Influence of prenatal nutrition and obesity on tissue specific fat mass and obesity-associated (*FTO*) gene expression

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Abstract

The recent discovery of an association between body composition, energy intake and the fat mass and obesity-associated (*FTO*) gene represents a promising new therapeutic target in obesity prevention. In a well, pre-established large animal model, we investigated the regulation of *FTO* gene expression under conditions either leading to obesity or increased risk of obesity related disorders: i) a sedentary 'Western' lifestyle and ii) prenatal exposure to nutrient restriction. Pregnant sheep were either fed to fully meet their nutritional requirements throughout gestation or 50% of this amount from early-to-mid gestation. Following weaning, offspring were either made obese through exposure to a sedentary obesogenic environment or remained lean. A significant positive relationship between placental *FTO* gene expression and fetal weight was found at 110 days gestation. In both the newborn and adult offspring, the hypothalamus was the major site of *FTO* gene expression. Hypothalamic *FTO* gene expression was upregulated by obesity and was further increased by prenatal nutrient restriction. Importantly, we found a strong negative relationship between the hypothalamic *FTO* gene expression and food intake in lean animals only that may imply *FTO* as a novel controller of energy intake. In contrast, *FTO* gene expression in the heart was downregulated in obese offspring born to nutrient restricted mothers. In addition, *FTO* gene expression was unaffected by obesity or prenatal diet in insulin-dependent tissues, where it changed with age possibly reflecting adaptations in cellular energetic activity. These findings extend information gained from human epidemiology and provide new insights into the regulation of *in vivo* energy metabolism to prevent obesity.

Reproduction (2010) **139** 265–274

Introduction

The global incidence of obesity is currently exhibiting a dramatic increase worldwide in children, adolescents, and adults. Obesity (i.e. body mass index (BMI) > 30 kg/m²) is a well-defined pathological state due to excessive accumulation of lipids in white adipose tissue; however, its main causes remain an area of intense debate (Blakemore & Froguel 2008, Dina 2008, O'Rahilly & Farooqi 2008). Very recently, genome-wide association studies reproducibly found genetic variants in the fat mass and obesity-associated (*FTO*) gene to be strongly associated with the risk of obesity (Dina *et al.* 2007, Price *et al.* 2008, Tan *et al.* 2008, Timpson *et al.* 2008, Villalobos-Comparan *et al.* 2008). They consistently found that *FTO* single nucleotide polymorphisms (SNP) between populations, especially for the SNP *rs9939609* carriers, are related to substantial rises in BMI, body weight, and/or waist circumference

(Timpson *et al.* 2008); although others have suggested it may only account for 1% of the heritability of obesity in humans (Dina 2008).

The main function of the product of the *FTO* gene is yet to be discovered. This gene is widely expressed in a range of human (Timpson *et al.* 2008) and animal (Gerken *et al.* 2007) tissues, with its expression being highest in the brain including the hypothalamic arcuate nucleus, one of the main appetite regulating centers (Gerken *et al.* 2007). In mice (Gerken *et al.* 2007) and rats (Fredriksson *et al.* 2005), hypothalamic *Fto* mRNA abundance is modulated by nutritional status in a species-dependent manner. In mice, starvation down-regulates *Fto* mRNA transcription (Gerken *et al.* 2007), whereas in rats the opposite effect is seen (Fredriksson *et al.* 2005). Although these results contradict each other, they, together with human data, show an association between energy intake and *FTO* polymorphisms

(Teng *et al.* 2002, Wardle *et al.* 2008, 2009), suggesting that the product of the *FTO* gene could be involved in energy balance regulation. In addition, the *FTO* gene is ubiquitously expressed in all peripheral tissues tested to date. This includes some of the key tissues or organs involved in the control of energy metabolism and cardiovascular function (i.e. liver, adipose tissue, skeletal muscle, heart, kidney, and pancreas; Gerken *et al.* 2007, Stratigopoulos *et al.* 2008, Wahlen *et al.* 2008). The extent to which alterations of *FTO* regulation in these organs can be involved in obesity and obesity-related disorders remain unknown. Interestingly, adipocyte *FTO* expression is upregulated with human obesity (Zabena *et al.* 2009) and *FTO* SNP allelic distribution correlates with some of the common features of obesity and insulin resistance syndrome, i.e. raised plasma leptin, adiponectin, C reactive protein (Do *et al.* 2008, Fisher *et al.* 2009). Finally, adipocyte *FTO* has also been suggested to play a role in the regulation of lipolysis (Do *et al.* 2008, Shoulders 2008).

