Research Paper

Reduced white fat mass in adult mice bearing a truncated Patched 1

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Hedgehog (Hh) signaling emerges as a potential pathway contributing to fat formation during postnatal development. In this report, we found that *Patched 1 (Ptc1)*, a negative regulator of Hh signaling, was expressed in the epididymal fat pad of adult mice. Reduced total white fat mass and epididymal adipocyte cell size were observed in naturally occurring spontaneous mesenchymal dysplasia (*mes*) adult mice (*Ptc1^{mes/mes}*), which carry a deletion of Ptc1 at the carboxyl-terminal cytoplasmic region. Increased expression of truncated *Ptc1*, *Ptc2* and *Gli1*, the indicators of ectopic activation of Hh signaling, was observed in epididymal fat pads of adult *Ptc1^{mes/mes}* mice. In contrast, expression of *peroxisome proliferator-activated receptor gamma*, *CCAAT/enhancer binding protein alpha*, *adipocyte P2* and *adipsin* were reduced in epididymal fat pads of adult *Ptc1^{mes/mes}* mice. Taken together, our results indicate that deletion of carboxyl-terminal tail of Ptc1 can lead to the reduction of white fat mass during postnatal development.

Key words: gene expression, mouse, Patched 1, spontaneous mesenchymal dysplasia, white fat mass, body weight

Introduction

Hedgehog (Hh) signaling plays a critical role during embryonic development and tumorigenesis [1, 2]. The canonical Hh signaling cascade is initiated through the binding of secreted Hh proteins to the membrane-bound receptor Patched-1 (Ptc1) or Patched-2 (Ptc2) to relieve patched-mediated repression of the signal transducer smoothened (Smo), leading to the activation of transcription factors Gli1, Gli2 and Gli3 to enhance transcription of downstream targets such as *Ptc1, Ptc2* and *Gli1* [1-5]. In addition to its critical roles during embryonic development and tumorigenesis, Hh signaling emerges as a potential mediator of fat formation and body weight gain during postnatal development [6, 7]. Injection of adult mice with sonic Hh (Shh)-IgG fusion protein (a Hh agonist) causes a significant increase in fat mass and body weight without affecting food consumption and fluid intake [8]. In agreement with this observation, inactivation of Hh signaling by injection of anti-Hh antibodies in normal adult mice fed with a high-fat diet and in obese leptin-deficient (*ob/ob*) mice fed with a low-fat diet can significantly reduce body weight gain in these animals [9]. These animal studies suggest that Hh signaling may stimulate fat formation (adipogenesis) and promote body weight gain in adult mice [8, 9]. In contrast to the above animal studies [8, 9], it has been reported that Hh can block adipogenesis in cultured mammalian adipocytic or multipotent mesenchymal cells [10-13]. This is highlighted by the recent study by Suh *et al* [13]. They found that fat-body-specific activation of Hh signaling inhibited fat formation in *Drosophila* and that Hh blocked adipogenesis in mammalian cells by regulating antiadipogenic gene expression. In addition, they found that Hh signaling molecules such as Gli1, Gli2 and Gli3 are expressed in white fat tissues (WAT) of adult mice and that expression of these genes are down-regulated in normal mice fed with high fat diet and in *ob/ob* mice. A model of Hh signaling in inhibiting fat formation has been proposed in which Hh activates Gli transcription factors (Gli1, Gli2 and Gli3), which then activate antiadipogenic gene expression to inhibit the expression of adipogenic genes such as *peroxisome proliferator-activated receptor gamma* (*PPARgamma*) [14, 15] and *CCAAT/enhancer binding protein alpha (CEBPalpha)* [16] in adipose tissues, resulting in inhibition of adipogenesis [6, 13]. According to this model, it is expected that ectopic activation of Hh signaling in adipose tissues will reduce fat formation in adults, which would be contradictory to observations reported by Martin *et al* [8]. Therefore, whether Hh signaling acts to stimulate or inhibit fat formation during postnatal development remains to be characterized *in vivo* using animal models such as mutant mice carrying deletions of Hh signaling molecules.

Ptc1 is a negative regulator of the Hh signaling during embryonic development [1, 4]. It is a Hh receptor protein containing twelve transmembrane domains and intracellular amino- and carboxyl-terminal regions [2, 17]. In the absence of Hh, Ptc1 represses Smo activity. This repression of Smo is relieved once after Hh binds to the extracellular loops within the transmembrane domains of Ptc1, resulting in the activation of Hh signaling downstream target genes to elicit its biological functions [2, 5, 17, 18]. It have been shown that the carboxyl terminus of Ptc1 is important for the inactivation of Hh signaling target genes and the localization and degradation of Ptc1 itself [19, 20]. Deletion of *Ptc1* in mice can mimic ectopic Hh signaling and cause embryonic lethality, indicating the essential role of Ptc1 during embryonic development [21]. Although Ptc1 has been shown to be a determinant of body weight *in utero* [21-23], there is no direct evidence that Ptc1 is also involved in controlling fat formation during postnatal development. The naturally occurring spontaneous mesenchymal dysplasia (*mes*) mouse line (*Ptc1mes/mes*), which has a deletion of the Ptc1 protein at the carboxyl-terminal cytoplasmic region and can bypass the embryonic lethality observed in a complete knockout of Ptc1 [22, 24], would be an excellent model to investigate the potential role of Ptc1 in fat formation during postnatal development.

