

## Tissue-specific expression of Sprouty1 in mice protects against high-fat diet-induced fat accumulation, bone loss and metabolic dysfunction

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### Abstract

We recently characterised Sprouty1 (Spry1), a growth factor signalling inhibitor as a regulator of marrow progenitor cells promoting osteoblast differentiation at the expense of adipocytes. Adipose tissue-specific Spry1 expression in mice resulted in increased bone mass and reduced body fat, while conditional knockout of Spry1 had the opposite effect with decreased bone mass and increased body fat. Because Spry1 suppresses normal fat development, we tested the hypothesis that Spry1 expression prevents high-fat diet-induced obesity, bone loss and associated lipid abnormalities, and demonstrate that Spry1 has a long-term protective effect on mice fed a high-energy diet. We studied diet-induced obesity in mice with fatty acid binding promoter-driven expression or conditional knockout of Spry1 in adipocytes. Phenotyping was performed by whole-body dual-energy X-ray absorptiometry, microCT, histology and blood analysis. In conditional Spry1-null mice, a high-fat diet increased body fat by 40%, impaired glucose regulation and led to liver steatosis. However, overexpression of Spry1 led to 35% ( $P < 0.05$ ) lower body fat, reduced bone loss and normal metabolic function compared with single transgenics. This protective phenotype was associated with decreased circulating insulin (70%) and leptin (54%;  $P < 0.005$ ) compared with controls on a high-fat diet. Additionally, Spry1 expression decreased adipose tissue inflammation by 45%. We show that conditional Spry1 expression in adipose tissue protects against high-fat diet-induced obesity and associated bone loss.

**Key words:** Sprouty: High-fat diet: Body fat: Obesity: Bone loss

### Background

Obesity is a major health problem worldwide with over a billion people overweight and 300 000 000 diagnosed clinically obese. The morbidity surrounding obesity relates to an array of metabolic abnormalities including insulin resistance, hypertension and hyperlipidaemia (i.e. 'metabolic syndrome')<sup>(1)</sup>. In the context of bone, several studies have shown that the metabolic syndrome is associated with a greater risk of fracture<sup>(2)</sup>, and adipocyte accumulation in the bone marrow has been associated with age-related osteoporosis<sup>(3–5)</sup>. Moreover, abnormalities in glucose, lipid and fluid homeostasis contribute to CVD and type 2 diabetes mellitus<sup>(6)</sup>. Research over the past decade has shown that obesity is a result of complex interaction among genes, physiology, behaviour, socio-cultural factors and the environment. Yet, diet plays an important role in the pathogenesis of obesity and a high-fat/energy diet is the most common factor contributing to rapid weight gain and accumulation of fat tissue. Excessive energy intake can initiate obesity in susceptible individuals and provoke metabolic and cardiovascular disorders stemming from

cellular stress due to chronic low-grade inflammation<sup>(1)</sup>. However, the molecular and genetic factors that ultimately determine the magnitude of obesity and the development of co-morbid conditions are still not well defined.

While osteoblasts and adipocytes arise from mesenchymal stem cells (MSC), a common progenitor cell type located in bone marrow, adipose tissue and other adult organs<sup>(7)</sup>, the lineage commitment of multipotent MSC depends on several factors including growth factor signalling. Adipogenesis is regulated by several growth factors starting from determination, commitment to preadipocytes and lipid accumulation stages<sup>(8)</sup> involving several receptor kinase signalling pathways such as epithelial growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF)<sup>(9,10)</sup>. Sprouty (Spry) and Spred (Spry-related protein) family proteins are evolutionarily conserved inhibitors of receptor tyrosine kinase signalling<sup>(11–13)</sup>. Spry regulates several signalling pathways, and affects common signal mediators, i.e. mitogen-activated protein kinase (MAPK), by suppressing the rat sarcoma (RAS)/MAPK

**Abbreviations:** aP2, fatty acid-binding protein promoter; aP2-Spry1, overexpression of Sprouty1 using the fatty acid-binding protein promoter; aP2-Spry1-KO, tissue-specific Sprouty1 deletion using the fatty acid-binding protein promoter; MSC, mesenchymal stem cells; Spry, Sprouty; Spry1, Sprouty1.

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pathway and generating a negative feedback loop during development<sup>(14)</sup>. The Sprouty1 (*Spry1*) gene is expressed in virtually all mouse tissues including adipose and bone marrow; there are no reports describing a role for *Spry1* in adipose tissue<sup>(15)</sup>.

Previously, we reported that *Spry1* functions as a regulatory switch modulating MSC lineage commitment, altering the balance between body fat and bone in mice<sup>(16)</sup>. In mice, tissue-specific *Spry1* deletion using the fatty acid-binding protein promoter (aP2-*Spry1*-KO) lowered bone mass, increased body fat, and led to a phenotype analogous to symptoms associated with diabetes, the metabolic syndrome and osteoporosis. Conversely, overexpression of *Spry1* using the aP2 promoter (aP2-*Spry1*) suppressed fat development and improved bone health. Additionally, aP2-*Spry1* mice did not lose bone or develop metabolic disorders associated with ageing. Given the role of *Spry1* in suppressing adipocyte differentiation, we tested whether *Spry1* expression could prevent high-fat diet-induced obesity, metabolic dysfunction and bone loss.

