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Thrombospondin-2 is an endogenous adipocyte inhibitor

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Abstract

The matricellular protein thrombospondin-2 (TSP2) inhibits proliferation and enhances osteoblastogenesis of multipotent mesenchymal progenitor cells (MPC). Osteoblastogenesis and adipogenesis are reciprocally regulated, thus we hypothesized that TSP2 could be an inhibitor of adipogenesis. TSP2 gene expression is up-regulated during MPC osteoblast differentiation and down-regulated during adipogenic differentiation through a cAMP-PKA pathway, relative to control cells. Next, the importance of TSP2 in adipogenesis was studied by comparing gene-targeted knockout mice that lack TSP2 protein (TSP2-null) and control wild-type mice. TSP2-null marrow-derived MPC show 25% increased adipocytes. Similarly, TSP2-null adipose-derived MPC show increased adipocytes (25–50%) and proliferation (2-fold) relative to wild-type cells. However, the increase in TSP2-null adipocytes is not due to an increase in MPC number, because the percentage of adipocytes relative to total MPC is still significantly increased. Surprisingly, there are only minor alterations in the adipogenic transcriptional program (PPAR- γ and C/EBP- α expression). Weight gain over time was evaluated in TSP2-null and control wild-type mice fed normal and high-fat diets. Female TSP2-null mice are 30% heavier than wild-type controls due to an increase in non-visceral adipose tissue, measured by dual-energy x-ray absorptiometry (DEXA) and direct weighing of fat pads. These results show that TSP2 is an endogenous matricellular protein produced by MPC that in addition to enhancing osteoblastogenesis and bone mass, inhibits adipocytes and decreases subcutaneous adiposity.

Keywords

thrombospondin-2; extracellular matrix; matricellular protein; mesenchymal progenitor; gene regulation; knockout mouse

1. Introduction

Adipocytes develop from multipotent mesenchymal progenitor cells (MPC). The process of adipogenic differentiation is highly-coordinated, characterized by distinct changes in transcription factor expression and alterations in genes associated with fatty acid metabolism and growth factor activity. Adipogenic induction results in the up regulation of transcription factors in the C/EBP family, including C/EBP α and C/EBP β . These transcription factors in turn activate transcription of PPAR γ , often referred to as a master regulator of adipogenesis as its activity is both necessary and sufficient for adipogenic differentiation. PPAR γ then

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orchestrates expression of genes required for fatty acid metabolism and adipocyte maturation (MacDougald and Lane, 1995).

In addition to changes in transcription factors, there are distinct changes in the expression of extracellular matrix (ECM) proteins that occur with the development of mature adipocytes. ECM proteins function not only for adhesion and structural integrity, but also bind to growth factors and interact with non-integrin cell surface receptors. Adhesive substrates and matrix stiffness inhibit adipogenesis but promote osteoblastogenesis of MPC (Engler et al., 2006; McBeath et al., 2004). We have recently reported that heparin promotes adipogenic differentiation by inhibiting focal adhesion formation (Luo et al., 2008). Mice deficient in the ECM protein secreted protein acidic and rich in cystine (SPARC), exhibit an increase in subcutaneous and visceral fat (Bradshaw et al., 2003) and adipogenic differentiation is enhanced in stromal cells isolated from bone marrow as well as white adipose tissue (WAT) of SPARC null mice. The molecular mechanism through which SPARC affects adipogenesis appears to be through stabilization of focal adhesions and promotion of integrin linked kinase activity which in turn results in stabilization of β -catenin (Nie and Sage, 2009). β -catenin is a component of the Wnt signaling pathway that is known to inhibit adipogenic differentiation (Ross et al., 2000). Intriguing recent studies demonstrated that osteocalcin, an ECM protein produced by osteoblasts, can regulate energy metabolism by acting on distally located pancreatic islet cells (Ferron et al., 2008; Lee et al., 2007). These findings demonstrate that ECM can influence adipogenesis through diverse mechanisms.