The *FTO* gene could thus have a primary role in the occurrence of obesity. To assess its biological role, tissue-specific gene expression needs to be studied within the context of exposure to obesogenic environments. In conjunction with energy imbalance and genetic predisposition, obesity, or risk of obesity-related disorders, can also originate from the prenatal energetic environment. Indeed, the nutritional status, during critical periods, in which the fetus grows and develops, is an important factor in determining body weight and energy homeostasis (McMillen *et al.* 2004, 2005, Symonds *et al.* 2005). The extent to which the *FTO* gene is developmentally regulated and may be reset by the *in utero* environment is yet to be determined and was thus the primary aim of the present study. We therefore examined *FTO* mRNA expression in a range of tissues sampled from mothers and offspring during critical stages of development, i.e. in the i) placenta at mid-gestation, ii) neonate at 7 days of age, and iii) lean and obese adults at 1 year. This analysis was undertaken in tissue samples taken from mothers fed to requirements and those nutrient restricted (NR) from early-to-mid gestation; a period we have previously established to have a pronounced impact on the control of food intake and organ endocrine sensitivity either in the neonate (Whorwood *et al.* 2001) or in the adult following obesity (Sebert *et al.* 2009, Sharkey *et al.* 2009).

Results

Study 1: placental expression of *FTO*

Maternal nutrient restriction during critical periods of gestation can affect placental function and metabolism (Gnanalingham *et al.* 2007). That said, in Scottish Blackface sheep, we found no significant effects of maternal nutrient restriction from 30 to 65 days gestation

on either placental (Control (C): ($n=4$): 630 ± 161 ; NR: ($n=5$): 636 ± 94 g) or fetal body (C: 2.08 ± 0.12 ; NR: 2.22 ± 0.20 kg) weight at 110 days gestation. Interestingly, *FTO* mRNA transcription was highly abundant in placental tissue as compared to the other tissues characterized in the present study (Fig. 1). Its expression was, however, unaffected by maternal nutrient intake during early-to-mid gestation (C: ($n=4$): 1.0 ± 0.3 ; NR: ($n=5$): 1.5 ± 0.4 -fold change; arbitrary units). Nevertheless, we observed a positive relationship between placental *FTO* mRNA abundance and fetal weight as determined at 110 days gestation (Fig. 2).

Study 2: influence of obesity and maternal nutrient restriction on *FTO* gene expression

Maternal nutrient restriction by 50% from early-to-mid gestation had no effect on either birth or bodyweight or organ mass up to 7 days of age (Table 1). As expected, post-weaning exposure to an obesogenic environment

Figure 1 Comparison of *FTO* gene expression in all major tissue types in offspring born to control fed animals sampled as either (A) neonates (i.e. 7 days of age), together with the 110 day placenta and (B) young adults (i.e. 1 year of age). Values represent means $\pm$ S.E.M.; different superscript within each age group indicates significant differences between tissues (a vs b $P < 0.05$; a vs c $P < 0.001$).

Figure 2 Significant positive relationship ($r^2=0.7$; $P=0.005$) between fetal body weight at 110 days gestation and placental *FTO* gene expression.

markedly increased total body weight as well as fat mass at 1 year of age (Table 1). The only organ that did not increase in size in proportion to the rise in total body mass was the heart. The prenatal environment had no influences on body mass and composition following obesity in NR obese (NRO) offspring.

We have previously described in more detail (Williams *et al.* 2007, Sebert *et al.* 2009, Sharkey *et al.* 2009) that maternal nutrient restriction during early-to-mid-gestation influenced the outcomes of juvenile obesity. Critically, concerning the control of food intake, NRO offspring ate substantially less than obese offspring born to control mothers (obese (O): 15.9 ± 0.5 MJ/24 h; NRO: 12.1 ± 0.8 MJ/24 h; $P<0.05$), an adaptation driven by the hypothalamus through upregulation of the energy sensing pathways (Sebert *et al.* 2009).

FTO gene expression and distribution

The *FTO* gene is a well-conserved gene between animal species (Fredriksson *et al.* 2005). Sequence data obtained for the *FTO* amplicon confirmed sequence homology to ovine *FTO* and to the *FTO* gene across species including humans (Fig. 3) suggesting comparative functionality. As in humans (Timpson *et al.* 2008), the ovine *FTO* gene was expressed in a wide variety of tissues, with maximal abundance in the hypothalamus, followed by skeletal muscle, and placenta. A majority of the other tissues analyzed showed an approximate tenfold lower expression when compared to the hypothalamus (Fig. 1A and B). Importantly, this hypothalamic predominance was observed both at birth and in adulthood. In addition, the lowest expression of *FTO* mRNA was measured in the kidney and pancreas.