**Methods**

*Mouse models*

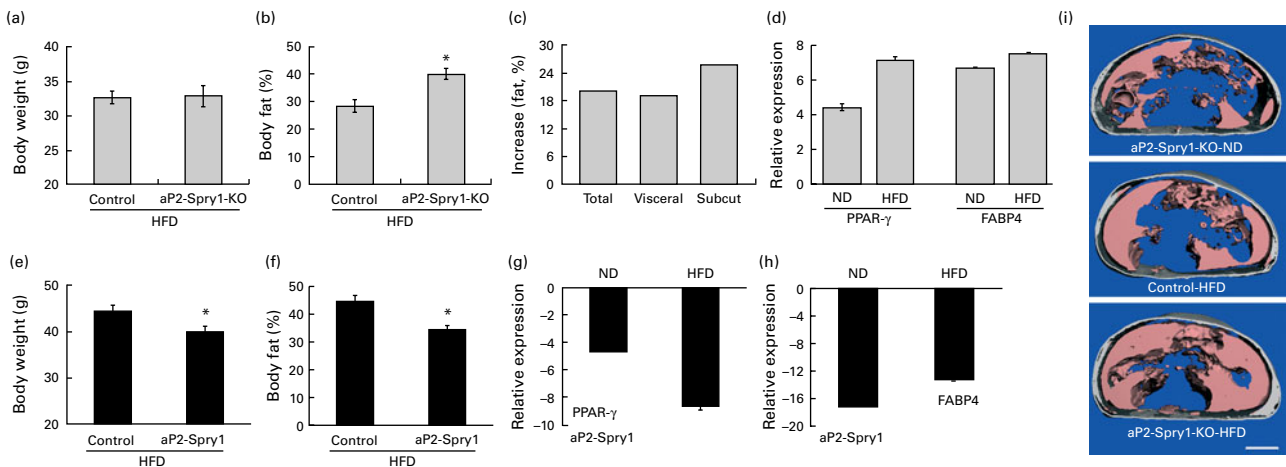
All experiments involving mice were approved by the Institutional Animal Care and Use Committee at Maine Medical Center Research Institute (Scarborough, ME, USA; 1003). Transgenic aP2-Cre (B6.Cg-Tg(Fabp4-cre)1Rev/J, stock 005069) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The *Spry1* transgenic mouse<sup>(17)</sup> and the conditional targeted *Spry1*-null allele<sup>(18)</sup> have been previously characterised. The double transgenic aP2-Cre*Spry1* experimental group is referred to as aP2-*Spry1*. Controls for comparison

were single transgenic aP2-Cre littermates. Genotyping primers and protocols were described previously<sup>(16)</sup>. The Cre recombinase (Cre)-mediated conditional *Spry1* null deletion was obtained by crosses to aP2-Cre, and are referred to as aP2-*Spry1*-KO, with aP2-Cre mice with wild-type *Spry1* as the control. Since the *Spry1* transgenic mouse and the conditional targeted *Spry1*-null allele were on different genetic backgrounds, FVB and C57BL/6, respectively, we maintained separate controls expressing aP2-Cre for the two groups. Consistent with our data showing a higher baseline weight and percentage of body fat after high-fat diet feeding (Fig. 1), comparison of strains has shown that compared with the FVB strain, mice on a C57BL/6 background have lower baseline body weight and percentage of body fat, and also have lower percentage of body fat following a high-fat diet (J Naggert, KL Svenson, RV Smith, B Paigen and LL Peters, personal communication).

At 5 weeks of age, mice were weaned and grouped based on their sex and genotype as controls and experimental group. Each group had a minimum of fifteen mice and mice were housed three to four per cage and allowed to feed *ad libitum* until 20 weeks of age. Diets were a standard chow diet (10% kJ fat, D12450B, hereto referred to as normal diet) or a high-fat diet (45% kJ fat, D12451; Research Diets, New Brunswick, NJ, USA), the compositions of which are presented in Table 1.

*Densitometry*

Whole-body dual-energy X-ray absorptiometry scans were conducted using a peripheral instantaneous X-ray imager (GE Lunar PIXImus; GE Healthcare, Waukesha, WI, USA).



**Fig. 1.** Sprouty1 (*Spry1*) expression protects from high-fat diet (HFD)-induced fat accumulation. Mice were fed a HFD for 15 weeks. (a–d) aP2-*Spry1*-KO mice and the corresponding controls are shown. (a) Average body weight and (b) percentage of body fat for the aP2-*Spry1*-KO and corresponding control groups at 20 weeks of age. Body fat calculations were determined by whole-body densitometry (dual-energy X-ray absorptiometry (DXA),  $n > 10$ ). \* Values were significantly different ( $P < 0.05$ ). (c) *In vivo* microCT image analysis for abdominal fat deposition of the aP2-*Spry1*-KO group ( $n > 4$ ) shows increased total, visceral and subcutaneous (subcut) fat compared with the controls on a HFD. (d) Changes in mRNA transcript levels of adipocyte markers, PPAR $\gamma$  and fatty acid binding protein 4 (FABP4), were quantified in the abdominal fat depots by quantitative RT-PCR. Shown are the aP2-*Spry1*-KO groups on both a normal diet (ND) and a HFD. (e–h) aP2-*Spry1* transgenic mice and the corresponding controls at 20 weeks of age on a HFD ( $n > 10$ ). (e) Total body weight and (f) percentage of body fat, quantified by DXA, were significantly decreased in the aP2-*Spry1* transgenics. \* Values were significantly different ( $P < 0.05$ ). Quantitative RT-PCR analysis was used to assess (g) PPAR- $\gamma$  and (h) FABP4 expression in the abdominal adipose tissue of the aP2-*Spry1* transgenics compared with the corresponding controls. (i) Transverse sections of the microCT-imaged abdomen from aP2-*Spry1*-KO and control mice at 20 weeks of age showing localisation of subcutaneous and visceral adipose tissue. Scale bar = 5 mm. aP2, fatty acid-binding protein promoter; aP2-*Spry1*, overexpression of *Spry1* using the aP2; aP2-*Spry1*-KO, tissue-specific *Spry1* deletion using the aP2.