Thrombospondin-2 (TSP2) is a large multi-modular ECM protein that belongs to the matricellular family — a group of proteins, including SPARC, that interact with cells and other structural ECM proteins but by themselves play minimal structural role (Bornstein et al., 2000). TSP2 has been shown to interact with multiple cell receptors, growth factors and ECM proteins and regulates apoptosis, cell proliferation and adhesion. TSP2 is highly expressed in MPC and mesenchymal-derived tissues including bone and adipose tissue. Previous work has shown that TSP2 mRNA is decreased in the mature adipocyte fraction when compared to the stromal-vascular fraction of isolated fat pads, which contains MPC, suggesting that TSP2 expression decreases with adipogenic differentiation (Voros et al., 2005). Interestingly, TSP2 is increased during osteoblastogenesis and promotes the osteoblast differentiation of marrow-derived MPC (Alford et al., 2009; Hankenson and Bornstein, 2002). However, whether TSP2 plays a role in adipogenic differentiation is unknown. Because of the reciprocal relationship between osteogenic and adipogenic fates, we hypothesized that TSP2 will inhibit adipogenic differentiation.

Herein we report that consistent with its role in promoting osteoblastogenesis, TSP2 expression increases during osteoblast differentiation and is inhibited during adipogenesis. MPC isolated from TSP2-null bone marrow and WAT display an increase in the amount of lipid containing adipocytes in vitro. Finally, female TSP2-null mice are heavier than wild-type controls due to an increase in non-visceral adipose tissue.

2. Results

2.1 TSP2 expression is differentially regulated during osteogenic and adipogenic differentiation

TSP2 is expressed at lower levels in mature adipocyte fractions when compared to the stromal vascular fraction of adipose tissue, suggesting that TSP2 expression may be down-regulated during adipogenic differentiation (Voros et al., 2005). Conversely, TSP2 expression increases during osteoblast differentiation of the preosteoblast cell line, MC3T3E1 (Alford et al., 2009). Adipocytes and osteoblasts are derived from mesenchymal progenitor cells (MPC), and differentiation to the two terminal fates is reciprocally regulated. To better understand the

regulation of TSP2 expression during MPC differentiation, ST2 cells, a multipotent bone marrow-derived mesenchymal cell line, were induced to undergo osteogenic and adipogenic differentiation and TSP2 mRNA as well as TSP2 secreted into the media was examined at 1, 3, 6, 9, and 12 days post induction (Figure 1). TSP2 mRNA levels initially decreased at day 1 post osteogenic induction, but similar to published results with MC3T3E1 cells (Alford et al., 2009), gene expression gradually increased such that at days 6, 9 and 12 there were 1.7-, 3.8-, and 6.9-fold increases in TSP2 mRNA expression in osteogenic induced cells relative to un-induced controls, respectively (Figure 1A). Under adipogenic induction, TSP2 mRNA decreased to 30% of un-induced controls by day 1 and remained at that low level at days 3, 6, 9 and 12 post-induction (Figure 1B). To determine whether the differences in TSP2 mRNA expression translated to the protein level, media was collected at the time of RNA harvest and TSP2 protein present in the media was analyzed by western blot. Our lab has previously demonstrated that TSP2 is secreted into the media of cultured cells (Alford et al., 2009). A similar pattern of gradual increase with osteogenic induction and rapid and sustained decrease with adipogenic induction relative to un-induced control cells was observed in TSP2 protein secreted into the media (Figure 1C). TSP2 is a relatively stable protein and some secreted TSP2 does accumulate in the media of the adipogenic cultures over time as new protein is produced; however, the amount of secreted TSP2 is much lower than osteogenic or control treatments. Therefore, TSP2 expression, at both the mRNA and protein levels, increases with osteogenic differentiation and decreases with adipogenic differentiation.