In this communication, we found that Ptc1 was expressed in epididymal white fat tissue (WAT) in adult mice. Loss of the carboxyl-terminus of Ptc1 in adult *Ptc1mes/mes* mice caused a significant reduction in WAT. The adipocyte size of epididymal WAT was also reduced in *Ptc1mes/mes* mice. Gene expression analyses showed that truncated *Ptc1*, *Ptc2* and *Gli1* were overexpressed in WAT of *Ptc1mes/mes* mice, indicating ectopic activation of Hh signaling in WAT. Consistent with reduced WAT, expression of adipogenic genes, *PPARgamma* and *CEBPalpha*, and the adipocyte marker genes, *adipocyte P2 (aP2)* [25] and *adipsin* [26], were reduced in WAT of adult *Ptc1mes/mes* males. Collectively, our results suggest that Ptc1 play a role in white fat formation during postnatal development. This study supports earlier findings of Suh *et al* and Spinella-Jaegle *et al* that Hh signaling inhibits fat formation in cultured cells [10, 13].

Materials and Methods

Animals and genotyping

Ptc1mes/+ mice (B6C3Fe-*a/a* strain) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained on a 14 hour light:10 hour dark cycle, with free access to normal chow (Lab diet, Catalog #5001, USA) and water, in the vivarium of the University of Louisville. *Ptc1+/+*, *Ptc1mes/+* and *Ptc1mes/mes* littermates were obtained from the intercross of *Ptc1mes/+* mice and genotyped as previously described [22].

Tissue collection and body, tissue and total WAT weight analyses

Mice at various ages were euthanized with carbon dioxide and the body weights of these mice were determined using an electronic balance. Liver, spleen, kidney, lung, heart and total WAT including subcutaneous, abdominal and intra-scapular fat in mice were collected and weighed using a balance. Since it took some time to collect WAT from subcutaneous regions of the whole animal which may damage the WAT histology and cause RNA degradation, WAT from epididymides were quickly harvested for histological and RNA analyses.

Histological analysis of WAT and determination of WAT size

Epididymal WAT was fixed in 4% paraformaldehyde, dehydrated and then embedded in paraffin. The paraffin-embedded tissues were sectioned at 7 μm thickness, stained with hematoxylin and eosin, and then examined under a microscope (Axioskop2, Zeiss, Thornwood, NY, USA). The average size of adipocyte cells from each mouse was determined by analyzing 40 randomly-selected adipocyte cells from 20 sections using the Zeiss Imaging Quantitative Software (Zeiss, Thornwood, NY, USA).

RNA isolation and RT-PCR

RNA was isolated from epididymal WAT of *Ptc1mes/mes* and control mice using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized at 42oC for 60 min using 1 μg of total RNA as template and random hexanucleotides as primers (Promega, Madison, WI, USA). PCR reactions with 1/10 of the cDNA were carried out in a buffer containing 1.5 mM $MgCl_2$ (94oC, 1 min; 52oC, 1 min; 72oC 1 min) for various numbers of PCR cycles (20-38 cycles), which can detect the differential gene expression (within the linear range of PCR products for each gene). The nucleotide sequences of PCR primers are as follows: *Ptc1* (Ptc1-F: 5′-tgtctggcatcagtgaggag-3′, Ptc1-R: 5′-gacaaggagccaga gtccag-3′, 190 bp; Ptc1-F2: 5′-gctggaggagaacaagcaac-3′, Ptc1-R2: 5′-cattgggggtagctggacagt-3′, 536 bp), *Ptc2* (5′-tgcctctctggagggcttcc-3′ and 5′-cagttcctcctgccagtgca -3′, 208 bp), *Gli1* (5′-actggggtgagttccccttc-3′ and 5′-tggcagggctctgactaact-3′, 492 bp), *Gli2* (5′-cccccctagcatcaatgagaa-3′ and 5′-tctgcacggattgtggatta -3′, 496 bp), *Gli3* (5′-ctacggcgactgagaggaag-3′ and 5′-tggcatcaattggtacagga-3′, 396 bp), *glucocorticoid-induced*