**Table 1.** Composition of the experimental diets

|                    | Normal diet | High-fat diet |
|--------------------|-------------|---------------|
| Protein            | 20          | 20            |
| Carbohydrate       | 70          | 35            |
| Fat                | 10          | 45            |
| Energy (%)         | 100         | 100           |
| Ingredient         |             |               |
| Casein             |             |               |
| kJ                 | 3347        | 3347          |
| kcal               | 800         | 800           |
| L-Cystine          |             |               |
| kJ                 | 50          | 50            |
| kcal               | 12          | 12            |
| Maize starch       |             |               |
| kJ                 | 5272        | 1218          |
| kcal               | 1260        | 291           |
| Maltodextrin       |             |               |
| kJ                 | 586         | 1674          |
| kcal               | 140         | 400           |
| Sucrose            |             |               |
| kJ                 | 5857        | 2891          |
| kcal               | 1400        | 691           |
| Soyabean oil       |             |               |
| kJ                 | 941         | 941           |
| kcal               | 225         | 225           |
| Lard               |             |               |
| kJ                 | 753         | 6686          |
| kcal               | 180         | 1598          |
| Vitamin mix V10001 |             |               |
| kJ                 | 167         | 167           |
| kcal               | 40          | 40            |

Mice at 20 weeks were weighed and anaesthetised with Avertin (tribromoethanol, 0.2 ml of 1.2% solution/10 g body weight). Data were analysed for whole body tissue as well as a region of interest (11 × 35 voxels) fixed at the central diaphysis of the left femur. Each study group of the transgenic or targeted strain was  $n > 8$ . Precision of readings was determined and the standard deviation as a percentage of mean was  $< 2\%$ .

### MicroCT analysis

The femurs were dissected, cleaned of soft tissue, fixed and stored in 70% ethanol at 4°C until analysis. Bones were loaded into 12.3 mm-diameter scanning tubes and imaged using a vivaCT 40 scanner (SCANCO Medical AG, Brüttsellen, Switzerland). Scans were integrated into three-dimensional voxel images (2048 × 2048 pixel matrices for trabecular and 1024 × 1024 pixel matrices for all other individual planar stack). A threshold of 220 was applied to all scans at high resolution ( $E = 55$  kVp,  $I = 145$  μA, integration time 300 ms). All trabecular measurements were made by drawing contours every twenty slices and using voxel counting for bone volume per tissue volume and sphere-filling distance transformation indices. Cortical thickness was measured at the femoral mid-diaphysis. Mice anaesthetised with Avertin for densitometry scans were used for *in vivo* abdominal adiposity scanning to avoid multiple anaesthetic administration. All procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Maine Medical Center. The torso was scanned at an isotropic voxel size of

76 μm (45 kV, 177 μA, 300 ms integration time) with a vivaCT 40 scanner (SCANCO Medical AG). Two-dimensional grey-scale image slices were reconstructed into a three-dimensional tomography. Scans were reconstructed between the proximal end of L1 and the distal end of L5. The head and feet were not scanned and/or evaluated because of the relatively low amount of adiposity in these regions, and to allow for a decrease in scan time and radiation exposure to the animals. The region of interest for each animal was defined based on skeletal landmarks from the grey-scale images. Precision of readings was determined and the standard deviation as a percentage of mean was  $< 2\%$ .

### Metabolic parameters

Blood glucose tolerance was measured on overnight fasted mice administered 1 g glucose/kg body weight by intraperitoneal injection and glucose clearance recorded at 0, 15, 30, 60 and 120 min. Blood collected terminally was used to analyse plasma levels of insulin and leptin using ELISA-based assay kits (no. 80-INSMS-E01 and no. 22-lepms-E01 respectively; Alpco Diagnostics, NH, USA).

### Tissue processing

Tissue samples were snap-frozen for RNA, extracted using TriReagent (Sigma-Aldrich Corporation, St Louis, MO, USA) and reverse transcribed using the qScript cDNA supermix (Quanta BioScience, Gaithersburg, MD, USA) for RT-PCR analysis. For histology, fresh tissue samples were fixed in 4% paraformaldehyde and processed. For immunostaining, paraffin sections were deparaffinised, antigen recovered using DakoCytomation Target Retrieval solution (DAKO, Glostrup, Denmark) and blocked in 2% bovine serum albumin. Primary antibodies were used at recommended dilutions, followed by detection using secondary antibody. Antibodies were anti-F4/80 (BioLegend, San Diego, CA, USA) and anti-platelet endothelial cell adhesion molecule (PECAM-1) (BD Pharmingen, San Diego, CA, USA; BD BioScience, Rockville, MD, USA).