2.2 Components of adipogenic induction cocktail have additive effects in down-regulating TSP2 expression

To explore the molecular mechanism of TSP2 down-regulation during adipogenesis, we evaluated the influence of components of the adipogenic cocktail in down-regulation of TSP2 in 3T3-L1 cells—a pre-adipocyte cell line in which the adipogenic differentiation program has been more thoroughly characterized (Darlington et al., 1998). Similar to ST2 cells, the combination, isobutyl-methylxanthine (IBMX), dexamethasone, and insulin (MDI) had profound effects in down-regulating TSP2 expression in 3T3-L1 cells reducing it to 12.6% of un-induced controls at 24 hours post-treatment (Figure 2A). When used alone, IBMX, dexamethasone and insulin reduced TSP2 expression to 46%, 51% and 64% of un-induced controls, respectively. Using two of these agents in combination resulted in greater decrease in TSP2 expression than using each one alone indicating that IBMX, dexamethasone and insulin have additive effects in down regulating TSP2 expression (Figure 2A).

IBMX is a phosphodiesterase inhibitor that acts to increase levels of cAMP therefore we examined the relative importance of the cAMP/PKA pathway in regulating TSP2 expression. To determine whether increasing cAMP levels is sufficient to inhibit TSP2 expression, cells were treated with increasing doses of 8-Br cAMP (a cAMP analog) for 24 hours and TSP2 mRNA levels were analyzed. Although treating cells with 0.5mM of 8-Br cAMP did not result in a statistically significant decrease in TSP2 expression, 2.5mM and 12.5mM of 8-Br cAMP reduced TSP2 mRNA to 22.4% and 11.5% of untreated controls, respectively, indicating that increased cAMP levels is sufficient to down regulate TSP2 expression in 3T3-L1 cells (Figure 2B). To determine whether PKA activity is required for down-regulating TSP2 expression, we treated cells with H89, which blocks PKA activity, or forskolin, a PKA activator. Treatment with IBMX or forskolin individually reduced TSP2 expression to 51% and 61% of untreated controls, respectively, and when used in combination, they reduced TSP2 levels to 12.4% of untreated controls. Although 0.5mM 8-Br cAMP, did not significantly reduce TSP2 expression, it acted synergistically with IBMX to reduce TSP2 mRNA to 14.7% of untreated controls. H89 by itself did not affect TSP2 expression but it completely abrogated the effect of IBMX and forskolin on down-regulating TSP2 expression by blocking PKA signaling (Figure 2C). These results suggest that increased PKA signaling decreases TSP2 expression.

2.3 TSP2-null bone marrow MPC (BmMPC) exhibit increased lipid accumulation

Since TSP2-null BmMPC display a delay in osteogenic differentiation (Hankenson et al., 2000) and adipogenic and osteogenic differentiation show reciprocal regulation, we next examined whether TSP2 plays a role in adipogenesis. First passage BmMPC from TSP2-null and wild-type control mice were induced to undergo adipogenic differentiation using routine adipogenic cocktails, isobutylmethylxanthine/dexamethasone/insulin (MDI) or MDI + the PPARgamma agonist troglitazone (MDI/T) and extent of adipogenic differentiation was evaluated at 4 and 10 days post induction by staining intracellular lipid droplets with Oil-Red-O and then measuring total extracted Oil-Red-O using spectrophotometry. TSP2-null BmMPC showed 24% and 25% more Oil-Red-O incorporation than wild-type controls at 4 and 10 days post-adipogenic induction, respectively (Figure 3A-C). Evaluation of adipogenic marker gene expression showed the well-established increases in C/EBPalpha, PPARgamma and FABP-4 expression following adipogenic induction at day 4 and 10 post-induction (MacDougald and Lane, 1995). However, there were no differences in gene expression between TSP2-null and wild-type BmMPC in any of the treatment groups at 4 days post adipogenic induction. At 10 days post induction, a 37% increase in C/EBP- α expression in MDI/T induced and a 15% increase in FABP-4 expression in uninduced TSP2-null BmMPC was detected when compared to wild-type MPC that received the same treatments. There was no significant difference in PPAR- γ expression between TSP2-null and wild-type BmMPC at 10 days post adipogenic induction (Figure 4). The increase in lipid accumulation in TSP2-null BmMPC without pronounced differences in C/EBPalpha, PPARgamma or FABP-4 expression suggests that TSP2 does not directly affect the adipogenic commitment program but rather may act through other, downstream mechanisms, such as inhibition of cell proliferation and/or fatty acid uptake, to influence lipid accumulation.