leucine-zipper protein (*Gilz*, 5'-tggggcctagtaacaccaag-3' and 5'-aagctgtaaccccacactgg-3', 364 bp), *CEBPalpha* (5'-atcccagagggactggagtt-3' and 5'-aagtcttagccggaggaa gc-3', 373 bp), *PARgamma* (5'- ccctggcaaagcatttgtat-3' and 5'-aatccttggccctctgagat-3', 403 bp), *aP2* (5'-tcacctggaagacagctcct-3' and 5'-tcgactttccatcccacttc-3', 344 bp), *adipsin* (5'-caagcgatggtatgatgtgc-3' and 5'-atccggtaggatgacactcg-3', 465 bp) and *beta-actin* (5'-ttgagaccttcaacacccc-3' and 5'-agccagagcagtaatctcc-3', 593 bp). All PCR products were confirmed by DNA sequencing.

Food intake measurement

Five adult wild type and *Ptc1^{mes/mes}* mice at the age of 23-27 weeks were singly housed with free access to water. The food present in a sealed food hooper was recorded weekly for consecutive two weeks using an electronic balance.

Glucose tolerance test

Glucose tolerance test was performed as described previously [23]. Briefly, overnight-fasted 24-26-week-old wild type, heterozygous and homozygous *Ptc1^{mes}* littermates were injected intraperitoneally with 2 grams/kg body weight of 20% D-glucose. Blood glucose levels at time 0 (immediately before the injection of glucose), 15, 30, 60, 120 and 180 minutes after injection of glucose were determined using a Glucometer Elite glucometer.

Statistical analysis

When applicable, *Student's t-test* was used to determine the statistical significance of difference between *Ptc1*^{mes/mes} and control littermates with *P* value less than 0.05. Data are presented as mean ± SEM of indicated numbers of mice in the figures.

Results

Expression of Hh signaling molecules *Ptc1*, *Ptc2*, *Gli1*, *Gli2* and *Gli3* in WAT of adult mice

To determine whether Hh signaling molecules such as *Ptc1*, *Ptc2*, *Gli1*, *Gli2* and *Gli3* are expressed in WAT of adult mice, we performed RT-PCR analyses using specific primers for each gene. As shown in Figure 1, specific PCR products for those genes were detected in RNA samples from epididymal WAT of adult mice. In addition, we also observed the expression of an antiadipogenic gene *Gilz* [27], adipogenic genes *CEBPalpha* and *PPARgamma*, and mature adipocyte marker genes *aP2* and *adipsin* in epididymal WAT (Figure 1).
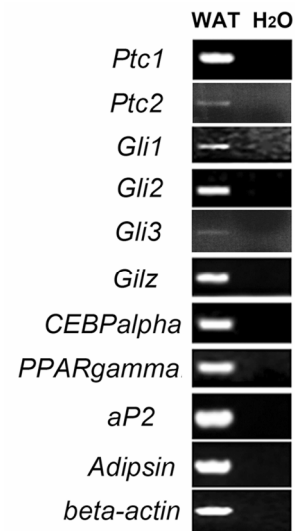

Figure 1. Expression of *Ptc1*, *Ptc2*, *Gli1 Gli2* and *Gli3* in mouse epididymal WAT. Epididymal WAT RNA from 8-week-old mice was subjected to RT-PCR analyses using specific primers for *Ptc1* (Ptc1-F and Ptc1-R), *Ptc2*, *Gli1*, *Gli2*, *Gli3*, *Gilz, PPARgamma*, *CEBPalpha*, *aP2* and *adipsin*. The numbers of PCR reaction cycles are 36 for *Ptc1*, 38 for *Ptc2* and 32 for other tested genes. Water was included as negative controls.

