FTO demethylase activity is essential for normal bone growth and bone mineralization in mice

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\textbf{ABSTRACT}

The Fto gene locus has been linked to increased body weight and obesity in human population studies, but the role of the actual FTO protein in adiposity has remained controversial. Complete loss of FTO protein in mouse and of FTO function in human patients has multiple and variable effects. To determine which effects are due to the ability of FTO to demethylate mRNA, we genetically engineered a mouse with a catalytically inactive form of FTO. Our results demonstrate that FTO catalytic activity is not required for normal body composition although it is required for normal body size and viability. Strikingly, it is also essential for normal bone growth and mineralization, a previously unreported FTO function.

\section{Introduction}

Genome wide association studies revealed a strong association between intronic variants in the human fat mass and obesity related (FTO) gene and body mass index (BMI) in multiple populations [1,2,3,4]. However, recent evidence suggests that FTO polymorphism effects on BMI are also mediated by enhancers located within FTO intron one that affect the expression of several neighbouring genes, including RPGRIP1L, IRX5 and IRX3 [5,6,7]. Moreover, there has been conflicting evidence from rodent models about FTO's physiological function [8,9,10,11,12,13]. A loss-of-function point mutation in the human FTO gene (FTO-R316Q) results in a fatal autosomal recessive phenotype, but there is insufficient patient data available to conclude if there is an effect of FTO-R316Q on adiposity [14]. Thus, the role of FTO in lean/fat body composition remains controversial.

Arginie-316 in human FTO corresponds to Arginine-313 in mouse, which is essential for FTO catalytic activity [15]. FTO is a DNA and RNA demethylase that partly co-localises with nuclear speckles [15,16]. N6-Methyladenosine, an abundant mRNA modification [17], is a major substrate and is selectively demethylated by FTO [16]. Notably, FTO controls mRNA stability via m\textsuperscript{6}A\textsubscript{m} demethylation of the 5′ mRNA cap [18,19]. However, it is not known if FTO exerts all of its functional effects through its demethylase activity, or if some effects are mediated via allosteric interaction with binding partners, or a scaffolding function.

To determine the physiological importance of FTO demethylase activity and clarify its role in body composition, we generated a mouse model for the human FTO-R316Q mutation (mouse FTO-R313A) using CRISPR/Cas9-facilitated genomic editing [20]. This approach has the advantage that the genomic locus is edited at a single codon on a homogenous genetic background (Suppl. Fig. 1A), excluding disruption of intronic regulatory elements or differences in local genetic background which may have confounded previous FTO loss-of-function studies [11,12,13].

\section{Methods}

\subsection{Animal husbandry}

Animals were housed under specific opportunistic pathogen-free conditions and body compositions were measured using DEXA, MRI, EE, and EMPReSS. Biochemical measurements were performed using ELISA, DEXA, and EMPReSS. All experiments were conducted in compliance with the guidelines of the local animal ethics committee. E-mail addresses: gregor.sachse@dpag.ox.ac.uk (G. Sachse), churchc@MedImmune.com (C. Church), m.stewart@har.mrc.ac.uk (M. Stewart), h.cater@har.mrc.ac.uk (H. Cater), l.teboul@har.mrc.ac.uk (L. Teboul), r.cox@har.mrc.ac.uk (R.D. Cox), frances.ashcroft@dpag.ox.ac.uk (F.M. Ashcroft).

Abbreviations: BMC, (bone mineral content); BMD, (bone mineral density); BMI (Body mass index); Cas9, (CRISPR associated protein 9); CRISPR, (clustered regularly interspaced short palindromic repeats); CV, (calorific value); DEXA, (Dual-energy X-ray absorptiometry); EE, (Energy expenditure); ELISA, (enzyme-linked immunosorbent assay); EMPReSS, (European Phenotyping Resource for Standardised Screens from EUMORPHIA); FTO, (Fat mass and obesity-associated protein); KO, (knockout); m\textsuperscript{6}A\textsubscript{m}, (N6\textsuperscript{,2}-O-dimethyladenosine); MRI, (magnetic resonance imaging); RER, (respiratory exchange ratio); SEM, (standard error of the mean); SOFF, (specific opportunistic pathogen-free); ssDNA, (single-stranded DNA); WAT, (white adipose tissue); WT, (wildtype)

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(SOPF) conditions, in individually ventilated cages. Mice were kept under controlled light (light 7 am–7 pm, dark 7 pm–7 am), temperature (21 ± 2 °C) and humidity (55 ± 10%) conditions. They had free access to water (9–13 ppm chlorine), and were fed ad libitum on a commercial diet (Rat and Mouse No. 3 Breeding diet, RM3; Diestex Int. Ltd., Witham, UK) containing 11.5 kcal% fat, 23.93 kcal% protein and 61.57 kcal% carbohydrate.