2.4 AdMPC from TSP2-null mice show increased proliferation and lipid accumulation

The stromal vascular (S/V) fraction of white adipose tissue contains cells with similar differentiation potential as bone marrow-derived MPC (Gimble and Guilak, 2003). To determine whether TSP2 has an effect on proliferation of AdMPC as previously demonstrated with marrow-derived MPC (Hankenson and Bornstein, 2002), TSP2-null and wild-type AdMPC were plated at equal density then were trypsinized and counted every 24 hours for 5 days. The total number of TSP2-null cells was 1.3-, 3.3-, 4.2- and 4.5-fold higher than wild-type controls at 48, 72, 96 and 120 hours, respectively (Figure 5A). This is consistent with published data on TSP2-null BmMPC which has shown increased proliferation relative to wild-type controls (Hankenson et al., 2000).

To evaluate whether TSP2 plays a role in adipogenic differentiation of AdMPC, TSP2-null and wild-type AdMPC were induced to undergo adipogenic differentiation using MDI or MDI/T and the extent of lipid accumulation at 4, 7, and 14 days post induction was measured using Oil Red-O incorporation (Figure 5B). A statistically significant increase in Oil Red-O staining was observed in TSP2-null AdMPC induced with MDI/T at days 4 and 7 and in MDI induced cells at day 7. There was also significantly more Oil Red-O incorporation in un-induced TSP2 null AdMPC at days 4 and 14 (Figure 5B). To determine whether the increase in Oil Red-O staining in TSP2-null cells was due to an increase in cell number, the number of Oil Red-O stained cells was counted and normalized to the total number of cells as determined by counting the number of DAPI stained nuclei. Similar to Oil Red-O absorbance, the number of Oil Red-O positive cells normalized to the total number of cells was significantly increased in MDI/T induced TSP2 null AdMPC at days 4, 7 and 10, in MDI treated cells at day 7 and in un-induced controls at day 4 and day 7 (Figure 5C). Taken together, these results show that in the absence of TSP2 there is an increase in the number of Oil Red-O positive cells in TSP2-null cultures, but that this increase appears to be independent of final cell number. Similar to the findings in

BmMPC, expression of C/EBP alpha, FABP4 and PPARgamma are similar between TSP2-null and WT AdMPC (Results not shown).

2.5 TSP2-null mice exhibit an increase in adipose tissue

Since TSP2-null MPC showed increased lipid accumulation in vitro, we asked whether TSP2 regulates overall adipose tissue development in vivo. Age and gender matched six-week old TSP2-null and wild-type control mice were placed on either control or high fat diet and were weighed weekly for 22 weeks (Figure 6). TSP2-null female mice gained significantly more weight than wild-type control female mice under control diet. The difference in weight became statistically significant at 17 weeks and was greatest at 22 weeks with TSP2-null mice weighing 30% more than wild-type controls (Figure 6A). Interestingly, there was no significant difference in weight between male TSP2-null and wild-type mice fed the control diet or in either male or female TSP2-null and wild-type mice fed a high fat diet (Figure 6B–D).

To determine whether the increase in weight in TSP2-null mice was due to an increase in adipose tissue, we measured body composition of TSP2-null and wild-type mice at the end of the 22 week study period using dual energy X-ray absorptiometry (DEXA). The percentage of total body fat was significantly higher in TSP2-null female mice on control diet when compared to gender matched wild-type controls (Figure 7A), but there was no difference in lean body mass (Figure 7B). No appreciable difference was detected in percent body fat in male mice fed control diet or male or female mice fed high fat diet (results not shown). These results are consistent with results that show significant differences in weight between TSP2-null and WT female mice fed a control diet and suggest that the explanation for increased body mass in TSP2-null mice is increased adipose tissue.