Reduced total WAT and adipocyte cell size in adult *Ptc1^{mes/mes}* mice

To investigate if there is any defect in WAT during postnatal development, total WAT, body and major organ weights of male *Ptc1*^{+/+}, *Ptc1*^{mes/+} and *Ptc1*^{mes/mes} littermates at various ages were determined (Figure 2). As shown in Figure 2a, total WAT weights were reduced in *Ptc1*^{mes/mes} male mice during postnatal development with a modest reduction at the age of 19-20 weeks and the remarkable reduction at the age of 33 or 38 weeks, when compared to their wild type and heterozygous littermates (Figure 2a). No significant changes in body weights were observed between male *Ptc1*^{mes/mes} and control wild type and heterozygous littermates during postnatal development, except that 19-20-week-old *Ptc1*^{mes/mes} males were slightly heavier than their controls (Figure 2b). The ratios of total WAT to body weights were also significantly reduced in 19-20-week-old or older *Ptc1*^{mes/mes} males (Figure 2c). In contrast, there were no significant changes in gross weights of other tested organs including liver, spleen, kidney, lung and heart (Figure 2d). The representative results of reduced total WAT of 33-week-old *Ptc1*^{mes/mes} male mice were shown in Figure 2e. Histological analyses showed that epididymal fat cells of these *Ptc1*^{mes/mes} mice were smaller than those of *Ptc1*^{+/+} mice (Figure 2f). Quantitative analyses showed that the sizes of epididymal WAT cells in 33-week-old *Ptc1*^{mes/mes} mice were significantly reduced (*P* < 0.01), when compared to those of *Ptc1*^{+/+} littermates (Figure 2g).
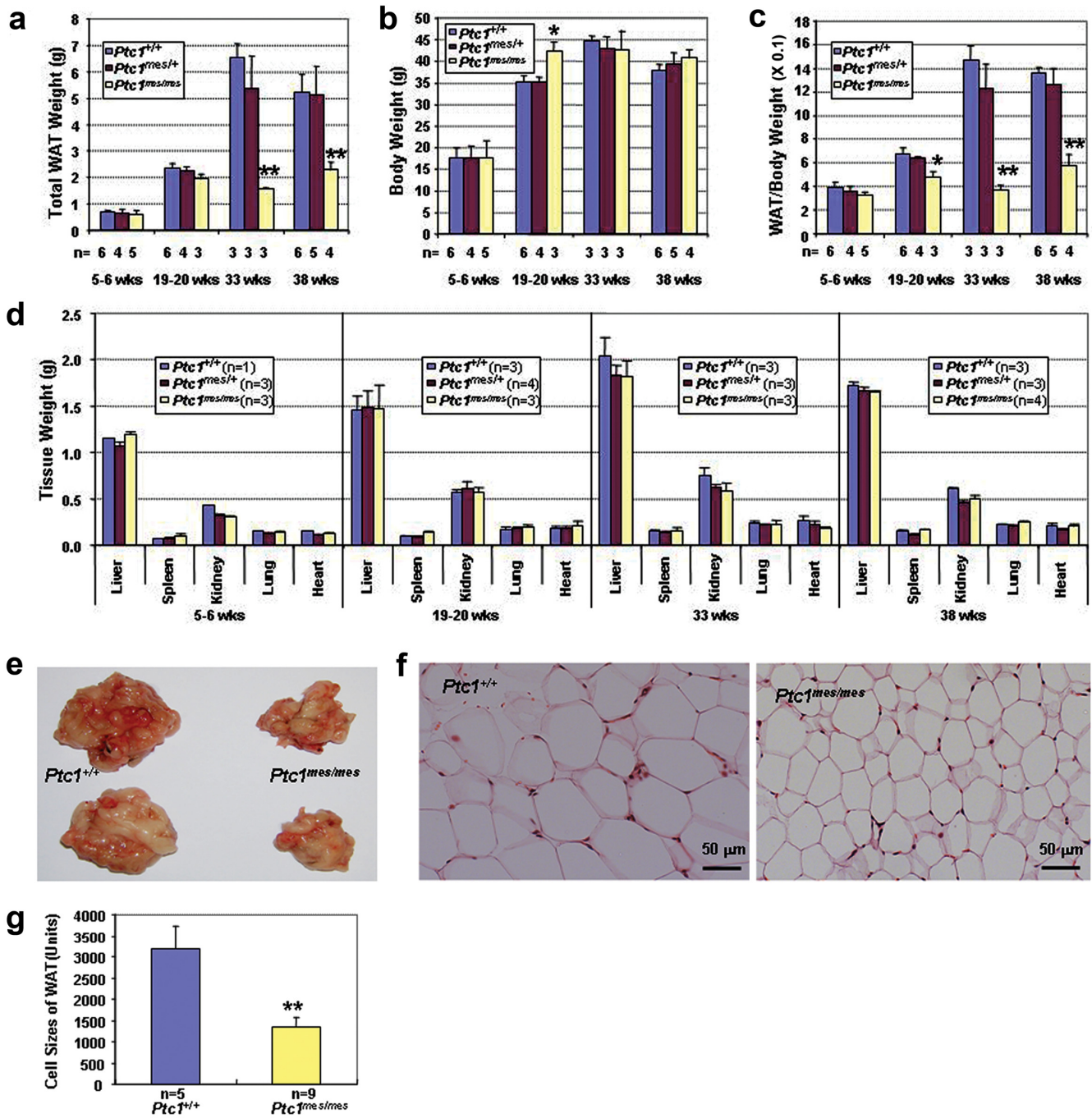
Figure 2. Reduced total WAT and adipocyte cell sizes in adult male $Ptc1^{mes/mes}$ mice, when compared to control $Ptc1^{+/+}$ littermates. **a-d.** Total WAT weights (**a**), body weights (**b**), the ratios of total WAT to body weights (**c**) and major organ weights (**d**) of $Ptc1^{+/+}$, $Ptc1^{mes/+}$ and $Ptc1^{mes/mes}$ male mice during postnatal development. **e.** Representative photos showing reduced total WAT in $Ptc1^{mes/mes}$ mice. Total WAT of two wild type and their $Ptc1^{mes/mes}$ littermates at the age of 33 weeks are shown. **f.** Histology of WAT from 33-week-old $Ptc1^{+/+}$ and $Ptc1^{mes/mes}$ littermates. Note the small adipocyte cell size in WAT of $Ptc1^{mes/mes}$ mice. **g.** Quantitative analyses showing the reduced cell size of WAT in 33-week-old $Ptc1^{mes/mes}$ mice, when compared to their wild type littermates. In **a-d** and **g**, data are presented as mean ± SEM of the indicated numbers of mice in the bar graphs. *$P < 0.05$, **$P < 0.01$, when compared to $Ptc1^{+/+}$ and/or $Ptc1^{mes/+}$ littermates.

