWG-6 v2.0 Expression BeadChips (Illumina, Inc., CA, USA, following the manufacturer’s protocol). Data were processed and analysed using ‘R’ statistical software (38) and BioConductor packages (39). Data were background corrected and then normalized using the ‘vsn’ package (40). Probes not detected in any sample were removed prior to statistical analyses performed using the ‘limma’ package (41). First, each diet group was considered separately, and genes dysregulated between Cited2−/− and Cited2+/+ embryos were identified (the model also included an effect for litter). Second, a two-factor ANOVA was performed on the whole data set to assess genotype and diet effects, as well as genotype–diet interactions. Microarray data have been submitted to GEO.

Quantitative real-time PCR
Quantitative RT–PCR were carried out using a Bio-Rad IQ5 (Bio-Rad, CA, USA) with Taqman® primer probe sets from Applied Biosystems [Mus musculus assays Mm00440826_m1 (for NM_001042502 Pitx2c)], and eukaryotic 18S rRNA. Expression levels were normalized to 18S RNA using the Ro method of analysis (42). All reactions were performed in triplicate and only samples for which the threshold cycle was within one Ct value of each other were included in the analysis.

Statistical analysis
Fisher’s exact test or chi-square test for categorical data and t-test for continuous data were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Two-way ANOVA (General Linearised Model, univariate analysis, with genotype and diet as fixed factors in SPSS17) was used to assess embryo weight and organ measurements.

A probability of Type I error (P) < 0.05 was taken as statistically significant.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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