Tissue-specific FTO gene expression

Hypothalamus. Prenatal nutrient restriction was not found to alter *FTO* gene expression in the newborn. However, *FTO* mRNA abundance doubled between birth and 1 year of age (Fig. 4A). *FTO* gene expression in the hypothalamus was amplified with obesity ($P<0.05$) and, importantly, prenatal exposure to maternal nutrient restriction in early pregnancy resulted in a further upregulation in *FTO* mRNA transcription induced by obesity ($P<0.01$ against O animals). Finally, we observed a significant negative correlation between food intake and *FTO* gene expression in lean animals at one year of age (Fig. 4B), a relationship lost with obesity.

Heart. No immediate effect of the prenatal diet on cardiac *FTO* gene expression was measured in the newborn. Nonetheless, in the 1-year-old NR offspring following obesity, cardiac *FTO* mRNA abundance was significantly downregulated compared to obese offspring born to control mothers (Fig. 5).

Table 1 Morphometric measurements in (A) newborn and (B) adult (1-year-old) offspring. Values are means with their S.E.M. from offspring born to control (C) or nutrient restricted (NR) mothers in neonatal and adult offspring that remained lean (L) or became obese (O).

	Body weight (kg)		Individual tissues/organs								
	Birth	7 days	PAT (g)	Fat (g/kg)	Kidney (g)	Liver (g)	Heart (g)	Pancreas (g)	Brain (g)		
(A)											
C (n=7)	3.3 ± 0.2	4.4 ± 0.3	43 ± 7	8.9 ± 0.8	30 ± 1	121 ± 5	33 ± 2	3.6 ± 0.4	53 ± 2		
NR (n=12)	3.2 ± 0.2	4.6 ± 0.3	49 ± 5	9.2 ± 1	27 ± 1	122 ± 9	36 ± 2	4.9 ± 0.4	53 ± 1		
	Individual tissues/organs										
	PAT		Kidneys		Liver		Heart		Pancreas		
	Weight (kg)	(g)	(g/kg)	(g)	(g/kg)	(g)	(g/kg)	(g)	(g/kg)	(g)	(g/kg)
(B)											
L (n=7)	58 ± 4	500 ± 93	10 ± 2	117 ± 9	2.0 ± 0.1	602 ± 36	10 ± 0.4	238 ± 10	4.1 ± 0.1	53 ± 3.5	0.9 ± 0.1
O (n=8)	91 ± 2 [†]	2690 ± 214 [†]	30 ± 3 [‡]	155 ± 7 [†]	1.7 ± 0.04	897 ± 59 [‡]	10 ± 0.3	309 ± 12 [*]	3.4 ± 0.1 [†]	60 ± 6.3	0.7 ± 0.1 [†]
NRO (n=8)	89 ± 2	2820 ± 212	30 ± 2	168 ± 7	1.9 ± 0.1	881 ± 50	10 ± 0.6	290 ± 8	3.3 ± 0.2	61 ± 4.5	0.7 ± 0.1

Significant differences between L and O groups indicated by: * $P<0.05$; $^{\dagger}P<0.01$; $^{\ddagger}P<0.001$. PAT, perirenal adipose tissue.

Sheep	9	TTCTATCAGCAGTGGCAACTGAAATATCCTAAACTGATTCTCCGAGAAGCTGCCAGCGTG	68
Human	146	TTCTATCAGCAGTGGCACTGAAATATCCTAAACTATTCTCCGAGAAGCCAGCAGTGT	205
Mouse	149	TTCTATCAGCAGTGGCAGCTGAAATACTCTAAACTGGTTTCCGAGAGGCCGGCAGCATA	208
Sheep	69	CCCAGTTGCTCCATAAAGAGGTTCAACAAGCCTTTCTCACACTGCACAAGCATGGCTGT	128
Human	206	TCTGAGGAGCTCCATAAAGAGGTTCAACAAGCCTTTCTCACACTGCACAAGCATGGCTGC	265
Mouse	209	CCAGAGGAGCTCCATAAAGAGGTTCCCGAGGCTTTCTCACACTGCATAAGCATGGCTGC	268
Sheep	129	TTATTTTCGAGATCTGGTGAGGATCCAGGGCAAAGACTTGCTCACTCCAGTCTCCACGCAT	188
Human	266	TTATTTTCGAGCTGGTGGATCCAAAGCAAAGATCTGCTCACTCCGATCTCTCGCAT	324
Mouse	269	TTATTTTCGAGCTGGTGAGGATCCAAAGCAAAGATGTGCTCACCCAGTCTCTCGCAT	327
Sheep	189	CCTCA	193
Human	325	CCTCA	329
Mouse	328	CCTCA	332

Figure 3 Sequence homology of the ovine *FTO* cDNA transcript with human (NM-001080432.1; 87% homology) and mouse (NM-011936.1; 82% homology).