### Quantitative RT-PCR

Primer sequences (see Table 2) and PCR conditions have been described previously<sup>(16)</sup>. Total RNA was extracted from cells using Tri-reagent and reverse transcribed using the qScript cDNA supermix (Quanta BioScience). Real-time quantitative PCR was performed on the iQ-Cycler (BioRad LifeScience, Hercules, CA, USA) using the iQ-SYBR green supermix (BioRad LifeScience). All runs were done in duplicates and results are an average of three separate runs, and quantification performed by normalising to β-actin or cyclophilin gene expression and further to control tissues.

### Statistical analysis

All data are reported as means and standard deviations. Group mean values were compared, as appropriate, by Student's

**Table 2.** Primer sequences used for quantitative RT-PCR

| Gene name                           | Forward primer (5'–3')   | Reverse primer (5'–3') |
|-------------------------------------|--------------------------|------------------------|
| <i>PPAR<math>\gamma</math>1 + 2</i> | TCATCTCAGAGGGCCAAGGA     | CACCAAAGGGCTTCCGC      |
| <i>FABP4</i>                        | TGGAAGCTTGCTCCAGTGA      | AATCCCCATTTACGCTGATG   |
| <i><math>\beta</math>-Actin</i>     | GGAGGAAGAGGATGCGGCA      | GAAGCTGTCTATGTTGCTCTA  |
| <i>Cyclophilin</i>                  | CTCGAATAAGTTTGACTTGTGTTT | CTAGGCATGGGAGGGAACA    |

*FABP4*, fatty acid binding protein 4.

unpaired two-tailed *t* test. A *P* value of <0.05 was considered significant.

## Results

### *Spry1*-expressing mice have lower body fat on a high-fat diet

On a high-fat diet, mice in all groups gained weight. The aP2-Spry1-KO and their control groups had a 5–10% increase in weight over their corresponding normal diet-fed mice with similar average weights (control 32.65 g, aP2-Spry1-KO 32.86 g; Fig. 1(a)). Although the final body weights were not significantly different between the control and experimental groups, percentage of body fat was considerably altered. aP2-Spry1-KO mice on a high-fat diet showed a 40% ( $P < 0.05$ ) increase in body fat over the controls (Fig. 1(b)). *In vivo* whole-body scan for abdominal fat deposition using microCT confirmed the changes in aP2-Spry1-KO mice, showing about 20% increase in total, visceral and subcutaneous fat over the controls on a high-fat diet (Fig. 1(c) and (i)). This increase in fat accumulation in aP2-Spry1-KO was associated with increased expression of adipogenic markers, PPAR- $\gamma$  and fatty acid binding protein 4 (FABP4) (Fig. 1(d)).

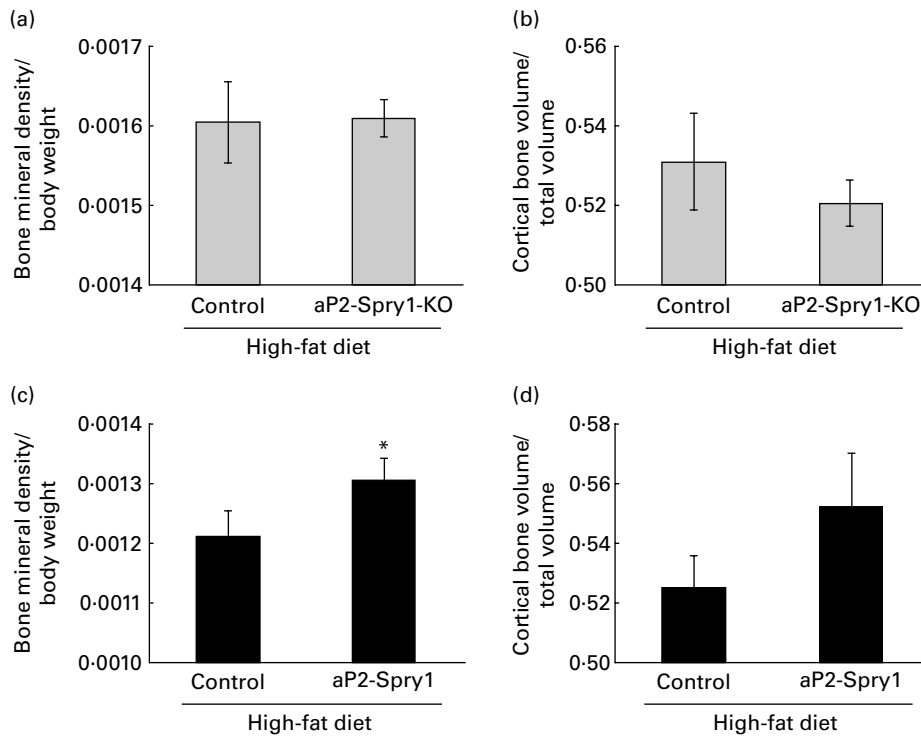
In the Spry1 gain-of-function group, the weight gain in the controls and aP2-Spry1 was about 15% ( $P < 0.05$ ) over normal diet-fed mice; however, the weight gain in aP2-Spry1 mice was significantly less than the single transgenic controls (Fig. 1(e)). The difference in body weight and body composition between the control groups for the Spry1 transgenic mouse and the conditional targeted Spry1-null allele is due to their different genetic backgrounds and therefore each group has its own control set on the matching genetic background. In aP2-Spry1 mice, body fat was 35% ( $P < 0.05$ ) lower compared with the single transgenic controls on a high-fat diet (Fig. 1(f)), indicating that mice expressing Spry1 in the fat tissue were protected from excessive fat accumulation. Additionally, there were fewer fat cells in the abdominal fat of Spry1-expressing mice compared with the controls, suggesting diminished hyperplasia (see also Fig. 4(b)). Overexpression of Spry1 in adipocytes was associated with decreased expression of the adipogenic markers PPAR- $\gamma$  (Fig. 1(g)) and FABP4 (Fig. 1(h)) both on a normal and a high-fat diet. Therefore, while loss of Spry1 promotes adipose accumulation, transgenic Spry1 expression in adipocyte lineages has a significant protective effect in reducing hyperplasia and hypertrophy of adipose tissue even during consumption of a high-fat diet.