To determine whether increased adipose tissue in TSP2-null mice occurs at specific sites, subcutaneous fat pads from interscapular and paralumbar regions, visceral fat pads from perirenal and reproductive (ovarian or testicular) regions, as well as brown fat and skin were isolated and weighed (Figure 7C,D). There was a 2.7-fold increase in both paralumbar and interscapular (subcutaneous) fat pads and 1.5 fold increase in skin weight in female TSP2-null mice on control diet when compared to wild-type controls. There was no statistically significant difference in visceral (perirenal and reproductive) or brown fat pad weights in either female or male TSP2-null and wild-type control mice (Figure 7C). With the exception of a decrease in ovarian fat pad weight in TSP2-null mice, there was no difference in fat pad or skin weights between TSP2-null and wild-type mice fed a high fat diet (Figure 7D).

3. Discussion

We have previously shown that TSP2 promotes osteogenic differentiation of BmMPC (Alford et al., 2009; Hankenson and Bornstein, 2002). Because of the well-established reciprocal relationship between osteogenic and adipogenic cell fates, in this study, we examined the role of TSP2 in adipogenesis, and show that TSP2 is down-regulated during adipogenesis and inhibits adipocyte development in vitro and adiposity in vivo.

TSP2 expression during MPC differentiation was examined and consistent with a role in promoting osteogenic differentiation and decreasing adipogenesis, TSP2 expression is up-regulated during osteogenic differentiation and is inhibited during adipogenic differentiation. The observation that TSP2 down-regulation occurs within 24 hours of adipogenic induction suggests that down regulation of TSP2 expression may be important for early adipocyte development and maturation. We also found that activation of cAMP-dependent PKA pathway during adipogenic differentiation plays a major role in down-regulating TSP2-expression. Although their mechanisms were not examined further in this study, dexamethasone and insulin

used in the adipogenic induction cocktail were also found to play a role in down-regulating TSP2 expression.

Our results demonstrate that TSP2-null MPC show an increase in lipid accumulation when compared to wild-type controls. By normalizing the number of lipid droplet containing adipocytes to the total number of cells, we found that TSP2 decreases lipid accumulation independent of TSP2 effects on cell proliferation. As well, there was no significant increase in mRNA expression of PPARgamma, a key transcription factor believed to be a master regulator of adipogenesis. C/EBPalph, an early adipogenic transcription factor that acts to up-regulate PPARgamma, only showed a slight increase in mRNA expression at day 10, well after the peak expression for C/EBPalph has occurred. The lack of pronounced differences in gene expression of these two master transcription factors between TSP2-null and wild-type MPC suggests that TSP2 decreases lipid accumulation in MPC not by directly regulating the adipocyte transcriptional program but rather through another mechanism.

Although the cellular and molecular mechanisms through which TSP2 influences adipogenesis were not investigated in this study, there are several possible considerations. First, TSP2 binds to CD36 (Fatty Acid Transporter), which is a Class B scavenger receptor and TSP2 could interfere with CD36 uptake of long-chain fatty acids and disrupt adipogenesis (Sfeir et al., 1997). Similarly, TSP2 also binds to the LDL lipoprotein receptor related protein, LRP1 (Yang et al., 2001). Conditional knockout mice lacking LRP1 in adipocytes show a decrease in adipose mass (Hofmann et al., 2007), and LRP1 influences in vitro adipogenesis (Masson et al., 2009). Thus TSP2 could block LRP1 function in adipogenesis.

Importantly, we also examined whether TSP2 plays a role in adipose tissue development in vivo and found that TSP2-null female mice weigh more than age and gender matched wild-type control mice. The increase in weight appeared to be due to an increase in non-visceral adipose. Adipose tissue is formed to store excess energy in the form of neutral triglycerides; thus, the TSP2-null female mice on control diet presumably have an increase in excess energy. Energy excess occurs either secondary to a decrease in energy expenditure or an increase in food intake. Future studies should examine whether an increase in food intake or a decrease in energy expenditure enables the increase in energy that is necessary for increased adipose tissue development in TSP2-null female mice. Furthermore the significance of TSP2 in regulating non-visceral adipose specifically should be pursued.