Pancreas. Pancreatic *FTO* gene expression was decreased with age ($P<0.05$). Interestingly, we measured a further reduction of its expression following obesity but no specific effect of the prenatal diet was seen (Table 2).

Kidney. Similar to the pancreas, renal *FTO* gene expression was unaffected by the prenatal diet. However, obese animals exhibited a significant upregulation of *FTO* mRNA abundance (Table 2).

Adipose tissues. *FTO* mRNA abundance was similar between fat depots at 1 year of age (e.g. in lean ($n=7$) – perirenal: 1.0 ± 0.12 ; pericardial: 1.33 ± 0.35 -fold change). Perirenal adipose tissue, the most abundant fat depot in our model, expressed more *FTO* mRNA at 7 days compared to 1 year of age ($P<0.05$). Obesity tended ($P=0.08$) to increase adipose tissue *FTO* mRNA abundance an adaptation that was independent of the maternal nutritional environment (lean (L) = 0.7 ± 0.1 ; O = 1.1 ± 0.2 ; NRO = 1.0 ± 0.2).

Liver and skeletal muscle. There was no effect of prenatal diet and the age-related changes between 7 days and 1 year of age in *FTO* mRNA abundance (i.e. decrease in liver and an increase in skeletal muscle) were unaffected neither by obesity nor maternal nutrition (Table 2) in both tissue types.

Discussion

Within the past year exciting new evidence from genome-wide association studies reproducibly highlighted the *FTO* SNP allelic distribution as a predictive factor for obesity in various human populations (Dina *et al.* 2007, Al-Attar *et al.* 2008, Freathy *et al.* 2008, Hotta *et al.* 2008, Hubacek *et al.* 2008, Timpson *et al.* 2008, Willers *et al.* 2008, Gonzalez-Sanchez *et al.* 2009). The mechanisms underlying the physiological relationship between *FTO* allelic variations and alterations of body weight homeostasis are yet to be elucidated. Tissue-specific genetic regulation of *FTO* transcription together with obesity development

represents a first step to assess its biological functions. Our study thus explored, for the first time, the regulation of the *FTO* gene transcription in experimental environments known to promote obesity-related metabolic

Figure 4 *FTO* gene expression in (A) the hypothalamus in the neonatal (i.e. 7 days of age) and adult sheep that were born to either control (C) fed mothers or those nutrient restricted between 30 and 80 days gestation (NR) and then remained lean (L) or became obese (O) and (B) significant negative relationship ($r^2=0.42$; $P=0.001$) with food intake in adult lean animals. Values represent means $\pm$ S.E.M.; * $P<0.05$, ** $P<0.01$.

Figure 5 *FTO* gene expression in the heart in the neonatal and adult sheep that were born to either control (C) fed mothers or those nutrient restricted between 30 and 80 days gestation (NR) and then remained lean (L) or became obese (O). Values represent means $\pm$ S.E.M., * $P < 0.05$.

disorders. We have thus demonstrated obesity-related alterations of *FTO* together with the novel finding that *FTO* gene expression is developmentally regulated in the hypothalamus and in the heart, a response dependent on the prenatal nutritional environment.

Obesity-related alterations

Overweight sheep offers an interesting alternative model to study the origins and consequences of human obesity (Sharkey *et al.* 2009) although it is recognized that the sheep is a ruminant and thus has a very different diet and digestive system to humans. Notwithstanding this point this model of environmental induction of obesity, excessive body fat deposition resulted in similar physiological and metabolic complications to those seen in human obesity including glucose intolerance, hyperinsulinemia, hyperleptinemia, and leptin resistance together with raised blood pressure (Williams *et al.* 2007, Sebert *et al.* 2009, Sharkey *et al.* 2009). In our model, one of the main obesity-related changes in *FTO*