### *Spry1*-expressing mice have less bone loss on a high-fat diet

Long-term high-fat diet feeding has a negative impact on skeletal remodelling<sup>(19,20)</sup>. Consistent with previous studies, areal bone mineral density in high-fat diet-fed mice was lower than the corresponding normal diet-fed mice (data not shown). Conditional deletion of Spry1 in the adipose tissue did not influence bone loss, since bone mineral density and bone volume were not significantly different between the control and aP2-Spry1-KO groups on a high-fat diet (Fig. 2(a) and (b)). However, significant less bone loss ( $P < 0.05$ ) on a high-fat diet occurred in the aP2-Spry1 transgenics compared with single transgenics (Fig. 2(c)). Mice in the aP2-Spry1 group retained about 8% higher bone mineral density, with a trend towards increased cortical bone volume (Fig. 2(d)). However, this effect was not seen in the femoral architecture, as there was no significant difference in trabecular bone volume or trabecular number between the aP2-Spry1 and corresponding control groups (data not shown). These results indicate that Spry1 expression in fat influences bone mass positively and reduces bone loss associated with body fat and weight gain typically seen in diet-induced obesity models.

### *Spry1*-expressing mice are protected from adverse metabolic effects from a high-fat diet

Regardless of the diet, aP2-Spry1-KO mice were predisposed to glucose intolerance, showing high blood glucose levels and decreased glucose clearance (Fig. 3(a)). While the controls developed glucose intolerance following high-fat diet feeding, the condition worsened in aP2-Spry1-KO mice ( $P < 0.05$ ). In the aP2-Spry1 transgenics, the control mice on the high-fat diet alone developed glucose intolerance ( $P < 0.05$ , Fig. 3(b)). Interestingly, glucose clearance in aP2-Spry1 mice on a high-fat diet was similar to the control mice on a normal diet (Fig. 3(b)). Consistent with these findings, blood serum levels of insulin in aP2-Spry1-KO (2.83 ng/ $\mu$ l) and respective control mice (2.8 ng/ $\mu$ l) were elevated (Fig. 3(c)) compared with mice on a normal diet (1.44 ng/ $\mu$ l, data not shown). In a pattern similar to insulin, plasma leptin was also elevated in the aP2-Spry1-KO (10.37 ng/ $\mu$ l) and control (9.7 ng/ $\mu$ l) groups, both on a high-fat diet (Fig. 3(d)). In contrast, in the Spry1 gain-of-function experiment, single transgenic control mice on a high-fat diet had markedly elevated insulin plasma levels (5.08 ng/ $\mu$ l) that were completely suppressed (70% decrease,  $P < 0.005$ ) in



**Fig. 2.** Sprouty1 (Spry1) expression protects against high-fat diet-induced bone loss. Bone parameter analysis on 20-week-old mice after high-fat diet feeding. aP2-Spry1-KO and the corresponding controls had no difference in (a) bone mineral density or (b) cortical bone volume. Bone mineral density was quantified by dual-energy X-ray absorptiometry scan and cortical bone volumes were quantified by microCT. aP2-Spry1 transgenic mice ( $n > 10$ ) lost less bone on a high-fat diet having higher (c) bone mineral density compared with single transgenics, and a trend towards higher (d) cortical bone volume. Values are means, with standard deviations represented by vertical bars. \* Values were significantly different ( $P < 0.05$ ). aP2, fatty acid-binding protein promoter; aP2-Spry1, overexpression of Spry1 using the aP2.

aP2-Spry1 mice (1.45 ng/ $\mu$ l) fed a high-fat diet (Fig. 3(e)). Moreover, leptin was more than 2-fold higher in the control mice (17.6 ng/ $\mu$ l) compared with the aP2-Spry1 transgenics (8.11 ng/ $\mu$ l) on a high-fat diet ( $P < 0.005$ , Fig. 3(f)).