The effects of TSP2 on body weight and adipose tissue development were only observed in female mice suggesting that TSP2 may alter adipose tissue regulation by female sex hormones. Interestingly, the absence of TSP2 has been shown to protect against ovariectomy-induced bone loss (Hankenson et al., 2005). It is well known that estrogen, produced by the ovaries, is a major regulator of adipose tissue development (Cooke and Naaz, 2004). It will be interesting to determine whether TSP2 can modulate the actions of estrogen or other female sex hormones on adipose tissue development. Intriguingly, this difference in weight gain and adiposity are not observed in female mice fed a high-fat diet, and in fact female TSP2-null mice show very little increase (approximately 10%) in body mass on a high-fat diet. At this point an explanation for the relative lack of weight gain by TSP2-null mice on a high-fat diet is not clear, and warrants further investigation.

Here we have described an inhibitory role for TSP2 on development of adipocytes in vitro and adipose in vivo and have characterized changes that occur in TSP2 expression levels with MPC differentiation. Future studies will address the mechanisms through which TSP2 affects adipose tissue development at molecular, cellular and whole animal levels.

4. Experimental Procedures

4.1 Mice

All procedures were approved by the institutional animal care and use committee. TSP2-null mice have been described previously and were generated by targeted disruption of the *Thbs2* gene which results in a functional TSP2-null phenotype (Kyriakides et al., 1998). Coisogenic wild-type 129/SvJ mice were used as controls for comparison. For high fat diet studies, one month old TSP2-null and wild-type control mice were fed control or high fat chow (D12450B or D12451, respectively, Research Diets Inc., New Brunswick, NJ) for 5 months. Mice were weighed weekly and at the end of the 5 month study period, they were anesthetized with pentobarbital sodium (50 mg/kg BW i.p.), and body composition was measured using dual-emission x-ray absorptiometry (DEXA) (PIXImus DEXA, General Electric, Madison, WI). Mice were then euthanized and subcutaneous and visceral fat pads as well as skin and brown fat was isolated and weighed.

4.2 Bone marrow-derived MPC (BmMPC) isolation

Whole bone marrow was harvested from femora and tibiae of three to four month old TSP2-null and wild-type control mice as previously reported (Hankenson et al., 2000). Single cell suspensions from three mice were pooled, and then plated onto four 100mm tissue culture plates in MPC media (alpha-minimum Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25ug/mL sodium ascorbate, and 100U/mL penicillin and 100ug/mL streptomycin). Cells were cultured for 8 to 10 days with half the media being replaced four days post plating, and every three days until harvest. When cultures reached 80% confluence, cells were trypsinized, and then plated onto 12-well tissue culture plates for differentiation experiments.

4.3 Adipose-derived MPC (AdMPC) isolation

Paralumbar fat pads from 3 to 5 mice were minced then digested for 30–60 minutes at 37°C with 2.5mg/ml collagenase II (Sigma-Aldrich). The digest was filtered through a 70µm filter and centrifuged at 500g for 6 min to pellet the stromal vascular fraction. The stromal vascular cells were then plated onto 10cm tissue culture plates in AdMPC media (Dulbecco's modified eagle medium (DMEM) + 10% fetal bovine serum + 100U/mL penicillin and 100ug/mL streptomycin) and passaged when they reached 80% confluence. Third passage cells were used for all experiments.

4.4 Cell culture and treatments

For adipogenic differentiation, 1×10^5 primary BmMPC or 5×10^4 ST2 and AdMPC were plated into each well of 12-well tissue culture plates. Three days post plating, BmMPC and ST2 cells were treated with induction media (MPC media containing MDI; 57µM isobutyl-methylxanthine, 1µM dexamethasone, and 1µg/mL insulin or MDI/T; MDI and 5µg/ml troglitazone) for three days then maintained in MPC media containing 1µg/mL insulin for the remaining duration of the experiments. AdMPC were induced two days post plating with the same induction cocktail in AdMPC media then were switched and maintained in SVF media with 1µg/mL insulin two days post induction. Cells were stained with Oil Red-O and DAPI and five representative images at 40X magnification were captured. Cells staining positive for DAPI and Oil Red-O were counted and compared to total DAPI positive cells. Oil Red-O was then extracted and quantified by measuring absorbance at 540nm in a Varioscan spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). For osteogenic differentiation, ST2 cells were treated and maintained in MPC media containing 10nM BMP2, 25µg/mL sodium ascorbate and 10mM β-glycerol phosphate.