gene expression was an upregulation in the entire hypothalamus. In mice, *FTO* is predominantly expressed in the arcuate nucleus of the hypothalamus (Gerken *et al.* 2007), a key neuronal centre for food intake control (Masuzaki *et al.* 2001) and therefore suggest a role for *FTO* in the control of food intake. It thus raises the question as to whether the alteration of hypothalamic *FTO* gene expression that we found partly characterized the dysregulation of the control of food intake related to obesity (Sebert *et al.* 2009). Intriguingly, we found hypothalamic *FTO* mRNA abundance in lean animals to be negatively related to daily energy intake. This relationship was lost with obesity but, the further elevation of *FTO* expression in the offspring born to NR mothers was associated with a significant reduction of food intake. In addition, together with findings from both human and animal studies, our study further supports a role for *FTO* in the control of food intake that appears to be reset following obesity. In rodents, hypothalamic *FTO* gene expression is associated with energy intake (Fredriksson *et al.* 2005, Gerken *et al.* 2007), while in humans, allelic distributions of *FTO* SNPs are similarly related to alterations of energy intake in childhood (Timpson *et al.* 2008, Wardle *et al.* 2009). It thus appears possible that the *FTO* SNP *rs9939609* promotes substantial perturbations in energy balance that ultimately leads to obesity.

Results from human studies further indicate that *FTO* gene expression in adipocytes can be upregulated in obesity (Kloting *et al.* 2008, Zabena *et al.* 2009), an adaptation dependent on duration and severity. In our ovine model, we found a trend for a similar response in perirenal adipose tissue, suggesting that with an extended period of obesity the same adaptation would occur. We also observed for the first time that adolescent-onset obesity was associated with local alterations of *FTO* gene expression in the kidney and pancreas. The extent to which such changes in *FTO* gene regulation are a cause or consequence of obesity and thus has a role in the etiology of the classical perturbations observed during the metabolic syndrome remains to be established.

Table 2 Fat mass and obesity-associated (*FTO*) gene expression in insulin and noninsulin-dependent tissues. Values are means with their S.E.M. from offspring born to control (C) or nutrient restricted (NR) mothers in neonatal and adult offspring that remained lean (L) or became obese (O).

Tissues/organs	7 days old		1-year-old		
	C (n=7)	NR (n=7)	L (n=7)	O (n=7)	NRO (n=8)
Insulin-dependent tissues					
Liver	1.0 $\pm$ 0.2	0.9 $\pm$ 0.1	0.5 $\pm$ 0.1*	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1
PAT	1.0 $\pm$ 0.1	1.2 $\pm$ 0.1	0.7 $\pm$ 0.1*	1.1 $\pm$ 0.2	1.0 $\pm$ 0.2
SM	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1	3.7 $\pm$ 0.9*	4.0 $\pm$ 0.4	3.6 $\pm$ 0.6
Noninsulin-dependent tissues					
Kidney	1.0 $\pm$ 0.1	0.8 $\pm$ 0.1	0.7 $\pm$ 0.3	1.2 $\pm$ 0.2 [†]	1.0 $\pm$ 0.2
Pancreas	1.0 $\pm$ 0.2	1.2 $\pm$ 0.2	0.7 $\pm$ 0.5	0.2 $\pm$ 0.1 [†]	1.3 $\pm$ 0.9

Significant differences with age between control animals (i.e. C; 7 days versus L; 1 year of age) indicated by *; * $P < 0.05$. Significant differences between L and O groups at 1 year of age indicated by [†]; [†] $P < 0.05$. PAT, perirenal adipose tissue; SM, skeletal muscle.

FTO as a critical factor for the fetal programming of obesity-related disorders?

The actual risk for obesity and cardiovascular diseases are now established to be directly influenced by the fetal nutritional environment (McMillen & Robinson 2005, Hanson & Gluckman 2008). Epigenetic alterations promoted by the early nutritional environment modulate, in part, genotype plasticity to enable the developing fetus to adapt to changes in nutrient supply (McMillen & Robinson 2005). *FTO* gene expression is upregulated in the hypothalamus following a combination of *in utero* exposure to nutrient restriction followed by juvenile obesity. In the present study, we also found *FTO* mRNA to be highly expressed in the placenta and to be positively related to fetal weight. This strong relationship suggests that *FTO* is one factor involved in regulating fetal energy supply a proposal that is supported by the observation in newborn humans in which, the rs9939609 SNP in *FTO* is positively associated with specific metabolic parameters including fat mass and visfatin concentration (Lopez-Bermejo *et al.* 2008).