#### Spry1-expressing mice on a high-fat diet show protective effects on other tissues

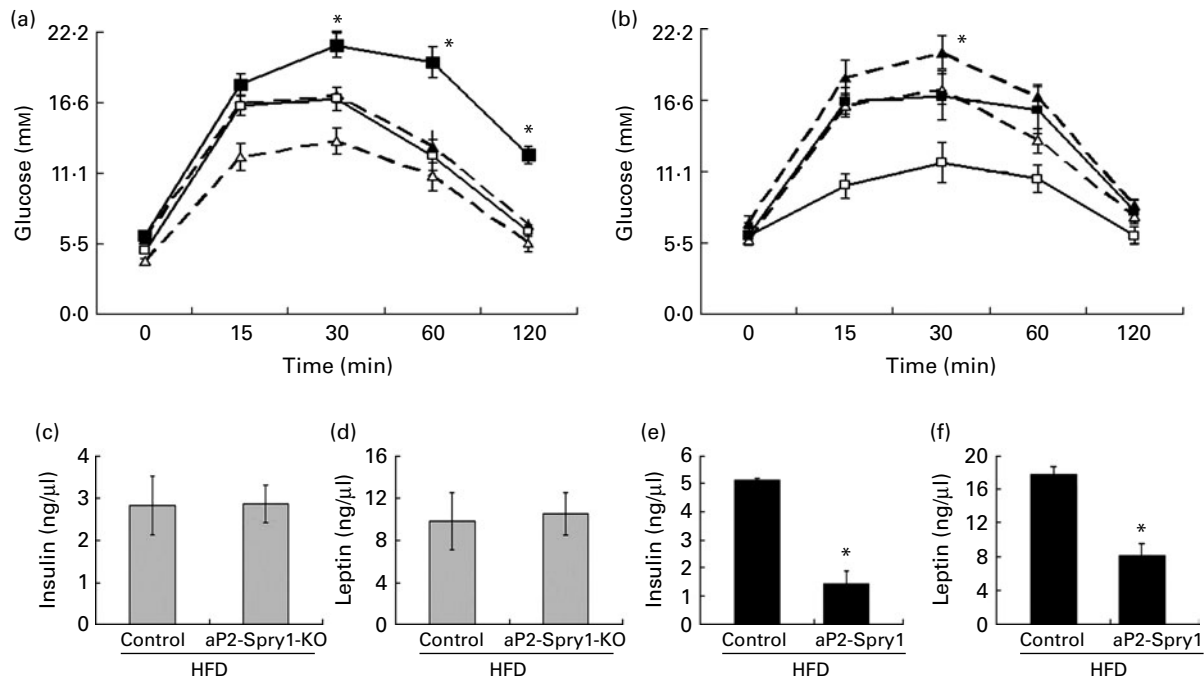
Imbalance in energy consumption and combustion results in lipid storage in other organs, most commonly the liver, resulting in fatty liver condition. Histology of the liver following high-fat diet feeding showed excessive deposition of fat indicative of hepatic steatosis in the aP2-Spry1-KO, their respective controls and the single transgenic control mice (Fig. 4(a) and (b)). However, the aP2-Spry1-overexpressing mice on a high-fat diet had no evidence of fatty liver (Fig. 4(b)). Since severe fatty liver is associated with inflammation, we examined macrophage infiltration in adipose tissue. Immunostaining for macrophages in the abdominal fat tissue of control mice fed a high-fat diet showed increased infiltration of macrophages and formation of cluster-like structures around the dead or dying adipocytes (Fig. 4(c)). Quantification indicated significantly higher numbers ( $P < 0.05$ ) of cluster-like structures in the control single transgenic adipose tissue compared with the aP2-Spry1 transgenics after high-fat diet feeding (Fig. 4(d)). Additionally, quantification of vascularisation in the abdominal fat tissue showed

significantly lower vessel area ( $P < 0.05$ ) in aP2-Spry1 mice compared with the controls on a high-fat diet (Fig. 4(e)), indicating a decrease in angiogenesis in the fat tissue. Spry1 expression reduced vascular density and macrophage infiltration, which are features commonly associated with obesity.

#### Discussion

The present study shows the effects of long-term high-fat diet feeding on body composition and metabolism in control or Spry1 conditional targeted or transgenic mice. Previously, we showed that fat tissue-specific expression of Spry1 resulted in reduced body fat, and in the present study, we further demonstrate that Spry1 expression can prevent fat accumulation even under high-fat diet feeding conditions. The present results confirm the hypothesis that Spry1 expression has long-term protective effects on body mass, bone density and metabolic parameters in mice exposed to high dietary fat. In the present study, we chose to use the high-fat diet feeding model for inducing obesity, as it is an effective and relevant model for the syndrome observed in humans. Indeed, diet-induced obesity in mice shares many features with human obesity and the resultant metabolic syndrome such as central adiposity, hyperinsulinaemia, insulin resistance and hypertension.