All animal studies were licensed by the Home Office under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (SI 4 2012/3039), UK. All mice were maintained in accordance with the UK Home Office Welfare guidelines and project licence restrictions. All studies were approved by the local Animal Welfare and Ethical Review Body at MRC Harwell, under the ethical guidelines issued by the Medical Research Council (Responsibility in the Use of Animals for Medical Research, July 1993). Mice were euthanized by Home Office Schedule 1 methods.

2.2. Mouse genetic modifications

The FTO-R313A allele was generated by microinjection of C57BL/6J pronuclei with Cas9 mRNA (100 ng/μl), guideRNA (TACCTCTGGCACGGTGTGAGTGG, 50 ng/μl), and FTO-R313A donor ssDNA (containing the 5’-→3’ C→T GTO-FTO-R313A mutation, length 172 nt, −69 to +103 from mutation, 50 ng/μl). Guided by the RNA oligo, the Cas9 enzyme cuts the genomic location of Fto exon 5, activating genomic repair mechanisms that lead to replacement of the original genomic sequence by the donor ssDNA carrying the R313A encoding mutation [21]. Because the mutation site lies within the guide sequence (Suppl. Fig. 1A), the guide fails to efficiently target the site after genomic exchange has happened [22].

One drawback of the CRISPR/Cas9 approach is potential damage to genomic off-target sites [23]. We identified 128 potential off-target sites for the guide RNA we employed (Zhang Lab CRISPR design tool (http://crispr.mit.edu/)), 15 of which were situated within genes. Only two of these off-target sites were located on the same chromosome as the Fto gene (UCSC genes NM_023395 and NM_176940, on chromosome 8) and thus would not be readily lost during backcrossing. However, PCR amplification of both these off-target sites, followed by sub-cloning and Sanger sequencing, confirmed that both were unchanged in R313A mice (Suppl. Fig. 1B).

F0 offspring were screened for mosaicism by sub-cloning and Sanger sequencing of PCR products. Two out of 27 pups were found to be mosaic. These were mated to C57BL/6J stock to produce non-mosaic F1 animals. Thus the strain has a pure C57BL/6J background. To avoid strain deviation due spontaneous mutations, it was maintained by crossing back to C57BL/6J. All experimental animals were F1 littermates of heterozygous parents.

Guide design and prediction of potential off-target sites were performed using the Zhang Lab CRISPR design tool (http://crispr.mit.edu/). Local genomic sequences around potential off-target sites were amplified using PCR, subcloned using Zero Blunt TOPO PCR Cloning Kit (ThermoFisher, Waltham, MA) and analysed by Sanger sequencing.

The FTO-R313A allele was genotyped by realtime-PCR followed by Sanger sequencing for genotype and wildtype littermates. SPSS software (version 20; IBM, North Castle, NY) and R (http://www.r-project.org/) were used for statistical analysis and plotting. P values above 0.05 were considered not significant (n.s.).

3. Results

3.1. Viability

The FTO-R313A mice showed reduced viability, the number of homozygous (R313A/R313A) and heterozygous (R313A/WT) animals being less than 50% and 70%, respectively, of that predicted by Mendelian inheritance (Table 1). Similarly reduced ratios were found previously for FTO knockout mouse strains [11,13]. Premature death occurred prenatally or perinatally, with only four animals (three R313A/R313A and one R313A/WT), dying between day 3 and weaning. This is reminiscent of what is found for human patients with the corresponding mutation [14].
3.2. Body weight and composition

Viable homozygous FTO-R313A mice consistently weighed ~20% less than age-matched littermate controls (Fig. 1). Heterozygous FTO-R313A animals displayed no body weight phenotype and were not phenotyped further. The reduction in R313A/R313A mouse body mass was associated with a reduction of lean mass at 9 and 17 weeks as well as fat mass at 9 weeks, as determined by non-invasive quantitative NMR (Echo-MRI) scanning (Fig. 2A,B). However, when normalised to total body weight there were no differences in fat or lean mass (Fig. 2A,B). These findings were corroborated by DEXA measurements at 20 weeks (Fig. 2C). Visceral versus sub-cutaneous fat-pad weights were also not different between genotypes (Fig. 2D).

3.3. Energy expenditure and respiratory exchange rate

Previous studies of FTO knockout mice have linked altered body composition to differences in energy expenditure [11] or carbohydrate/fat utilisation, as exemplified by a significant change in the respiratory exchange rate [13]. However, no significant differences, after adjustment for lean mass, were found between R313A/R313A mice and littermate controls (Fig. 3A-D).