Enhanced expression of Hh signaling target genes (amino region of *Ptc1*, *Ptc2*, and *Gli1*) and reduced expression of adipogenic genes *PPARgamma* and *CEBPalpha* as well as adipocyte marker genes *aP2* and *adipsin* in epididymal WAT of adult *Ptc1*^mes/mes mice

It has been reported that *Ptc1^mes* mutant mice carry a deletion of a 32 bp DNA fragment in the exon 22 of *Ptc1* allele [22] (Figure 3a). To confirm this, we isolated RNA from epididymal WAT of 8-10-week-old wild type and *Ptc1^mes/mes* mice and performed RT-PCR analyses. As shown in Figure 3a, a positive DNA band covering exons 22 and 23 (using Ptc1-F and Ptc1-R primers) was detected in wild type, but not *Ptc1^mes/mes* mice. These results confirmed the absence of the carboxyl-terminus of *Ptc1* mRNA in epididymal WAT of *Ptc1^mes/mes* mice. Since Ptc1 is a known downstream target gene of Hh signaling, RT-PCR analyses were also performed to determine whether expression of the amino region of the Ptc1 gene will be upregulated in epididymal WAT of *Ptc1^mes/mes* mice. As shown Figure 3a, positive DNA bands covering exons 15 and 18 of *Ptc1* (using Ptc1-F2 and Ptc1-R2 primers) were detected in wild type mice and even more pronounced

in *Ptc1^mes/mes* mice. Similar *Ptc1* expression patterns were observed in epididymal WAT from 33-week-old mice (Figure 3b), indicating that the amino-terminal *Ptc1* mRNA is overexpressed in WAT of *Ptc1^mes/mes* mice.

To determine whether there is mis-expression of other Hh signaling molecules and WAT marker genes in epididymal WAT, semi-quantitative RT-PCR analyses were performed on RNA samples isolated from 33-week-old *Ptc1^mes/mes* mice. As shown in Figure 3b, expression levels of the Hh signaling downstream target genes *Ptc1*, *Ptc2* and *Gli1* were increased in epididymal WAT of *Ptc1^mes/mes* mice, when compared to those of *Ptc1^+/+* mice. Expression of *Gli2*, but not *Gli3*, was also increased in epididymal WAT of *Ptc1^mes/mes* mice. In contrast, reduced expression of *PPARgamma* and *CEBPalpha*, two known adipogenic genes, were observed in *Ptc1^mes/mes* mice, while there was no obvious change in the expression level of *Gilz*, an antiadipogenic gene [27], between *Ptc1^mes/mes* and *Ptc1^+/+* mice (Figure 3b). In addition, expression of WAT marker genes *aP2* and *adipsin* were also reduced in *Ptc1^mes/mes* mice (Figure 3b).
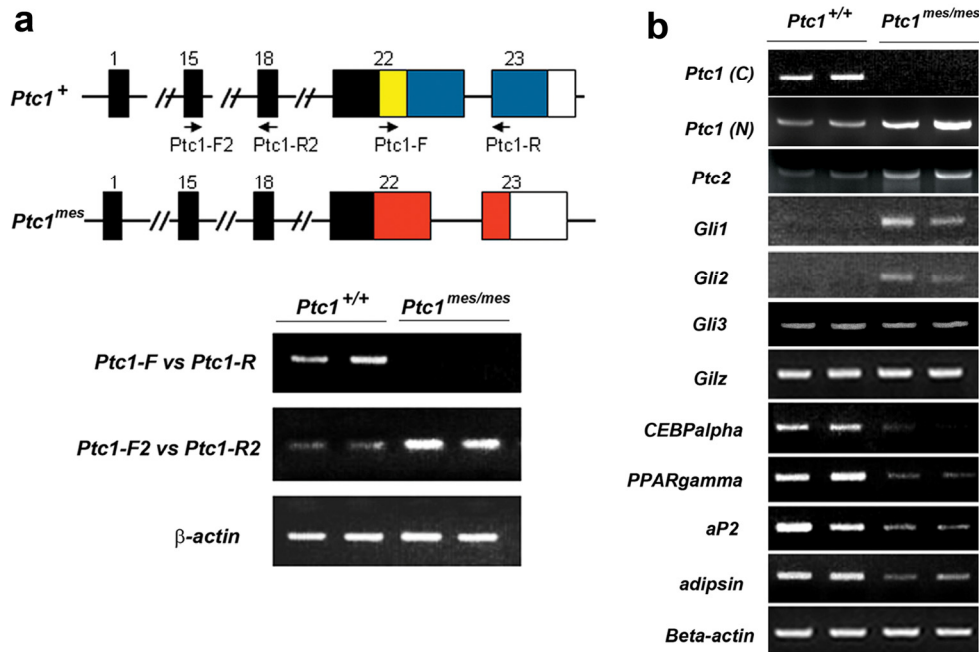