We did not, however, find any alterations in *FTO* mRNA abundance in any of the neonatal tissues examined. Nonetheless, the developmental origins of adult health and diseases remain a complex physiological process. Cell-specific adaptations to changes in the fetal nutritional environment appear to be dependent not only on the *in utero* environment but also to a potential mismatch between the pre- and post-natal nutritional environments (Hanson & Gluckman 2008, Vickers *et al.* 2008). Our results, therefore, suggest that adolescent onset of obesity is necessary to induce the phenotypic responses programmed by maternal nutrient restriction. However, future work will need to incorporate lean offspring born to NR mothers in order to fully confirm our hypothesis. Nevertheless, previous works from our group have already seen no differences in the control of food intake or metabolism in these offspring when they remain lean (Gopalakrishnan *et al.* 2005, Hyatt *et al.* 2007). Two of our main findings are that *FTO* gene expression in NR offspring is reset in the hypothalamus and heart following adolescent-onset obesity. The period of early-to-mid gestation in the sheep can be compared to the first trimester of human pregnancy and represents a period of maximal placental growth that coincides with critical periods of heart morphogenesis and neuronal network remodeling, including hypothalamic networks. Maternal nutrient restriction was therefore specifically chosen in order to establish the long-term adaptations upon the functions of these organs following obesity. The main characteristics of our particular model is a reduction of food intake following obesity (Sebert *et al.* 2009), without substantial weight loss, and intra-cardiac accumulation of triglycerides (Chan *et al.* 2009) in conjunction with marked adipose tissue-specific stress and inflammation responses (Sharkey *et al.* 2009). Taken together, these

adaptations suggest an amplified response to obesity in offspring born to NR mothers. Interestingly, *FTO* was one of the few genetic factors, co-expressed together with modulators of energy sensing e.g. *PRKA* (AMPK), *ACACA* (*ACC-alpha*), and *PPARGC1A* (*PGC-1 alpha*), for which hypothalamic gene expression in the adult is promoted by maternal nutrient restriction (Sebert *et al.* 2009). We thus suggest that during this critical developmental window, early maternal nutrient restriction induced long-term adaptation towards hypothalamic and cardiac energy sensing. These findings could now enable us to gain a better understanding of the developmental origins of obesity and metabolic diseases. We therefore raise the question as to whether the developmentally-induced modulations of *FTO* expression are related to a consistent resetting of energy sensing. Using bioinformatic sequence homology analysis, the *FTO* gene has been shown to encode a 2-oxoglutarate-dependent DNA demethylase (Gerken *et al.* 2007, Sanchez-Pulido & Andrade-Navarro 2007). This indicates a novel molecular link between energy generated via the Krebs cycle through modulation of intra-cytoplasmic concentrations of 2-oxoglutarate concentration, and gene transcription via DNA demethylation. It could therefore be very likely that cell metabolism may regulate *FTO* gene expression and this clearly requires further investigation. Tissue-specific biological functions of the *FTO*-related peptide remain to be fully established. However, a recently developed *FTO*-deficient mouse (Fischer *et al.* 2009) provides new insights into the biological systems regulated by *FTO*. Systemic depletion of the *FTO* gene resulted in major growth impairment of white, but not brown, adipose tissue leading to lipodystrophy and resistance against obesity. These mice were also hyperphagic and exhibited substantial alterations in energy expenditure leading to raised heat production. The extent to which similar roles are present in large mammals remains to be established but we clearly observed that the early nutritional environment can have a modulating role.

An improved understanding of the primary mechanisms regulating body weight homeostasis and energy balance is critical to current public health concerns with respect to obesity. In this regard, we have found that regulation of the *FTO* gene undergoes profound developmental adaptations that are dependent on maternal nutritional status, age, and fat mass. It is highly expressed in the hypothalamus and represents a new molecule involved in the control of food intake. Furthermore, *FTO* gene expression is modified by obesity both in the hypothalamus and the heart following exposure to prenatal NR coincident with early organogenesis. As to whether the specific alterations of *FTO* gene expressions we observed, following NR, either contributes directly or are a compensatory factor towards obesity development requires further investigation. In addition to fetal programming of *FTO* gene expression,

we found obesity-dependent effects on *FTO* gene expression in the hypothalamus, adipose tissue, kidney, and pancreas that could represent an important adaptation in obesity development and obesity-related diseases such as hypertension and type II diabetes. There is a clear need for further research efforts to be prioritized in this area. Taken together, our unique findings lends further support to previous small animal studies (Fredriksson *et al.* 2005, Gerken *et al.* 2007, Fischer *et al.* 2009) for the *FTO* gene to be classified as a novel controller of food intake and as a cellular energy sensor that is developmentally and nutritionally regulated. Importantly, we confirm some strong links between *FTO* and obesity, in a large animal model, that may promote the discovery of new pathways for targeting therapeutic strategies designed to prevent obesity and its associated metabolic complications and social and financial implications.