For pharmacomimetic and inhibitor studies, 3T3-L1 cells were plated at a density of 1×10^5 cells per well into each well of a 6 well tissue culture plate in L1 media (DMEM, 10% fetal bovine serum, 100U/mL penicillin, 100ug/mL streptomycin) and cultured for 3 days. Cells were then treated with the indicated combinations and concentrations of drugs for 24 hours.

4.5 Quantitative RT-PCR

RNA was extracted using RNeasy RNA extraction kit (QIAGEN) according to the manufacturer's instruction and 1 μ g of total RNA was reverse transcribed in a 30 μ L reaction. 2 μ L of the cDNA generated was used for quantitative real-time PCR reaction using SybrGreen for detection on either a MJR Opticon or an ABI 7500 Fast. Relative change in expression of genes of interest was determined using the $2^{-\Delta\Delta C(T)}$ method using β -actin expression as an endogenous control. Primer sets are available from the corresponding author upon request.

4.6 Western Blot Analysis

Prior to harvesting adipogenic and osteogenic induced ST2 cells for RNA extraction associated with Figure 1, total media was collected and equivalent volumes for each treatment condition and time were resolved on 8% SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with a mouse TSP2 polyclonal antibody. After incubating with HRP-conjugated secondary antibody, membranes were developed with ECL Chemiluminescent reagent.

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Figure 1. TSP2 expression is reciprocally regulated during adipogenic and osteogenic differentiation

ST2 cells were induced to undergo osteogenic and adipogenic differentiation for 12 days. (A,B) Cells were harvested at the indicated time points following induction and TSP2 mRNA expression relative to un-induced control cells harvested at the same time was measured by quantitative real-time PCR. (C) Media was collected prior to cell harvest for RNA extraction and the amount of TSP2 protein secreted into the media was estimated by loading equivalent volumes of media on SDS-PAGE and then resolving TSP2 using western-blot analysis. Results shown are mean $\pm$ S.D. of two independent experiments each done in duplicate. # significantly different from control treated cells; $p < 0.05$.

Figure 2. Components of adipogenic induction cocktail additively down-regulate TSP2 expression (A) Confluent 3T3-L1 cells were treated with the indicated combinations of adipogenic induction cocktail for 24 hours then TSP2 mRNA was measured using qPCR. (M, IBMX; D, Dexamethasone; I, Insulin) (B) 3T3-L1 cells were treated with either 100mM IBMX or increasing doses of 8Br-cAMP (0.5mM, 2.5mM and 12.5mM) for 24 hours and TSP2 expression was analyzed by qPCR (C) 3T3-L1 cells were treated with the indicated combination of IBMX (100μM), forskolin (10μM), 8-Br cAMP (0.5mM) or H89 (20μM) for 24 hours and TSP2 mRNA levels were analyzed by qPCR Data shown are mean ± S.D. of two independent experiments each done in duplicate. # = TSP2-null significantly different from control treated cells; $p < 0.05$.

Figure 3. TSP2 Inhibits lipid accumulation in bone marrow MPC

BmPC isolated from bone marrow of wild-type and TSP2-null mice were induced to undergo adipogenic differentiation using MDI or MDI/T then stained with Oil Red-O at 4 and 10 days post induction. (A) Image of representative Oil Red-O stained plate at 10 days post adipogenic induction and (B) representative 10X and 20X magnification images of cells induced with the indicated treatments are shown. (C) To quantify lipid accumulation, Oil-Red O was extracted from MDI/T induced cells at 4 and 10 days post adipogenic induction and absorbance was measured at 540 nm. The results shown are representative of two independent experiments each performed in duplicate. For each experiment BmMPC were obtained from 3 wild-type and 3 TSP2-null mice and whole marrow pooled to yield MPC. (Error bars represent standard deviation. # = TSP2-null significantly different from wild-type; #, $p < 0.05$; ##, $p < 0.005$).