Figure 3. Increased expression of *Ptc1*, *Ptc2*, *Gli1* and *Gli2* and reduced expression of *PPARgamma*, *CEBPalpha*, *aP2* and *adipsin* in epididymal WAT of *Ptc1^mes/mes* mice. **a.** Representative RT-PCR analyses showing the loss of carboxyl-terminal *Ptc1* mRNA (using Ptc1-F and Ptc1-R primers, 36 cycles) and increased expression of amino-terminal *Ptc1* mRNA (using Ptc1-F2 and Ptc1-R2 primers, 32 cycles) in WAT of two 8-10-week-old *Ptc1^mes/mes* mice. Deletion of 32 bp coding sequence within the exon 22 of *Ptc1* in *Ptc1^mes/mes* mice is highlighted by a yellow box. Two sets of RT-PCR primers for Ptc1 mRNA are indicated by arrows. **b.** Representative semi-quantitative RT-PCR showing the increased expression of amino-terminal *Ptc1*, *Ptc2*, *Gli1 and Gli2* and the reduced expression of *PPARgamma*, *CEBPalpha*, *aP2* and *adipsin* in epididymal WAT of 33-week-old *Ptc1^mes/mes* mice. The numbers of PCR reaction cycles for carboxyl-terminal *Ptc1* [Ptc1 (C): using primers Ptc1-F and Ptc1-R], amino-terminal *Ptc1* [*Ptc1-(N):*using primers Ptc1-F2 and Ptc1-R2], *Ptc2, Gli1, Gli2, Gli3, Gilz, CEBPalpha, PPARgamma, aP2, adipsin* and *beta-actin* were 36, 38, 38, 26, 26, 38, 36, 28, 28, 20, 20 and 32, respectively. Experiments were repeated twice on independent RNA samples.

No significant changes in food consumption and glucose tolerance in adult *Ptc1^mes/mes* mice

To determine if there is any difference in food consumption by *Ptc1^mes/mes* and their control *Ptc1^+/+* mice, we performed food intake experiments. As shown in Figure 4a, there was no statistically significant change in food consumption in either adult *Ptc1^mes/mes* or *Ptc1^+/+* mice during the consecutive two weeks. The average food intake by adult *Ptc1^mes/mes* mice at each tested week was also not significantly different from *Ptc1^+/+* control mice (Figure 4a). To test if there is any abnormality in serum glucose concentrations in adult *Ptc1^mes/mes* mice, glucose tolerance experiments were performed. As shown in Figure 4b, no statistical significance of difference in serum glucose concentrations among adult *Ptc1^+/+*, *Ptc1^mes/+* and *Ptc1^mes/mes* mice at each tested time point after the injections of high doses of glucose.
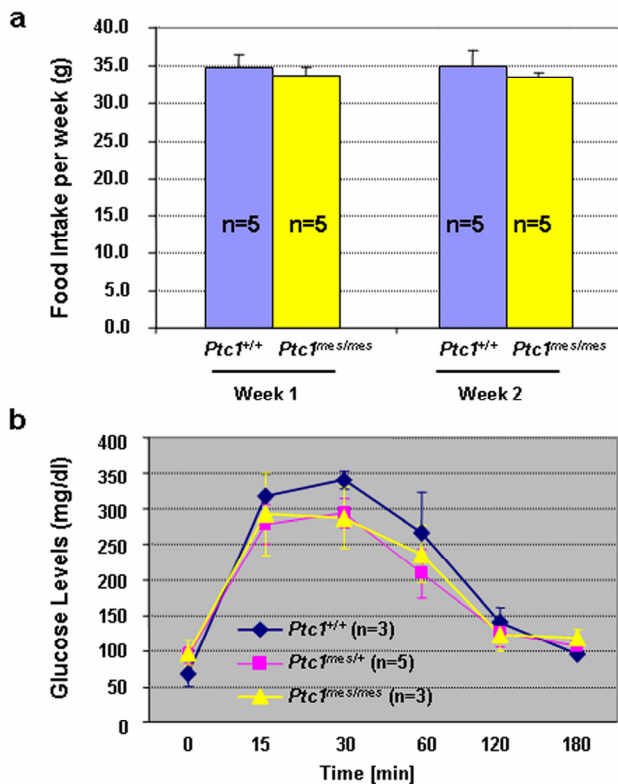