3.4. Skeletal and bone phenotype

No obvious skeletal malformations were observed in R313A/R313A mice. However, adult body size was decreased (Fig. 4A,B), and can explain most (if not all) of the reduction in lean mass, fat mass and total body mass (Fig. 1, Fig. 2A,B). Tail length was reduced by 10% in R313A/R313A mice compared to wt/wt littermates (Fig. 4A,B). However, adult body size was decreased (Fig. 4A,B). Tail length was reduced by 10% in R313A/R313A mice compared to wt/wt littermates (Fig. 4A,B). The shorter body and bone length of R313A/R313A mice was associated with a profound reduction in bone mineral density (BMD) and bone mineral content (BMC) (Fig. 5A,B). In particular, the BMD of R313A/R313A mice was 3.0 standard deviations below that of their wt/wt littermates, which is a greater difference than that used to define osteoporosis in human patients [25]. Despite these strong effects on bone mineralization, the blood profile of R313A/R313A mice was largely normal, including plasma calcium, phosphorus, parathyroid hormone and growth hormone levels (Table 2). However, alkaline phosphatase activity, an indicator of osteoblast function [26], was noticeably reduced in R313A/R313A mice.

Like R313A/R313A mice, homozygous FTO knockout mice are smaller than wt/wt. [11,13]. We therefore performed DEXA scanning on FTO knockout animals to determine if they also show a bone phenotype, as this was not reported in previous studies. Fig. 5C,D show that BMD and BMC were reduced in FTO knockout mice, but not in wildtype or heterozygous littermates. Interestingly, mice with a homozygous I367F mutation in FTO, which reduces catalytic activity of FTO by 80%, or heterozygous I367F/I367F mice [10], had normal body size [10] and unaltered BMD and BMC (Fig. 5E,F). Interestingly in this context, the HIF prolyl hydroxylase inhibitor IOX3 has been shown to reduce FTO expression in vitro and effects BMD/BMC when administered in vivo [27].

4. Discussion

Our data demonstrate that lack of FTO enzymatic activity results in a marked reduction of bone mineral density and bone mineral content, comparable to that seen in osteoporosis [25]. These changes were not seen in heterozygous FTO knockout animals or in homozygous FTO-I367F/I367F mice [10], which retain roughly 50% and 20% of wildtype demethylase activity respectively. This indicates a relatively small amount of catalytic activity is sufficient to rescue the bone phenotype. The mechanism behind the BMD and BMC reduction remains to be elucidated, but reduced alkaline phosphatase levels in FTO-R313A suggest osteoblast function might be affected.

The catalytic activity of FTO is also required for normal body size, as...
R313A/R313A mice had reduced body length and body mass, like global FTO knockout mice. Again, only a minimum amount of FTO catalytic activity is necessary, as heterozygous FTO-R313A mice, FTO-I367F/I367F mice [10] and FTO overexpressing mice [12] all have normal body length. Reduced size and bone mineralization also occur in multiple mouse models of premature aging [30,31,32,33] and in mouse models with substantial fetal growth restriction [34]. Unfortunately, studies on mouse models with growth retardation but bone-unrelated phenotypes rarely report bone mineralization data [35,36,37].

The enzymatic activity of FTO was also essential for normal peri-natal viability. As viability was also reduced in heterozygous FTO-R313A animals, this seems unrelated to the bone phenotype. The cause of sub-viability remains enigmatic, as studies of FTO knockout embryos did not reveal any obvious malformations or defects [11]. However, the reduced viability and smaller body size mimic the phenotype of the corresponding autosomal recessive human mutation [14].

Our results further show that FTO demethylase activity is not essential for normal body composition. Germline deletion of FTO also had no effect on body composition in one study [13], although reduced fat mass has been reported in another FTO knockout model [11]. The range of previous FTO knockout phenotypes (reviewed in Merkestein & Sellayah, 2015 [38]), may reflect disruption of intronic regulatory elements or differences in strain background, environmental factors and experimental protocols. In contrast, FTO overexpression results in a clear gene-dose-dependent increase in body fat and food intake [12], suggesting excess levels of FTO enhance appetite and thereby alter body composition. A role for FTO demethylase activity in adipose tissue cannot be excluded, however, as FTO-R313A overexpression was unable to rescue an adipogenesis phenotype in FTO knockout embryonic cells [39]. Also, acute loss of FTO in adulthood resulted in a sudden cessation of lean mass growth followed by a compensatory rise in fat mass [13]. Because FTO-R313A/R313A mice have a constitutive germline loss-of-function, potentially, effects on adipogenesis may be compensated during development.