Figure 4. Minor differences in expression of adipogenic marker genes exist between TSP2-null and wild-type BmMPC

BmMPC from TSP2-null and wild-type control mice were induced to undergo adipogenic differentiation with either MDI or MDI/T. Cells were harvested at 4 and 10 days post adipogenic induction and expression of adipogenic differentiation marker genes C/EBP- α , PPAR- γ , and FABP-4 was analyzed by qPCR. The results shown are averages of two independent experiments each performed in duplicate. Error bars represent standard deviation. # = TSP2-null significantly different from wild-type; $p < 0.05$

Figure 5. Adipose tissue derived MPC from TSP2-null mice show increased proliferation and increased adipogenic differentiation

(A) AdMPC were plated at 50,000 cells/well in 12 well tissue culture plates then were trypsinized and counted at the indicated time points. Statistically significant increase in TSP2-null cell numbers relative to wild-type controls was observed at 48, 72, 96, and 120 hours post plating. Values shown are mean $\pm$ SEM of five independent experiments conducted on five independent age-matched cell harvests. # = TSP2-null significantly different from wild-type; $p < 0.05$. (B) AdMPC were induced to undergo adipogenesis with either MDI or MDI/T then were fixed and stained with DAPI and Oil Red-O at days 4, 7 and 14 post induction. Five representative images were captured at 40X magnification from each well then Oil Red-O was extracted and quantified by measuring absorbance at 540 nm. (C) The number of Oil Red-O stained cells in captured image were counted and normalized to the total number of cells (DAPI staining nuclei) to account for differences in cell proliferation between TSP2 null and wild-type MPC. Values are mean $\pm$ SEM of 3 independent experiments from 3 independent harvests. # = TSP2-null significantly different from wild-type; $p < 0.05$.

Figure 6. TSP2-null mice are heavier than wild-type control mice but do not gain appreciably more weight on high fat diet

Six-week-old TSP2-null and wild-type mice from at least four litters were randomly placed on either a control or a high fat diet for 22 weeks. Mice were weighed weekly and the average body weight for each group ($\pm$ SEM) at each time point is shown. Control diet: (A) Female (wild-type $n=5$, TSP2-null $n=5$); (B) Male (wild-type $n=8$; TSP2-null $n=6$) High calorie diet: (C) Female (wild-type $n=5$, TSP2-null $n=5$); (D) Male (wild-type $n=9$; TSP2-null $n=6$). A statistically significant increase in body weight was observed at weeks 17 through 22 for female TSP2 null mice fed the control diet relative to wild-type control mice on the same diet. (# = TSP2-null significantly different from wild-type; $p < 0.05$).

Figure 7. TSP2-null female mice on control diet have an increase in adipose tissue

Total body composition of TSP2-null and wild-type female mice fed either a control diet or a high fat diet for 22 weeks starting at 6 weeks of age was measured using dual-emission x-ray absorptiometry (DEXA). **(A)** The percentage of fat tissue is depicted as mean $\pm$ SEM. **(B)** The total lean mass is depicted as mean $\pm$ SEM. Wild-type (control diet, female $n=5$) and high calorie diet (female $n=5$) and TSP2-null (control diet, female $n=5$ and female $n=5$); # = significantly different from wild-type; $p < 0.05$. Fat pads and skin from TSP2-null and wild-type mice fed either **(C)** control or **(D)** high fat diet were harvested and weighed. A statistically significant increase in paralumbar and interscapular (subcutaneous) fat pad as well as skin weight was observed in female TSP2-null mice on control diet. Values are mean $\pm$ SEM. Wild-type (control diet male $n=7$, female $n=5$ and high calorie diet male $n=9$, female $n=5$) and TSP2-null (control diet male $n=6$, female $n=5$) and high calorie diet male $n=6$, female $n=5$); # = TSP2-null significantly different from wild-type; $p < 0.05$.