Figure 4. No significant change in (**a**) food intake and (**b**) glucose tolerance between adult *Ptc1^mes/mes* and their control (*Ptc1^+/+* or *Ptc1^mes/+*) mice. **a.** Food consumption by five adult wild type and *Ptc1^mes/mes* mice at the age of 23-27 weeks was recorded weekly for consecutive two weeks. **b.** Overnight-fasted 24-26-week-old littermates were subjected to glucose tolerance experiments as described in *Materials and Methods*. Data are presented as mean ± SEM of the indicated numbers of mice in the bar graphs. *Student's t-test* was performed to determine the significant difference. *P* value was greater than 0.05 between *Ptc1^mes/mes* and controls (*Ptc1^+/+* or *Ptc1^mes/+*) at each time point in the figure.

Discussion

In our ongoing study to determine the role of Ptc during ovarian and uterine development in *Ptc^mes/mes* mice, we observed that total white fat masses in adult female *Ptc^mes/mes* mice were less than their wild type littermates (unpublished data). Since female *Ptc^mes/mes* mice are being used to characterize their ovarian and uterine phenotypes, male *Ptc^mes/mes* and their control littermates were used to investigate the potential defects in WAT during postnatal development in this report. We found that *Ptc1* was expressed in adult WAT and that loss of the carboxyl-terminus of Ptc1 in adult mice caused a significant reduction in total white fat mass and adipocyte cell size (Figure 1-2). Reduced white fat mass observed in adult *Ptc1^mes/mes* mice can be due to the reduced cell sizes of adipocyte cells (Figure 2) and the reduced adipocyte cell numbers in adult *Ptc1^mes/mes* mice. The latter is indicated by the observation that the reduced levels of adipose cell sizes (40% of controls) are less than the reduced levels of total WAT weights in 33-week-old *Ptc1^mes/mes* (about 75% of controls), even though it is difficult and almost impossible to determine the exact numbers of total adipocyte cells in adult mice. Since we did not observe significant changes in food intake and blood glucose concentrations in adult *Ptc1^mes/mes* mice (Figure 4), the reduced white fat mass (reduced adipocyte cell size and cell numbers) is probably not due to reduced food consumption and aberrant glucose metabolism observed in *Ptc1^+/-* mice [28] as well as other hormonal signaling such as insulin signaling during adipogenesis [29]. Rather, the reduced white fat mass is likely due to the defective Hh signaling during adipogenesis in *Ptc1^mes/mes* mice during postnatal development. By RT-PCR analyses, we found that many Hh signaling molecules are expressed in epididymal WAT (Figure 1), which is consistent with the presence of Gli transcription factors in perigonadal fat pads reported previously [13]. Importantly, we found that truncated *Ptc1*, *Ptc2* and *Gli1*, the Hh signaling downstream target genes, are overexpressed in epididymal WAT of *Ptc1^mes/mes* mice (Figure 3b), indicating the ectopic activation of Hh signaling in WAT to regulate its downstream gene expression for fat formation. Consistent with reduced white fat mass in *Ptc1^mes/mes* mice, we also observed the reduced expression of adipocyte marker genes *aP2* and *adipsin*. These reduced expression of adipocyte marker genes likely results from the decreased expression of adipogenic genes *PPARgamma* and *CEBPalpha*. It has been reported that Shh can inhibit adipocyte differentiation in 3T3-L1 cells, C3H10T1/2 and primary mouse calvaria cells [10, 13]. Overexpression of *Gli1* and reduced expression of adipocyte markers,

aP2 and/or *adipsin*, and adipogenic transcription factors, *PPARgamma* and *CEBPalpha*, have been observed in Shh-treated 3T3-L1 or C3H10T1/2 cells [10, 13]. Moreover, reduced expression of *Gli1* and *Gli2* has been reported in *ob/ob* mice and normal mice fed with a high fat diet to induce obesity [13]. Collectively, our results are consistent with the recent findings from cultured cells [10, 13], indicating that Hh signaling plays a role in inhibiting WAT formation in adult mice.