Materials and Methods

Animals, diets and experimental design

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the local ethics committee of the University of Nottingham and

Aberdeen. The present paper utilized tissue samples obtained from two independent studies previously performed which were designed to analyze the nutritional regulation of placental development and the fetal programming of obesity and related disorders as summarized in Fig. 6. These two independent studies thus provide an excellent opportunity to answer our hypothesis but cannot be interpreted as a single study.

Study 1: influence of nutrient restriction during the period of maximal placenta growth on placental *FTO* gene

Nine singleton-bearing Scottish Blackface sheep were either fed a control diet ($n=4$) or a 50% of this amount i.e. NR ($n=5$) during the period of maximal placenta growth in sheep i.e. between 30 and 65 days of gestation, as previously described (Brennan *et al.* 2005). At 110 days of gestation, all mothers were euthanized, the fetus weighed, and placentomes randomly sampled for molecular analysis.

Study 2: influence of maternal nutrient restriction followed by adolescent-onset obesity on *FTO* gene expression in resultant offspring

FTO gene expression was assessed in a range of tissue samples collected from animals previously used to study the early nutritional programming of food-intake regulation (Sebert *et al.* 2009). Briefly, at day 30 of gestation, 25 twin-bearing Welsh

Figure 6 Summary of the experimental protocol from conception through to adulthood. See Materials and Methods section for further details.

Mountain sheep were randomly allocated into two groups to receive either a control (7 MJ/day: C: $n=15$) or NR (50% of C, $n=10$) diet until day 80 of gestation (Fig. 6). Thereafter, all sheep were fed 100% of their calculated metabolisable energy requirements up to term (12–13 MJ/day near term; *Agricultural and Food Research Council* 1993). All offspring were delivered spontaneously at term (~ 147 days). The female to male ratio was (1:1) in the control and (2:1) in the NR group. Seven control offspring and 12 NR offspring were tissue sampled at 7 days of age. For ethical considerations, in order to reduce the number of mothers entered into the study, eight of the newborn control twins (twin 1) were allocated to a separate independent study. The remaining offspring (C: $n=15$; NR: $n=8$) were reared by their mothers until weaning (~ 10 weeks of age). During lactation, all mothers were fed a diet of hay *ad libitum*, together with a fixed amount of concentrate pellets, sufficient to fully meet their own metabolizable requirements, as well as those required to maintain lactation. From weaning to 12 months of age, the O ($n=8$) and NR ($n=8$) offspring were exposed to an 'obesogenic' environment: i.e. group housed in a barn at a stocking rate of 17 animals per 50 m² with *ad libitum* access to hay and concentrate pellets, as previously described (Sebert *et al.* 2009). The remaining control offspring ($n=7$) were reared in a nonobesogenic standard 'free-living' environment: i.e. put out in the pasture at a stocking rate of 17 animals per 3000 m² with free-grazing allowance and a fixed amount of concentrate pellets (400 g/day). Neonatal offspring sampled at 7 days of age were thus grouped according to the maternal nutritional environment: i.e. control or NR. The remaining post weaning 'free-living' offspring were defined as L ($n=7$). Offspring raised in the obesogenic environment were redefined as O ($n=8$) or NRO ($n=8$). Just prior to dissection, at 12 months of age, adult offspring were individually penned and their daily energy intake recorded (i.e. food offered minus food refusal over 24 h) in order to assess some aspects of food intake regulation (Sebert *et al.* 2009).

Post-mortem analysis

Study 1

At dissection (110 day gestation), individual placentomes were dissected and weighed in order to calculate total placental mass for each fetus. Fetal body weight was also recorded. Representative A type placentomes (that constitute $\sim 80\%$ of all placentomes at this stage of gestation) were randomly sampled, snap frozen in liquid nitrogen and stored at -80°C . Approximately 100 mg of maternal caruncular placentome were dissected for RNA extraction.

Study 2

All offspring were tissue sampled in the morning between 0900 and 1100 h. The 1-year-old offspring were euthanized as previously described after an overnight fast (Williams *et al.* 2007). Seven day old offspring could not be subjected to a similar period of food withdrawal as this would have meant prolonged physical separation from their mothers and, as such, would have been unethical. Following euthanasia, the whole brain was immediately recovered and the entire

hypothalamus dissected, snap frozen in liquid nitrogen and stored at -80°C as previously described (Sebert *et al.* 2009). In addition, the perirenal adipose tissue depot, that is the most abundant depot in young sheep (Alexander & Bell 1975), heart, kidney, liver, pancreas, and skeletal muscle (*Longissimus dorsi*) were rapidly sampled, snap frozen in liquid nitrogen and immediately stored at -80°C until molecular analyses.

