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 in17), the mechanisms by which environmental factors interact with genetic variants to cause birth defects is only beginning to be understood (reviewed in18). 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. However, 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
...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 dysregulation 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−/−...
embryos from mothers fed with high-fat diet compared with control diet. In Cited2−/− embryos, Pitx2 expression for both diets was comparable with 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 a
significance in understanding and preventing birth defects
(18). The results presented here identify a hitherto unknown
gene–environment interaction between a genetic deficiency
in \textit{Cited2} and a maternal high-fat diet that affects
\textit{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
\textit{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 differ-
ent environmental challenges. Notably palatal clefting is an
important consequence of maternal obesity (21). It is also strik-
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ing 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 \textit{Cited2}-deficient mice. This specificity is note-
worthy as previous experiments have reported that maternal
folic acid supplementation will reduce the penetrance of
neural tube defects in \textit{Cited2}-deficient mice (2).
Consistent with previous reports we found that \textit{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 \textit{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 inter-
pret 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 \textit{Cited2} embryos from the high-fat
group lies at least in part in the reduced expression of
\textit{Pitx2c}. Downregulation of \textit{Pitx2c}, specifically in the high-fat
maternal diet \textit{Cited2} embryos, was identified in an
unbiased whole-embryo transcriptional-profiling experiment,
and quantitative PCR showed that high-fat diet reduces the
\textit{Pitx2c} isoform in \textit{Cited2}-deficient embryos. \textit{Pitx2c} is necess-
ary for normal cardiopulmonary laterality, and aortic arch
development (4,33–35). The mechanism of palatal clefting
and \textit{Pitx2c}’s involvement or otherwise is not understood and
will require further study.
While previous studies indicate that \textit{Cited2} is necessary for
\textit{Pitx2c} transcription and is present at the \textit{Pitx2c} promoter
\textit{in vivo} (4), the experiments reported here do not clarify
whether \textit{Pitx2c} transcription or transcript stability, or its

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Effect of genotype and maternal diet on embryo and placental weight and organ volumes. Genotypes are \textit{Cited2}+/+ (WT), \textit{Cited2}+/- (HET) and \textit{Cited2}–/– (NULL). The significance of genotype, diet and genotype–diet interaction is indicated for each data set. P is the probability of a type I error. NS, not significant. R is Pearson’s coefficient of correlation. N is the number. (A and B) Embryo and placenta weight by genotype. (C) Correlation of embryo and placental weight for all genotypes. Analysis by genotype indicated that this correlation was strongest in wild-type and in \textit{Cited2}+/- embryos (N = 41; R = 0.3158; P = 0.04 for wild-type, and N = 41; R = 0.3585; P = 0.02 \textit{Cited2}–/– embryos). The correlation was weaker in \textit{Cited2}+/+ embryos (N = 87; R = 0.1447; P = 0.18). (D–F) Kidney, adrenal and thymus volumes by genotype. Data for control diet (CD, open circles) and for high-fat diet (HFD, closed circles) are shown as mean ± standard error, with the number of observations in each group indicated either above (CD) or below (HFD). Adrenal glands were absent in all \textit{Cited2}–/– embryos (43 from CD, and 52 from HFD), and volumes were taken as 0.}
\end{figure}
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 affecting chromatin structure. This hypothesis could be explored by varying the 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 show exaggerated sensitivity to hyperglycaemia. A limitation to this 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 mice have offspring with laterality defects (24). Moreover, unpublished work (N.A.B. et al.) indicates that exposure of 8.5 dpc mouse embryos in culture to glucose results in an increased frequency of left-turned embryos, left-looped and heterotaxic hearts and a reduction of asymmetric Pitx2c expression, in a dose-dependent fashion. These possibilities could be investigated in future studies by varying the dietary components, the timing for dietary intervention (pre- or post-conception) and using different genetic backgrounds with different susceptibility to hyperglycaemia or obesity. Another issue for future study is to determine whether low-calorie diets can ameliorate the phenotype.

Notably, mutations in genes in the NODAL pathway (including NODAL, GDF1, CFC1, TDGF1, FOXH1, SMAD2) have been reported in human congenital heart disease (37). 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

Diet

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 micorsinulin 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. Ptx2c mutant embryos (Ptx2c-neo) 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 rRNA 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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Conflict of Interest statement. None declared.

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