Since we did not observe overexpression of *Gilz*, an antiadipogenic gene, which has been reported by Suh *et al* in cultured cells [13], it is likely that the action of Hh signaling in adult white fat formation is via the inhibition of adipogenic gene transcription, rather than the stimulation of antiadipogenic gene expression. Our gene expression results indicate that Gli1 and Gli2, but not Gli3, may be two major transcription factors involved in adult WAT formation. These Gli transcription factors may inhibit transcription of adipogenic genes *PPARgamma* and *CEBPalpha* in adipocyte cells, resulting in reduced WAT formation. Or, they could directly regulate adipocyte gene expression such as *aP2* in WAT, given that Gli1 can inhibit *aP2* promoter activity in C3H10T1/2 cells [10]. Future studies using molecular approaches such as gel-shift mobility and promoter reporter analyses will determine these possibilities. In addition, further molecular analyses of Hh signaling downstream genes in Hh agonist- or antagonist-treated 3T3-L1 cells over-expressing the amino region of Ptc1 (with a deletion of C-terminal residues of Ptc1) or mouse embryonic fibroblast cells isolated from *Ptc1^mes/mes* embryos will likely provide more details about the mechanism about the action of C-terminal tail of Ptc1 during adipogenesis.

We observed that there was a gradual reduction of total WAT in *Ptc1^mes/mes* males during postnatal development with no significant reduction of total WAT weights at the age of 5-6 weeks, modest reduction in 19-20-week-old mice and remarkable reduction in 33-week-old or older mice. This gradual reduction of WAT observed in our studies is contradictory to the previous finding of the increased adipose weights in Shh-treated mice [8]. One possible reason may be due to the differential expression levels of Hh target genes such as *Ptc1*, *Ptc2* and *Gli1* in white adipocyte cells in mice during postnatal development. Future gene expression analyses of *Ptc1^mes/mes* mice at different developmental stages and Shh agonist-treated mice will be required to address this possibility.

Increased body weights have been observed in *Ptc1* mutant embryos [21-23] and Shh-treated normal mice [8]. Inhibition of body weight gain has also been reported in mice injected with antibodies against Hh proteins [9]. Ectopic activation of Hh signaling in *Ptc1^mes/mes* mice would lead to general stimulation of cell proliferation (other than adipocyte cells), resulting in an increase of body weight. However, we did not observe a significant increase of the body weight in *Ptc1^mes/mes* mice during postnatal development, except those mice at the age of 19-20 weeks after birth (Figure 2). This phenomena is unlikely due to the reduction of food consumption as well as potential aberrant glucose metabolism displayed in *Ptc1^+/-* mice [28], since food consumption and serum glucose levels in adult *Ptc1^mes/mes* mice appears to be comparable with those of control littermates (Figure 4). Considering that the major organ weights including liver, heart, kidney, lung and spleen were not significantly different between *Ptc1^mes/mes* and their control mice (Figure 2d), it is also unlikely the case that the general stimulation of cell proliferation by Hh signaling observed in embryos [22] plays a significant role in body weight gain during postnatal development. Instead, Hh signaling may be active only in a subset of target tissues such as WAT to elicit its biological function in adult *Ptc1^mes/mes* mice. Interestingly, *Ptc1^mes/mes* mice at the age of 33 weeks or older were not lighter than their control littermates, even though there was a significant reduction of total white fat mass (Figure 2). In light of no significant changes in major organ weights in *Ptc1^mes/mes* mice, it is possible that hyperplasia in other tissues and/or cells other than WAT and tested organs exist in *Ptc1^mes/mes* mice, which may offset the reduced WAT weights. In support of this, we did notice that *Ptc1^mes/mes* mice at that age appear to be rounder and bigger than their controls (unpublished data). Considering that activation of Hh signaling can push adipocyte precursor cells toward an osteogenic fate at the expense of adipogenesis [10-13, 30], it is likely that *Ptc1^mes/mes* mice will display aberrant bone formation, which will affect the body weight during postnatal development. Recently, epidermal hyperplasia has been observed in adult *Ptc1^mes/mes* mice [18], which will likely be another factor to offset the reduced WAT weight. Nevertheless, it appears that white fat mass is not a major factor that directly contributes to the body weight gain during postnatal development.

In conclusion, we found that *Ptc1* was expressed in the epididymal fat pad of adult mice. Reduced total white fat mass and white adipose cell size were observed in adult *Ptc1^mes/mes* mice. Truncated *Ptc1*, *Ptc2* and *Gli1* were overexpressed in WAT of *Ptc1^mes/mes*, suggesting ectopic activation of Hh signaling in WAT. Consistent with reduced WAT, expression of adipogenic genes, *PPARgamma* and *CEBPalpha*, and adipocyte marker genes, *aP2* and *adipsin,* were reduced

in WAT of adult *Ptc1^mes/mes^* mice. Our results suggest that deletion of the carboxyl terminal tail of Ptc1 can lead to a reduction of white fat mass during postnatal development.

Acknowledgements

Conflict of Interests

The authors have declared that no conflict of interest exists.

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