Determination of FTO expression and mRNA abundance

Total RNA was extracted from all tissues using Qiazol lysis reagent and RNeasy extraction kit (Qiagen Ltd) which included a DNase I digestion (RNase-Free DNase Set, Qiagen Ltd). In the case of adipose tissue the lipid was first discarded (Sebert *et al.* 2005). One microgram of total RNA was then reverse transcribed into cDNA (Superscript II RT, Invitrogen Ltd). *FTO* gene expression was determined using the entire hypothalamus as has previously been used to study the regulation of food intake in rats (Teske *et al.* 2006, Sahu 2008), mice (Sellayah *et al.* 2008), and sheep (Warnes *et al.* 1998, Sebert *et al.* 2009). It is recognized that the hypothalamus is not a homogeneous entity but includes many nuclei that may exhibit differential regulation of *FTO* gene expression (Gerken *et al.* 2007). The advantage of using the entire hypothalamus is that it enabled us to obtain quantitative information with regard to the effect of age, maternal diet, obesity, and to compare *FTO* gene expression between tissues.

The *FTO* primers utilized were; forward: 5'-ACA CAT GGC TTC CCT ACC TG-3', reverse: 5'-GAG GAT GCG AGA GAC TGG AG-3' were designed from the published ovine *FTO* sequence (gene bank accession number: EU972419). The PCR product was gel extracted (QIAquick Gel Extraction Kit, Qiagen Ltd) and sequenced (Fig. 6) in order to confirm amplicon specificity and to generate a standard curve (tenfold serial dilutions) for real-time PCR analysis.

Gene expression was determined by a real-time PCR thermocycler (Quantica, Techne Incorporated, Barloworld Scientific Ltd, Stone, UK) using the Quantitect SYBR green-based PCR kit (Qiagen Ltd; Mostyn *et al.* 2006). 18S rRNA; (forward: 5'-GAT GCG GCG GCG TTA TTC C-3', reverse: 5'-CTC CTG GTG GTG CCC TTC C-3') and Acidic Ribosomal Protein P0 (*RPL0*) (forward: 5'-AG CAA GTG GGA AGG TGT AAT-3', reverse: CCC ATT CTA TCA TCA ACG GGT A-3') were used as housekeeping genes and results calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). We also assessed the suitability of a number of other house keeping genes including cyclophilin although only *RPL0* was shown not to be influenced by the *in vivo* experimental conditions. There was no difference in any of the results obtained when using either 18S or *RPL0* therefore we have only presented results when using 18S. To enable inter-tissue comparisons, the mRNA abundance measured in the 7-day-old hypothalami was chosen as a reference to enable a direct comparison between tissues; this was also the case for the 1-year-old hypothalamus samples. However, to analyze intra-tissue-specific modifications, the mean mRNA abundance of values obtained in the control

group at 7 days of age for the related tissue was used and therefore, acted as an internal reference for each separate tissue being examined.

Statistical analyses

Statistical analyses were performed using SPSS v14.0 for Windows (SPSS Inc., Chicago, IL, USA). To assess the data for normality, we performed a Kolmogorov–Smirnov test. Data were all found to be normally distributed and therefore analyzed using parametric tests as follow: Study 1: unpaired Student's *t*-tests were used to compare FTO expression between groups. Study 2: 1) tissue comparisons in the 7-day-old and separately in the 1-year-old offspring were made by one-way ANOVA with Bonferroni *post-hoc* tests. 2) In order to assess the effects of age (i.e. C (7-day-old) versus L (1-year-old)) paired Student's *t*-test (comparison of heterozygote twins) were used, 3) both the effect of maternal nutrient restriction (i.e. 7-day-old C versus NR and 1-year-old O versus NRO animals) and of postnatal obesity (L versus O) we used an unpaired Student's *t*-test. Data are presented as means $\pm$ S.E.M., with a *P* value <0.05 indicating statistically significant differences between groups.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the European Union Sixth Framework Program for Research and Technical Development of the European Community – The Early Nutrition Programming Project (FOOD-CT-2005-007036), the Nutricia Research Foundation (2009-35), the British Heart Foundation, the Medical Research Council, and the Universities of Nottingham and Alberta.

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Received 29 April 2009

First decision 5 June 2009

Revised manuscript received 15 September 2009

Accepted 28 September 2009