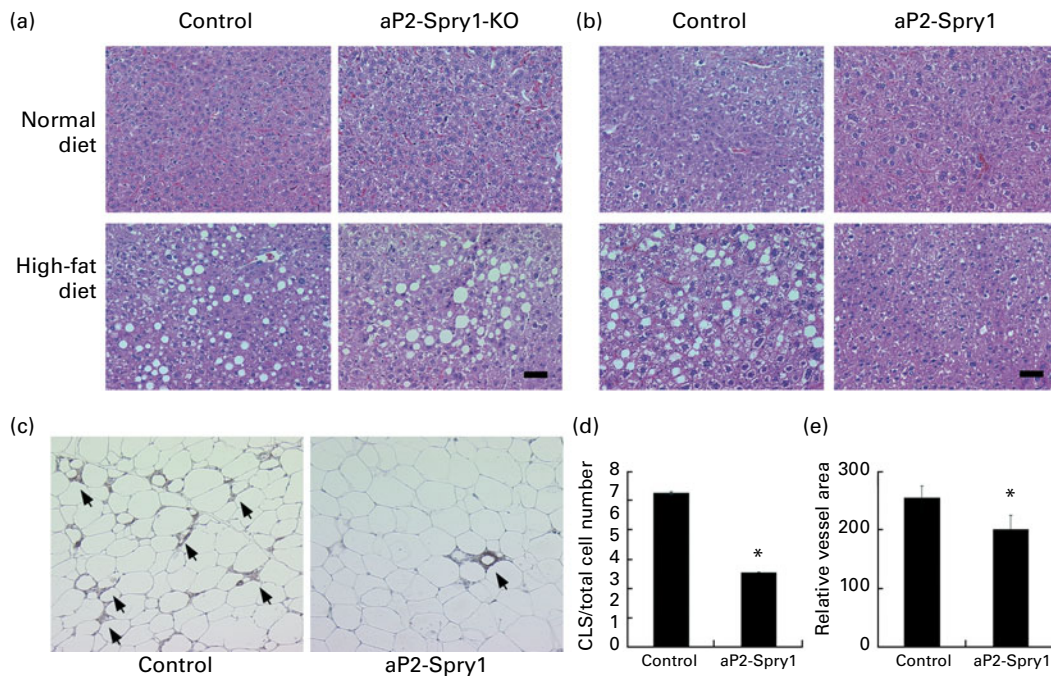
In the high-fat diet model, aP2-Spry1-KO gained more weight and accumulated more body fat compared with aP2-Spry1



**Fig. 3.** Sprouty1 (Spry1) expression protects from diet-induced metabolic abnormalities. The glucose tolerance test was performed on (a) aP2-Spry1-KO (—△—, control: normal diet (norm); —▲—, control: high-fat diet (HFD); —□—, aP2-Spry1-KO norm; —■—, aP2-Spry1-KO: HFD) and (b) aP2-Spry1 (—△—, control: norm; —▲—, control: HFD; —□—, aP2-Spry1 norm; —■—, aP2-Spry1: HFD) overexpressing mice fed a norm or a HFD at 20 weeks of age. Blood glucose levels were recorded at 0, 15, 30, 60 and 120 min after intraperitoneal injection of glucose to overnight fasted mice ( $n > 8$ ). All groups underwent blood serum analysis to quantify circulating (c, e) insulin and (d, f) leptin after HFD feeding ( $n > 8$ ). Values are means, with standard deviations represented by vertical bars. \* Values were significantly different ( $P < 0.005$ ). aP2, fatty acid-binding protein promoter; aP2-Spry1, overexpression of Spry1 using the aP2.

transgenics. The difference in fat gain demonstrates the localised effect of Spry1 expression in the adipose tissue compartment by repressing adipose tissue accumulation, and thereby establishes *in vivo* the protective function of Spry1 expression under conditions of high-fat diet feeding. In growing animal models, a high-fat diet has deleterious effects on bone mineral status including bone mineral content, structure and mechanical properties<sup>(19,20)</sup> and leads to increased bone resorption in mice, with only modest effects on bone formation<sup>(21)</sup>. Since osteoblast and adipocytes share a common progenitor, it is somewhat surprising that bone formation is not more significantly altered by high-fat diet feeding in mice, although Spry1 play an additional role in lineage allocation of osteogenic progenitors by promoting bone formation as described previously<sup>(16)</sup>. However, there are several possible mechanisms that may be operative. First, high-fat diet feeding in older mice leads to more marked suppression of bone formation and reduced osteoblast function than in young mice<sup>(20)</sup>. Second, recent studies have shown that activation of PPAR- $\gamma$ , which is a critical determinant of cell fate in marrow progenitors, not only causes impaired osteoblast differentiation but also stimulates bone resorption through induction of *c-fos*<sup>(22)</sup>. Regardless, Spry1 expression can antagonise the effects of PPAR- $\gamma$  in MSC via transcriptional repression brought about by the binding of transcriptional co-activator with the PDZ-binding motif (TAZ), and thus direct lineage allocation into the osteogenic lineage<sup>(16)</sup>. Whether, Spry1 also has a direct effect on osteoclast-mediated resorption remains to be determined.

Given the strong interactions and influence between bone, fat and brain, it is not surprising that targeted expression of Spry1 in adipose tissue influences bone metabolism especially by the adipokines, cytokines and growth factors. In addition, aP2 promoter is expressed in other cell types including brown adipose tissue, macrophages, part of the brain, peripheral nervous system and skeletal muscle and active in marrow progenitors, suggesting that Spry1 may influence marrow stromal cell fate<sup>(23,24)</sup>. Insulin resistance is implicated in the metabolic syndrome and is tightly interwoven with obesity. Although insulin has been shown to enhance bone formation<sup>(25,26)</sup>, elevated insulin levels in patients with type 2 diabetes paradoxically have increased fracture risk and, in some cases, lower bone density<sup>(27,28)</sup>. Likewise, we see insulin negatively correlated with bone volume in the high-fat diet-fed aP2-Spry1-KO, their respective controls and the controls for the aP2-Spry1, all of which had elevated serum insulin, while in aP2-Spry1 transgenic mice, there was no relationship between insulin levels and bone mineral density. Lower bone volume in aP2-Spry1-KO mice can also be attributed to hyperinsulinaemia which is associated with increased bone resorption, high levels of serum leptin and insulin. Leptin and adiponectin are heavily influenced by obesity and can adversely affect bone metabolism in a complex manner<sup>(29,30)</sup>. In the present study, leptin negatively correlated with bone mineral density and bone volume:total volume, consistent with previous reports of leptin negatively influencing bone metabolism via a hypothalamic stimulation of the sympathetic nerve system<sup>(31–34)</sup>. Put together, high-fat