We saw no change in RER in homozygous FTO catalytic null mice, in contrast to the reduction observed in FTO knockout animals [13]. This suggests FTO may regulate RER via a non-catalytic function, such as allosteric effects on binding partners, recruitment of factors to different sub-cellular locations, or a scaffolding function. A role for FTO that is
unconnected to its catalytic activity may also help explain some of the phenotypic variability seen in FTO loss-of-function mouse models [10,11,13], in which both catalytic and non-catalytic phenotypes overlap.

5. Conclusions

FTO catalytic activity is needed for normal body size and viability, although it is not required for normal fat/lean body composition, energy expenditure or respiratory quotient. Lack of enzymatic activity results in substantial osteoporotic changes that are rescued by as little as 20% of control catalytic levels. The key findings necessitate a thorough re-interpretation of previous FTO loss-of-function studies and introduce FTO as a novel regulator of bone growth and mineralization.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2017.11.027.
Fig. 5. Effect of FTO on bone density and mineral content.

A,B. Bone mineral density (A) and bone mineral content (B) of 20 week homozygous FTO-R313A mice (R313A/R313A, n = 17(f), n = 11(m)) and littermate controls (wt/wt, n = 16(f), n = 11(m)), measured by DEXA.

C,D. Bone mineral density (C) and bone mineral content (D) of homozygous FTO knockout mice (ko/ko, n = 30(f), n = 32(m)), littermate heterozygous FTO knockout mice (wt/ko, n = 19(f), n = 17(m)) and littermate controls (wt/wt, n = 7(f), n = 8(m)) for both genders at 24 weeks of age.

E,F. Bone mineral density (E) and bone mineral content (F) of male homozygous FTO-I367F mice (I367F/I367F, n = 15) and littermate controls (wt/wt, n = 14) at 24 weeks of age.

Bone mineral density values in A, C and E were adjusted for body mass by multiple linear regression. Data points indicate individual animals, horizontal bars mean ± SEM.
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Author contributions

GS, CC, RC and FMA designed research; GS, CC, MS, HC and LT performed research and analysed data; GS, RC and FMA wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Table 2

Plasma biochemistry of FTO-R313A mice.

| 20 Weeks terminal bleed |  | Male |  |
|-------------------------|----------------|--------|---|
|                         | (n = 11) | (n = 11) | (n = 4) | (n = 5) |
| Sodium (mM)             | 146.6 ± 0.5 | 145.8 ± 0.4 | n.s. | 149.0 ± 0.4 | 149.6 ± 0.5 | n.s. |
| Potassium (mM)          | 4.9 ± 0.1   | 4.9 ± 0.2   | n.s. | 5.0 ± 0.2   | 5.1 ± 0.3   | n.s. |
| Chloride (mM)           | 113.2 ± 0.6 | 112.6 ± 0.5 | n.s. | 112.6 ± 0.7 | 113.8 ± 0.8 | n.s. |
| Urea (mM)               | 10.3 ± 0.7  | 10.8 ± 0.5  | n.s. | 9.9 ± 0.8   | 12.4 ± 0.8  | n.s. |
| Creatinine (µM)         | 16.5 ± 0.9  | 15.5 ± 0.5  | n.s. | 15.8 ± 0.8  | 15.3 ± 1.1  | n.s. |
| Calcium (mM)            | 2.60 ± 0.02 | 2.60 ± 0.02 | n.s. | 2.64 ± 0.02 | 2.68 ± 0.04 | n.s. |
| Inorganic phosphorus (mM) | 2.3 ± 0.1 | 2.5 ± 0.1 | n.s. | 2.3 ± 0.1 | 2.3 ± 0.1 | n.s. |
| Alkaline Phosphatase (U/L) | 117 ± 3 | 102 ± 3 | p = 0.003 | 85 ± 1 | 58 ± 4 | p = 0.004 |
| PTH (µg/mL)             | 216 ± 47    | 260 ± 64    | n.s. | 210 ± 52    | 160 ± 35    | n.s. |
| GH (ng/mL)              | 6.5 ± 2.0   | 6.1 ± 2.7   | n.s. | 6.2 ± 2.3   | 12.6 ± 7.0  | n.s. |

| 12 Weeks tail bleed |  | Male |  |
|---------------------|----------------|--------|---|
|                      | (n = 11) | (n = 12) | (n = 4) | (n = 5) |
| Calcium (mM)         | 2.33 ± 0.05 | 2.31 ± 0.04 | n.s. | 2.40 ± 0.03 | 2.43 ± 0.02 | n.s. |
| Inorganic phosphorus (mM) | 2.0 ± 0.1 | 2.0 ± 0.1 | n.s. | 2.2 ± 0.1 | 2.2 ± 0.1 | n.s. |

PHT: Parathyroid hormone; GH: Growth hormone.

* Calcium corrected for plasma albumin [Ca]corr = [Ca] + 0.02 mM × (40 – [Alb] × g⁻¹ L⁻¹).

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