**Fig. 4.** Sprouty1 (Spry1) expression protects from conditions of fatty liver and inflammation. (a) Liver tissue samples were collected from the aP2-Spry1-KO group and the respective control on a normal or high-fat diet. Histological staining with haematoxylin–eosin shows adipocyte ghost cells, which are remnants of lipid-filled adipocytes characteristic of fatty liver condition. Scale bar = 50  $\mu$ m. (b) aP2-Spry1 transgenics and single transgenic controls were compared similarly. Scale bar = 50  $\mu$ m. (c) Immunohistochemistry using F4/80 antibody to detect macrophage infiltration in abdominal adipose tissue in controls and aP2-Spry1 mice on a high-fat diet. The arrows indicate cluster-like structure (CLS) formation indicative of macrophages surrounding the dead or dying adipocytes. (d) CLS were quantified and normalised to total cell numbers for each group indicated. The CLS were significantly decreased in aP2-Spry1 adipose tissue ( $P < 0.05$ ). (e) Adipose tissue sections were stained for platelet endothelial cell adhesion molecule (PECAM-1) to quantify the vasculature. aP2-Spry1 transgenic mice had reduced vascular area compared with the controls ( $P < 0.05$ ). Values are means, with standard deviations represented by vertical bars. \*Values were significantly different ( $P < 0.05$ ). aP2, fatty acid-binding protein promoter; aP2-Spry1, overexpression of Spry1 using the aP2.

diet-induced bone loss is complex and could involve several factors: (1) lineage allocation of MSC; (2) receptor activator of NF- $\kappa$ B ligand (RANKL)-induced bone loss from immune cell activation; (3) bone resorption from activation of PPAR $\gamma$ ; (4) direct effects of NEFA on bone formation. So Spry1 has an impact on lineage allocation between adipocytes and osteoblasts but also may have an impact on osteoclast activity through antagonism of PPAR $\gamma$  or via macrophage elaboration of cytokines. In addition, NEFA may regulate both resorption and formation. Thus, Spry1 overexpression would antagonise PPAR $\gamma$  activation of bone resorption and therefore prevent high-fat diet-induced bone loss. On the other hand, deletion of Spry1 may have little effect on bone resorption via PPAR $\gamma$  and therefore may cause little change with a high-fat diet but could still have an impact on bone mass through non-cell autonomous effects on osteoblasts.

An additional factor is the excessive lipid storage in the liver brought about as a consequence of peripheral resistance to insulin and transport of fatty acids from adipose tissue to the liver leading to the pathogenesis of fatty liver disease<sup>(35)</sup>. Histology of the liver in the aP2-Spry1-KO, their respective controls and single transgenic Spry1 control mice showed excessive deposition of lipid indicative of non-alcoholic fatty liver or liver steatosis. This condition is also related to insulin resistance, type 2 diabetes mellitus and the metabolic syndrome. Spry1 expression prevented liver steatosis, insulin

resistance, diabetes and associated metabolic dysfunction, showing a protective role on multiple levels.

Chronic lipid overload associated with intra-abdominal obesity is an inducer of cellular stress and perpetuates the inflammatory cascade. Clinical and animal studies have demonstrated that obesity, insulin resistance and metabolic dysfunction are associated with a low-grade chronic inflammation with increased levels of circulating pro-inflammatory cytokines and adipokines<sup>(1,36)</sup>. Adipose tissue, in general, contains a resident population of innate immune cells, particularly macrophages and T-lymphocytes<sup>(37)</sup>. In obese individuals, infiltration of macrophages disrupts adipocyte homeostasis, acts as an additional trigger for the immune response, and contributes to insulin resistance and hepatic steatosis associated with obesity in mice and human subjects<sup>(35,38,39)</sup>. In the present study, this immune response was repressed in mice expressing Spry1, demonstrated by fewer cluster-like structures in abdominal tissue. These data demonstrate that Spry1 expression suppresses fat mass accumulation and thereby suppresses the associated inflammatory response and macrophage infiltration. Consistent with the role of Spry1 as a feedback inhibitor of receptor tyrosine kinase signalling regulating the RAS/MAPK pathway inhibiting angiogenesis<sup>(14,40)</sup>, there was decreased vessel area in the adipose tissue expressing Spry1. Therefore, Spry1 expression in fat tissue has dual consequences, (1) inhibiting adipocyte differentiation

mediated by PPAR $\gamma$  inhibition and (2) inhibiting adipose tissue-mediated angiogenesis resulting in decreased macrophage infiltration and inflammation in the adipose tissue.

### Conclusion

In conclusion, the present results demonstrate the protective effects of *in vivo* Spry1 expression in adipose tissue on bone fat and hepatic tissue, even during consumption of a high-fat diet. Future studies in animals should explore the role of Spry1 as a possible intervention in diet-induced obesity and ovariectomy and/or age-related bone loss.

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